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An in vitro assay for the selective endoplasmic reticulum associated degradation of an unglycosylated secreted protein

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Abstract

The endoplasmic reticulum (ER) represents the first compartment into which nascent secreted proteins traffic, and not coincidentally the ER lumen houses a high concentration of factors that facilitate protein folding, such as molecular chaperones. To off-set the potentially lethal consequences of mis-folded secreted protein accumulation, aberrant proteins may be selected for degradation via a process known as ER associated degradation (ERAD). After their selection ERAD substrates are retro-translocated back to the cytoplasm and then degraded by the 26S proteasome. Key features of the selection, retro-translocation, and degradation steps that constitute the ERAD pathway were elucidated through the development of an in vitro ERAD assay. In this assay the fates of two yeast proteins can be distinguished after their translocation, or import into ER-derived microsomes. Whereas a wild type, glycosylated protein ("GpαF") is stable, a non-glycosylated version of the same protein ("pαF") is rapidly degraded when microsomes containing radiolabeled forms of these substrates are incubated in cytosol and ATP. The purpose of this chapter is first to discuss the experimental findings from the use of the in vitro assay, and then to describe the assay in detail. Finally, future potential uses of the in vitro system are illustrated.

Keywords: ERAD; proteasome; Sec61; Translocation; Molecular chaperones; Degradation; Hsp70; Protein quality control; Yeast; Secretion

1. Introduction

Based on the sequencing of eukaryotic genomes it has been estimated that ~25–33% of all proteins enter or interact with organelles that comprise the secretory pathway. The first step in the biogenesis of the vast majority of these proteins is their translocation, or import into the endoplasmic reticulum (ER). After import through the Sec61-containing translocation pore in the ER membrane, the nascent secreted protein folds into its final three-dimensional conformation, which is aided by the action of ER associated enzymes that directly catalyze folding and by molecular chaperones [1]. Although most molecular chaperones do not directly catalyze protein folding, they prevent the formation of off-pathway aggregates and thus enhance folding efficiency. However, the overall efficiency of protein folding can be compromised because of cellular stresses, because of translational errors, or because of genetic mutations. If the concentration of mis-folded proteins rises then the unfolded protein response (UPR) may be induced and/or protein aggregates may accumulate in the ER. Prolonged UPR induction and/or protein aggregate accumulation can result in cell death [2].

To prevent the accumulation of these potentially toxic proteins, and to ensure that only folded, mature proteins traffic beyond the ER, mis-folded or improperly processed secreted proteins are subject to ER protein quality control. One aspect of the ER quality control check-point is the degradation of aberrant secreted proteins, and for a long time an ER-resident quality control

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protease was sought, but to no avail. It was also unclear how substrates for this protease might be selected from wild type proteins in the ER.

Early hints into the identity of the ERAD protease came from studies on the degradation of the cystic fibrosis transmembrane conductance regulator (CFTR), the protein that when mutated leads to cystic fibrosis. It was found that CFTR—either in its nascent, pre-folded state or containing a disease-causing mutation that hastens its degradation—was a substrate for the proteasome [3,4], a large (~2.1 MDa) multi-catalytic protease that resides in the cytoplasm. Because the majority of CFTR faces the cytoplasm, and because a large number of proteasomes are associated with the ER membrane [5], it seemed logical that the proteasome was involved in CFTR turnover. Nevertheless, it remained unclear how soluble secreted proteins, which reside entirely within the ER, were destroyed.

To this end, we utilized a well-established system to translocate a radiolabeled precursor of a yeast mating pheromone, pre-pro α factor (ppαF), into yeast ER-derived microsomes in vitro [6–8]. Normally, the signal (pre) sequence is removed by signal peptidase in the ER, the pro region becomes triply glycosylated, and the resulting protein (3GpαF) is packaged into COPII vesicles for transport to the Golgi [9]. However, previous work had established that an unglycosylated version of the protein, in which the three, core oligosaccharyl consensus sites in the pro region were mutated, was rapidly degraded in the secretory pathway [10]. Based on this information, we also translocated the mutated form of ppαF that could not be glycosylated ("ΔGppαF") into yeast microsomes. After signal sequence cleavage, ΔGppαF is converted into pro α factor ("pαF"). We initially found that both 3GpαF and pαF were stable in yeast microsomes, but surmised that the bona fide intracellular milieu might not have been established and therefore added yeast cytosol to a final concentration of 5 mg/ml. When ATP was also present, we observed a robust and rapid degradation of the mutant (pαF) but not wild type (3GpαF) substrate, indicating that a mutated, but not wild type secreted, protein had been selected for proteolysis [11].

As a first step toward determining the protease responsible for the degradation of pαF, which we too assumed resided in the ER lumen, a centrifugation step at an early time-point (~5 min) of the degradation reaction was introduced. Surprisingly, we found that the pαF (but not the wild type 3GpαF) had been exported from the vesicles back into the cytosolic fraction (Fig. 1; note the pαF signal in the supernatant when cytosol and ATP are present). PαF is also protease-accessible, indicating that it had not been packaged into transport vesicles [11]. This was the first observation of polypeptide retro-translocation from the ER, and has also been referred to as "dislocation" in the literature [12]. Moreover, these

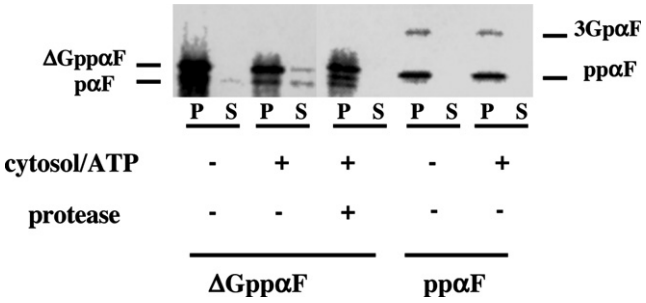

Fig. 1. Selective export of pαF from ER-derived yeast microsomes. In vitro ERAD reactions were set-up as described in the text and a centrifugation step was introduced to separate microsomes (P) from the cytosolic (S) fractions. PαF is only present in the supernatant when cytosol and ATP are present, and this material is accessible to exogenously added protease, demonstrating that it is not encapsulated within a secretory vesicle. In contrast, 3GpαF is not retro-translocated to the cytosol. Reproduced from [11], by copyright permission of The Rockefeller University Press.

results suggested that the site of degradation for soluble ERAD substrates was in the cytoplasm, and not in the ER. The results also suggested that perhaps the proteasome, which plays a role CFTR turn-over and resides in the cytoplasm (see above), might also be required for the degradation of soluble proteins. Data supporting this premise are displayed in Fig. 2, showing that the degradation of pαF is attenuated by ~2.5-fold in cytosol prepared from a proteasome ("*pre1/pre2*") mutant strain (compare pαF signal in lanes 2 and 4) [13]. The ability of the proteasomse to degrade membrane [12,14] and soluble ERAD substrates after their retro-translocation [15; also see Chapter from R. Sifers, this volume] was corroborated in the same year by several other groups in studies utilizing both yeast and mammalian systems. Notably, mammalian ERAD substrates are also degraded via ERAD when expressed in yeast [16,17], suggesting that the machinery for this pathway is conserved.

The in vitro assay has also been utilized to establish or further dissect several fundamental aspects of the ERAD pathway. First, the contribution of ER lumenal molecular chaperones to ERAD substrate selection and

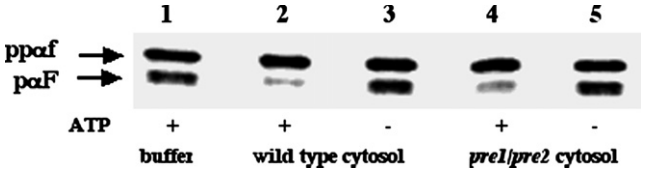

Fig. 2. Cytosol prepared from strains mutated for two proteasomal subunits exhibits reduced ERAD efficiency. P/pαF-containing microsomes were incubated with buffer, wild type cytosol, or *pre1pre2* mutant cytosol in the presence or absence of an ATP-regenerating system, as indicated. In this experiment, the amount of pαF remaining when the incubation was performed with the mutant cytosol was 2.5-fold higher than when performed with wild type cytosol (it should be noted that residual proteasome activity is present in cytosols prepared from the *pre1pre2* mutant). Reproduced with permission from [13], Copyright 1996, The National Academy of Sciences.