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Cytoplasmic peptide:*N*-glycanase cleaves *N*-glycans on a carboxypeptidase Y mutant during ERAD in *Saccharomyces cerevisiae*

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ABSTRACT

Background: Endoplasmic reticulum (ER)-associated degradation (ERAD) is a pathway by which misfolded or improperly assembled proteins in the ER are directed to degradation. The cytoplasmic peptide:*N*-glycanase (PNGase) is a deglycosylating enzyme that cleaves *N*-glycans from misfolded glycoproteins during the ERAD process. The mutant form of yeast carboxypeptidase Y (CPY*) is an ERAD model substrate that has been extensively studied in yeast. While a delay in the degradation of CPY* in yeast cells lacking the cytoplasmic PNGase (Png1 in yeast) was evident, the *in vivo* action of PNGase on CPY* has not been detected.
Methods: We constructed new ERAD substrates derived from CPY*, bearing epitope tags at both N- and C-termini and examined the degradation intermediates observed in yeast cells with compromised proteasome activity.
Results: The occurrence of the PNGase-mediated deglycosylation of intact CPY* and its degradation intermediates was evident. A major endoproteolytic reaction on CPY* appears to occur between amino acid 400 and 404.
Conclusions: The findings reported herein clearly indicate that PNGase indeed releases *N*-glycans from CPY* during the ERAD process *in vivo*.
General Significance: This report implies that the PNGase-mediated deglycosylation during the ERAD process may occur more abundantly than currently envisaged.

1. Introduction

In eukaryotes, proteins that pass through the secretory pathway are synthesized in the endoplasmic reticulum (ER). The folding status of these proteins is closely monitored by a sophisticated protein surveillance system, which is referred to as ER quality control (ERQC) [1]. In this system, correctly folded/assembled proteins are delivered to their respective destinations, while aberrant proteins that fail to form a functional folding/complex are degraded by a specific degradation process called ER-associated degradation (ERAD) [1,2]. In ERAD, misfolded proteins are recognized by chaperone proteins in the ER, and are transported back to the cytosol. In most cases, these aberrant proteins are degraded in a ubiquitin–proteasome-dependent manner [3].

The cytoplasmic peptide:*N*-glycanase (PNGase; Png1 in yeast) is a deglycosylating enzyme that is highly conserved throughout eukaryotes [4]. It has been shown that this deglycosylation enzyme is involved in ERAD and cleaves *N*-glycans from misfolded glycoproteins during their proteasomal degradation [5–8]. In mammalian cells, some ERAD substrates are presumed to be deglycosylated by the PNGase during the degradation process [7,9,10]. In budding yeast *Saccharomyces cerevisiae*, a ricin toxin A-chain non-toxic mutant (RTAΔ) and its transmembrane protein derivative, RTL (RTAΔ-transmembrane-Leu2), have been identified as Png1-dependent ERAD substrates [6–8,11].

The vacuolar protease carboxypeptidase Y (CPY) (Gly255Arg) mutant (CPY*) is the most extensively studied ERAD substrate in yeast [12]. CPY contains four *N*-glycosylation sites, which are all occupied [13] and, as a result, CPY* is frequently used as a model glycoprotein substrate in studies of the molecular mechanism of glycoprotein ERAD [14]. In a previous study, a deglycosylated form of the intact CPY* was not detected in wild-type cells, whereas the degradation of CPY* was found to be delayed in *png1*Δ cells [5]. In a subsequent study, it was also reported that Png1 is also required for the efficient degradation of CTL* (a Leu2-bearing, transmembrane version of CPY*) [8]. These data clearly indicate that Png1 is involved in the degradation of CPY*/CTL*, while experimental evidence for the occurrence of "deglycosylated CPY*" has not been reported. The objective of this study was to attempt to sort out this seemingly puzzling situation. Our working hypothesis was that the proteolysis of CPY* during ERAD may hinder the detection of the deglycosylation of CPY*. In order to facilitate the detection of putative proteolytic products, a new CPY*-derived ERAD model substrate (hereafter referred to as V5-CPY*-HA) was constructed, in which both N- and C-terminal epitope tags were added. Using this new construct, a putative deglycosylated CPY* N-terminal fragment was observed in a proteasomal mutant, *cim5-1*. Moreover, the

Abbreviations: PNGase, peptide:*N*-glycanase; ERAD, ER-associated degradation; CPY, carboxypeptidase Y; Endo H, endoglycosidase H

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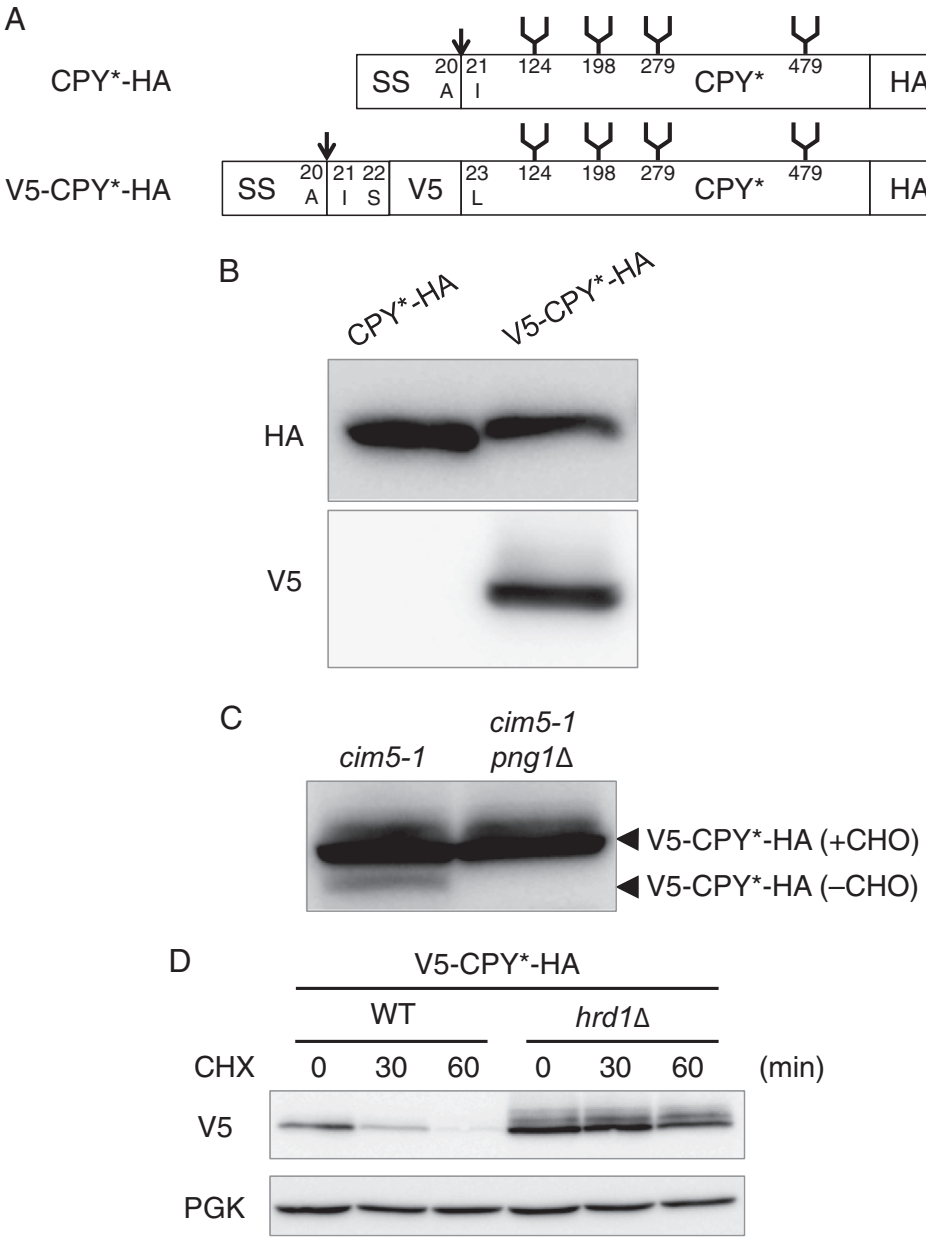


Fig. 1. Detection of intact de-*N*-glycosylated CPY* in *cim5-1* strain. (A) Schematic representations of CPY*-HA and V5-CPY*-HA. SS, signal sequence; V5, V5 epitope, which is located between Ser22 and Leu23; HA, HA epitope, which is located in C-terminus. Arrows indicate signal sequence cleavage site. Numbers (124, 198, 279 and 479) represent the potential *N*-glycosylation sites. (B) Western blotting analysis of CPY*-HA and V5-CPY*-HA. CPY*-HA or V5-CPY*-HA was expressed in wild-type cells. Extracts from these cells were resolved by SDS-PAGE and CPY*-HA and V5-CPY*-HA were visualized by immunoblotting using anti-HA and V5 antibodies, respectively. (C) Western blotting analysis of V5-CPY*-HA using proteasome mutant. V5-CPY*-HA was expressed in *cim5-1* and *cim5-1 png1*Δ cells. Extracts from these cells were resolved by SDS-PAGE and V5-CPY*-HA was visualized by immunoblotting using anti-V5 antibody. (D) Cycloheximide (CHX) decay assay for V5-CPY*-HA. V5-CPY*-HA was expressed in WT and *hrd1*Δ cells. CHX was added at $t = 0$ min. Samples were collected at the indicated times and were subjected to SDS-PAGE, followed by immunoblotting using anti-V5 antibody. The immunoblot was also probed with anti-PGK antibody as a loading control.

