

N-terminal ubiquitination and general techniques for monitoring ubiquitin conjugation and protein degradation

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1. SUMMARY

An important step in the ubiquitin proteolytic cascade is specific recognition of the substrate by a member of the ubiquitin ligases family of proteins - an E3, that is followed by generation of the polyubiquitin degradation signal. For most substrates, it is believed that the first ubiquitin moiety is conjugated, via its C-terminal Gly76 residue, to an ϵ -NH₂ group of an internal Lys residue. Recent findings indicate that for several proteins, the first ubiquitin moiety is fused linearly to the α -NH₂ group of the N-terminal residue. An important biological question relates to the evolutionary requirement for an alternative mode of ubiquitination. In this chapter we describe methods that will enable researchers to identify this novel mode of ubiquitination.

To demonstrate that a protein is targeted via ubiquitination of its N-terminal residue, one or more of the following criteria should be fulfilled:

1. A lysine-less (naturally occurring or generated via site directed mutagenesis of all of its internal lysine residues) protein is conjugated in and degraded in a cell-free reconstituted system in vitro in a ubiquitin-dependent manner.
2. The same protein is degraded in cells in vivo following its ubiquitination, and inhibition of the proteasome leads not only to its stabilization, but also to accumulation of specific high molecular mass ubiquitin adducts.
3. Selective chemical modification of the N-terminal residue of the protein either in vivo (by site directed mutagenesis of up to the first four N-terminal residues that will result in acetylation of the N-terminal residue by NAT) or in vitro (by carbamylation) should stabilize it.
4. Fusion of a long tag such as 6 x Myc or HA or GFP to the N-terminal residue but not to the C-terminal residue of the protein, will stabilize it, both in vitro and in vivo.
5. Truncation of the N-terminal segment of the test protein (first 15–30 residues) will stabilize it, both in vitro and in vivo. Experimental establishment of some of these criteria is described in the following subheadings.

2. MATERIALS

2.1. Conjugation and Degradation of Protein Substrates in an In Vitro, Cell-Free Reconstituted Ubiquitin System

1. New Zealand white rabbits (preferably females) of approx 2 kg body wt (2–3 mo old) for preparation of reticulocyte lysate.
2. Phenylhydrazine, 2-deoxyglucose, and 2,4-dinitrophenol (Sigma).
3. Cultures cells (HeLa, 293, Cos) for preparation of nucleated cell extract
4. MgCl₂, NaN₃, and NaF (Sigma).
5. Nitrogen Cavitation Bomb (Parr Instrument Company, 211 53rd Street, Moline, IL 6 1265- 9984. Tel: 1-800-872-7720 or 309-762-7716; Fax: 309-762-9453)
6. Diethylaminoethyl cellulose (DEAE cellulose: DE-52, Whatman).
7. Ammonium sulfate, enzyme grade.

8. Dialysis tubing.
9. Purified protein dissolved (at ~1–10 mg/ml) in water or a buffer. Make sure that the buffer does not contain free amino or hydroxyl groups, Tris-HCl, for example, as this may result in iodination of these groups. Because the buffer is in large molar excess over the protein, the protein will not be labeled.
10. Radiolabeled Na^[125]I at a specific activity of 100–350 mCi/ml and unlabeled NaI (GE Healthcare).
11. Chloramine-T and Na-metabisulfite stock solutions of 10 and 20 mg/ml, respectively (both from Sigma; freshly dissolved in 0.05 M NaPi, pH 7.5).
12. Desalting column (low molecular mass exclusion gel filtration matrix; GE Healthcare).
13. cDNA template coding for the test protein and driven by the RNA polymerase promoters SP6, T7, or T3. The cDNA can code for a lysine-less (naturally occurring or generated via site directed mutagenesis of all of its internal lysine residues) protein, an N-terminal segment-truncated protein, an N- or C-terminally tagged protein, or any protein in which the N-terminal domain was manipulated so that conclusion can be drawn as for the possibility that it is targeted via N-terminal ubiquitination.
14. [³⁵S]Methionine (1000 Ci/mmol at 10 mCi/ml; GE Healthcare).
15. In vitro translation–transcription kit (reticulocyte lysate or wheat germ extract (TNT[®]; Promega).
16. RNasin[®], ribonuclease inhibitor (40 U/μl; Promega).
17. Nuclease-free water.
18. Dithiothreitol (DTT).
19. Krebs - Ringer phosphate solution.
20. 20 mM 2-hydroxyethyl piperazine-N'-(2-ethanesulfonic acid) (HEPES), pH 7.5.
21. ATP regenerating system: 0.5 mM ATP, 10 mM creatine phosphate, and 2.5–10 μg creatine phosphokinase.
22. ATP_γS (Sigma).
23. Ubiquitin and ubiquitin aldehyde (UbAl) (BioMol).
24. Hexokinase (Roche), ammonium sulfate slurry. Centrifuge the slurry and resuspend to a concentration of 10 mg/ml in 20 mM Tris-HCl buffer, pH 7.6. Dilute in the same buffer. Stock solution in the buffer can be stored at 4°C for at least 4 wk.

