Video Article
Assaying Proteasomal Degradation in a Cell-free System in Plants

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Abstract

The ubiquitin-proteasome pathway for protein degradation has emerged as one of the most important mechanisms for regulation of a wide spectrum of cellular functions in virtually all eukaryotic organisms. Specifically, in plants, the ubiquitin/26S proteasome system (UPS) regulates protein degradation and contributes significantly to development of a wide range of processes, including immune response, development and programmed cell death. Moreover, increasing evidence suggests that numerous plant pathogens, such as *Agrobacterium*, exploit the host UPS for efficient infection, emphasizing the importance of UPS in plant-pathogen interactions.

The substrate specificity of UPS is achieved by the E3 ubiquitin ligase that acts in concert with the E1 and E2 ligases to recognize and mark specific protein molecules destined for degradation by attaching to them chains of ubiquitin molecules. One class of the E3 ligases is the SCF (Skp1/Cullin/F-box protein) complex, which specifically recognizes the UPS substrates and targets them for ubiquitination *via* its F-box protein component. To investigate a potential role of UPS in a biological process of interest, it is important to devise a simple and reliable assay for UPS-mediated protein degradation. Here, we describe one such assay using a plant cell-free system. This assay can be adapted for studies of the roles of regulated protein degradation in diverse cellular processes, with a special focus on the F-box protein-substrate interactions.

Video Link

The video component of this article can be found at http://www.jove.com/video/51293/

Introduction

The ubiquitin/26S proteasome pathway is emerging as a widespread mechanism for control of diverse biological reactions, including transcriptional regulation, cell-cycle progression and signal transduction, receptor down-regulation or endocytosis, among others processes[1-4]. In this pathway, the target protein is tagged by ubiquitin residues which are first attached *via* a thiolester bond to ubiquitin-activating enzyme E1 and then translocated to a cysteine amino acid residue of ubiquitin-conjugation enzyme E2; finally, E2 interacts with ubiquitin ligase E3, resulting in polyubiquitination of the protein substrate. Ultimately, the polyubiquitinated proteins are recognized and degraded by the 26S proteasome. In this mechanism, the E3 enzyme specifies the substrate and acts as the key regulatory component of the ubiquitin/26S proteasome system (UPS). E3 ligases can act independently, such as RING domain ligases, or as part of a multisubunit SCF (Skp1/Cullin/F-box protein) complex, such as F-box domain ligases. SCF-mediated proteasomal degradation pathways are involved in regulation of transcription, cell cycle, signal transduction[5-10] and many other major cellular functions.

Besides these critical roles in regulation of cellular processes, UPS takes the central stage in many plant-pathogen interactions. For example, increasing evidence suggests that several plant pathogens, including *Agrobacterium tumefaciens,* rely on the host UPS for to facilitate the infection process[11]. Agrobacterium elicits neoplastic growths on plants, which represent its natural hosts, and it can also transform a wide range of other eukaryotes, from fungi[1,2] to human cells[12,13]. During its infection, Agrobacterium exports a DNA element (T-DNA) and several virulence (Vir) proteins into the host cell[12-13]. One of these proteins is VirF, the first F-box protein found to be encoded by a prokaryotic genome[14]. As part of the SCF ubiquitin ligase complex, VirF, and its functional host homolog VBF[15], facilitate Agrobacterium infection *via* the UPS-mediated protein degradation, which presumably facilitates uncoating of the invading bacterial T-DNA from its accompanying bacterial and host proteins, VirE2 and VIP1, respectively[16,17]. Interestingly, many F-box proteins, including VirF, are intrinsically unstable due to their own proteolysis, which is mediated by autoubiquitination activity[18,19] or by other E3 ligases for which F-box proteins may serve as substrates[20-23].

When studying biochemical activities of F-box proteins, other ubiquitin ligases, and/or their substrates, it would be very useful to employ a simple and reliable assay for proteasomal degradation. Here we describe one such protocol for analyzing protein stability in a cell-free system. In this assay, the stability of the UPS substrate is analyzed in the presence or absence of one of the essential components of the proteasomal degradation pathway, such as an F-box protein, in a cell-free system. Generally, we express the tested protein(s) in plant tissues, prepare cell-free extracts from these tissues and monitor the amounts of the protein(s) of interest by western blotting. The UPS-dependent mechanism of protein degradation is demonstrated by inclusion of specific proteasomal inhibitors and/or using coexpression of dominant-negative form of an

SCF component, Cullin. Whereas we illustrate this assay using proteasomal degradation of the Arabidopsis VIP1[17] protein by the F-box protein VBF[15], it may be employed to investigate the stability of any other proteasomal substrates.

