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Membrane targeting and binding of the 74-kDa form of mouse L-histidine decarboxylase via its carboxyl-terminal sequence

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Abstract The role of the C-terminal region of the 74-kDa form of L-histidine decarboxylase (HDC) in the targeting to the endoplasmic reticulum (ER) was investigated in COS-7 cells. The deletion of a 10-kDa segment (residues 578–662) of the C-terminal end of HDC, especially a 20 amino acid sequence (residues 588–607), abrogated the targeting to the ER. The C-terminal 10-kDa portion is sufficient to target the green fluorescent protein (GFP) to the ER. The 74-kDa form of HDC synthesized in an in vitro translation system post-translationally associated with the heterogeneous canine microsomal membranes. These results suggest that the C-terminal 10-kDa portion of HDC contains a signal necessary for HDC to be targeted to the ER membrane.

Key words: Histidine decarboxylase; C-terminal peptide; Targeting; Endoplasmic reticulum; COS-7 cell

1. Introduction

The transfer of newly synthesized peptides to the endoplasmic reticulum (ER) is generally controlled by the signal recognition particle (SRP)/SRP receptor system [1–3], but an alternative pathway controlled by the insertion sequence at the C-terminal hydrophobic domain is recently reported [4–7], although its mechanism is still unclear.

L-Histidine decarboxylase (HDC; EC 4.1.1.22) is a dimer consisting of two identical 53–55-kDa subunits [8–11], while the size of cDNA-deduced HDC is around 74 kDa [12–14]. We previously showed that the 74-kDa form expressed in Sf9 cells was recovered in the $100\,000\times g$ precipitate fraction, while the mutant 54-kDa form that lacks the C-terminal region of the 74-kDa form preferentially was recovered in the supernatant fraction [15,16]. Furthermore, in rat basophilic leukemia cell line, RBL-2H3 cells, the endogenous 74-kDa form of HDC was translated in the cytosol and then translocated to the ER, where it was post-translationally processed to the 53-kDa form [17]. In this cell line, the 74-kDa form in the cytosol was demonstrated to be rapidly degraded by ATP/ubiquitin-dependent proteosome system [18]. These results indicate that the 74-kDa HDC, which is translated in the cytosol, is targeted to the ER membranes. However, the 74-kDa HDC has no N-terminal signal sequence and no hydrophobic domain at the C-terminal region [14]. Therefore it is assumed that this protein is inserted into the ER membrane without the participation of the SRP/SRP receptor or the C-terminal anchor domain-dependent system. The present paper describes the sequence at the C-terminal region responsible for the targeting of the 74-kDa HDC to the ER membrane.

2. Materials and methods

2.1. Materials

Anti-GST-fusion HDC antiserum was prepared as described previously [19]. COS-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum.

2.2. Construction of expression vector

The partial amino acid sequence of the carboxyl-terminal 10-kDa region deduced from the nucleotide sequence of HDC cDNA [14] is shown in Fig. 1. Plasmids, pcDNA3/HDC5, pcDNA3/HDC6 and pcDNA3/HDC7 were generated by subcloning *Eco*RI fragments of vHDC5, vHDC6 and pVLHDC1 [15] into *Eco*RI site of pcDNA3 (Invitrogen, Carlsbad, CA, USA), respectively. The 3′-deletion mutants of HDC cDNA were generated using PCR amplification with Vent DNA polymerase (New England Biolabs, Beverly, MA, USA) and the appropriate primers containing an *Eco*RI site and a stop codon. All the plasmids were constructed by inserting *Eco*RI fragments of the PCR products into the *Eco*RI site of pcDNA3. The constructed vectors were named based on the numbering of the amino acid residues mutated to the stop codon (Fig. 1). To produce the green fluorescent protein (GFP)-fusion vectors, various C-terminal segments of HDC were amplified by PCR using oligonucleotides complementary to the 5′- and 3′-ends of the desired fragment. The oligonucleotides had *Eco*RI sites at their 5′-end to facilitate cloning into the expression vector, pEGFP-C1 (Clontech Laboratories, Palo Alto, CA, USA).

2.3. Transient expression

COS-7 cells were transfected with the various constructs using a CellPhect Transfection Kit (Pharmacia Biotech) according to the manufacturer's instructions. The cells were harvested 48 h after transfection and assayed for expression of HDC activity and HDC protein.

2.4. Subcellular fractionation

Cells transfected with the various vectors were homogenized in 10 mM potassium phosphate, pH 6.8, containing 1.5 mM $MgCl_2$,

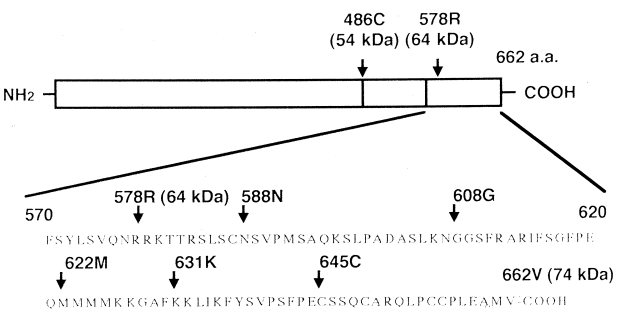

Fig. 1. Carboxyl-terminal sequence of HDC. The carboxyl-terminal region of HDC is represented with one-letter codes for amino acids. The mutated amino acid residues in the mutant HDCs numbered relative to the stop codon are indicated by arrows.

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Abbreviations: HDC, L-histidine decarboxylase; ER, endoplasmic reticulum; PDI, protein disulfide isomerase; GFP, green fluorescent protein

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10 mM KCl, 0.2 mM DTT, 0.01 mM pyridoxal 5′-phosphate, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 10 μg/ml E-64, 0.1 mM benzamidine and 10 μg/ml aprotinin with a Teflon-glass homogenizer. The homogenate was centrifuged at 100 000×*g* for 1 h at 4°C to obtain the supernatant fraction and the particulate fraction.

2.5. Immunoblot analysis

Immunoblot analysis and immunofluorescence observation were performed according to the procedures described in a previous paper [17].

2.6. In vitro transcription and translation

Both pBSHDC5 and pBSHDC7 were linealized with *Sal*I, and in vitro RNA was transcribed using T7 RNA polymerase (Toyobo, Osaka, Japan). In vitro translations were performed using a rabbit reticulocyte lysate (Amersham, Buckinghamshire, England) according to the manufacturer's instructions. The translations were performed in a volume of 20 μl, with 10 μl of rabbit reticulocyte lysate, 1 μl of 2.5 M potassium acetate, 0.5 μl of 25 mM magnesium acetate, 2 μl of 10× translation mixture, 1 μl of RNA (1 μg/μl), 20 μCi of [^{35}S]methionine (1000 Ci/mmol) (Du Pont-New England Nuclear, Boston, MA, USA) and ribonuclease-free H_2O at 30°C for 80 min. Commercially prepared canine microsomal membranes (Promega, Madison, WI, USA) were added at the beginning (0.5 μl) or after (4 μl) the translation by adding 1 μl of 1 mM cycloheximide.

2.7. Assay for the post-translational targeting of HDC protein

In vitro translation mixtures (21 μl) prepared as described above were incubated at 30°C for 90 min with or without 4 μl of canine pancreatic microsomal membranes. To separate the microsomal membranes, 10 μl of rat microsomal membranes (5 mg protein/ml) and 35 μl of 0.2 M Na_2CO_3 were added to the reaction mixtures (25 μl) which were incubated at 4°C for 30 min. The mixtures were added to 125 μl of 2.5 M sucrose, and then 400 μl of 1.25 M sucrose containing 0.1 M Na_2CO_3 and 400 μl of 0.25 M sucrose containing 0.1 M Na_2CO_3 were carefully layered over them sequentially. The samples were spun at 120 000×*g* for 1.5 h at 4°C, and the resulting upper (containing the microsomal membranes) and lower layers were collected, respectively. Both layers were treated with 10% trichloroacetic acid, and the resulting precipitates were collected, solubilized and subjected to SDS-PAGE. Radio-labeled HDC protein was analyzed with a Fujix BAS 2000 Bio-Imaging Analyzer.

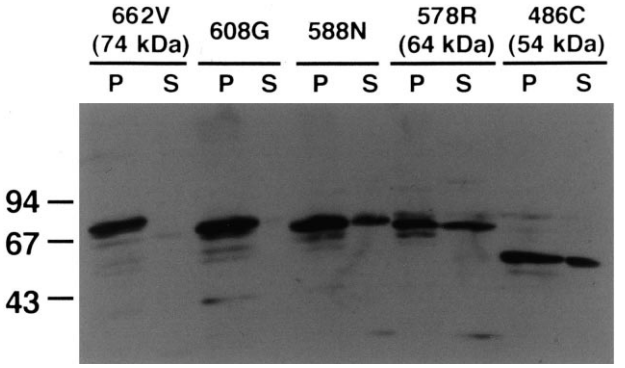

Fig. 2. Immunoblot analyses of the various carboxyl-terminal deletion mutants expressed in COS-7 cells. Cells transfected with the vectors indicated were fractionated into particulate (P) and supernatant (S) fractions as described in Section 2. Each fraction was subjected to SDS-PAGE and immunoblot analysis with anti-GST-fusion HDC antibody. The molecular weight marker is indicated on the left.

3. Results

3.1. Requirement of the C-terminal portion of the 74-kDa HDC for the targeting to the particulate fraction

To investigate which portion of the peptide at the C-terminal end contains the information necessary to target the HDC to the particulate fraction, we analyzed the distribution of the various C-terminal deletion mutants in the particulate and the supernatant fractions of COS-7 cells by immunoblot analysis using anti-GST-fusion HDC antibody. As a result, the 74-kDa form of HDC (662V HDC) and the mutant 608G HDC were entirely recovered in the particulate fraction. Similar results were obtained in the mutants 622M HDC, 631K HDC and 645C HDC (data not shown). In contrast, the mutants 588N HDC, 578R HDC and 486C HDC (corresponding

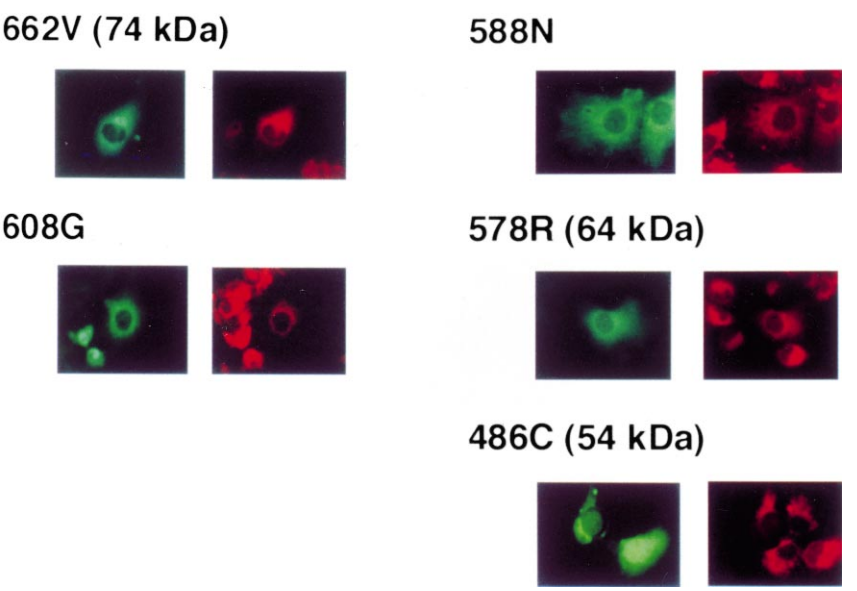

Fig. 3. Immunofluorescence study of HDC and its carboxyl-terminal deletion mutants. An immunofluorescence study was performed with anti-GST-fusion HDC antibody (1:500) (left) and anti-PDI antiserum (1:500) (right) and stained with secondary antibody, anti-rabbit IgG antibody conjugated with FITC (1:200) and anti-mouse IgG antibody conjugated with rhodamine (1:150). Cells were transfected with the vectors indicated. After fixation with 100 mM sodium phosphate, pH 7.4, containing 2% paraformaldehyde, 0.1% glutaraldehyde and 3% sucrose, cells were permeabilized with 0.1% of Triton X-100. Thereafter cells were stained.

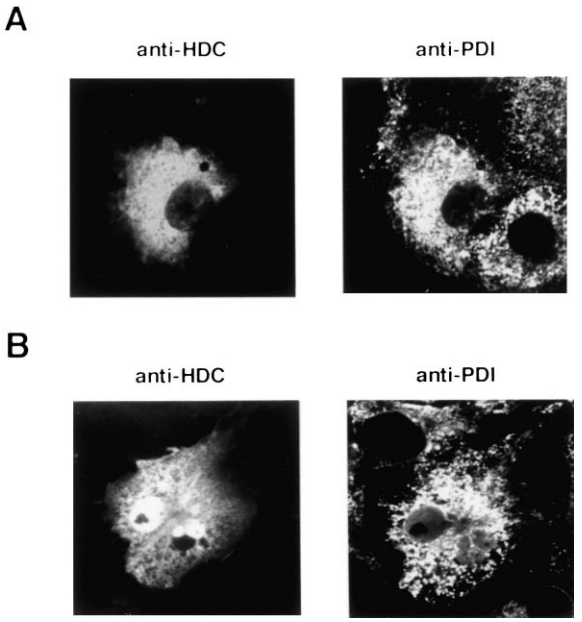

Fig. 4. Confocal microscopic observation of HDC and PDI. Cells expressing the 74-kDa HDC (A) or 54-kDa HDC (B) were double-stained with anti-GST fusion HDC antiserum (left) and with anti-PDI antibody (right) as described in the legend to Fig. 3. The fluorescent images were observed with a confocal microscope.

to the 64-kDa and 54-kDa form of HDC, respectively), were recovered in the supernatant fraction (Fig. 2).

3.2. *Immunofluorescent study of the intracellular localization of the mutant HDCs*

In the cells expressing 662V HDC or the mutant 608G HDC, a reticular pattern of immunofluorescent signal was observed, which is similar to that of protein disulfide isomerase (PDI), a marker protein of the ER (Fig. 3). The signals of mutants 622M, 631K or 645C HDCs were the same as those of 662V HDC (data not shown). However, the reticular pattern of signal was obscure in the cells expressing mutant 588N, 578R and 486C HDCs, where the signals were widespread in the cytoplasm.

The co-localization of the 74-kDa form of HDC and PDI was confirmed by confocal microscopy (Fig. 4A), but the staining of the 54-kDa form was distinct from that of PDI (Fig. 4B).

3.3. *Intracellular localization of the GFP-fusion C-terminal segment of HDC*

To ensure the potential of the C-terminal sequence of HDC in the translocation of a heterologous cytosolic protein to the ER, we constructed vectors that code GFP-fusion carboxyl-terminal segment of HDC and expressed them in COS-7 cells. Under the conditions in which approximately 50% of GFP

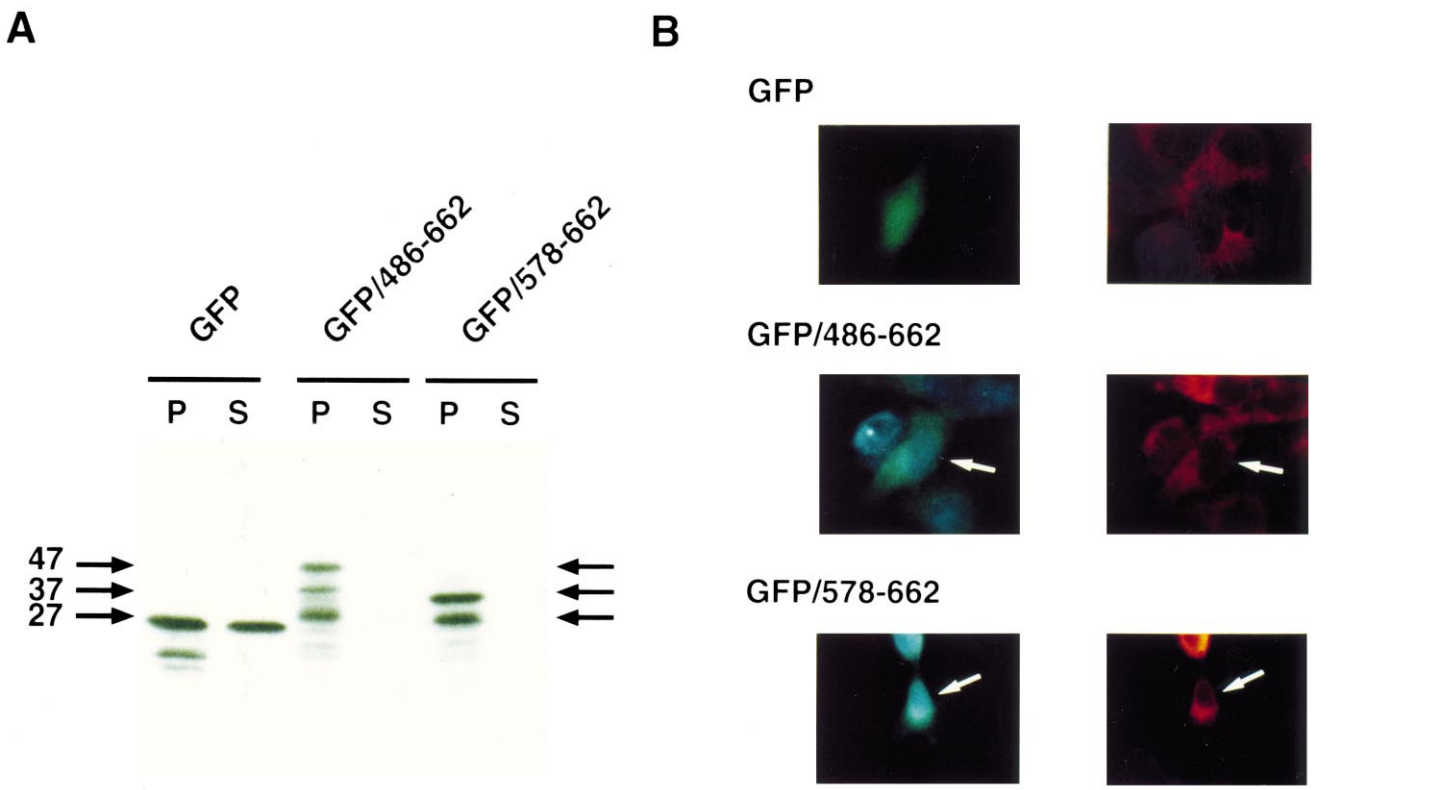

Fig. 5. Translocation of GFP by the carboxyl-terminal sequence of HDC. A: Cells transfected with pEGEP-C1, pEGFP-C1/HDC486–662, or pEGFP-C1/HDC578–662 were fractionated into particulate and supernatant fractions as described in Section 2. Each fraction was subjected to SDS-PAGE and immunoblot analysis with anti-GFP antibody (1:1000). The molecular weights of GFP, GFP-fusion HDC578–662 and GFP-fusion HDC486–662 are indicated as 27, 37, and 47 kDa with the arrows, respectively. B: GFP and GFP-fusion HDC proteins were detected by their intrinsic fluorescence (left). The counter-staining of PDI was performed (right) as described in the legend to Fig. 3. The transfection and fixation of the cells was performed as described in the legend to Fig. 3. The cells expressing GFP-fusion HDC were indicated by white arrows.

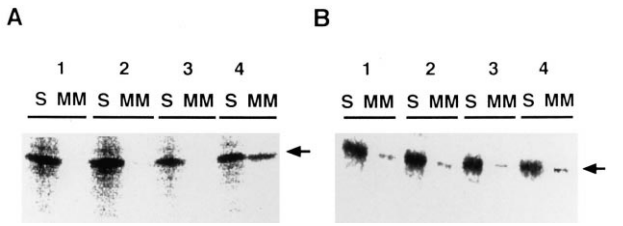

Fig. 6. Post-translational targeting of HDC. [^{35}S]Methionine-labeled 74-kDa (A) and 54-kDa (B) HDC were synthesized, respectively, using an in vitro translation system with rabbit reticulocyte lysates. The microsomal membrane fraction (MM) and supernatant fraction (S) were isolated by the alkaline flotation method described in Section 2. To perform the co-flotation, rat microsomal membranes were added to the translation mixtures (lanes 2, 3 and 4). Control was performed without microsomal membranes (lane 1). The canine pancreatic microsomal membranes were added to the translation mixtures during the translation reaction (lane 3), or after the addition of cycloheximide and incubated for 90 min at 30°C (lane 4).

protein was recovered in the supernatant fraction of COS-7 cells, neither GFP-fusion C-terminal 10-kDa nor 20-kDa segments of HDC were recovered in the supernatant fraction (Fig. 5A). Compared with the whole cell fluorescent pattern of GFP, the signals of both GFP-fusion C-terminal segments demonstrated the reticular pattern corresponding to that of PDI (Fig. 5B). These results indicate that the C-terminal region of HDC has a potential to translocate a heterologous cytosolic protein to the ER.

3.4. Post-translational targeting of the 74-kDa form HDC

To clarify whether the targeting of HDC is a post-translational or co-translational phenomenon, we examined the association of nascent [^{35}S]methionine-labeled 74-kDa HDC with canine pancreatic microsomal membranes exogenously added to the in vitro translation assay mixture (Fig. 6A). As a result, [^{35}S]methionine-labeled 74-kDa HDC was associated with the canine microsomal membranes post-translationally (lane 4) but not co-translationally (lane 3). On the other hand, the [^{35}S]methionine-labeled 54-kDa HDC did not associate with the canine microsomal membranes under any conditions examined (Fig. 6B).

4. Discussion

The observations reported here provide, for the first time, information on the mechanism of the intracellular trafficking of mouse HDC and indicate that the 74-kDa form of HDC is targeted to the ER via its C-terminal 10-kDa portion, especially a 20 amino acid segment (residues 588N–607G).

There are several different mechanisms by which proteins are targeted. One, which has been well characterized, is the pathway depending on the SRP/SRP receptor [20,21]. Alternatively, proteins without N-terminal signal sequence are targeted by a C-terminal hydrophobic domain termed an insertion sequence [22]. Since HDC contains neither an N-terminal signal sequence nor a C-terminal hydrophobic domain [14], it is possible that the 20 amino acid segment (residues 588N–607G) is a novel ER-targeting signal. Further, this segment does not share any sequence homology with other proteins except for HDC. Since its amino acid sequence is highly conserved in HDCs from mouse, rat and human [12–14], it is possible that the segment is responsible for the HDC-specific membrane targeting.

In a previous paper, we showed that the membrane topology of the HDC in RBL-2H3 cells is similar to that of PDI [17]. In the present study, we found that the molecular species of HDC which coexisted with PDI is the 74-kDa form in COS-7 cells (Figs. 3 and 4), indicating that the 74-kDa form of HDC locates in the ER. On the other hand, the recombinant 54-kDa HDC was recovered in the cytosol of COS-7 cells (Figs. 2 and 4) as in the case of Sf9 cells [15,16]. Since the recombinant 54-kDa form lacks the C-terminal portion that contains the putative targeting signal, it should be recovered in the cytosol of the expressed cells. This result also indicates that the N-terminal 54-kDa region does not contain the putative targeting signal.

Based on the present observation that the targeting of the 74-kDa HDC is mediated by the 20 amino acid segment (residues 588N–607G), further examination is required to determine the role of this unique sequence in the association of HDC with the ER. However, we found that a GFP-fusion 20 amino acid segment in place of the C-terminal 10-kDa region (Fig. 6) was not targeted to the ER in COS-7 cells (data not shown). Subsequently we speculate that the association of the 20 amino acid segment with the ER requires other segments in the 10-kDa region at least in the case of GFP-fusion constructs. To confirm this hypothesis, proteoliposome experiments would be useful, but at present this is difficult because we have not succeeded in the purification of the 74-kDa HDC. It is also important to search for the targeting factors which play a role in the translocation of HDC from free polysomes to the ER. Very recently, we have shown that the [^{35}S]methionine-labeled 74-kDa HDC in RBL-2H3 cells is susceptible to rapid degradation by the ATP-ubiquitin dependent proteosome system [18]. Therefore, we hypothesize that the targeting plays an important role in the control of the cellular level of HDC consisting of two identical 53–55-kDa subunits, which is formed from the precursor 74-kDa form by post-translational processing in the lumen of the ER [17].

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