2.2. Conjugation and Degradation of Proteins in Cells In Vivo

1. Cultured cells in a monolayer or in suspension.
2. [³⁵S]Methionine (1000 Ci/mmol at 10 mCi/ml; GE Healthcare).
3. Methionine-free medium (the same medium in which the cells are grown. Dulbecco's modified Eagle medium (DMEM), minimum essential medium (MEM), or Richter's Improved MEM Insulin (RPMI) medium are the media that are most frequently used for most cells and can be substituted for each other for the short labeling period).
4. Dialyzed serum (the same serum in which cells are grown).
5. Lactacystin (or its lactone homolog, clastolactacystin b-lactone) or MG 132 (Z-Leu-LeuLeu-H), or epoxomicin, or Z-Leu-Leu-Leu-vinyl sulfone, or Z-Leu-Leu-Leu-B (OH)₂ dissolved in DMSO. Stock solutions are of approx 10 mM and final concentration in cultured cells is approx 10–20 μM. Please consult the catalogs of the suppliers (such as BioMol).
6. Cycloheximide (Sigma).
7. CHO-E36 and CHO-ts20 E1 ts mutant cells.
8. Potassium phosphate and potassium chloride (Sigma).
9. Buffer A: 5 mM potassium phosphate, pH 7.0, 1 mM DTT.
10. Buffer B: 20 mM Tris-HCl, pH 7.2, 1 mM DTT.

3. METHODS

3.1. Preparation of Cell Extracts for Monitoring Conjugation and Degradation

To conjugate or degrade a protein substrate *in vitro*, one has to utilize the appropriate cell extract. Rabbit reticulocyte lysate contains all the enzymes required for degradation of most proteins and can be therefore used in most cases. Reticulocytes contain a relatively small number of proteins and do not have lysosomes from which proteases can leak during preparation of the extract. Unlike the case in cultured cells lysate, one can obtain reticulocyte lysate in a relatively large amount. Also, the lack of requirement for tissue culture media and sera make this lysate significantly less expensive than its nucleated cultured cells counterpart. All these attributes make this lysate an ideal extract in which one can test conjugation and proteolysis of the studied protein. For monitoring conjugation and degradation of labeled proteins in the crude extract, it is not necessary to deplete ATP from the cells prior to the preparation of the extract. This will be necessary, however, in order to reconstitute the cell-free proteolytic system and to monitor dependence of the proteolytic process on the addition of exogenous ubiquitin. It will also be necessary in order to monitor conjugation of labeled or tagged ubiquitin to different substrates. Depletion of ATP from cells leads to deubiquitination of most proteins. Once such an ATP-depleted lysate is fractionated over the anion-exchange resin diethylaminoethyl (DEAE)-cellulose, ubiquitin is eluted in fraction I, the unadsorbed, flow-through material that contains also certain E2 enzymes. Fraction II, the high-salt eluate, contains E1, the remaining E2s, all the E3s, and the 26S proteasome.

3.1.1. Preparation of Reticulocyte Lysate

1. Inject rabbits subcutaneously with 10 mg/kg of phenylhydrazine (dissolved in phosphate buffered saline [PBS]) on days 1, 2, 4, and 6.
2. Bleed the rabbits from the ear artery or vein or from the heart (following anaesthesia) on d 8. Induction of reticulocytosis is dramatic and >90% of the circulating red blood cells are reticulocytes as determined by methylene blue or brilliant cresyl blue staining.
3. Wash the cells three times with ice-cold PBS and using a Pasteur pipet, aspirate carefully the thin layer of white blood cells ("buffy coat") that overlays the pelleted red blood cells.
4. Lyse the cells in 1.6 volumes (of pelleted cells volume) of ice-cold H₂O × 2 (double distilled water) containing 1 mM DTT
5. Centrifuge at 80,000 × g for 1 h at 4°C to remove particulate material.
6. Collect the supernatant and freeze in aliquots at -70° C.
7. To deplete ATP, cells are washed twice in PBS and resuspended in one volume of Krebs-Ringer phosphate buffer containing 20 mM 2-deoxyglucose and 0.2 mM 2,4-dinitrophenol. Following incubation accompanied by gentle shaking for 90 min at 37°C, the cells are washed twice in PBS, and lysed and centrifuged as described in steps 4 and 5.

