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Functions of Characteristic Cys-Gly-His-Cys (CGHC) and Gln-Glu-Asp-Leu (QEDL) Motifs of Microsomal ER-60 Protease[1]

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The human ER-60 protease cDNA was expressed in *Escherichia coli* BL21 (DE3) cells using the pET-20b(+) T7 promoter. The recombinant ER-60 protease was obtained in a water-soluble form and purified through four sequential chromatographies. The ER-60 protease contains two CGHC motifs. When an alanine residue was substituted for the N-terminal cysteine residue in both motifs, the protease activity was not lost. However, when the C-terminal cysteine residue in both motifs was replaced by a serine residue, the cysteine protease activity, which was inhibited by *p*-chloromercuribenzoic acid (*p*CMB) but not by diisopropyl fluorophosphate (DFP), changed to serine protease activity, which was inhibited by DFP but not by *p*CMB. These results indicate that the C-terminal cysteine residue(s) of the CGHC motifs may constitute the active site(s) of ER-60 protease. The ER-60 protease has a C-terminal QEDL sequence, which was proved to serve as an ER-retention signal by deletion of the QEDL sequence. However, because QEDL could not serve as the ER-retention signal for protein disulfide isomerase or ERp72, it is suggested that amino acid residue(s) of ER-60 protease, other than the QEDL sequence itself, is complimentarily responsible for the ER retention of this protein.

Key words: active site, cysteine protease, endoplasmic reticulum, retention signal, site-directed mutagenesis.

A number of secretory and membrane proteins when newly synthesized are properly folded and assembled into oligomers in the lumen of the endoplasmic reticulum (ER) (*1*). The folding is assisted by molecular chaperones (*2, 3*). When polypeptides are folded or assembled improperly, they are rapidly degraded in ER by ER-resident proteases (*4, 5*). However, the protease(s) responsible for this ER degradation has not been well studied.

ER-60 protease (*6, 7*), which is also known as ERp61 (*8*), HIP70 (*9*), Q2 (*10*), GRP58 (*11*), and ERp57 (*12, 13*), was first purified from rat liver ER and found to degrade ER-resident proteins such as protein disulfide isomerase (PDI), calreticulin, and lysozyme (*14*). ER-60 protease, a cysteine protease, has characteristic CGHC and QEDL motifs in its primary structure (*15, 16*). Another microsomal cysteine protease, ER-72 protease (ERp72), is also known to contain the CGHC motifs (*17, 18*). However, these cysteine proteases do not contain the well-conserved amino acid sequence found around the cysteine residue of the active site of the papain family (*16*). Hence, it is assumed that microsomal ER-60 and ER-72 proteases may be classified into a new family of cysteine proteases with a novel active site structure. ER-60 protease contains seven cysteine residues, four of which constitute two copies of CGHC. However, ER-72 protease contains no cysteine residue other than the six constituting three CGHC motifs. This suggests that the active site cysteine residue of ER-60 protease is located in the CGHC motif(s). On the other hand, the mechanism underlying the retention of ER-60 protease in ER remains obscure. Generally, the C-terminal KDEL sequence of the protein is known to be the retention signal that keeps the protein in ER (*19, 20*). The C-terminal QEDL sequence of ER-60 protease may be regarded as a retention signal as well as KDEL (*11*). On the other hand, QEDL was reported not to function as an ER-retention signal (*12*). In addition, on substitution of QEDL for KDEL and KEEL of PDI and ERp72, these proteins were not retained in ER (*21*). It is necessary to examine the function of the C-terminal QEDL of ER-60 protease.

In this study, by site-directed mutagenesis of the recombinant human and rat ER-60 proteases, we demonstrate that the C-terminal cysteine residue of the CGHC motif functions as the active site cysteine residue, and that the C-terminal QEDL is required to keep the ER-60 protease in ER.

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Abbreviations: CBB, Coomassie Brilliant Blue R-250; DFP, diisopropyl fluorophosphate; DMEM, Dulbecco's minimum essential medium; ER, endoplasmic reticulum; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PBS, phosphate-buffered saline; *p*CMB, *p*-chloromercuribenzoic acid; PDI, protein disulfide isomerase; PMSF, phenylmethylsulfonylfluoride; PVDF, polyvinylidene difluoride.

