

CPY* and the Power of Yeast Genetics in the Elucidation of Quality Control and Associated Protein Degradation of the Endoplasmic Reticulum

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1	CPY*, a Malfolded Secretory Protein Is Retained in the Endoplasmic Reticulum and Degraded in the Cytosol	42
2	Endoplasmic Reticulum to Cytosol Retrotranslocation: A New Cellular Mechanism	43
3	Carbohydrate Trimming: A Tool of the Endoplasmic Reticulum Quality Control of Glycoproteins	44
4	Soluble Proteins Require Endoplasmic Reticulum-Luminal Chaperones for Degradation	46
5	Sec61p, Part of the Retrotranslocation Channel?	46
6	Endoplasmic Reticulum-Associated Protein Degradation: Ubiquitin, the Proteasome and Other Helpers	47
7	Modular CPY*-Based Membrane Substrates Broaden the Picture	48
8	Yeast Genomics Discovers New Players	50
	References	51

Abstract CPY* is a mutated and malfolded secretory enzyme (carboxypeptidase yscY, Gly255Arg), which is imported into the endoplasmic reticulum but never reaches the vacuole, the destination of its wild type counterpart. Its creation, through mutation, had a major impact on the elucidation of the mechanisms of quality control and associated protein degradation of the endoplasmic reticulum, the eukaryotic organelle, where secretory proteins start the passage to their site of action. The use of CPY* and yeast genetics led to the discovery of a new cellular principle, the retrograde transport of luminal malfolded proteins across the ER membrane back to their site of synthesis, the cytoplasm. These tools furthermore paved the way for our current understanding of the basic mechanism of malfolded protein discovery in the ER and their ubiquitin-proteasome driven elimination in the cytosol (ERQD).

1

CPY*, a Malfolded Secretory Protein Is Retained in the Endoplasmic Reticulum and Degraded in the Cytosol

During the establishment of the yeast *Saccharomyces cerevisiae* as a model organism to study the function of proteolysis in eukaryotic cell physiology via biochemical and genetic means, the first protease mutant defective in the activity of one of the vacuolar proteases, carboxypeptidase yscY (CPY), was isolated [79]. As a protein of the hydrolytic vacuolar (lysosomal) compartment, CPY is synthesized in the cytosol as a pre-pro-enzyme and thereafter enters the secretory pathway: after import into the endoplasmic reticulum (ER) the pre-(signal)-sequence is cleaved off and the enzyme is folded. During these processes, disulfide bonds are formed and CPY is modified with four N-linked carbohydrate chains, yielding p1-CPY. After outer chain mannosylation in the Golgi-apparatus, CPY enters the vacuole where the pro-sequence is cleaved to yield the mature form of the enzyme [64, 47]. The fact that mutated CPY had never matured to the vacuolar wild type form indicated that the mutation had either destroyed the maturation site of this serine protease, thus preventing its cleavage into the mature form in the vacuole, or that the mutation prohibited secretion of the protein to its vacuolar location [51]. Sequencing of the mutant gene uncovered that a mutation, Gly255Arg, in a highly conserved site of all serine proteases, two amino acids away from the active site serine, had occurred [20]. Incubation of the mutant protein with trypsin *in vitro* leads to its rapid degradation, in contrast to wild type pro-CPY, which is cleaved to its mature size. This indicates that the mutant protein is completely differently folded as compared to the wild type protein. This malfolded pro-CPY protein was named CPY* [20]. Even though CPY* contains the pro-sequence, which could direct it to the vacuole, it never reaches this organelle. This was very surprising at the time, as on one hand the vacuole is the working place of CPY, on the other hand it represents the gut of the cell responsible for degrading cellular proteins in an unspecific way. Instead, CPY* is retained in the endoplasmic reticulum (ER) and rapidly degraded, with a half life of 15–20 min [20]. Two developments, which had merged in 1991, made the subsequent discoveries possible: (a) the elucidation of the ubiquitin system, which through tagging selected proteins with the 76-amino acid protein ubiquitin, targets them for degradation [72, 29] and (b) the discovery of the proteasome as the proteolytic machinery, which degrades the ubiquitin-tagged proteins *in vivo* [27, 78, 80]. The establishment of yeast in the elucidation of the physiological function of vacuolar [77, 70, 78] and ubiquitin-proteasome linked proteolysis [32, 72] and the finding of CPY* as a rapidly degraded, malfolded secretory protein [20, 65, 61] were crucially important for the dissection of ER quality control and the associated cytosolic degradation pathway (ERQD).