3.1.2. Preparation of Extract From Cultured Cells

All procedures are carried out at 4°C.

1. Wash cells three times in 20 mM HEPES, pH 7.5-saline buffer, and resuspend to a concentration of 10⁷-10⁸/ml in 20 mM HEPES, pH 7.5, that contains also 1 mM DTT.
2. Cavitate cells in a high-pressure nitrogen chamber. For HeLa cells, the best conditions are 1000 psi for 30 min. However, these conditions may vary among different cell species. Make sure that most of the cells are disrupted by visualizing the suspension in a light microscope before and after cavitation. Following disruption, one should observe intact nuclei (that are not broken) and cell debris.
3. Centrifuge the homogenate successively at 3000 × g and 10,000 × g for 15 min, and then at 80,000 × g for 60 min. The supernatant is collected and frozen at -70° C.
4. To deplete ATP, cells are washed twice in HEPES-saline buffer and resuspended in Krebs-Ringer phosphate buffer (to a density of 10⁷ cells/ml in the presence of 2-deoxyglucose, 2,4-dinitrophenol (as described earlier), 20 mM NaF, and 10 mM of NaN₃. Following incubation for 60 min at 37° C, cells are washed twice in HEPES-saline, resuspended in HEPES-DTT (1 mM), and lysed and centrifuged as described in Subheading 3.1.1.

3.2. Fractionation of Cell Extract to Fraction I and Fraction II

As described earlier, fractionation of the lysate into Fraction I and Fraction II separates ubiquitin from many of the other components of the system, thus enabling one to examine whether the enables to monitor the dependence of conjugation and degradation on the addition of exogenous ubiquitin and certain E2 enzymes. To fractionate the lysate (see protocol 2), ATP-depleted lysate is resolved on a DEAE-cellulose column. In the ATP-depleted lysate, all the ubiquitin is free. It was released from conjugates by isopeptidases during the incubation in the presence of the glycolysis and respiration inhibitors. In the absence of ATP, reconjugation is inhibited. Under these conditions, ubiquitin is resolved in fraction I, and fraction II is dependent for its conjugating and proteolytic activities on the addition of exogenous ubiquitin. In cell extracts from which ATP was not depleted, the ubiquitin that is still conjugated to endogenous protein substrates will adsorb to the anion-exchange resin DEAE (via the protein substrate moiety) and will elute in fraction II. During incubation, this bound ubiquitin fraction will be released by the activity of isopeptidases and will be available for conjugation to other proteins, including the test substrate we examine. Therefore, it will be difficult to demonstrate ubiquitin-dependent conjugation and degradation in fraction II that is prepared from an extract from which ATP was not depleted. In addition, the bound ubiquitin fraction, when released, will dilute any added labeled or tagged ubiquitin, and thus decrease the detectable signal in the biosynthesized ubiquitin adducts.

All procedures are carried out at 4°C.

1. Swell the resin in 0.3 M potassium phosphate, pH 7.0, for several hours. Use enough resin to adsorb all the proteins in the extract that can be bound. As a rule, use 0.6 resin volume per volume of reticulocyte lysate or 1ml resin/approx 5 mg of protein of nucleated cell extract (in principle, one can use also a chromatographic system such as fast protein liquid chromatography [FPLC, Pharmacia-Amersham Biotech] with a MonoQ column, although, for resolution of large quantities, the DEAE resin procedure is advantageous).
2. Load the resin onto a column and wash with 10 column volumes of buffer A.
3. Load the extract. Once all the material is loaded, elute fraction I with buffer A. When resolving reticulocyte lysate, collect only the dark red fraction. When resolving cell extract, collect only the fractions with the highest absorption at 280 nm. Freeze fraction I in aliquots at -70°C.
4. Wash the column extensively with buffer A containing 20 mM KCl. When resolving reticulocyte lysate, make sure all the hemoglobin is eluted. When resolving nucleated cell extract, wash until the absorbency at 280 returns to baseline.
5. Elute fraction II with 2.5 column volumes of a buffer B containing 1 M KCl.
6. Add ammonium sulfate to saturation (~70 g/l of solution) and swirl on ice for 30 min.
7. Centrifuge at 15,000 rpm for 15 min.
8. Resuspend pellet in 0.2–0.3 the volume of the original extract. At times, it will be impossible to dissolve all the proteins. This is not essential. They will be dissolved during dialysis.
9. Dialyze against two changes of buffer B. Dialysis should be carried out on ice. Remove particulate material by centrifugation at 15,000 rpm for 15 min. Freeze in aliquots at -70°C.

