RECONSTITUTING ENDOPLASMIC RETICULUM-ASSOCIATED DEGRADATION (ERAD) IN RABBIT RETICULOCYTE LYSATE

MICHELE ARDUENGO, PH.D., PROMEGA CORPORATION

Here we highlight several peer-reviewed publications in which endoplasmic reticulum-associated degradation was reconstituted in rabbit reticulocyte lysate cell-free systems.

Introduction

Conditions such as environmental stress, viral infection and absence of required partner proteins can result in aberrant folding of proteins that are being synthesized in the rough endoplasmic reticulum (RER). The RER has a "quality control" system that detects these abnormal proteins and targets them for degradation using the ubiquitin/proteasome pathway. This process, ER-Associated Degradation (ERAD), requires ATP and is distinct from the lysosomal degradation pathway in cells (1). Misfolded proteins are transported from the lumen of the ER back to the cytoplasm (retrotranslocation) where they are ubiquitinated and subsequently degraded by the proteasome (2). Accumulation of aberrantly folded proteins has been linked to a variety of human diseases such as cystic fibrosis, type I diabetes, and genetic liver disease, among others (3,4). Therefore, ERAD is an important cellular process to understand from a therapeutic viewpoint.

Promega Rabbit Reticulocyte Lysate, Untreated, contains no hemin, making it suitable for ERAD assays.

Suitability of Rabbit Reticulocyte Lysate for Studying ERAD

Rabbit reticulocyte lysate (RRL) has been used to reconstitute the ERAD activity in a variety of systems (5). RRL presents advantages over intact-cell systems for such study. First, only the protein of interest will be labeled in the RRL system, making its degradation easy to follow (5). Second, a variety of microsomal membranes ranging from canine microsomal membranes to semipermeabilized cells can be used in conjunction with the system to reconstitute the activity (5,6). Because hemin inhibits proteasome activity, degradation studies are best performed in RRL that does not contain exogenous hemin, and researchers have reported that RRL well suited for studies of degradation is usually poor for translation and vice versa (5). Additionally, since ERAD is ATP-dependent, the RRL will need to be supplemented with an ATP regeneration system (5), and some authors report that excess unlabeled methionine seems to aid in reconstituting ERAD activity (7).

Promega Rabbit Reticulocyte Lysate, Untreated (Cat.# L4151), contains the cellular components necessary for protein synthesis (tRNA, ribosomes, amino acids, initiation, elongation and termination factors) but has not been treated with micrococcal nuclease. It is not supplemented with creatine phosphate, creatine phosphokinase, DTT, potassium acetate, magnesium chloride or hemin, making it suitable for degradation assays once optimized for a particular experimental system and supplemented with an ATP regeneration system.

Here we highlight four peer-reviewed publications in which ERAD was studied in cell-free degradation assays using either laboratory-prepared or commercially available rabbit reticulocyte lysate.

Svedine, S. *et al.* (2004) Carbohydrates act as sorting determinants in ER-associated degradation of tyrosinase. *J. Cell Sci.* 117, 2937-49.

A cell-free system using semipermeabilized melanocytes and Rabbit Reticulocyte Lysate, Untreated (Cat.# L4151), was used to reconstitute tyrosinase ERAD (8). Wildtype and albino mutant tyrosinase were translated in Rabbit Reticulocyte Lysate, Nuclease Treated (Cat.# L4960) supplemented with canine microsomal membranes or semipermeabilized melanocytes (SP-melanocytes). Semipermeabilized cells were created by treatment with low concentrations of digitonin to permeabilize the plasma membrane, while leaving the organelle membranes intact. Proteasomes were washed from the SP-melanocytes and added back as required. After translation, SP-melanocytes were isolated, and resuspended in Rabbit Reticulocyte Lysate, Untreated (Cat.# L4151), in the presence of an ATP regenerating system. Both the wildtype and mutant tyrosinase were degraded, with the mutant protein degraded at a higher rate than the wildtype protein.

Gusarova, V. *et al.* (2001) Apoprotein B degradation is promoted by molecular chaperones hsp90 and hsp70. *J. Biol. Chem.* 276, 24891–900.

An RRL degradation system was used to determine if the chaperone protein hsp90 is required for the degradation of Apoprotein B (apoB; 9). For this study the shorter apoB48 species, expressed in mammalian intestine and rodent liver, was used. To determine if apoB48 would be ubiquitinated in vitro, the protein was synthesized using the TNT® SP6 Coupled Reticulocyte Lysate System (Cat.# L4600) in the presence of canine microsomal membranes. HA-tagged ubiquitin and an ATP regeneration system were added at the end of translation and ubiquinated apoB48 was immunoprecipitated (9). For ERAD studies, microsomes from the translation reaction were pelleted and resuspended in either laboratory-prepared RRL or rat hepatic cytosol containing ATP and an ATP regeneration system. Both systems degraded the protein, although there were some differences between the systems in response to various degradation inhibitors. To assess the role of hsp90 in the degradation of apoB48, geldanamycin (GA), an antibiotic that competes for the ATP binding site on hsp90, was added to pelleted canine microsomal membranes before the degradation assay. There was no significant degradation of apoB48 in the GA-treated system, indicating that GA inhibited degradation. This finding led the authors to conclude that hsp90 was required for apoB48 degradation.

Teckman, J.H., Gilmore, R. and Perlmutter, D.H. (2000) Role of ubiquitin in proteasomal degradation of mutant αl-antitrypsin Z in the endoplasmic reticulum. *Am. J. Physiol. Gastrointest. Liver Physiol.* 278, G39–48.

RRL has also been used to reconstitute the degradation of α1-antitrypsin Z [(α1AT)Z] (7). Individuals who are homozygous recessive for a mutation that results in a Glu^{342} to Lys substitution have increased susceptibility to liver disease (7). The amino acid substitution disrupts proper protein folding of (α1AT)Z, and individuals susceptible to the liver disease do not appear to be able to efficiently degrade the misfolded protein. Mutant and wildtype (α1AT)Z were synthesized in Rabbit Reticulocyte Lysate System, Nuclease Treated (Cat.# L4960) in the presence of canine microsomal membranes. Iodinated mutant and wildtype protein degradation was then examined using laboratory-prepared RRL assay system. The mutant (α1AT)Z was degraded efficiently. Mutant protein produced using salt-washed or puromycin-treated, salt-washed microsomes was also degraded, indicating that the full complement of RER proteins was not required for degradation. To further elucidate the components required for degradation in the cell-free RRL degradation assay, RRL was fractionated over a sucrose gradient. Two fractions, FRI, containing ubiquitin and the ubiquitin-conjugating enzyme E2-F1, and FRII, containing the proteasome, were obtained. Complete degradation was achieved when degradation was performed using both fractions. However when FRII alone was used, a small amount of protein was degraded, indicating that both ubiquitin-dependent and independent mechanisms exist for (α1AT)Z degradation (7).

Wilson, C.M., Farmery, M.R. and Bulleid, N.J. (2000) Pivotal role of calnexin and mannose trimming in regulating the endoplasmic reticulum-associated degradation of major histocompatibility complex class I heavy chain. *J. Biol. Chem.* 275, 21224–32.

Several mechanisms have been suggested to control targeting ER proteins for degradation, including regulating the trimming of N-linked oligosaccharide chains. Oligosaccharide side chains can be modified by mannosidase I in the ER. Inhibiting this activity seems to stabilize misfolded proteins (6). Wildtype MHC class I heavy chain and a mutant heavy chain lacking the N-linked glycosylation site but capable of assembling into functional MHC Class I molecules, were translated in Flexi® Rabbit Reticulocyte Lysate System (Cat.# L4540, lacking DTT to support disulfide bond formation) in the presence of semipermeabilized cells (SP) derived from the human cell line HT1080 (6). The SP cells were isolated from the translation reaction and resuspended in untreated, laboratory-prepared RRL containing no hemin but supplemented with an ATP regeneration system. The wildtype protein was degraded more quickly than the mutant, indicating that glycosylation is important for ERAD (6).

Summary

These studies and others like them show that rabbit reticulocyte lysate can provide a powerful cell-free system for reconsituting ERAD and ubiquitination for a variety of proteins using proteins synthesized in the presence of canine microsomal membranes or semipermeabilized cells. Researchers routinely prepare their own hemin-free RRL for such studies, but commerically available RRL such as Rabbit Reticulocyte Lysate System, Untreated, can also be used for these degradation assays. ■

ERAD in Rabbit Reticulocyte Lysate

References

1. Kostova, Z. and Wolf, D.H. (2003) *EMBO J.* **22**, 2309–17.
2. Zhang, K. and Kaufman, R.J. (2004) *J. Biol. Chem.* **279**, 25935–8.
3. Kaufman, R.J. (2002) *J. Clin. Invest.* **110,** 1389–98.
4. Sifers, R.N. (2003) *Science* **299,** 1330–1.
5. Carlson, E. *et al.* (2005) *Meth. Mol. Bio.* **301**, 185–205.
6. Wilson, C.M., Farmery, M.R. and Bulleid, N.J. (2000) *J. Biol. Chem.* **275**, 21224–32.
7. Teckman, J.H., Gilmore, R. and Perlmutter, D.H. (2000) *Am. J. Physiol. Gastrointest. Liver Physiol.* **278**, G39–48.
8. Svedine, S. *et al.* (2004) *J. Cell Sci.* **117,** 2937–49.
9. Gusarova,V. *et al.* (2001) *J. Biol. Chem.* **276**, 24891–900.

Protocols

Rabbit Reticulocyte Lysate System Technical Manual #TB232, Promega Corporation.
www.promega.com/tbs/tm232/tm232.html

Ordering Information

Product	Size	Cat.#
Rabbit Reticulocyte Lysate System, Untreated	1ml	L4151

Related Products

Product	Size	Cat.#
Rabbit Reticulocyte Lysate System, Nuclease Treated*	5 × 200µl	L4960
Canine Pancreatic Microsomal Membranes	50µl	Y4041
TnT® SP6 Coupled Reticulocyte Lysate System*	40 reactions	L4600
TnT® T7 Coupled Reticulocyte Lysate System*	40 reactions	L4610
TnT® T3 Coupled Reticulocyte Lysate System*	40 reactions	L4950
TnT® T7/SP6 Coupled Reticulocyte Lysate System*	40 reactions	L5020
TnT® T7/T3 Coupled Reticulocyte Lysate System*	40 reactions	L5010
Flexi® Rabbit Reticulocyte Lysate System	5 × 200µl	L4540

*For Laboratory Use.