2

Endoplasmic Reticulum to Cytosol Retrotranslocation: A New Cellular Mechanism

The isolation of yeast mutants defective in the degradation of CPY* started to give crucial insights into the quality control and degradation pathway [45]. After mutagenesis of a yeast strain carrying the *prc1-1* allele encoding CPY*, mutants were isolated on the basis of defective CPY* degradation, which was made visible on colony immunoblots of mutated strains using CPY* antibodies [20, 45]. The first series of seven mutant alleles giving rise to a disturbed ER quality control and degradation process of CPY* were named *der1* to *der7* (*der*, *d*egradation of the *ER*) [45, 37]. Analysis of the *der2* mutant led to a breakthrough in our knowledge of how ER-associated degradation works. The *DER2* gene was identified as the gene encoding the ubiquitin-conjugating enzyme (E2) Ubc7p [31]. The participation of an ubiquitin-conjugating enzyme in the degradation of CPY* immediately pointed to the participation of the ubiquitin-proteasome system (UPS) in the degradation of the misfolded enzyme. Analysis of CPY* degradation in proteasome mutants indeed confirmed the requirement for the 26S proteasome in the degradation process [31]. As the 26S proteasome had only been found in the cytosol and the nucleus of cells [44], and Ubc7p only in the cytosol [42], a retrograde transport of CPY* from the ER lumen back to the cytoplasm had to be postulated. The appearance of polyubiquitinated and at the same time glycosylated CPY* on the cytoplasmic side of the ER membrane substantiated this idea [31]: CPY* had obviously been imported into the ER and N-glycosylated, somehow recognized as being unable to fold, transported back out of the ER and polyubiquitinated for subsequent proteasomal degradation (Fig. 1). These findings had set the stage for a new biological mechanism. It violated the dogma that proteins, which had entered the endoplasmic reticulum, were trapped in the secretory pathway unable to return back into the cytoplasm [5]. The findings of Hiller et al. in 1996 [31], using CPY* as a substrate, had set the frame around a mosaic composed of two processes: (a) protein quality control in the endoplasmic reticulum and (b) degradation of misfolded proteins in the cytoplasm via the ubiquitin-proteasome system. Both processes are linked via a transport step of the misfolded protein back out of the ER into the cytoplasm. Additional work was and still is required to fill the gaps in knowledge in the mosaic. Here also CPY* continues to serve as an excellent model substrate. By introducing a fifth glycosylation site at the very C-terminus in addition to the four N-glycosylation sites of CPY*, it could be shown that this CPY* molecule receives five carbohydrates. This CPY* derivative was found to be degraded as the authentic CPY* molecule. As glycosylation occurs 12–14

amino acids away from the translocon in the lumen of the ER [54], the CPY* molecule containing the fifth glycosylation at the very C-terminus must have entered the ER lumen completely prior to its degradation via the ubiquitin-proteasome system [59]. Thus, a targeting of ER luminal CPY* back to some translocation channel must occur, followed by retrotranslocation and, after polyubiquitination, degradation by the proteasome (Fig. 1).

3

Carbohydrate Trimming: A Tool of the Endoplasmic Reticulum Quality Control of Glycoproteins