3.3. Labeling of Proteolytic Substrates

In most cases, monitoring the conjugation and/or degradation of a specific protein substrate requires its labeling. The fate of the protein can also be followed via Western blot analysis using specific antibodies directed against the test protein (Western blot analysis is not described here. Yet, the conjugation and degradation assays for labeled proteins [described below] can be applied in an almost identical manner for unlabeled proteins, followed via immune techniques). Two methods of labeling have proved to be useful, iodination and biosynthetic incorporation of labeled amino acid such as [³⁵S]methionine. Iodination is utilized mostly when a purified recombinant or a pure commercial protein are available. The main advantage of the method is the high specific radioactivity that can be attained. The disadvantage of the method is that one needs a pure protein. Also, during iodination, unless it is carried out using the Bolton–Hunter reagent, the protein can be damaged from the oxidizing agent (chloramines T) used to oxidize the iodide. In addition, during storage, the labeled substrate may be subjected to radiochemical damage from the isotope. A different method of labeling utilizes incorporation of ³⁵S-labeled methionine to a protein that is synthesized in a cell-free system from its corresponding mRNA. The generated protein is native; however,

the specific activity obtained is relatively low. Also, the labeled protein is contained in the crude extract in which it is synthesized and it is not pure. This extract contains, among other proteins, enzymes of the ubiquitin system that may interfere in the reconstitution of a cell-free system from purified components.

3.3. 1. Radioiodination of Proteins

1. Add the following reagents in the following order to 1.5ml microcentrifuge (Eppendorf) tubes. The volume of the reaction mixture can vary from 20 to 100 μ l.
 - a. NaPi, pH 7.5, final concentration of 100 mM
 - b. Protein substrate, 10–500 μ g.
 - c. 50 nmol of unlabeled NaI.
 - d. 0.1–2.0 mCi of radiolabeled Na¹²⁵I.
 - e. 10–50 μ g of chloramine-T solution.
2. Vortex-mix once and incubate for 1–2 min at room temperature.
3. Add 20–100 μ g of Na-metabisulfite solution and mix.
4. To remove unreacted radioactive iodine, resolve the mixture over a desalting column equilibrated with 10 mM Tris-HCl, pH 7.6, and 150 mM NaCl. Collect fractions (in a fraction collector or manually) of approx 10% of column volume each. The radioactive protein is typically eluted in fraction 4 (void volume of the column which is ~35% of the column's total volume).
5. Store in aliquots at –18°C.

3.3.2. Biosynthetic Labeling of Proteins

This is the most frequently used procedure to label substrate proteins and follow their fate in vitro. To label proteins biosynthetically, one can first synthesize the mRNA on the cDNA template, using the appropriate RNA polymerase. Following digestion of the cDNA, the extracted mRNA can be translated in vitro in reticulocyte or wheat germ extracts. Alternatively, one can use a coupled transcription–translation cell-free extract that synthesizes the mRNA and translates it simultaneously. Such systems are available commercially (TNT[®]; Promega). Biosynthesis is carried out basically according to the manufacturer's instructions. In principle, it is preferred to use wheat germ extract. This extract lacks many, although not all, of the mammalian E3 enzymes. Therefore, in most cases, a protein synthesized in this extract can be used in experiments in which a cell-free system is reconstituted from purified enzymes, and in particular, when the role of a specific E3 is tested. A protein synthesized in reticulocyte lysate may be “contaminated” in many cases with its cognate endogenous E2 or E3 enzyme(s). This enzyme, which is being carried to the reconstituted system, may interfere with the examination of the role of an exogenously added E2 or E3 in the conjugation of the translated protein. Yet, at times, one must use the reticulocyte lysate, as the translation efficiency in the wheat germ extract is extremely low. In that case, the “contaminating” E2 or E3 in the reticulocyte lysate can be inactivated by N-ethylmaleimide (NEM; 10 min incubation at room temperature in a final concentration of 10 mM of freshly prepared solution). Because E1, all known E2s, and some of the E3s (HECT domain-containing) have an essential –SH group, the alkylating agent inactivates them. The NEM is then neutralized by the addition of DTT (final concentration of 7.5 mM). It should be noted that this procedure can also denature/inactivate the substrate. In most cases, however, the substrate can still be utilized and reproduces faithfully the behavior of the native substrate.