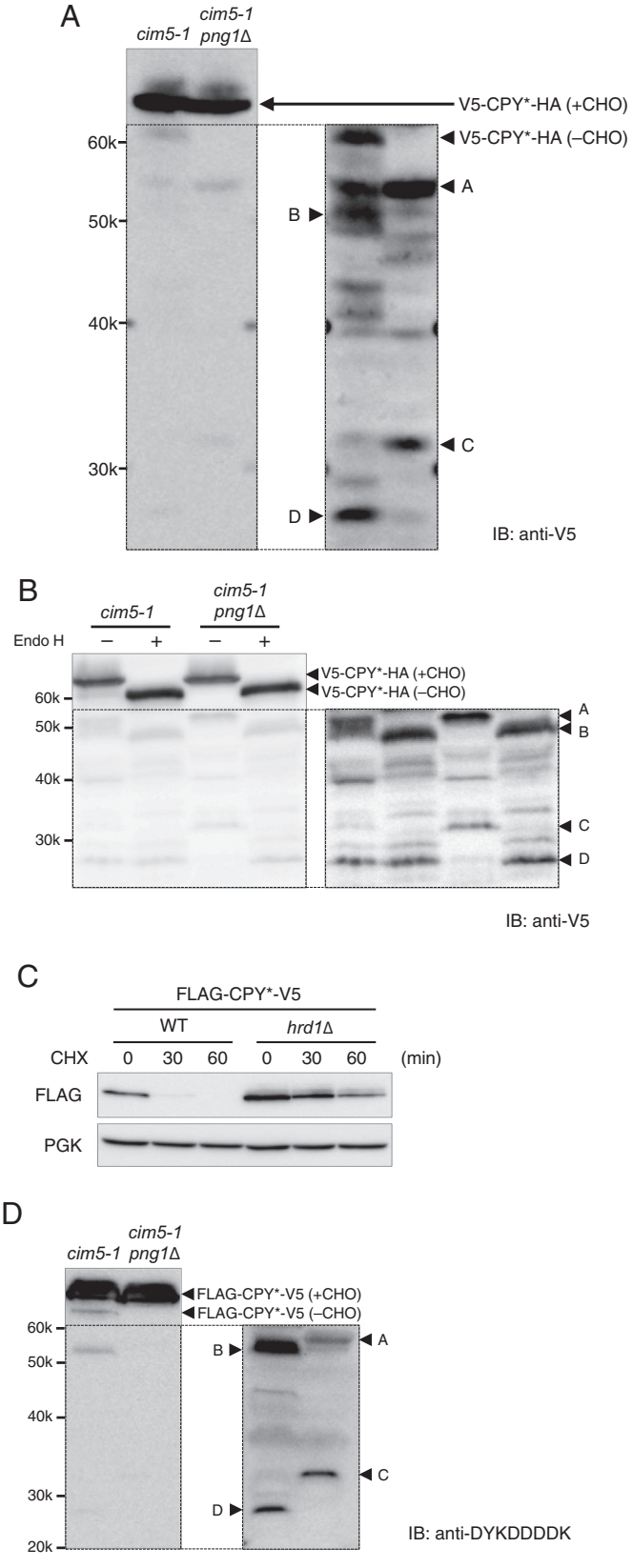
occurrence of the deglycosylated form of the intact protein was also detected in *cim5-1* cells, indicating that it would be possible to observe the PNGase-deglycosylated intact protein under conditions in which proteasomal degradation is compromised. Interestingly, this fragment was an *N*-glycosylated protein in a *cim5-1 png1*Δ double mutant, clearly indicating that the cytoplasmic PNGase is, indeed, involved in the deglycosylation of CPY* *in vivo*.

2. Materials and methods

2.1. Yeast strains and media

We used the following yeast strains: BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), AHY201 (*hrd1*Δ::hphNT1 BY4741) (this study), AHY202 (*png1*Δ::hphNT1 BY4741) [15], AHY208 (*cim5-1* BY4741) (this study), AHY210 (*cim5-1 png1*Δ::hphNT1 BY4741) (this study), AHY262 (*pdr5*Δ::natNT2 BY4741) (this study), AHY263 (*pdr5*Δ::natNT2 *png1*Δ::hphNT1 BY4741) (this study), AHY292 (*pdr5*Δ::natNT2 *hrd1*Δ::hphNT1 BY4741) (this study), AHY293 (*pdr5*Δ::natNT2 *cue1*Δ::hphNT1 BY4741) (this study) and AHY294 (*pdr5*Δ::natNT2 *ubc7*Δ::hphNT1 BY4741) (this study). The transfer of *cim5-1* gene to BY4741 from *cim5-1* (CMY765) was performed essentially as described previously [16]. In this preparation, the ORF region (1–1404) of *cim5-1* was amplified from the *cim5-1* (CMY765) [17] genome. The PCR product was cloned into pCR2.1-TOPO (Invitrogen). The promoter and ORF region of *URA3* was amplified from pRS316. The PCR product was cloned into 3′ side of *cim5-1* of pCR2.1-TOPO-*cim5-1* plasmid. A partial *cim5-1* (1001–1525) was amplified from *cim5-1*

genome. The PCR product was cloned into the 3′ side of URA3 of pCR2.1-TOPO-*cim5-1-URA3* plasmid. The *cim5-1*(1–1404)-*URA3*-*cim5-1*(1001–1525) was amplified and the resulting PCR product was transformed into BY4741 wild-type cells. Transformed cells were selected by –Ura media and the correct integration of the construct into the genome was confirmed by PCR. The transformed cells were then spread on 5-Fluoroorotic acid (5-FOA)-containing media to eliminate the *URA3* gene. The absence of the *URA3* gene and occurrence of *cim5-1* allele in AHY208 cells, isolated on 5-FOA containing media, were confirmed by PCR and DNA sequencing. Standard yeast media and genetic techniques



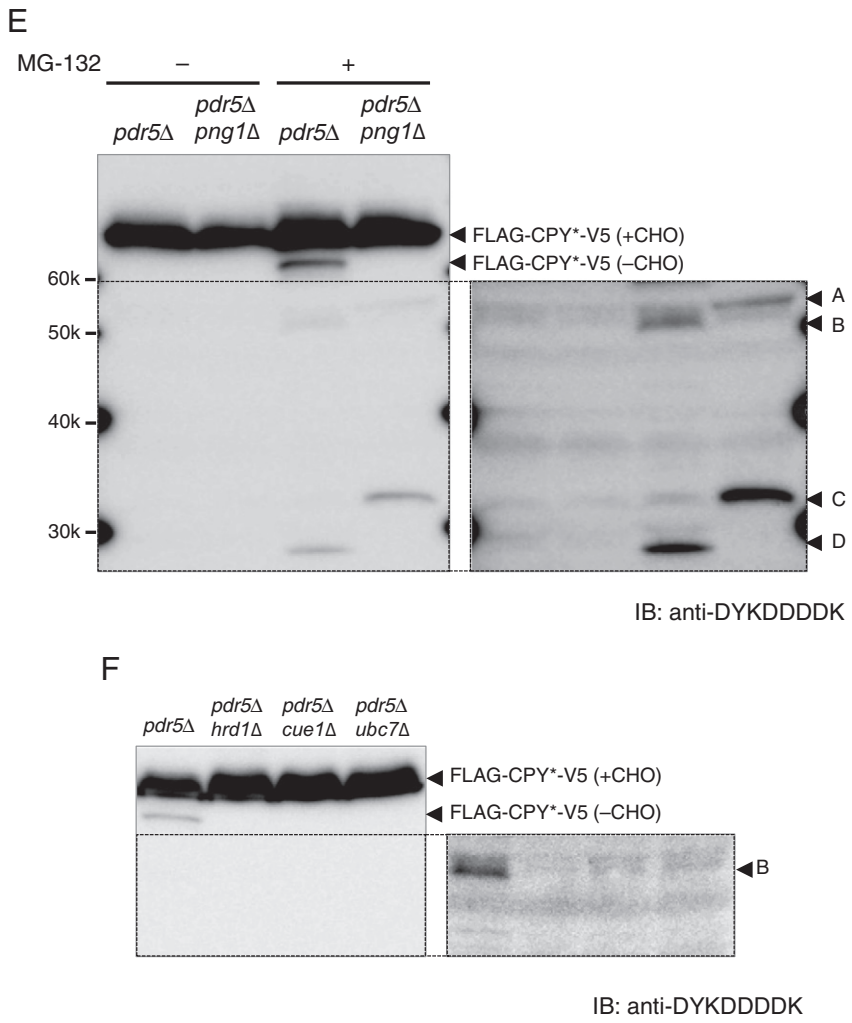


Fig. 2. Proteolytic intermediates of CPY* were deglycosylated by Png1 in a proteasome mutant. (A) Western blotting analysis of V5-CPY*-HA in proteasome mutants. V5-CPY*-HA was expressed in *cim5-1* and *cim5-1 png1*Δ cells. Extracts from these cells were resolved by SDS-PAGE using large gel (20 cm × 26 cm) and V5-CPY*-HA was visualized by immunoblotting, using anti-V5 antibody. Right panel: long exposure of the region below 60 k to detect degradation intermediates. (B) Endo H treatment of V5-CPY*-HA degradation intermediates in *cim5-1 png1*Δ cells. V5-CPY*-HA was expressed in *cim5-1* and *cim5-1 png1*Δ cells. Cell extracts were mock-treated (−) or digested (+) with Endo H, and resolved by SDS-PAGE. V5-CPY*-HA was visualized by immunoblotting using anti-V5 antibody. Right panel: long exposure of the region below 60 k. (C) CHX decay assay for FLAG-CPY*-V5. FLAG-CPY*-V5 was expressed in WT and *hrd1*Δ cells. CHX was added at $t = 0$ min. Samples were collected at the indicated times. Samples were subjected to SDS-PAGE, followed by immunoblotting using anti-DYKDDDDK antibody. The immunoblot was also probed with anti-PGK antibody as a loading control. (D) Western blotting analysis of FLAG-CPY*-V5 in proteasome mutants. FLAG-CPY*-V5 was expressed in *cim5-1* and *cim5-1 png1*Δ cells. Extracts from these cells were resolved by SDS-PAGE and FLAG-CPY*-V5 was visualized by immunoblotting using anti-DYKDDDDK (FLAG-epitope) antibody. Right panel: long exposure of the region below 60 k. (E) Western blotting analysis of FLAG-CPY*-V5 using MG-132 in *pdr5*Δ cells. FLAG-CPY*-V5 was expressed in *pdr5*Δ and *pdr5*Δ *png1*Δ cells. Cells were incubated with DMSO (−) and MG-132 (+) for 1 h. Extracts from these cells were resolved by SDS-PAGE and FLAG-CPY*-V5 was visualized by immunoblotting using anti-DYKDDDDK antibody. Right panel: long exposure of the region below 60 k. (F) Western blotting analysis of FLAG-CPY*-V5 using MG-132 in *pdr5*Δ *hrd1*Δ, *pdr5*Δ *cue1*Δ and *pdr5*Δ *ubc7*Δ cells. FLAG-CPY*-V5 was expressed in *pdr5*Δ, *pdr5*Δ *hrd1*Δ, *pdr5*Δ *cue1*Δ and *pdr5*Δ *ubc7*Δ cells. These cells were treated with 50 μM MG-132 for 1 h before extraction. Extracts from these cells were resolved by SDS-PAGE and FLAG-CPY*-V5 was visualized by immunoblotting using anti-DYKDDDDK antibody. Right panel: long exposure of the region below 60 k.

were used [18,19]. Where indicated, cells bearing *pdr5*Δ mutation were incubated with 50 or 100 μM MG-132 (3175-V; Peptide Institute, Inc. Japan) to inhibit proteasomal degradation.

2.2. Plasmid construction

The pRS315-CPY*-HA was generously provided by Davis Ng (NUS Singapore) [20]. pRS315-V5-CPY*-HA and pRS315-FLAG-CPY*-V5 were generated by site-directed mutagenesis (Quikchange, Stratagene, CA, USA) according to the manufacturer's protocols. Truncation mutants of pRS315-V5-CPY* and alanine mutants of pRS315-V5-CPY*-HA were also generated by site-directed mutagenesis. Detailed information on primer sequences is available upon request. The DNA sequences of the constructs were confirmed using BigDye ver. 3.1 and an ABI DNA sequencer (3730xl).

2.3. Preparation of yeast cell extracts and western blotting

Preparation of yeast cell extracts and western blotting were carried out as previously described [8]. Antibodies were used at the following dilutions: 1:10,000 for anti-PGK (22C5; Invitrogen, CA, USA), 1:20,000 for anti-V5 (46-0705; Invitrogen), 1:10,000 for anti-DYKDDDDK (018-22381; Wako Chemical Co. Japan), and 1:1000 for anti-HA (sc-7392; Santa Cruz Biotechnology, Inc., CA, USA).

2.4. Cycloheximide (CHX) decay assay

Strains harboring the V5-CPY*-HA expression plasmid were grown and CHX (01810; Sigma) was added to the cultures (final concentration, 4 μg/ml). The cultures were collected at the indicated times, and the cells were subjected to western blotting analysis.

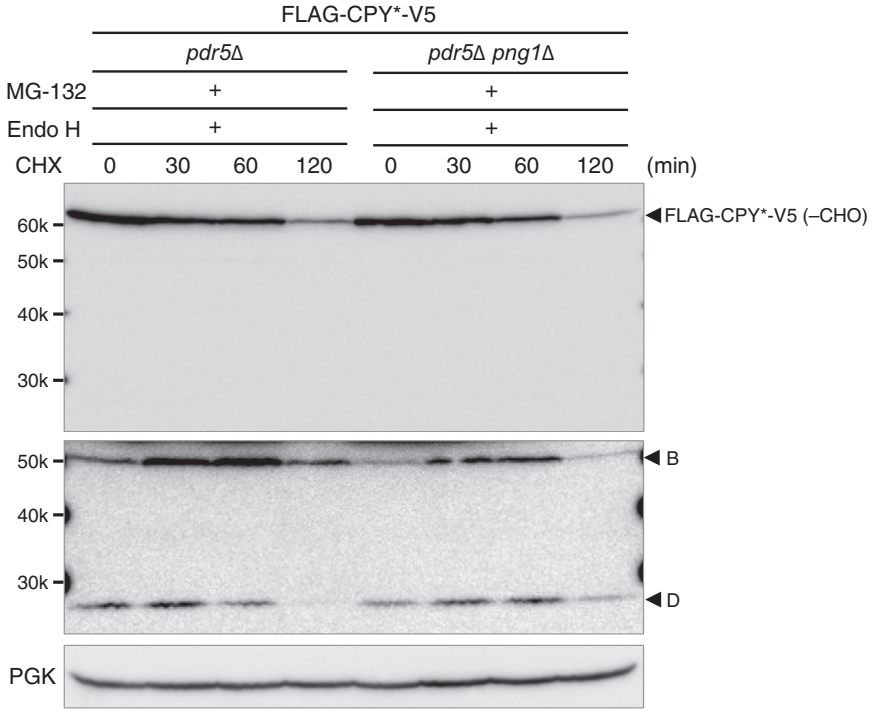


Fig. 3. CHX decay assay for FLAG-CPY*-V5 using *pdr5*Δ and *pdr5*Δ *png1*Δ cells. FLAG-CPY*-V5 was expressed in *pdr5*Δ and *pdr5*Δ *png1*Δ cells. These cells were treated with 100 μM MG-132 for 1 h before CHX decay assay. CHX was added at $t = 0$ min. Samples were collected at the indicated times and treated with Endo H. Samples were subjected to SDS-PAGE, followed by immunoblotting using anti-DYKDDDDK antibody. Middle panel: long exposure of the region below 60 k. The immunoblot was also probed with anti-PGK antibody as a loading control.

3. Results

3.1. The deglycosylated form of CPY* was detected in a proteasome mutant

It has been reported that the cytoplasmic PNGase cleaves an *N*-glycan from RTAΔ, a non-toxic mutant of the ricin toxin A-chain, which is degraded in a PNGase-dependent manner [6,7]. In sharp contrast, the analogous deglycosylated form of CPY* was not detected in wild-type yeast cells, although its degradation was delayed in *png1*Δ cells [5]. In addition, the degradation of the CPY*-derived transmembrane ERAD substrate, CTL*, was also compromised in *png1*Δ cells [8], indicating that Png1 is required for their efficient degradation. The issue of whether Png1 is actually involved in the deglycosylation of CPY*, however, remains unclarified.

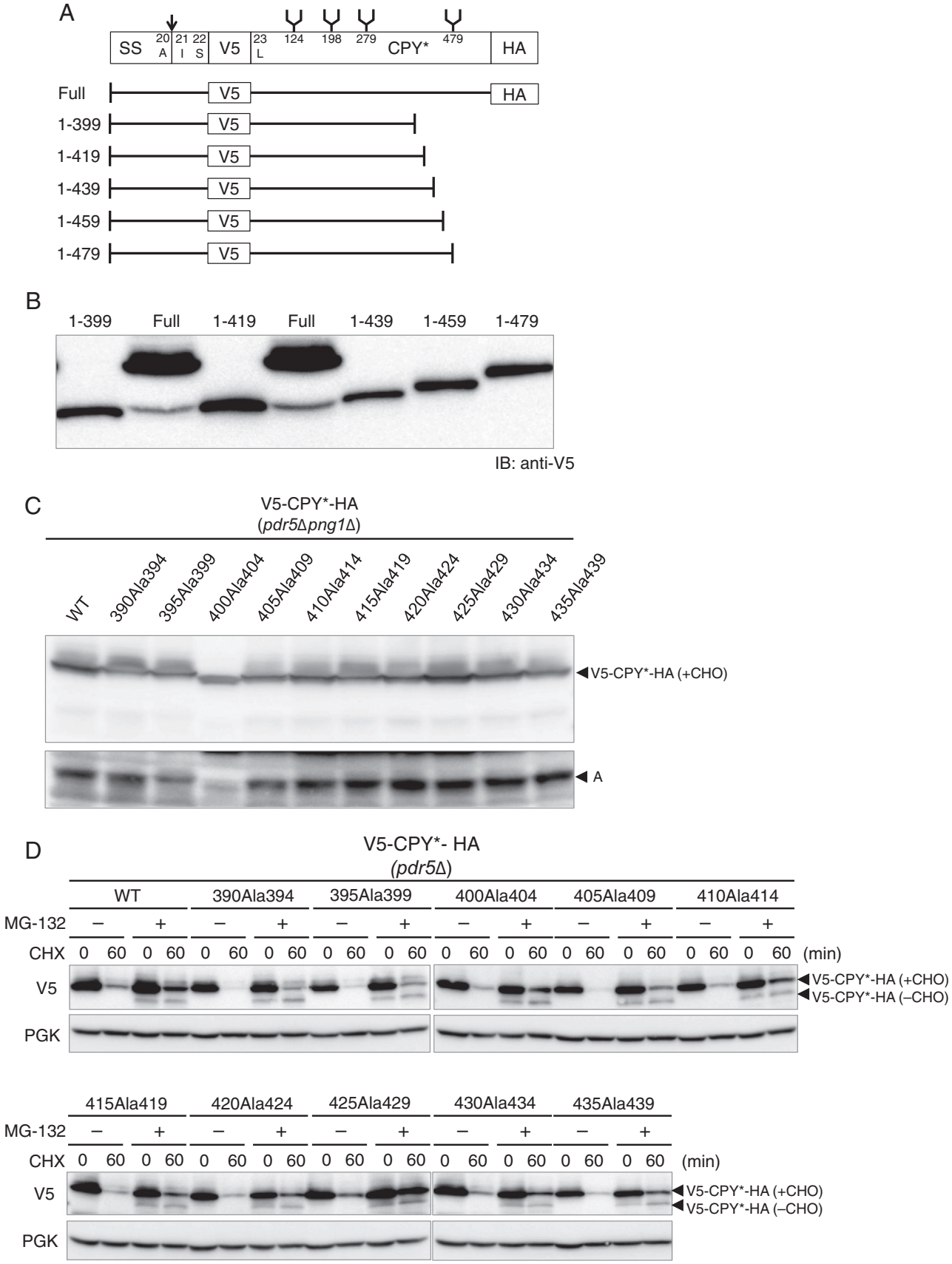
We rationalized that the lack of detection of deglycosylated CPY* may be due to its proteolysis, which would hinder the detection of a deglycosylated product with an intact protein. To validate this hypothesis, we constructed a new CPY*-derived protein with tags at both the N- (V5) and C-termini (HA), in order to detect intermediates produced during proteolytic degradation. An N-terminal V5 tag was inserted between Ire21 and Ser22 of the CPY*-HA expression plasmid to avoid any disruption in signal sequence recognition/cleavage (Fig. 1A). We confirmed the specific detection of CPY*-HA and V5-CPY*-HA by means of anti-tag antibodies. As shown in Fig. 1B, CPY*-HA was detected by means of an anti-HA antibody but not an anti-V5 antibody. On the other hand, V5-CPY*-HA was detected by both antibodies. Interestingly, a faint band equivalent to a deglycosylated V5-CPY*-HA was observed in *cim5-1*, but not in *cim5-1 png1*Δ cells (Fig. 1C). This tentative deglycosylated band did not show a further shift upon treatment with Endo H, thus confirming that the band is indeed a deglycosylated form of the protein and not a product by proteolytic cleavage (data not shown). These results suggest that Png1 acts on the *N*-glycan on the new CPY* construct. In order to confirm that the V5-CPY*-HA was also degraded by ERAD, cycloheximide (CHX)-decay assay was carried out using the new CPY* construct in wild-type and *hrd1*Δ cells. As shown in Fig. 1D, the protein was significantly stabilized in *hrd1*Δ cells, in which ERAD of CPY* was impaired [14]. This result clearly indicates that the new construct remains an ERAD substrate in yeast.

3.2. Proteolytic intermediate of CPY* was deglycosylated in a proteasome mutant

After confirming the occurrence of intact deglycosylated V5-CPY*-HA, a more detailed analysis was carried out by western blotting in order to gain additional insights into the deglycosylation of CPY*. As shown in Fig. 2A, a number of degradation intermediates were produced from V5-CPY*-HA in both *cim5-1* and *cim5-1 png1*Δ cells (Fig. 2A; fragments A and C in *cim5-1 png1*Δ cells and fragments B and D in *cim5-1* cells). The samples were treated with Endo H, in order to confirm the *N*-glycosylation status of these fragments. As shown in Fig. 2B, the N-terminal fragments of V5-CPY*-HA (fragments A and C) were found to be *N*-glycosylated in *cim5-1 png1*Δ cells. After digestion with Endo H, the bands corresponding to fragments A and C were shifted to bands equivalent to fragments B and D from *cim5-1* cells

Fig. 4. Analysis of the main proteolytic cleavage site for CPY*.Schematic representation of truncation mutants of V5-CPY*. V5-CPY*-HA was shown in Fig. 1A. (B) Western blotting analysis of truncation mutants of CPY*. V5-CPY*-HA was expressed in *cim5-1 png1*Δ cells and truncation mutants of V5-CPY* were expressed in *png1*Δ cells. Extracts from these cells were resolved by SDS-PAGE and V5-CPY*-HA and truncation mutants of V5-CPY* were visualized by immunoblotting using anti-V5 antibody. (C) Western blotting analysis of alanine mutants of CPY*. Wild-type and alanine mutant form of V5-CPY*-HA were expressed in *pdr5*Δ *png1*Δ cells. These cells were treated with 50 μM MG-132 for 1 h before extraction. Extracts from these cells were resolved by SDS-PAGE and V5-CPY*-HA was visualized by immunoblotting using anti-V5 antibody. Lower panel shows long exposure. (D) CHX decay assay for alanine mutants of V5-CPY*-HA using *pdr5*Δ cells. Wild-type and alanine mutants of V5-CPY*-HA were expressed in *pdr5*Δ cells. These cells were treated with 50 μM MG-132 for 1 h before CHX decay assay. CHX was added at $t = 0$ min. Samples were collected at the indicated times. Samples were subjected to SDS-PAGE, followed by immunoblotting using anti-V5 antibody. The immunoblot was also probed with anti-PGK antibody as a loading control.

(Fig. 2B). Moreover, bands corresponding to the deglycosylated form of intact CPY* were again observed in *cim5-1* cells (Fig. 2A, long exposure, V5-CPY*-HA (-CHO)). These results suggest that Png1 indeed cleaves *N*-glycan(s) from intact CPY* or N-terminal degradation intermediates of V5-CPY*-HA. These results clearly indicate that Png1 removes *N*-glycans from CPY* *in vivo*.



We also constructed another CPY*-derived protein bearing tags at both the N- (FLAG) and C-termini (V5), FLAG-CPY*-V5, to examine the effect of epitope-tagging on the proteolytic cleavage of CPY*. The CHX-decay assay confirmed that FLAG-CPY*-V5 was significantly stabilized in *hrd1*Δ cells, suggesting that this protein also serves as an ERAD substrate in yeast (Fig. 2C). The patterns of the N-terminal fragments produced from V5-CPY*-HA and FLAG-CPY*-V5 were quite similar (compare Fig. 2D and A). Furthermore, in *cim5-1 png1*Δ cells, these N-terminal fragments of FLAG-CPY*-V5 were also found to be glycosylated as evidenced by an Endo H-treatment (data not shown). In addition to a proteasome mutant, we used MG-132, a proteasome inhibitor, to determine whether the fragmentation of CPY* is a strain-specific event or not. Pdr5 is an ATP-binding cassette transporter that is required for multi drug resistance, and *pdr5*Δ cells have been used to render yeast cells sensitive to various drug treatments [21]. Accordingly, we generated *pdr5*Δ or *pdr5*Δ *png1*Δ cells in order to treat cells with MG-132 to determine whether proteasomal activity was inhibited. As shown in Fig. 2E, similar N-terminal fragments of FLAG-CPY*-V5 (fragments A, B, C and D) were detected in these cells only when MG-132 was added to the media.

While we observed CPY* fragments in cells with compromised proteasome activity, it is still possible that it may be generated during cell lysis. To eliminate such a possibility, we tested several ERAD-defective cells, i.e. *pdr5*Δ *hrd1*Δ, *pdr5*Δ *cue1*Δ and *pdr5*Δ *ubc7*Δ strains, for the occurrence of CPY* fragments. As shown in Fig. 2F, no CPY* fragment was observed in these cells, even in cells that had been treated with MG-132 (Fig. 2F). This result indicates that the functional ERAD process is prerequisite for the fragmentation of CPY*, and therefore the proteolytic processing is clearly not an artifact produced during the cell lysis. Collectively, these results indicate that the proteolytic cleavage of CPY* is not a tag or a strain-specific event.

In sharp contrast to the case of N-terminal fragments, the C-terminal fragments of V5-CPY*-HA or FLAG-CPY*-V5 were not detected by anti-HA and V5 antibodies, respectively (data not shown).

3.3. The stability of the N-terminal fragments of CPY is not significantly affected by the deletion of PNG1 under condition of proteasome inhibition*

After confirming the presence of a deglycosylated fragment of CPY* under the conditions of proteasome inhibition, we next examined the issue of whether Png1 is critical for the efficient degradation of the N-terminal fragment of CPY*. Accordingly, we performed a CHX-decay assay to examine the degradation of the N-terminal fragment of CPY*. To precisely quantitate the amount of protein bands, the samples were subjected to an Endo H-treatment. As shown in Fig. 3, no significant delay in degradation was observed for intact FLAG-CPY*-V5 as well as other fragments in the presence of MG-132, while a slight delay in the formation and disappearance of fragment D was observed in *png1*Δ cells. These results indicate that, under conditions of proteasomal inhibition, no significant change in degradation efficiency occurred in *png1*Δ cells.

3.4. The proteolytic cleavage of CPY is predicted to occur at an amino acid residue around 400–404*

Since N-terminal CPY* fragments were detected as degradation intermediates, we next attempted to identify the cleavage site of CPY*. To this end, we constructed various truncation mutants of CPY* (V5-CPY* (1–399, 1–419, 1–439, 1–459, and 1–479)). Asparagine 479 is the fourth *N*-glycosylation site of CPY*. The CPY* mutants were expressed in *png1*Δ cells and V5-CPY*-HA (full-length) was expressed in *cim5-1 png1*Δ cells, respectively. As shown in Fig. 4B, the migration of the N-terminal fragment of V5-CPY*-HA was similar to that from the V5-CPY*(1-419) mutant, suggesting that the cleavage site of CPY* is most likely located in the vicinity of the 420th amino acid residue. To identify the cleavage site of CPY* more precisely, we constructed alanine mutants of CPY*, where the 10 indicated amino acid residues were all converted into Ala residues, i.e., V5-CPY*-HA (390Ala394), V5-CPY*-HA(395Ala399), V5-CPY*-HA(400Ala404), V5-CPY*-HA(405Ala409), V5-CPY*-HA(410Ala414), V5-CPY*-HA (415Ala419), V5-CPY*-HA(420Ala424), V5-CPY*-HA(425Ala429), V5-CPY*-HA(430Ala434), and V5-CPY*-HA(435Ala439). As shown in Fig. 4C, among the CPY* mutants tested, only V5-CPY*-HA (400Ala404) produced significantly decreased amounts of the intermediate, suggesting that the cleavage site of CPY* is likely located between the 400th and 404th amino acid residues. It was not possible, however, to identify the specific amino acid residue responsible for the cleavage, as the point mutants tested (V5-CPY*-HA(G400A, A401G, E402A, V403A or D404A) did not exhibit a strong defect in the formation of degradation intermediates equivalent to V5-CPY*-HA (400Ala404) (data not shown). We also performed CHX-decay assays using alanine mutants of V5-CPY*-HA. All of the alanine mutants showed delayed degradation and occurrence of the deglycosylated form was evident when cells were treated with MG-132 (Fig. 4D), indicating that all mutants remained as ERAD substrates and that the retrotranslocation step was not drastically affected by these alanine mutations. Collectively, it can safely be concluded that the cleavage site of CPY* is located between the third (residue 279) and fourth (residue 479) *N*-glycosylation site.

4. Discussion

While it has been suggested that Png1 can enhance the efficiency of CPY* degradation [5,8], its direct involvement as a deglycosylating enzyme on the degradation of CPY* has not been experimentally demonstrated. In the present study, we investigated the issue of whether Png1 indeed deglycosylates CPY* during the ERAD process. Using some newly established CPY* constructs, it was found that even a deglycosylated form of the intact CPY* could be detected. In addition, the N-terminal proteolytic degradation fragments were also shown to be deglycosylated by Png1. These results clearly indicate that Png1 cleaves *N*-glycans from CPY* *in vivo*. Both the glycosylated and deglycosylated CPY* fragments at around 50 k were detected in *cim5-1* cells, suggesting that a portion of the CPY* was proteolytically processed prior to its deglycosylation. Therefore, we assume that this uncharacterized proteolysis makes detecting the deglycosylated intact CPY* a difficult task. While a further delay in the degradation of CPY* was not observed as the result of the deletion of the *PNG1* gene under the condition of proteasome inhibition, the possibility that some of the degradation of proteolytic products would be affected by *png1*Δ mutants in other strain backgrounds cannot be excluded. In any event, our results clearly indicate that Png1 is involved in the deglycosylation of CPY* *in vivo*.

In terms of the uncharacterized proteolytic processing of CPY*, the findings indicate that the main cleavage likely occurs at an amino acid residue in the region between 400 and 404, while it is still possible that the lack of cleavage for V5-CPY*-HA(400Ala404) may simply be a result of an altered recognition by a putative endoprotease due to the alanine mutation. In either case, since this cleavage still occurs in MG-132 treated cells or proteasomal mutants, it is likely that an as yet uncharacterized endopeptidase activity that is independent of proteasomes is involved in this proteolysis. At this moment, however, the possibility that this reaction is actually proteosome-catalyzed cannot be completely excluded, since such activity might have been somehow unaffected under the experimental conditions employed in this study. In any event, it seems reasonable to assume that endoproteolytic reactions for ERAD substrates may be more common than currently thought. Further studies will be needed to clarify the nature of the endoproteolytic activity.

It is noteworthy that we were not able to detect the equivalent C-terminal fragment of CPY*, suggesting that the C-terminal fragment is rapidly catabolized after the proteolysis. Interestingly, the findings indicate that the CPY* cleavage site (fragment A in Fig. 2A; around 50 k) is

most likely located between amino acid residues of 400 and 404, therefore suggesting that the cleavage site of CPY* is between the third and the fourth *N*-glycosylation site. Therefore, assuming that the all of the *N*-glycosylation sites are occupied, at least one *N*-glycan would be expected to be attached to the C-terminal fragment of CPY*. How the glycosylated C-terminal CPY* fragment is degraded so efficiently in the absence of Png1 remains to be determined.

Cytoplasmic PNGase is highly conserved throughout eukaryotes. While it was found that mutants of the cytoplasmic PNGase and its orthologue in budding yeast [5] or plants [22] exhibited no significant phenotypes, it has been suggested that this protein has important functions in higher eukaryotes [23,24]. Most recently, human patients with mutations in the *NGLY1* gene, a mammalian orthologue of the cytoplasmic PNGase [25] have been identified [26,27]. These patients exhibited multiple symptoms including developmental delay, movement disorders, hypotonia, the absence of tears and abnormal liver functions, clearly suggesting the functional importance of this protein in human biology. In order to provide mechanistic insights into these phenotypic consequences, characterization of enzyme dependent, as well as independent functions [28] will be imperative. In the present study, we were able to show that cytoplasmic PNGase can act on CPY*, and the previous failure to detect deglycosylated intermediates may be due to endoproteolytic reactions involving ERAD substrates. To unveil the overall processes on CPY* degradation, the characterization of degradation intermediates as well as the factor(s) involved in the degradation reactions will be imperative. While the proteins involved in the recognition/retrotranslocation of misfolded glycoproteins have been well defined [29], proteins involved in the ERAD process after retrotranslocation are relatively poorly defined, and will require further clarification.

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