Protocol

1. Protein expression

1. **Choice of expression system**

   Select the system, i.e., vectors and vector delivery method, best suited for expression of the protein of interest in the specific model organism/cell. Note that our assay requires expression of the tested proteins in easily detectable amounts, which is best achieved by transient transformation of large numbers of cells. In plants, for example, this is best accomplished using binary plasmids as expression vectors and Agrobacterium as delivery system.

2. **Construction of binary expression vectors**

   Clone the coding sequence(s) of the protein(s) of interest into an expression vector either alone or in translational fusion to an epitope tag. Use vectors suitable for the chosen expression system and standard molecular biology procedures for gene cloning. For Agrobacterium-mediated gene delivery, employ binary vectors and introduce them, also using standard protocols, into an Agrobacterium strain, such as EHA105, for subsequent inoculation of plant tissues.

3. **Choice of plant species**

   Select the plant species in which the protein(s) of interest will be expressed. Note that, while any plant species susceptible to Agrobacterium-mediated genetic transformation can be used, our plant species of choice is *Nicotiana benthamiana*, which is easily grown, highly susceptible to Agrobacterium, and has large leaves which are easily inoculated.

4. **Plant growth**

   Grow one plant for 4 to 6 weeks in a pot with Pro-Mix BX in a pot (20 cm x 20 cm x 20 cm) under environmentally-controlled conditions (e.g., growth chamber) of long day (i.e., 16 h of 130-150 µE m-2 s-1 light at 23°C and 8 h dark at 20°C) and 40-65% relative humidity.

   1.5.2 Fertilize occasionally with commercially available products per manufacturer's instructions. Once the plant is grown, select leaves with the size of 50 mm x 70 mm or larger (these length measurements do not include the petiole) for Agrobacterium inoculation.

5. **Inoculation with Agrobacterium**

   Grow Agrobacterium strain harboring the binary construct expressing the tested protein(s) from step 1.3 overnight at 28°C in YEP medium (1% peptone, 1% yeast extract, and 0.5% NaCl) supplemented with appropriate antibiotics (e.g., 100 mg/L streptomycin and 10 mg/L rifampicin for pPZP-RSC2-based vectors[24-25]).

   1.6.2 Centrifuge the cells, resuspended to OD_{600} =0.5 in infiltration buffer [10 mM $MgCl_2$, 10 mM Mes (pH 5.6), 100 µM acetosyringone], and incubate for 2 h at room temperature. Infiltrate the culture into the abaxial side of a leaf, using a 1-mL needleless syringe; note that the leaves are inoculated in situ, while is attached to the plant.

   1.6.3 After infiltration, grow the plant for 72 h under the light regime of 16 h 130-150 µE m-2 s-1 light at 23°C/8 h dark at 20°C before harvesting.

6. **Application of proteasomal inhibitors**

   To support the notion that the tested protein(s) is degraded via a proteasomal pathway, use specific proteasomal inhibitors MG132 and lactacystin[28-29], which should reduce or even block degradation. Four hours before harvesting (see Step 2.1), infiltrate the Agrobacterium-inoculated leaf areas with 10 µM MG132 (EMD Millipore) or 10 µM lactacystin (Sigma-Aldrich) or mock-treat the leaf with the respective solvents, i.e., 0.1% DMSO or distilled water.

2. Preparation of cell-free extracts

1. **Leaf harvesting**

   Harvest the inoculated areas of the leaf, usually ca. 200-400 mg of fresh weight, and grind them into fine powder in liquid nitrogen. Alternatively, bead-beat the tissues, using any bead-beater (e.g., Biospec) or a dental amalgamator (e.g., TPC Advanced Technologies) directly in the degradation buffer (see Step 2.2).

2. **Protein extraction**

   Prepare total protein extract by placing the ground tissue into 500 µL of degradation buffer [50mM Tris-HCL (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 5 mM adenosine 5´-triphosphate, and 1x protease inhibitor cocktail (Sigma-Aldrich)]. Note that the protease inhibitor cocktail mainly affects serine, cysteine, aspartic, and metalloproteases and does not interfere with the 26S protease. Clarify the extract by two sequential centrifugations at 12,000 x g for 5 min.

3. **Protein degradation reaction**

Transfer equal volumes of extracts, usually 20 µL, to microfuge tubes and incubate them at room temperature for increasing periods of time. Typically, sample time zero, and 5, 10, 15, 20, and 30 min time points. Stop reactions by boiling in SDS gel-sample buffer, and analyze them by western blotting.

3. Detecting protein degradation by immunoblotting

1. **Gel electrophoresis**

   3.1.1 Resolve the protein samples by SDS-polyacrylamide gel electrophoresis and electrotransfer the resolved proteins to a nitrocellulose membrane according to the standard protocol[30].