When monitoring conjugation, the in vitro translated substrate can be used without further processing. This is also true in many cases when the degradation of the labeled substrate is followed by monitoring its disappearance in PhosphorImager-analyzed gels after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). However, as the degradation of certain proteins is not always efficient, it may be difficult to follow with accuracy the disappearance of 10–30% of a labeled protein band in a gel. In this case, it will be necessary to monitor the release of radioactive material into Trichloroacetic acid (TCA)-soluble fraction. In that case the excess of unincorporated labeled methionine in the preparation of the translated protein must be removed. This can be achieved via chromatography over DEAE exactly as described above for fractionation of lysate into resin-unadsorbed (Fraction I) and adsorbed fractions (Fraction II). The vast majority of the labeled proteins will resolve in Fraction II, while the labeled amino acid will be eluted in Fraction I. If the labeled protein is eluted in Fraction I, changing the pH may lead to its adsorption. Alternatively, extensive dialysis of the labeled protein against a solution (20 mM Tris-HCl, pH 7.6, 150 mM NaCl) that contains also 1 mM of unlabeled methionine will also remove efficiently the labeling amino acid.

The protein is synthesized according to the manufacturer's instructions.

3.4. Conjugation of Proteolytic Substrates In Vitro

To demonstrate that the degradation of a certain protein proceeds in a ubiquitin-independent manner, it is essential to demonstrate the intermediates in the process, ubiquitin-protein adducts. Typically, incubation of the labeled protein in a complete cell extract in the presence of ATP will lead to the formation of high molecular mass adducts that can be detected following resolution of the mixture in SDS-PAGE. To increase the amount of the adducts generated, one can use two approaches, independently or simultaneously. The nonhydrolyzable ATP analog, adenosine-5'-O-(3-thiotriphosphate) (ATP γ S) can be used instead of ATP. The ubiquitin-activating enzyme, E1, can catalyze activation of ubiquitin in the presence of the analog, as it utilizes the α -3 high-energy bond of the nucleotide that is cleavable also in the analog. In contrast, assembly and activity of the 26S proteasome complex requires the γ bond that cannot be cleaved in the analog. Caution should be exercised, however, when utilizing the ATP analog. Often, phosphorylation of the target protein is required in order for the ubiquitin ligase to recognize it and target it with ubiquitin. In these cases, the analog cannot substitute for the hydrolyzable native nucleotide, ATP. An additional approach to increase the amount of generated conjugates in a cell-free system is to use ubiquitin aldehyde (UbAl), a specific inhibitor of certain ubiquitin C-terminal hydrolases, isopeptidases. This derivative is available from BioMol.

1. Add the following reagents to 0.5ml microcentrifuge (Eppendorf) tubes. The volume of the reaction mixture can vary from 10.0 to 50.0 μ l. Addition of all the reagents should be carried out on ice:
 - a. 50 mM Tris-HCl, pH 7.6
 - b. 5 mM MgCl₂
 - c. 2 mM DTT
 - d. 5.0–30 μ l of reticulocyte lysate or 50–200 μ g of complete cell extract protein
 - e. 2.5–10 μ g of ubiquitin
 - f. 0.5–2.0 μ g UbAl
 - g. ATP-regenerating system or 2 mM ATP γ S (see preceding paragraph)
 - h. For depletion of endogenous ATP, the system should contain, instead of the ATP-regenerating system, 10 mM 2-deoxyglucose and 0.25–1.0 μ g of hexokinase
 - i. Substrate. Use either a labeled protein (25,000–100,000 cpm) or an unlabeled substrate in an amount that is sufficient for detection by Western blot analysis (100–2000 ng)
2. Incubate the mixture for 30 min at 37°C and resolve via SDS-PAGE (7.5–10% acrylamide).
3. Detect high molecular mass conjugates by PhosphorImager analysis (labeled proteins) or via enhanced chemiluminescence (ECL) following Western blot (for unlabeled substrates) using a specific primary antibody against the test protein and a secondary tagged antibody.