EXPERIMENTAL PROCEDURES

Materials—EXPLE ^{35}S ^{35}S protein labeling mix (37.0 TBq/mmol) and Enlightning were purchased from Du Pont, NEN. [^{32}P]ATP (>185 TBq/mmol) and Cy5-streptavidin were obtained from Amersham. The ZAP-cDNA Synthesis Kit and Gigapack II Gold Packaging Extract were obtained from Stratagene. The Dye Termination Cycle Sequencing Kit was from Perkin Elmer, Applied Biosystems Division. Tli DNA polymerase was purchased from Promega Biotek. Bovine liver PDI was obtained from Takara Shuzo (Kyoto) and purified by hydroxyapatite column chromatography. Bovine serum albumin was purchased from Miles Laboratories. Leupeptin was obtained from Peptide Institute (Osaka). The DEAE Toyopearl 650 prepacked column, AF-heparin Toyopearl 650M resin, TSK gel G3000SW HPLC column, and TSK gel DEAE-5PW HPLC column were obtained from TOSOH (Tokyo). The HTP cartridge (hydroxyapatite), polyvinylidene difluoride (PVDF) protein sequencing membranes, and Bio-Lyte (*3-10*) were obtained from Bio-Rad. Carboxypeptidase Y (103 units/mg) was purchased from Oriental Yeast (Osaka). Anti-PDI serum was obtained from Stressgen Biotechnologies. Pansorbin was purchased from Calbiochem-Novabiochem. The biotin-anti-rabbit IgG (H+L) goat polyclonal antibodies were from Vector. Protein A-Sepharose CL-4B (binding capacity, approx. 20 mg human IgG/ml) was purchased from Sigma. All other chemicals were of reagent grade.

Cloning of Human ER-60 Protease cDNA—Unless otherwise stated, the DNA manipulations were performed as described by Sambrook *et al.* (*22*). A cDNA library in Uni-ZAP XR was prepared from poly(A)$^+$ RNA of Chang liver cells (human), according to the instruction manual (Stratagene). A premade library, Hep G2 in Uni-ZAP XR, was obtained from Stratagene. These libraries were screened with two 5′-^{32}P-labeled synthetic oligonucleotide probes, 5′-TCAGACGTGTTGGAACTGACGGACGAAAAC-3′ (corresponding to the coding sequence for an N-terminal 10 amino acid sequence, SDVLELTDEN, of rat ER-60 protease), and 5′-AGCATCAAGGAATGTCTTTGCCACCATCAT-3′ (corresponding to the complementary sequence of the coding sequence for the partial amino acid sequence, MMVAKTFLD, of rat ER-60 protease) (*6, 15*). Hybridization was carried out for 22 h at 42°C. The filters were washed and exposed to Kodak XAR-5 X-ray film. Among the 1×10^5 plaques screened in the Chang liver library, two positive clones were identified. From the Hep-2 library, six clones were obtained. The *Eco*RI-*Xho*I insert from the longest λ-phage screened from each library was excised *in vitro* as a pBluescript SK(−) plasmid according to the instruction manual provided by Stratagene. Plasmid DNAs were prepared by the alkali method and purified with Magic™ Miniprep DNA Purification Resin (Promega Biotek). The DNA sequences of both strands were determined by the dideoxy chain termination method of Sanger *et al.* (*23*) and the fluorescence dideoxy chain termination method (Perkin Elmer, Applied Biosystems).

Construction of Expression Plasmids for Human ER-60 Proteases in Escherichia coli—A plasmid was constructed for expression in *E. coli*, designated as pET-hER60 for human ER-60 protease. The recombinant plasmid encodes 481 amino acids of the human ER-60 protease, which correspond to amino acid residues 25–505. pET-hER60 was constructed by ligation of a nucleotide fragment, which was prepared by PCR, into the *Nde*I site of pET-20b(+) (Novagen). The PCR was carried out using a template, pBluescript, including the full-length cDNA of human ER-60 protease and the following two primers: 5′ oligonucleotide, 5′-TGTCCGACGTGCTAGAACTCACGGACGAC-3′ (corresponding to part of the recognition sequence for *Nde*I and the sequence for human ER-60 protease), and 3′ oligonucleotide, 5′-GGCTAAAGCTTTAGAGATCCTCCTGTGCCTTCTTC-3′ (corresponding to the recognition sequence for *Hin*dIII and part of the human ER-60 protease sequence). The amplified PCR product was digested with *Hin*dIII and inserted into pET-20b(+), which was digested with *Nde*I, blunt-ended, then digested with *Hin*dIII.

The resultant expression plasmid was used as the template for site-directed mutagenesis. To change Cys-57 or Cys-406 to alanine (C57A or C406A), or Cys-60 and Cys-409 to serine (C60S/C409S), PCRs were performed with the following mutagenic primers by the method of Ito *et al.* (*24*).

C57A: 5′-TGGGCTGGACACTGCAAGAGA-3′
C406A: 5′-CCCTTGGGCTGGTCATTGTAA-3′
C60S/C409S: 5′-GGTGTGGACACTCCAAGAGACTTGCA-3′
5′-GGTGTGGTCATTCTAAGAACCTGGAG-3′

The amplified PCR fragments were digested with *Xba*I and *Hin*dIII, then inserted into pET-20b(+). A mutant in which both Cys-57 and Cys-406 were changed to alanine (C57A/C406A) was generated by ligating the *Nhe*I-*Xba*I N-terminal fragment of C57A into C406A, from which the *Nhe*I-*Xba*I fragment was removed. All mutants were confirmed by sequence analysis.