The lumen of the ER contains a highly active protein quality control machinery [18, 17]. For N-glycosylated proteins, trimming of the Glc₃Man₉GlcNAc₂ oligosaccharides is an important process of the quality control mechanism. The N-glycans are matured by stepwise removal of the three terminal glucose residues via α -glucosidases I and II. Finally, α 1,2-mannosidase I releases a mannose residue from the inner branch of the N-glycan, giving rise to Man₈GlcNAc₂ [28, 17]. It is thought that this process sets the timer for folding, and when unsuccessful, for degradation of the N-glycosylated secretory protein. When the mannose-9 residue is cleaved off the Man₉GlcNAc₂ structure, the protein is retained in the ER and delivered for elimination. Mutant analysis using CPY* as malformed protein has shown that indeed α -glucosidase I, found in the *der* screen as Der7p [37], and glucosidase II [40] as well as α 1,2-mannosidase I [46] are required for degradation of the CPY* protein, thus substantiating the proposed quality control mechanism of N-glycosylated proteins (Fig. 1). In a systematic study of the four N-glycans of CPY* in ERQD, substantial differences in their signaling function were found: of the four N-linked carbohydrate chains at positions Asn13, Asn87, Asn168, and Asn368, only the presence of the Asn368-linked glycan is necessary and sufficient for efficient degradation of CPY* [49a, 65a]. Recent studies have shown that degradation of CPY* also requires the lectin-like protein Htm1p/Mnl1p [39, 53]. It is proposed that Htm1p/Mnl1p recognizes the trimmed Man₈GlcNAc₂ structure of CPY* and other N-glycosylated malformed proteins, retaining them in the ER and finally delivering them to proteasomal degradation.

One of the *DER* genes required for CPY* degradation is *DER5*, encoding the Ca²⁺/Mn²⁺ pump Pmr1p: a *DER5* deletion considerably slows down CPY* degradation [16]. Pmr1p is localized to the Golgi but is also required for maintaining normal Ca²⁺ levels in the ER [66]. A second Ca²⁺ pump required for undisturbed degradation of CPY* was found to be Cod1p, localized in the ER membrane [73]. Both Pmr1p and Cod1p are required for Ca²⁺ homeostasis of

the ER [13, 73]. In contrast to CPY*, degradation of nonglycosylated ER substrates was not affected by the absence of both Ca^{2+} pumps; Ca^{2+} homeostasis in the ER must be linked to the quality control mechanism of N-glycosylated proteins [73]. α 1,2-Mannosidase I is a Ca^{2+} -dependent enzyme. Indeed, analysis of N-linked oligosaccharides in $\Delta pmr1\Delta cod1$ double mutants uncovered a large portion of protein-linked sugar being of the untrimmed $\text{Man}_9\text{GlcNAc}_2$ type [73]. This indicates that one function of ER-calcium in the degradation of CPY* rests in its ability to render α 1,2-mannosidase I active and thus allow proper ER quality control of N-glycosylated proteins.

4

Soluble Proteins Require Endoplasmic Reticulum-Lumenal Chaperones for Degradation

The search for luminal chaperones of the ER required for degradation of CPY* uncovered Kar2p (BiP in mammalian cells) [57]. Besides CPY*, the degradation of other soluble mutated ER proteins such as mutated pro- α -factor is also dependent on Kar2p [10] and the interacting DnaJ-like proteins Jem1p and Scj1p [55]. It is proposed that the Kar2p machinery prevents soluble malformed proteins from aggregation in the ER lumen, thus facilitating their retrograde export into the cytosol for degradation [55, 69] (Fig. 1). The targeting mechanism responsible for retrotranslocation of CPY* and all other malformed proteins to some retrotranslocation channel for export into the cytosol is not yet known.

5

Sec61p, Part of the Retrotranslocation Channel?

Mutant studies using CPY* as ERQD substrate indicate that the translocon protein Sec61p, which forms the import channel for secretory proteins into the ER, is also part of the export channel delivering CPY* to the cytosolic ubiquitin-proteasome machinery [57]. Also genetic interaction studies point to Sec61p as being part of the CPY* export channel [58]. These studies also indicate a composition of the export channel, which is different from the import channel (Fig. 1). Studies on a variety of other ERQD proteins point to the participation of Sec61p in the retrotranslocation process as well [76, 56, 3; 14]. The appearance of glycosylated CPY* in the cytoplasm [31, 41] points to a diameter of the retrotranslocation channel, which must be larger than the pore size of the import channel, which has only to accommodate a single

polypeptide chain. However, recent experimentation seems to indicate that the use of Sec61p in retrotranslocation may not be a unique principle [38, 82]. Final proof for the nature of a retrotranslocation channel will only come from isolation of such a channel in the process of protein export.

6

Endoplasmic Reticulum-Associated Protein Degradation: Ubiquitin, the Proteasome and Other Helpers

Retrotranslocation of CPY*—and the majority of proteins destined for endoplasmic reticulum-associated protein degradation (ERAD) [9, 61, 48, 49, 34]—is followed by polyubiquitination and proteasomal degradation. The biochemical search for the number of ubiquitin-conjugating enzymes (Ubcs) that had overlapping specificity with the *DER2* gene product Ubc7p in the polyubiquitination process of CPY* uncovered two additional members: Ubc6p [31], an integral ER membrane localized E2, the active site facing the cytoplasm [74], and Ubc1p, a cytosolic E2 [21]. Of the three ubiquitin-conjugating enzymes, Ubc7p has the strongest influence on the degradation of CPY*. Interestingly, the soluble cytoplasmically localized Ubc7p gains its activity for polyubiquitinating CPY* and other proteins only after binding to an ER membrane protein, Cue1p [4].

On the basis of the mutant screen using CPY* for the discovery of components of the ubiquitination and degradation machinery, Der3p/Hrd1p was uncovered [6]. It is a polytopic ER membrane protein containing six transmembrane domains with its N- and C-terminus facing the cytoplasm [15]. It contains a RING-H2 finger domain in its C-terminus [7] and turned out to be the ubiquitin-protein ligase (E3) polyubiquitinating CPY* [15] as well as other proteins destined for ER degradation via the proteasome [1]. In a search for mutants defective in regulated degradation of the ER membrane-located enzyme hydroxymethylglutaryl (HMG) CoA reductase, an additional membrane protein, named Hrd3p, was found, which interacts with Der3p/Hrd1p [25, 23, 15] and which was also found to be required for the degradation of CPY* [58]. It is thought to be a device signaling the presence of misfolded proteins in the ER to the cytoplasmically located ubiquitination and degradation machinery [23, 15]. Der3p/Hrd1p represents an ubiquitin-protein ligase, which is responsible for polyubiquitination of a certain set of misfolded ER proteins, among them CPY* (Fig. 1). A second, polytopic ER membrane-located ubiquitin-protein ligase is Doa10p [68], which is responsible for polyubiquitination of a different set of ER proteins destined for proteasomal degradation [75, 24, 38].

As the 26S proteasome contains six different ATPases in the base of its 19S cap (regulator) complex, it was thought that pulling of the ubiquitinated mal-folded proteins away from the ER and delivering them to the 20S proteasome core complex for degradation was carried out by these ATPase subunits. Using CPY* as substrate, four research groups at nearly the same time uncovered that transport of the malformed substrate from the ER to the proteasome requires the AAA-ATPase Cdc48 (p97 in mammals) and two additional complexing proteins, Ufd1p and Npl4p [2, 81, 41, 62]. Mutations in the components of this trimeric complex lead to a failure of delivery of CPY* into the cytosol, leaving ubiquitinated CPY* bound to the ER [41]. The requirement of the trimeric Cdc48 complex for ERAD has been shown for all tested proteins so far, which require polyubiquitination for degradation [2, 81, 8; 62, 24, 38] (Fig. 1).

7

Modular CPY*-Based Membrane Substrates Broaden the Picture

The use of CPY* and different other substrates during time to study ERQD had given insight into a basic machinery, which was equally necessary for elimination of all substrates tested. For glycosylated proteins, this machinery constitutes of the glucosidases I and II, α -mannosidase I, and Htm1p/Mnl1p (EDEME) for quality control assessment. On the cytoplasmic side of the ER membrane, the ubiquitin-conjugating enzyme Ubc7p, depending on the substrate, either Der3/Hrd1p or Doa10p as ubiquitin-protein ligases, the trimeric AAA-ATPase complex Cdc48 (p97)-Ufd1-Npl4p, and the 26S proteasome, were shown to be required for the degradation of all substrates tested so far (Fig. 1). There was a discrepancy in the use of ER-lumenal and cytoplasmic chaperones for degradation of malformed soluble and membrane proteins [57, 60, 10, 30, 55, 83] and in the use of an ER membrane protein, Der1p, required for CPY* ERAD but not membrane protein ERAD [45, 60, 30, 36]. The construction of three topologically different modular substrates all containing CPY* as the malformed protein in the lumen of the ER shed more light on the question of which ERQD system components are generally used for recognition and degradation of topologically different proteins containing the same malformed recognition domain. The set of molecules used consisted of CPY*, a CPY* molecule linked to a transmembrane domain (CT*), and transmembrane-linked CPY* containing a strongly folding cytoplasmic domain, the green-fluorescent protein (GFP) (CTG*) [69] (Fig. 2). As previously found for several completely different substrate species containing different malformed domains, the basic machinery required for degradation of the three topologically different CPY* substrates (CPY*, CT*, CTG*)