There are several ways to demonstrate that the high molecular mass adducts generated are indeed ubiquitin conjugates of the test protein.

1. It is expected that the adducts will not be generated in an ATP-depleted system.
2. Generation of the conjugates of the specific substrate should be inhibited reversibly by the addition of increasing amount of methylated ubiquitin (MeUb; available from BioMol). This reductively methylated derivative of ubiquitin lacks free amino groups and therefore cannot generate polyubiquitin chains. It serves therefore as a chain terminator in the polyubiquitination reaction, and consequently as an inhibitor in this reaction.
3. Adducts can be precipitated from the reaction mixture with an antibody directed against the test protein, and following SDS-PAGE, can be detected with an antiubiquitin antibody (available from BioMol, Boston Biochemicals, Chemicon, Zymed, Sigma and several other suppliers). Alternatively, the reaction can be carried out in the presence of HA-, Myc-, or His-tagged ubiquitin, and the immunoprecipitate can be detected with one of the appropriate antibodies against the tag.
4. A cell-free system can be reconstituted from purified or isolated components of the ubiquitin system and the formation of conjugates can be followed, depending on the addition of these components.

Rather than adding a complete cell extract, it is possible to add Fraction II (50–200 μg ; derived from ATP-depleted cells) and free or tagged ubiquitin (2–5 μg ; same amount as added to supplement the complete extract; see above). Because Fraction II is devoid of ubiquitin, formation of conjugates that is dependent on the addition of exogenous ubiquitin will strongly suggest that the high molecular mass derivatives generated are indeed ubiquitin adducts of the test substrate. Since not all E2 enzymes are present in fraction II, it may be necessary, at times, to add to the reconstituted system or purified UbcH5a, b, or c, UbcH7, or UbcH8 (available from BioMol). In most cases, one of the UbcH5 enzymes will be able to reconstitute activity.

3.5. Degradation of Proteolytic Substrates In Vitro

With several exceptions, cell free systems for monitoring degradation of proteolytic substrates are similar to those used for monitoring their conjugation. In proteolytic assays, however, unlike in conjugation assays, ATP (and not ATP γ S) must be used, as activity of the 26S proteasome complex is dependent on cleavage of the high energy β - γ bond (see above). ATP is added along with ATP-regenerating system as described above. Also, UbA1 is not added. Following incubation for 2–3 h at 37°C the mixture is resolved via SDS-PAGE and disappearance of the substrate can be monitored either via PhosphorImager analysis (in case the protein substrate is radioactively labeled), or via Western blot analysis (in case of unlabeled substrate). Control reactions are complete mixtures that have been incubated on ice, and mixtures that were incubated at 37°C in the absence of ATP. At times, degradation efficiency is low, and it is difficult to follow the reduction in the amount of a protein band in gel analysis. In these cases, it is necessary to monitor the appearance of radioactivity in trichloroacetic acid (TCA)-soluble fraction. Here, only radioactive substrate can be used. Radio-iodinated proteins can be used directly. In vitro translated proteins must undergo DEAE fractionation or extensive dialysis in order to remove the excess of unincorporated labeled methionine (see above). At the end of the incubation, a carrier protein (10–25 μl of 100 mg/ml solution of bovine serum albumin) is added, followed by the addition of 0.5ml of ice-cold TCA (20%). Following mixing, the reaction is incubated on ice for 10 min and centrifuged (5 min at 15,000 \times g). The supernatant is collected and the radioactivity is determined in either β -scintillation counter (for methionine) or a γ -counter (for iodine-labeled substrates). Control reactions are complete mixtures that have been incubated on ice, and mixtures that were incubated at 37°C in the absence of ATP.

3.6. Involvement of the Ubiquitin System in the Degradation of Proteins In Vivo: Effect of Specific Proteasomal Inhibitors and Inactivation of E1 on the Stability of Proteins in Intact Cells

All the known proteolytic substrates of the ubiquitin system are degraded, following generation of the covalently conjugated polyubiquitin chain, by the 26S proteasome complex. The opposite notion, that all substrates of the 26S proteasome must be ubiquitinated prior to their recognition by the enzyme is true in all but one established case, that of ornithine decarboxylase, ODC. This enzyme is degraded by the 26S complex without prior ubiquitination. A noncovalent association with another protein, antizyme, renders ODC susceptible to degradation by the proteasome. The core catalytic subunit of the 26S enzyme is the 20S proteasome complex and inhibition of this complex inhibits all proteolytic activities of the 26S proteasome. To test whether a certain protein substrate is degraded by the 20S proteasome, it is possible to inhibit the enzyme, both in vitro and in vivo. Inhibition of the 26S proteasome in a cell free system requires higher concentrations of the inhibitor (two- to fivefold) compared to the concentrations required to inhibit the enzyme in cultured cells. Also, as noted earlier, for accumulation of ubiquitin adducts in cell free systems, it is possible to inhibit the activity of the proteasome by the utilization of the nonhydrolyzable ATP analog adenosine-5'-O- (3-thiotriphosphate) (ATP γ S; see above). Stabilization of a protein under such conditions is a strong indication that the protein is indeed degraded by the 26S proteasome. Furthermore, inhibition of the 20S proteasome may lead to accumulation of ubiquitin adducts of the test protein that cannot be detected under conditions of rapid degradation when the proteasome is active. Detection of such intermediates serves as a strong evidence that the protein is degraded by the 26S proteasome complex following tagging by ubiquitin.

3.7. Determination of the Stability (Half-Life) of a Protein in Cells: Effect of Proteasome Inhibitors

1. Wash cells twice in a methionine-free medium at 4°C.
2. Add methionine-free medium that contains dialyzed serum (serum is added in the concentration used for growing the cells).
3. Incubate for 1 h (to remove endogenous methionine) and remove the medium (by aspiration for adherent cells and following centrifugation at 800 \times g for 10 min for cells in suspension) and add fresh

methionine-free medium with serum. To save on labeled methionine, for adherent cells add medium to barely cover the cells (1–1.5ml for a 60 mm dish. For cells in suspension, resuspend cells to 2×10^6 /ml).

4. Add labeled methionine (50–250 μ Ci/ml) and continue the incubation for 0.5 h (pulse).
5. Add the inhibitor to the experimental dishes. Lactacystin and its lactone inhibitor should be added to a final concentration of 5–20 μ M while MG 132 to a final concentration of 50–100 μ M. The inhibitor should be added for 1 h (the last hour of the labeling period).
6. Remove the labeling medium (that contains also the inhibitor(s) in some of the samples).
7. Add ice-cold complete medium that contains, in addition to the inhibitor, also 2 mM of unlabeled methionine and wash the cells twice.
8. Add prewarmed complete medium (that contains the inhibitor and 2 mM of unlabeled methionine) and continue the incubation for the desired time periods (chase).
9. Withdraw samples at various time points and monitor degradation/stabilization of the target protein by immunoprecipitation followed by PhosphorImaging analysis. High molecular mass conjugates of the labeled protein should be precipitated by the specific antibody directed against the target protein under study. To avoid proteolysis of the conjugates by ubiquitin C-terminal hydrolases, it is recommended to dissolve the cells in a detergent-containing lysis buffer at 100°C. Also, the buffer should contain 10 mM NEM to inhibit the ubiquitin recycling enzymes.

Instead of using pulse-chase labeling and immunoprecipitation, one can use cycloheximide (20–100 μ g/ml diluted from 20–100 mg/ml freshly dissolved solution) to stop protein synthesis and follow its degradation via Western blot analysis. The advantage of the approach is that it does not necessitate the use of radioactive material and immunoprecipitation, and one can load whole cell extract onto the gel. The utilization of the proteasome inhibitors is similar to that described above for the pulse-chase experiment. The disadvantage is that the potential interference of a drug in the proteolytic process. Thus, if the drug inhibits the synthesis of a short-lived ubiquitin ligase, E3, involved in the process, the test protein can be stabilized or further destabilized, dependent on the role of the ligase.

A complementary approach to the utilization of proteasome inhibitors, stabilization of the protein and accumulation of ubiquitin adducts, is the use of cells that harbor a temperature sensitive mutation in the ubiquitin-activating enzyme E1, the first enzyme in the ubiquitin proteolytic cascade. At the nonpermissive temperature the cells fail to conjugate the target proteins which are consequently stabilized. Such cells can be, for example, the CHO-E36 (WT) and CHO-ts20 (E1 ts mutant). The experimental approach used can be either pulse-chase labeling and immunoprecipitation or cycloheximide chase.