   3.1.2 Determine protein concentration using the Bradford method (Bio-Rad), and load 50-80 µg of total protein per lane. Make sure that all samples are loaded equally. For loading controls, compare intensities of a ubiquitous protein species, such as putative RuBisCo large chains which migrate as a major band with a relative electrophoretic mobility of around 50 kDa; they can be detected on Coomassie blue-stained gels or on Ponceau S- or fluorescent SYPRO Ruby-stained nitrocellulose membranes.

2. **Blocking**

   Block the membrane with 5% skim milk in TBST (10 mM Tris-HCl, 140 mM NaCl, 0.05% Tween 20, pH 7.4) for 1 h at room temperature.

3. **Primary antibody**

   Dilute primary anti-epitope antibody in 1% skim milk in TBST to the concentration recommended by the manufacturer and incubate with the blocked membrane for 1 h at room temperature or for overnight at 4°C with gentle agitation.

4. **Rinsing**

   Rinse the membrane is rinsed in 20 ml TBST for 15 min once, and twice for 5 min at room temperature with gentle agitation.

5. **Secondary antibody**

   Dilute secondary antibody (e.g., anti-rabbit IgG) conjugated with horse radish peroxidase (HRP) in 1% skim milk in TBST as recommended by the manufacturer and incubate with the membrane for 1 h at room temperature with gentle agitation.

6. **Detection**

   Rinse the membrane again as described in Step 3.4. After the final rinse in TBST, visualize the proteins of interest with an HRP chemiluminescent substrate, most commonly using an ECL kit.

Representative Results

Figure 1, adapted from Zaltsman *et al.*[17], illustrates representative experiments for detection of proteasomal degradation in a cell-free system. Specifically, we demonstrate destabilization of a plant defense-related protein VIP1 by the VBF F-box protein *via* the SCF[VBF] pathway in *N. benthamiana*. Arabidopsis VBF and HA-tagged VIP1 (HA-VIP1) proteins were transiently coexpressed, and HA-VIP1 content protein within extracts of the expressing leaves was analyzed by western blotting. This analysis demonstrated that VIP1 amounts were reduced to a significant degree when coexpressed with VBF, but remained relatively stable in the absence of VBF coexpression (**Figure 1A**). Note that a slight reduction in the VIP1 content without VBF may be due to the low level of the endogenous tobacco VBF activity. The proteasomal degradation mechanism of VIP1 destabilization by VBF was inferred from its inhibition by MG132 (**Figure 1A**). Quantification of the results in **Figure 1A** demonstrated almost complete ($\geq 90\pm 5$%) VIP1 by VBF, which was blocked by MG132 (**Figure 1B**). Note that slightly higher levels of VIP1 in the presence of MG132 most likely are explained by inhibition of the endogenous VBF activity[17].
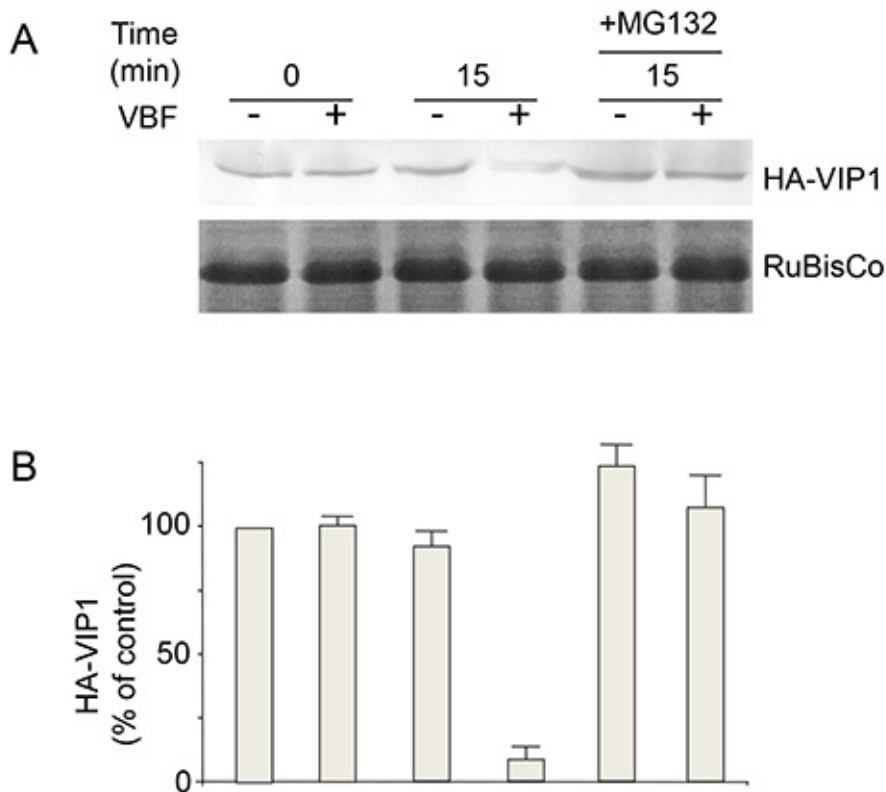
Figure 1. **VBF promotes proteasomal degradation of VIP1.** HA-VIP1 was expressed alone or coexpressed with VBF in *N. benthamiana* leaves. The resulting protein extracts were incubated for the indicated time periods and analyzed by western blotting using anti-HA antibodies. The putative RuBisCo large chain was used as loading control (**A**). Quantified western blot signal was expressed as percent of the signal obtained in the absence of VBF at the start of the incubation period. The data represent average values of three independent experiments with indicated standard deviations (**B**). This figure was adapted from Zaltsman *et al.*[17]