Expression and Purification of the Recombinant Human ER-60 Proteases—The expression plasmid, pET-hER60, was transformed into the *E. coli* host strain, BL21(DE3) (Novagen), grown as 800-ml cultures in the presence of 500 μg/ml ampicillin at 37°C, and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 30°C for 2 h. The 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 phenylmethylsulfonylfluoride (PMSF), 50 mM KCl, and 10% glycerol (buffer A), then centrifuged at $5,000\times g$ for 10 min at 4°C. Purification was then carried out according to the procedure described previously (*6*). The supernatant was subjected to AF-heparin Toyopearl 650M column chromatography. From the effluent obtained with 175 or 400 mM KCl in buffer A, the recombinant ER-60 protease was separately purified through chromatographies on a TSK gel G3000SW HPLC column, a TSK gel DEAE-5PW HPLC column, and a HTP-cartridge. The HTP-cartridge chromatography was performed with a 25-ml linear gradient of 10-200 mM potassium phosphate buffer, pH 7.4, at the flow rate of 0.5 ml/min. The purified recombinant ER-60 protease was stored in multiple aliquots at −80°C.

Amino Acid Sequencing—N-terminal amino acid sequencing was carried out with a Protein Sequencer Model 477A (Applied Biosystems), as described previously (*6*).

For determination of the C-terminal amino acid, the recombinant ER-60 protease (600 μg of protein) was digested with 3 μg of carboxypeptidase Y in 50 μl of 0.1 M pyridine/acetic acid buffer, pH 5.6, at 25°C for 1, 2, 5, 10, 20, 30, 60, and 120 min, and the released amino acids were determined with an Amino Acid Analyzer Model 835 (Hitachi, Tokyo).

Preparation of Polyclonal Antibodies—The purified recombinant human ER-60 protease protein (100 μg) emulsified with complete Freund's adjuvant was injected intradermally into a male rabbit weighing about 2 kg. First and second booster injections (100 μg each) were given with the complete adjuvant 4 and 8 weeks after the initial injection, respectively. The third booster (100 μg) was given with the incomplete adjuvant 4 weeks after the second one. Serum was collected 7 days after the last injection.

Immunoblot Analysis—Proteins separated by two-dimensional PAGE or SDS-PAGE were blotted onto a PVDF membrane, then immunostained with anti-recombinant human ER-60 protease rabbit serum or anti-PDI rabbit serum, using a Proto Blot™ Immunoblotting System (Promega Biotec) or Renaissance Chemiluminescence Reagent (DuPont NEN).

Assay of Proteolytic Degradation by Protein Disulfide Isomerase—The stored recombinant ER-60 protease was dialyzed overnight against 10 mM bis(2-hydroxyethyl)-iminotris (hydroxymethyl)methane (bis-Tris)/HCl buffer, pH 7.0, at 4°C. Then the ER-60 protease (2 μg) was incubated for 3 h at 37°C with PDI (2 μg) as a substrate in 10 mM bis-Tris/HCl, pH 6.3, in a final volume of 10 μl. The reaction products were analyzed by SDS-PAGE. The proteins were stained with Coomassie Brilliant Blue R-250 or immunostained with anti-PDI rabbit serum using a chemiluminescence immunodetection kit.

Construction of Expression Plasmids for Rat ER-60 Proteases in COS Cells—Cloning of the cDNA of rat ER-60 protease (accession number, D63378) has been described (*15*). The cDNA of rat ER-60 protease was isolated from the plasmid, pBluescript-rER60, on a 5′-*Eco*RI and 3′-*Xho*I restriction fragment, then cloned into the complementary site in pcDNA3 (Invitrogen). The resultant expression plasmid, pcDNA3-rER60, was used as the template for site-directed mutagenesis by PCRs. For generation of a set of ER-60 protease C-terminal mutants, PCRs were performed with a forward primer, 5′-CTTTGCTGTAGCTAG-CCGTAAAACC-3′, corresponding to the ER-60 protease sequence (967-991), and the following reverse primers, which contain mutations and a *Xho*I site.

KDEL: 5′-GTTTGGCTCTCGAGTTAGAGCTCATCTT-TTGCCTTCTTCT-3′
AAGL: 5′-GTTTGGCTCTCGAGTTAGAGGGCCTGT-GCTGCCTTCTTCT-3′
QEDL-deletion: 5′-GTTTGGCTCTCGAGTTATGCCT-TCTTCT-3′

The PCR fragments were digested with *Nhe*I and *Xho*I, then ligated between the *Nhe*I and *Xho*I restriction sites of pcDNA3-rER60. All mutants were confirmed by sequence analysis.

Expression of Recombinant Rat ER-60 Proteases in COS Cells—COS cells were plated at a density of 9×10^3 cells/cm^2 in Dulbecco's minimum essential medium (DMEM) containing 10% fetal calf serum. After incubation for 24 h, the cells were transfected with the wild-type and mutant expression plasmids by the method of Chen and Okayama (*25, 26*).

Metabolic Labeling and Immunoprecipitation—At 48 h after transfection, the cells were washed with serum-free DMEM, then preincubated in methionine- and cysteine-free DMEM containing 5% dialyzed serum for 1 h. The preincubated cells were metabolically labeled with 100 μCi [^{35}S]methionine and cysteine/ml DMEM for 30 min, then the radioactive medium was replaced with the complete medium containing methionine and cysteine, and the labeled cells were incubated for the indicated times. After removal of the medium, the cells were washed three times with ice-cold phosphate-buffered saline (PBS), then solubilized in lysis buffer comprising 0.05% SDS, 1% Nonidate P-40, 150 mM NaCl, 50 mM Tris/HCl buffer, pH 7.5, 2 mM PMSF, 0.2 mg/ml soybean trypsin inhibitor, and 0.2 mg/ml leupeptin (*14*). The lysate and medium were incubated with 5 μl of a 10% suspension of Pansorbin for 30 min, then centrifuged at 14,000 rpm and 4°C for 10 min. The precleared supernatant (0.2 ml) was incubated with 20 μl of anti-rat ER60F serum (*6*) and 15 μl of 25% skimmed milk for 16 h at 4°C. The immunocomplexes were collected on 10 μl of Protein A-Sepharose (50% suspension in PBS) by shaking for 1 h, and then washed four times with 0.05% Tween 20 in PBS. The immunocomplexes were boiled in Laemmli's sample buffer (*27*), then analyzed by SDS-PAGE (10% acrylamide gel). The gels were fixed and equilibrated in a mixture of Enlighting, ethanol, and distilled water (5 : 2 : 3) before fluorography.

Laser Scanning Immunofluorescence Microscopy—COS cells were grown on polylysine-coated cover glasses, then transfected as described above. At 48 h after transfection, the cells were fixed with 2% formaldehyde and 0.1% glutalaldehyde, permeabilized with 1% Triton X-100 in PBS, then treated with 1 mg/ml $NaBH_4$. The cells were then triple stained using anti-rat ER60F serum, biotin-anti-rabbit IgG (H+L) goat polyclonal antibodies and Cy5-streptavidin for rat ER-60 protease protein, tetramethylrhodamine-conjugated wheat germ agglutinin for Golgi apparatus, and fluorescein isothiocyanate-conjugated concanavalin A for ER.

The specimens were examined with a MRC-1024 laser scanning confocal imaging system (Bio-Rad) attached to a Nikon microscope.

Miscellaneous—SDS-PAGE was performed according to the method of Laemmli (*27*). All SDS-PAGE analyses were carried out under reducing conditions. Two-dimensional PAGE was carried out by the method of O'Farrell (*28*).

Protein concentrations were determined using a protein assay kit from Bio-Rad, with γ-globulin as a standard.

RESULTS

cDNA Cloning of Human ER-60 Protease—The longest clone obtained from the Chang liver cell cDNA library was 1,760 nucleotides in length, consisting of a 22-nucleotide 5′ untranslated region, a 1,515-nucleotide open reading frame, a 204-nucleotide 3′-untranslated region, and a 19-nucleotide poly(A)$^+$ tail (Fig. 1). The nucleotide sequence of the open reading frame in the clone from the Hep G2 cDNA library was identical to that of the Chang liver cell cDNA

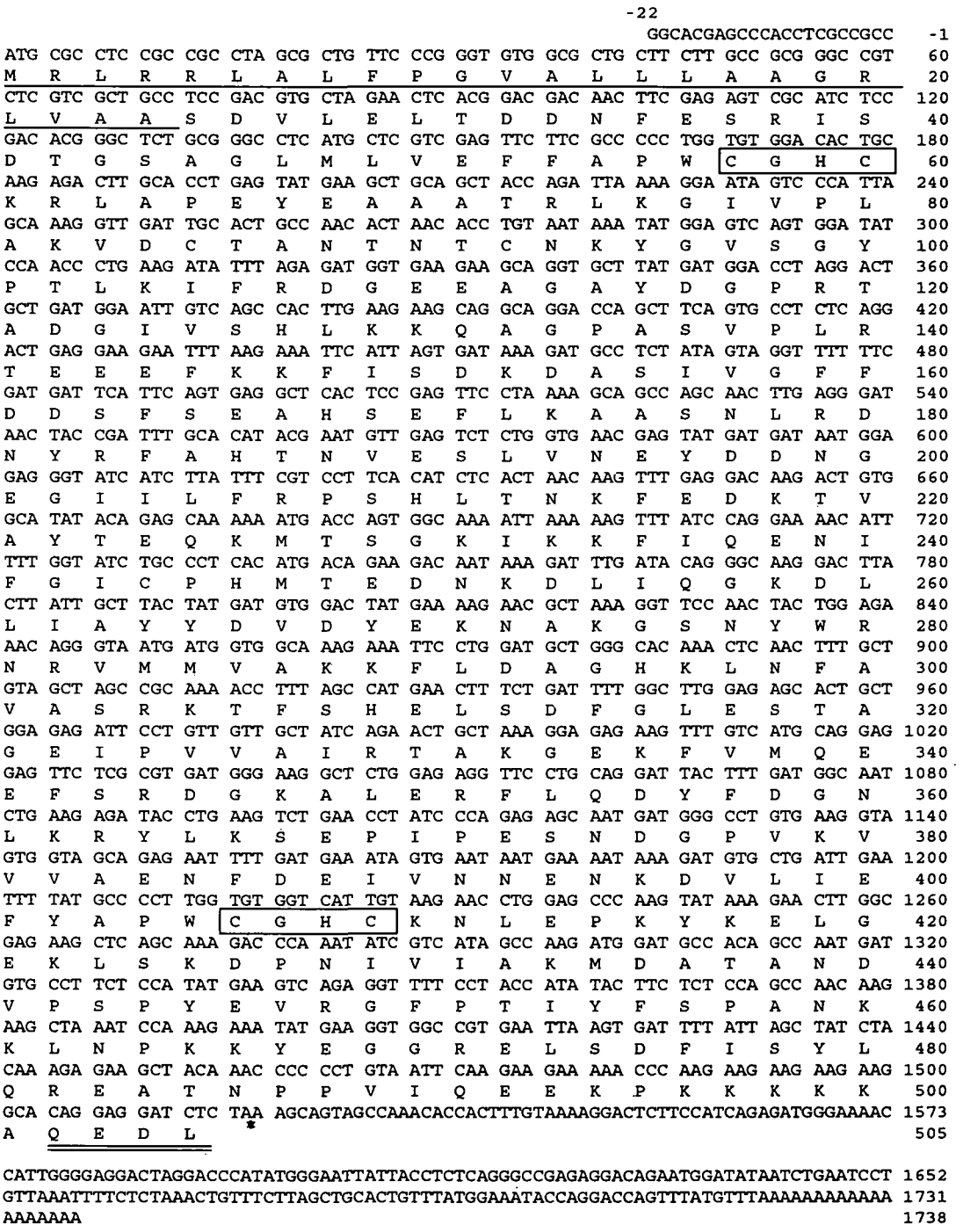

Fig. 1. **Sequence of the cDNA and the deduced primary structure of human ER-60 protease.** Amino acid residue 1 is the putative initiator methionine. The putative signal peptide is underlined. The C-terminal QEDL sequence is double-underlined. The two CGHC motifs are boxed. The star denotes the stop codon.

(data not shown). The ER-60 protease protein was composed of 505 amino acid residues, as deduced from its nucleotide sequence, with a signal sequence composed of 24 amino acid residues at its N-terminal, and a QEDL sequence at its C-terminal. Two CGHC motifs were present in the protein. The homology between the human and rat ER-60 proteases (DDBJ/EMBL/Genbank accession number, D63378) was 90% in the nucleotide sequence and 92% in the amino acid sequence. However, the amino acid sequences around the two CGHC motifs were well conserved in the proteins. The cDNA sequence may be the same as that of the human thiol-dependent reductase (*12, 29, 30*).

Expression of the Recombinant Human ER-60 Protease in E. coli—Expression plasmid pET-hER60 was constructed by linking the cDNA fragment encoding the human ER-60 protease to the downstream site of the pET-20b(+) T7 promoter, then expressed in *E. coli* BL21 (DE3) cells

(Fig. 2A). Coomassie Brilliant Blue (CBB)-staining of the SDS gel indicated that the *E. coli*-synthesized ER-60 protease protein comprised a few percent of the total bacterial proteins even before IPTG induction (Fig. 2A, lane 3). However, the human ER-60 protease protein, which was expressed in a water-soluble form (Fig. 2A, lane 5), comprised about 17% of the total proteins 2 h after the IPTG induction (Fig. 2A, lane 4). Under such conditions, a part of the recombinant protein was found to be degraded on Western blotting analysis (Fig. 2A, lane 7). The degradation could not be prevented by replacement of the expression vector with pKK 233-2 (Pharmacia) or pMAL-p2 (New England BioLab), by the use of protease inhibitors, or by expression at lower temperatures (data not shown). The soluble fraction of the *E. coli* extract containing the recombinant human ER-60 protease was applied to a heparin column. Approximately three-fourths of the total recombinant protein was eluted with 175 mM KCl, and the rest with 400 mM KCl (Fig. 2B, lanes 3 and 4). The protein obtained with 400 mM KCl, as in the case of the rat liver ER-60 protease (*6*), was subjected to G3000 gel column chromatography, and eluted at a position corresponding to 60 kDa. The protein was purified by DEAE-5PW column chromatography with elution with 90-100 mM NaCl, followed by hydroxyapatite column chromatography with elution with 200 mM potassium phosphate buffer. The effluent was stored at −70°C. Under these conditions, the enzyme remained stable. The purified preparation gave a single band on SDS-PAGE with silver staining (Fig. 2B, lane 7). On two-dimensional PAGE, a single spot with a pI value of 6.1 was observed (Fig. 2C). The purified recombinant human ER-60 protease was confirmed to be intact by analyzing its N-terminal and C-terminal amino acid sequences, which were found to be SDVLELTDDN and DL, respectively (*6, 15*). Methionine derived from the initiation codon was removed during expression in *E. coli*.

CGHC Motifs as the Active Sites of ER-60 Protease— The wild-type recombinant human ER-60 protease purified from the transformed *E. coli* BL21(DE3) degraded PDI (Fig. 3A, lanes 3 and 7) like the rat liver ER-60 protease (*6*). The proteolytic activity appeared to be low. This may be due to the substrate, PDI, since true substrates have not

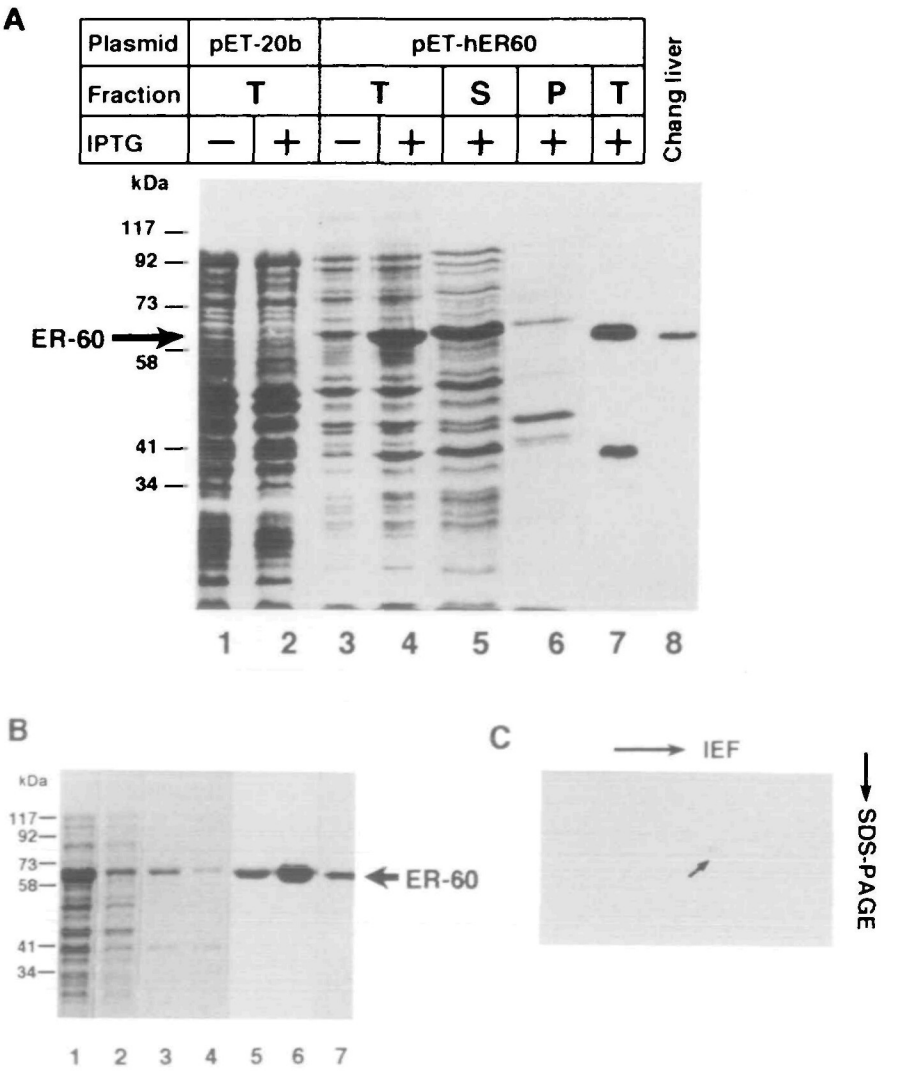

Fig. 2. **SDS-PAGE and Western blotting analysis of the expressed human ER-60 proteases.** Panel A: Expression of human cDNA was carried out as described under "EXPERIMENTAL PROCEDURES." The proteins were electrophoresed on a 10% gel, and then stained with CBB (lanes 1-6), or immunostained with anti-recombinant human ER-60 protease serum (lanes 7 and 8), as described under "EXPERIMENTAL PROCEDURES." Lanes 1 and 2, total proteins (T) of BL21-(DE3) cells carrying pET-20b(+); lanes 3, 4, and 7, total proteins of BL21(DE3) cells carrying pET-hER60; lanes 5 and 6, Triton X-100 soluble (S) or insoluble proteins (P) of BL21(DE3) cells carrying pET-hER60, respectively; lane 8, total proteins of Chang liver cells (2 μg of protein). Induction of the recombinant proteins was carried out by incubation with IPTG for 2 h as described under "EXPERIMENTAL PROCEDURES" (lanes 2, and 4-7). Panel B: Purification of the recombinant human ER-60 protease was carried out as described under "EXPERIMENTAL PROCEDURES." An extract of BL21(DE3) cells carrying pET-hER60 (lane 1), the unabsorbed fraction (lane 2), fractions eluted from a heparin column with 175 mM KCl (lane 3), and 400 mM KCl (lane 4), and fractions obtained on TSK gel G3000-SW column chromatography (lane 5), DEAE-5PW column chromatography (lane 6), and HTP column chromatography (lane 7) were electrophoresed on a 10% gel. The proteins were stained with CBB (lanes 1-6) or a silver staining kit (Bio-Rad) (lane 7), as described under "EXPERIMENTAL PROCEDURES." Panel C: Two-dimensional PAGE of the recombinant human ER-60 protease obtained on HTP column chromatography was carried out, and the protein was transferred to a PVDF membrane, then immunostained with an anti-recombinant human ER-60 protease serum as described under "EXPERIMENTAL PROCEDURES." First dimension, isoelectric focusing (IEF) on a 5% polyacrylamide gel containing 2% Bio-Lyte 3-10; second dimension, SDS-PAGE (10% gel).

yet been identified. The reaction product of 54 kDa stained with CBB was identified as a PDI fragment on Western blotting analysis with anti-PDI antiserum. Its N-terminal amino acid sequence was determined to be APDEEVHV-LV, which is the same as that of bovine PDI. The reaction was inhibited by *p*-chloromercuribenzoic acid (*p*CMB) (Fig. 3A, lanes 4 and 8). The recombinant enzyme was thus reconfirmed to be a cysteine protease. ER-60 protease contains seven cysteine residues, four of which constitute two CGHC motifs. However, ER-72 protease contains no cysteine residue other than the six constituting three CGHC motifs (*18*). Therefore, the common CGHC motifs of these two cysteine proteases were assumed to include an active center cysteine residue(s) of the proteases (*17*). Next, we prepared a recombinant human ER-60 protease with site-directed mutations in the CGHC motifs. First, the N-terminal cysteine residues of the CGHC motifs were replaced by alanine residues. The double-mutated enzyme with Cys-57 and Cys-406 both modified to alanine (C57A/C406A) showed similar activity to the wild-type enzyme (Fig. 3B, lane 2). The single-mutated enzymes, C57A and

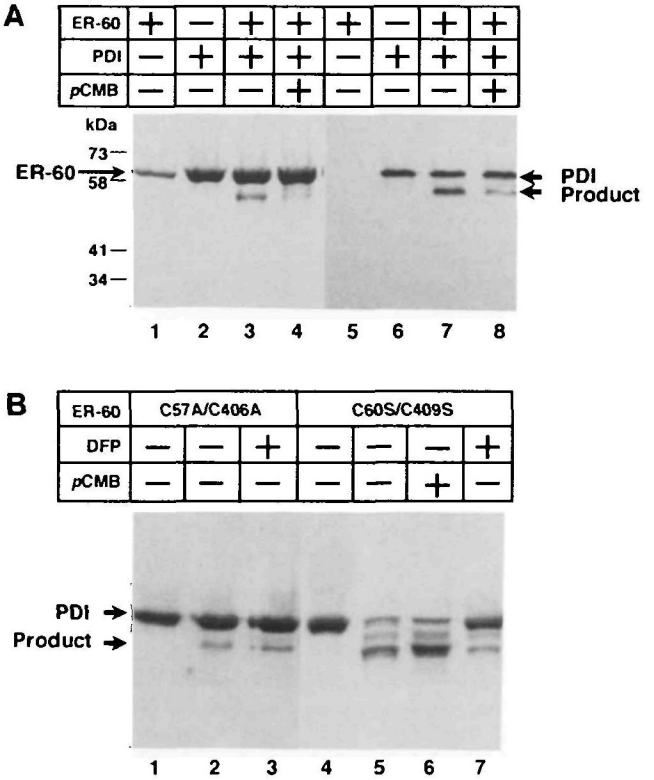

Fig. 3. **Proteolytic degradation of PDI by the recombinant human ER-60 proteases.** Protease activity was assayed as described under "EXPERIMENTAL PROCEDURES." Panel A: The recombinant wild-type human ER-60 protease (ER-60) (1 μg of protein) (lanes 1 and 5), PDI (2 μg of protein) (lanes 2 and 6), or ER-60 and PDI (lanes 3, 4, 7, and 8) were incubated at 37°C for 3 h with (lanes 4 and 8) or without *p*CMB (lanes 1–3 and 5–7). Samples were subjected to SDS-PAGE (10% gel). Proteins were stained with CBB (lanes 1–4) or by Western blotting with anti-PDI serum (lanes 5–8) as described under "EXPERIMENTAL PROCEDURES." Panel B: C57A/C406A or C60S/C409S and PDI were incubated at 37°C for 3 h with (lane 6) or without *p*CMB (lanes 2, 3, 5, and 7), and then subjected to SDS-PAGE (10% gel). Proteins were stained with CBB. Recombinant mutant ER-60 proteases were treated without (lanes 2, 5, and 6) or with (lanes 3 and 7)10 mM DFP in 250 mM phosphate buffer, pH 7.4, at 20°C for 30 min, dialyzed against 10 mM bis-Tris/HCl, pH 7, at 4°C overnight, then used for the proteolytic reactions. Lanes 1 and 4 are 0 time controls.

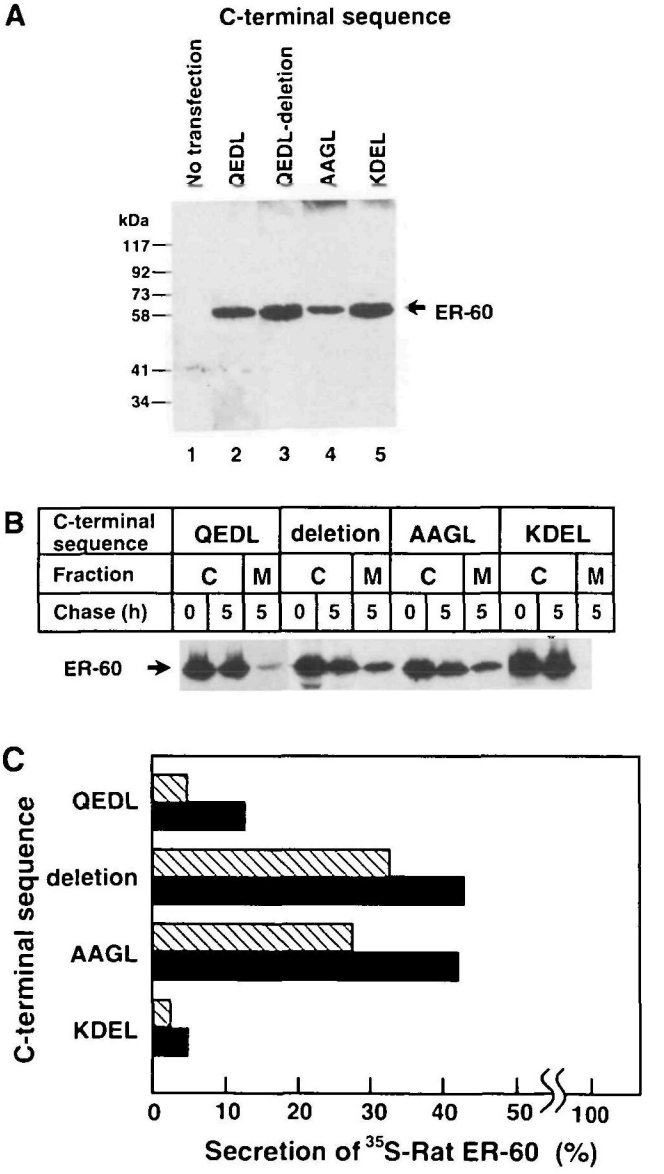

Fig. 4. **Expression and secretion of the C-terminal tetrapeptide mutants by COS cells.** COS cells were transfected with expression plasmids for the wild-type (QEDL) and C-terminal tetrapeptide mutants of rat ER-60 protease as described under "EXPERIMENTAL PROCEDURES." "deletion" refers to deletion of the C-terminal tetrapeptide. Panel A: After transfection, the cells were collected and subjected to Western blotting analysis with anti-rat ER60F serum. Panel B: After transfection, the cells were labeled with [^{35}S]methionine and -cysteine for 30 min, and then chased as described under "EXPERIMENTAL PROCEDURES." The cell lysate (C) and medium (M) were immunoprecipitated with anti-rat ER-60F serum, and then subjected to SDS-PAGE and fluorography. Panel C: The secretion rates of the wild type and mutant rat ER-60 proteases were calculated from the values obtained on scanning of the fluorogram with a densitometer, AE-6920 (ATTO, Tokyo), as follows: [ER-60 protease in medium at chase time 3 h (hatched column) or 5 h (solid column)/ER-60 protease in cells at 0 time] $\times$ 100. