

# Catalytic cysteine residues of ER-60 protease

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**Abstract** ER-60 protease contains two CGHC motifs that appear to include an active site cysteine residue(s). Its proteolytic activity was lost with a double mutation of the C-terminal cysteines of the two motifs to alanine, but not with a single mutation of the C-terminal cysteine of either of the motifs to alanine. This suggests that these C-terminal cysteines independently constitute the catalytic active site. A mutation of both histidine residues in the two CGHC motifs to serine did not abolish the proteolytic activity, suggesting these histidine residues in the CGHC motifs do not constitute the catalytic dyad of ER-60 protease.

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**Key words:** Endoplasmic reticulum; Cysteine protease; Catalytic dyad; Site-directed mutagenesis

## 1. Introduction

ER-60 protease (ER-60), which is an endoplasmic reticulum (ER)-resident protein in animal cells, has been shown to be a multifunctional protein with proteolytic activity and disulfide bond-dependent folding activity toward polypeptides [1–3]. ER-60 has been shown to be a cysteine protease, which is inhibited by *p*-chloromercuribenzoate (*p*CMB), and cysteine protease inhibitors such as *N*-acetyl-leucyl-leucyl-norleucinal, *N*-acetyl-leucyl-leucyl-methioninal, E-64 and leupeptin [2]. ER-60 contains seven cysteine residues, four of which constitute two copies of the CGHC motif [4]. Another microsomal cysteine protease, ER-72 protease (ERp72), is also known to contain CGHC motifs [5,6]. However, ERp72 contains no cysteine residue other than the six constituting three CGHC motifs. This suggests that the active site cysteine residue(s) of ER-60 protease is located in the CGHC motifs. Previously, the N-terminal cysteine residues of the CGHC motifs were shown not to be responsible for the proteolytic activity by the replacement of these cysteine residues with alanine [7]. In addition, when the C-terminal cysteine residues in the two motifs were replaced by serine residues, the cysteine protease activity, which was inhibited by *p*CMB but not by diisopropyl fluorophosphate (DFP), changed to serine protease activity, which was inhibited by DFP but not by *p*CMB [7].

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**Abbreviations:** ER-60, ER-60 protease; ER, endoplasmic reticulum; *p*CMB, *p*-chloromercuribenzoate; DFP, diisopropyl fluorophosphate; PDI, protein disulfide isomerase; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio- $\beta$ -D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue R-250; bis-Tris, bis-(2-hydroxyethyl)iminotris (hydroxymethyl) methane

These results suggest that the C-terminal cysteine residue(s) of the CGHC motifs might constitute the active site(s) of ER-60 protease.

In this study, we demonstrate that the C-terminal cysteine residues in the CGHC motifs independently function as active site cysteine residues through site-directed mutagenesis of the recombinant human ER-60 proteins, in which the C-terminal cysteine residues in the two CGHC motifs were replaced with alanine.

## 2. Materials and methods

### 2.1. Materials

The Dye Termination Cycle Sequencing Kit was from Perkin Elmer, Applied Biosystems Division. Tli DNA polymerase was purchased from Promega Biotek. Bovine liver protein disulfide isomerase (PDI) was obtained from Takara Shuzo (Kyoto) and purified by hydroxyapatite column chromatography. The AF-heparin Toyopearl 650M resin and TSK gel G3000SW HPLC column were obtained from Tosoh (Tokyo). The HTP cartridge (hydroxyapatite) was obtained from Bio-Rad. All other chemicals were of reagent grade.

### 2.2. Construction and expression of plasmids

Plasmids for expression of the recombinant mutant ER-60s in *Escherichia coli* were constructed by ligating a nucleotide fragment, which was formed by polymerase chain reaction (PCR), into the *Nde*I and *Xba*I sites of pET-20b(+) (Novagen, WI), as described previously [7]. PCR was carried out using a template pET-HER60 [7], including an open reading frame for human ER-60 protease (other than the N-terminal signal sequence), and mutagenic primers. All mutants were sequenced by the fluorescence dideoxy chain termination method (Perkin Elmer, Applied Biosystems). The expression plasmids were transformed into AD494(DE3) or BL21(DE3) (Novagen), grown in 800 ml of LB broth in the presence of 500  $\mu$ g/ml carbenicillin at 37°C, and then induced with 0.4 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) at 30°C for 2 h. The proteins of *E. coli* carrying the expression plasmids were separated by SDS-polyacrylamide gel electrophoresis (PAGE) [8] and stained with Coomassie brilliant blue R-250 (CBB).

### 2.3. Purification of recombinant ER-60

*E. coli* cells were collected by centrifugation, disrupted by sonication in 40 ml of 20 mM HEPES buffer, pH 6.8, containing 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 mM KCl, and 10% glycerol (buffer A), and then centrifuged at 5000 $\times$ *g* for 10 min at 4°C. Purification was then carried out according to the procedure described previously [1,7]. Briefly, the supernatant was subjected to AF-heparin Toyopearl 650M column chromatography. From the effluent obtained with 400 mM KCl in buffer A, the recombinant mutant ER-60 protease was separately purified through chromatographies on a TSK gel G3000SW HPLC column and a HTP cartridge. The purified recombinant mutant ER-60 protease was stored in multiple aliquots at –80°C.

### 2.4. Assaying of proteolytic degradation

The purified recombinant mutant ER-60 proteases were dialyzed overnight against 10 mM bis-(2-hydroxyethyl)iminotris (hydroxymethyl) methane (bis-Tris)/HCl buffer, pH 7.0, at 4°C. Then, the mutant recombinant ER-60 proteases was incubated for 3 h at 37°C with 2  $\mu$ g of PDI, as a substrate, in 10 mM bis-Tris/HCl buffer, pH

6.3, in a final volume of 11  $\mu$ l. The reaction products were subjected to SDS-PAGE, and then analyzed by Western blotting using anti-PDI rabbit serum and Renaissance Chemiluminescence Reagent (Du Pont NEN), as previously described [7]. Anti-PDI serum was prepared by injecting the purified bovine PDI intradermally into a male rabbit weighing about 2 kg.

### 3. Results and discussion

In the previous study, we determined the proteolytic activity of recombinant mutant ER-60, of which the C-terminal cysteine residues in the CGHC motifs were replaced with serine, and demonstrated that one or both of these cysteine residues comprised the active center of ER-60 [7]. However, it has not been confirmed whether the proteolytic activity was abolished by a single or double mutation of the C-terminal cysteine residues of the CGHC motifs to alanine, since the intact proteins with these mutations were not expressed in *E. coli* BL21(DE3), which was used as a host strain [7]. These ER-60 mutants were rapidly degraded in BL21(DE3) cells after synthesis. Hence, we examined the expression of these mutant enzymes (C60A/C409A, C60A, and C409A) (Fig. 1) in another *E. coli* strain, AD494(DE3) (Novagen, WI), which is devoid of thioredoxin reductase [9]. The intact C60A/C409A, C60A, and C409A enzymes, with an apparent molecular size of 60 kDa, were expressed after IPTG induction (Fig. 2A). Most of each mutant enzyme protein was expressed in a water-soluble form. The mechanism by which the stable mutant enzymes were produced in AD494(DE3), but not in BL21(DE3), remains unclear. The mutant enzymes were purified from cell lysates through three sequential column chromatographies on AF-heparin Toyopearl 650M, TSK gel G3000SW, and hydroxyapatite columns (Fig. 2B). At every step of column chromatography, the mutated ER-60 proteases exhibited the same elution profile as the recombinant wild type ER-60 [7]. This indicates that the recombinant mutant ER-60 proteases were properly folded. The C60A/C409A enzyme showed no proteolytic activity, suggesting the C-terminal cysteine residue(s) of the CGHC motif(s) was the active site cysteine residue(s) (Fig. 2C, lane 4). However, the single-mutated enzymes, C60A and C409A, exhibited proteolytic activity, suggesting that the two copies of the CGHC motif were independently responsible for the proteolytic activity and that both the C-terminal cysteine residues may independently function as the active site cysteine residues (Fig. 2C, lanes 5 and 6). The cleavage site(s) of PDI by the wild type, C60A and C409A enzymes could not be identified, since N-terminal amino acid sequences of the major products, 54-kDa fragments, produced by these enzymes were the same as that of bovine PDI (data not shown). The relative

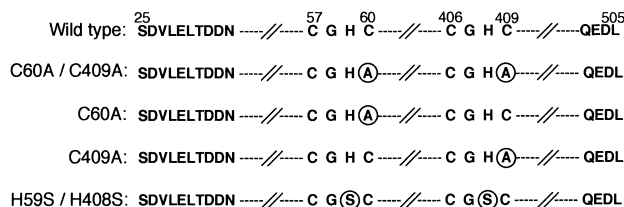


Fig. 1. Schematic diagram of CGHC motif mutants of human ER-60. The N-terminal signal sequence (amino acids 1–24) was deleted from the recombinant ER-60. The numbers in the diagram indicate amino acid residue numbers. The amino acid residues in the CGHC motifs which were mutated from cysteine or histidine residues are circled.

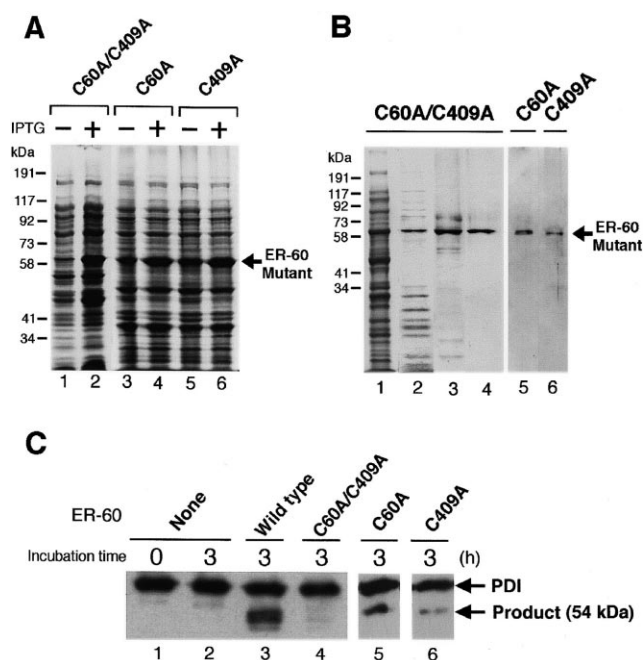


Fig. 2. Expression, purification and protease activity of recombinant ER-60s with mutations of C-terminal cysteine residues in the CGHC motifs. A: Expression of mutant ER-60 in AD494(DE3) was carried out as described in Section 2. The total proteins of AD494(DE3) cells, which carries plasmids for the expression of the C60A/C409A (lanes 1 and 2), C60A (lanes 3 and 4), or C409A enzyme (lanes 5 and 6), were separated by SDS-PAGE (10% gel) before (lanes 1, 3 and 5) or after 0.4 mM IPTG induction for 2 h (lanes 2, 4 and 6). Proteins were stained with CBB. B: Purification of the C60A/C409A (lanes 1–4), C60A (lane 5), or C409A enzyme (lane 6) was carried out as described in Section 2. The supernatant obtained from the disrupted cells on centrifugation (lane 1) was subjected to sequential column chromatographies on a heparin column (lane 2), a TSK gel G3000SW column (lane 3), and a HTP column (lanes 4, 5 and 6). The proteins were separated by SDS-PAGE and then stained with CBB. C: Protease activity was assayed as described in Section 2. PDI (lanes 1 and 2), or PDI and 5  $\mu$ g of wild type (lane 3), 5  $\mu$ g of C60A/C409A (lane 4), 0.7  $\mu$ g of C60A (lane 5), or 2.9  $\mu$ g of C409A enzyme (lane 6) were incubated at 37°C for 0 (lane 1) or 3 h (lanes 2–6). Samples were subjected to SDS-PAGE (10% gel). Proteins were immunoblotted with anti-PDI serum.

activities of the wild type, C60A and C409A enzymes were calculated from the values obtained on scanning of the 54-kDa products on the fluorogram with a densitometer, AE-6920 (Atto, Tokyo). The activities per the same amount of C60A and C409A proteins were 98 and 10% of that of the wild type enzyme, respectively. These results suggest that the major activity of the wild type enzyme depends on that by Cys-409. It is difficult to determine kinetic parameters such as  $K_m$ ,  $K_i$  and turnover number of mutant enzymes, since we have not obtained any synthetic substrates for quantitative analysis of proteolytic activity. Although luminal ER proteins PDI [10], ERp72 [6], and P5 [11] are also known to contain CGHC motifs, the properties of their enzyme activities are different. ERp72 was shown to have similar proteolytic activity to that of ER-60 [5]. Such proteolytic activity has not been found for PDI, but autodegradation activity of bovine or recombinant human PDI has been demonstrated [12]. Furthermore, all the cysteine residues of the CGHC motifs were shown to be necessary for the autodegradation of PDI [12]. On the other hand, PDI or ER-60 is known to facilitate the

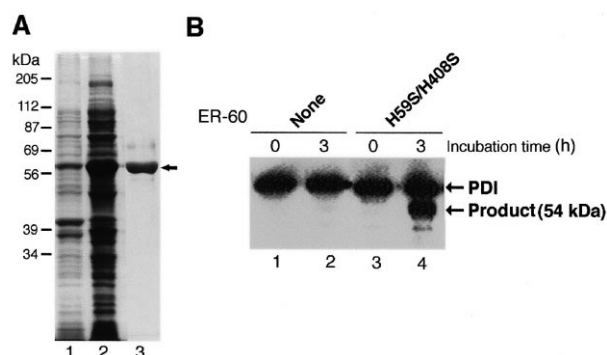


Fig. 3. Expression, purification and protease activity of the recombinant ER-60 with mutations of histidine residues in the CGHC motifs. A: Expression and purification of the H59S/H408S enzyme were carried out as described in Section 2. The proteins in the pellet (lane 1) and supernatant (lane 2) fractions obtained from the disrupted cells on centrifugation, and the purified H59S/H408S enzyme (lane 3) were separated by SDS-PAGE and then stained with CBB. B: Protease activity was assayed as described in Section 2. PDI (lanes 1 and 2), or PDI and 5  $\mu$ g of H59S/H408S enzyme (lanes 3 and 4) were incubated at 37°C for 0 (lanes 1 and 3) or 3 h (lanes 2 and 4). Samples were subjected to SDS-PAGE (10% gel). Proteins were immunoblotted with anti-PDI serum.

formation and rearrangement of disulfide bonds in unfolded proteins [3,13]. In this case, the two individual CGHC motifs of PDI can both function independently [14]. The N-terminal cysteine residues in the CGHC motifs of PDI are essential for catalysis of the formation and rearrangement of disulfide bonds, whereas the C-terminal cysteine residues in the CGHC motifs are only essential for catalysis of the formation of disulfide bonds [15]. It remains unknown what conformation or structure of the proteins causes such a difference in the function of the cysteine residues in CGHC motifs between ER-60 and PDI.

In general, cysteine protease is known to have a catalytic dyad of cysteine and histidine residues [16]. In order to determine whether His-59 or His-408 in the two copies of the CGHC motif is the histidine residue comprising a catalytic dyad, we prepared a recombinant mutant ER-60 protease

with His-59 and His-408 both modified to serine (H59S/H408S) (Fig. 1). Large amounts of the intact H59S/H408S enzymes were expressed in BL21(DE3) (Fig. 3A, lane 2) and purified by the same procedures as those used for purification of the cysteine mutant enzymes. The purified enzyme gave a single band on a SDS gel (lane 3). The H59S/H408S enzyme showed proteolytic activity (Fig. 3B), suggesting the histidine residues in the two copies of the CGHC motif do not constitute the catalytic dyad of ER-60.

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