Discussion

This assay relies on the expression of the tested proteins in plant tissues; thus, the potential proteasomal degradation process obviously occurs already within the living tissues. We assay protein destabilization, however, only in the extracts, with the time zero sample serving as the initial reference point. Hence, we define it as a cell-free assay.

One important aspect for the success of this assay is the correct choice of the expression vector from which the tested protein(s) will be produced. Unless specific antibodies against the tested protein are available, it should be epitope-tagged for detection by western blotting. The tagged protein should be expressed from an *Agrobacterium* binary vector. Whereas many such vectors are available[26], we suggest using vectors derived from our modular pSAT plasmid system[24], which allows one-step insertion of expression cassettes into the pPZP-RCS2 binary plasmids[24-25]. Another advantage of the pSAT system is that it allows expression of multiple genes from the same vector[25], which is especially useful for experiments to assay several proteasomal substrates or to study the effects of interactors of the tested substrate; for example coexpression of the *Agrobacterium* VirD5 protein that interacts with the proteasomal substrate VirF results in VirF protection from proteasomal degradation[22]. Importantly, the expanding pSAT series of vector already includes vectors for epitope tagging[27]. Regardless of the chosen cloning strategy, the sequence encoding the epitope-tagged protein of interest should be introduced in a binary vector for subsequent expression in plant tissues. Because this assay uses transient expression, the binary vector does not have to contain selection markers for stable transformation, although their presence is not detrimental to the assay efficiency.

Although coexpression of several proteins of interest is best achieved using multigene expression vectors, it can also be done by combining equivalent volumes of liquid cultures of two or three *Agrobacterium* strains, each of which carries a separate binary construct.

Another critical point is that, in many cases, the proteasomal degradation assay involves expression not only of the proteasomal substrate, but also of a component of the SCF pathway. For example, the experimental goal might be to test whether an F-box protein recognizes and targets for degradation a specific substrate. In this case, this epitope-tagged substrate should be coexpressed with the untagged F-box protein.

For more detailed analysis of the data, the extent of protein degradation can be easily quantified by measuring the relative intensity of the western signals using the latest ImageJ software (NIH)[31]. When performing such quantification, it is important not to over-develop the western blots to avoid signal saturation. Data quantification also requires that all samples are loaded on the SDS-polyacrylamide gel equally in respect to their protein content as protein degradation in this assay is detected by reduction in the intensity of the appropriate protein band. This is achieved by determination of the protein content of each cell extract and by subsequent verification of equal loading of each lane (see step 3.1). Furthermore, in any given time-course experiment, all samples should be derived from the same batch of extract helps to ensure equal loading.

The cell-free assay for proteasomal degradation described here uses plants as an experimental system. However, the same experimental design could be used for any other organism. The assay also can be extended by further focusing on the SCF pathway. For example, the SCF-dependent mechanism of protein degradation can be demonstrated using a dominant-negative form of the SCF component CULLIN1 (CUL1[DN]). Specifically, a mutant of Arabidopsis CUL1 with deleted amino acid residues between positions 1 and 420 has been shown to interact with the Skp1/ASK1 component of the SCF complex, but not with RBX1, which represents the catalytic core of SCF[22]. Inhibition or reduction of substrate degradation following coexpression of CUL1[DN] indicates involvement of the SCF pathway.

Our assay utilizes proteins of interest expressed in plant tissues, in their natural environment. One alternative to this approach is to purify recombinant proteins, add them to plant extracts, and follow their degradation by western analysis[32]. We do not recommend this tactic as methodology of choice because recombinant proteins do not always faithfully reflect the native biological properties; however, if expression *in planta* is not feasible, the use of recombinant proteins certainly represents a valuable alternative.

Disclosures

No conflicts of interest declared.

Acknowledgements

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