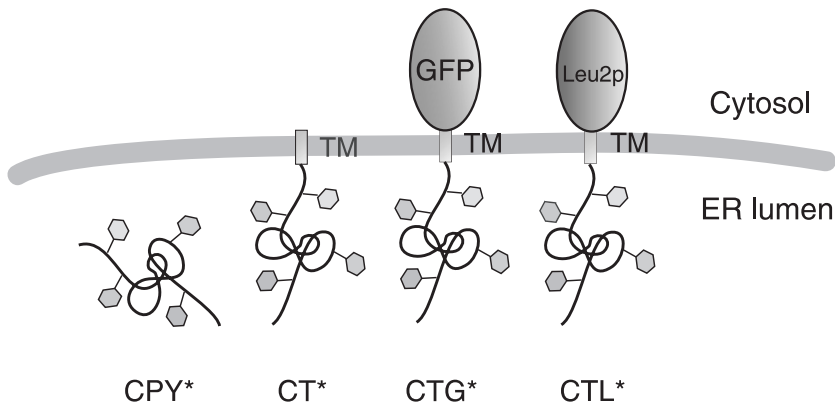


Fig. 2 Schematic presentation of CPY* and its variants

consisted of the ubiquitin conjugating enzyme Ubc7p, the ubiquitin-protein ligase Der3/Hrd1p, the trimeric Cdc48-Ufd1-Npl4p complex, and the 26S proteasome [69]. The ER luminal Hsp70 chaperone Kar2p was only required for degradation of soluble CPY* and not for degradation of any of the membrane-bound CPY* species [69]. When testing the requirement of cytoplasmic chaperones for degradation of the topologically different CPY* species, it turned out that degradation of CTG* containing the tightly folding GFP domain was crucially dependent on the presence of the cytoplasmic Hsp70 chaperones of the Ssa family. Neither degradation of soluble CPY* nor of membrane bound CPY* without cytoplasmic domain required Ssa1p. The most likely explanation for this finding is that unfolding of the tightly folding GFP domain of CTG* is dependent on these cytoplasmic Hsp70 chaperones to allow ER removal and/or proteasomal degradation of this protein species. A less but clearly observable effect on degradation of CTG* was also found for the DnaJ orthologs Hlj1p, Cwc23p, and Jid1p as well as for the Hsp100 member of chaperones, Hsp104 [69]. The integral ER membrane protein Der1p was only required for degradation of soluble CPY* and not for degradation of any other membrane-bound CPY* species [69]. The finding of an interaction of a mammalian ortholog of Der1p, Derlin-1, with membrane-bound MHC class I molecules in association with the viral US11 protein for degradation upon cytomegalovirus infection of cells is therefore somewhat surprising [82]. One may assume that recruitment of the Der1 ortholog to a membrane protein is virus specific, and may even be specific for the US11 protein: the second cytomegalovirus protein, US2, which also targets MHC class I heavy chains for degradation [76], does not seem to work in conjunction with Derlin-1 [50].

For the moment, one may conclude that a machinery exists for ERAD of all misfolded proteins, which indeed consists of the ubiquitin-conjugating enzyme Ubc7p, the ubiquitin protein ligase Der3/Hrd1p and/or Doa10p, the trimeric Cdc48-Ufd1-Npl4p complex, and the 26S proteasome (Fig. 1). The chaperone requirement for degradation may vary, whereby only degradation of soluble proteins seems to require ER luminal Kar2p/BiP. Also, the use of additional ubiquitin conjugating enzymes as Ubc1p or Ubc6p may vary from substrate to substrate. Obviously the use of the ubiquitin-protein ligases Der3/Hrd1p and Doa10p is also substrate-dependent but not necessarily exclusive for one or the other malformed ER protein [24, 38]. Using CPY* as ERQD substrate, the requirement of a cytoplasmic peptide: N-glycanase (PNGase), for undisturbed proteasomal degradation of the malformed protein was shown [67]. It is thought that PNGase cleaves off the carbohydrate residues from N-glycosylated ERQD substrates to allow their efficient elimination by the proteasome [33, 35].

8

Yeast Genomics Discovers New Players

The elucidation of the yeast genome with its roughly 6,000 open reading frames had been followed by the construction of yeast deletion libraries, which consist of approximately 5,000 individual clones each deleted for a single nonessential gene (i.e., EUROSCARF deletion library, Frankfurt, Germany). Such a deletion library can be used for genome-wide screens of mutants defective in a certain cellular process. As cells can tolerate a defect in ERQD as long as the unfolded protein response (UPR) is intact (CPY* and other misfolded proteins induce the UPR) [45, 21, 71], the existence of such a deletion library made it possible to search for new components of ERQD by performing a genomic screen. As cell growth is one of the most sensitive indicators of alterations in cell physiology due to mutations, a growth test on the basis of degradation of CPY* was developed. The fact that CTG*, carrying CPY* in the ER lumen and GFP in the cytosol behaved as an excellent ERQD substrate, the cytosolic GFP domain was exchanged for the Leu2 protein (3-isopropylmalate-dehydrogenase), leading to the new substrate CTL* [11, 52] (Fig. 2). Cells carrying a *LEU2* deletion can only grow when CTL* is present, the cytoplasmic Leu2p domain of the fusion protein being able to complement the leucine auxotrophy of cells: Strains with *leu2* auxotrophy but otherwise wild type for ERQD are unable to grow in media lacking leucine. Only when ERQD is defective is CTL* stabilized and able to complement the *LEU2* deficiency and thus allow growth [11, 52]. Screening of the nearly 5,000 individual deletion mutants of the EUROSCARF

deletion library expressing CTL* resulted in finding most of the known ERQD components [52]. In addition, however, the search resulted in the discovery of a variety of new mutants defective in ERQD. Among these were mutants deleted in genes of the ubiquitin-like (UBL) and ubiquitin-associated (UBA) domain proteins Rad23p and Dsk2p as well as the mannose-6-phosphate receptor-like domain protein Yos9p [11, 52]. Interestingly Yos9p is only required for degradation of N-glycosylated proteins, not for degradation of nonglycosylated ERQD substrates. Its localization in the ER may lead to the proposal that Yos9p is a lectin-like protein, which acts in concert with or sequentially with Htm1p/Mnl1p in the quality control process of N-glycosylated proteins [11]. Dsk2p and Rad23p were shown to be able to bind ubiquitinated proteins via their UBA domain and dock onto the 19S cap subunit of the proteasome via their UBL domain [12, 22, 63, 19, 26, 43]. In contrast to mutants defective in the trimeric Cdc48 complex in which polyubiquitinated CPY* remains bound to the ER, in $\Delta dsk2\Delta rad23$ double mutants, a substantial amount of polyubiquitinated CPY* is found in the soluble fraction of cells. At the moment, the most plausible explanation for Dsk2p and Rad23p action in ERAD is their function as receptors shuttling polyubiquitinated ERQD substrates from the trimeric Cdc48 complex to the proteasome. By keeping malformed ER luminal and especially malformed ER membrane proteins with their exposed hydrophobic domains complexed to soluble partners in the cytoplasm until their degradation in the proteasome, the cell avoids aggregation and precipitation of these malformed proteins in the cytoplasm, preventing diseased cell states. CPY* and yeast genetics have indeed paved the way to our understanding of the basic mechanisms of protein quality control of the ER and ER-associated degradation (Fig. 1). They will certainly continue.

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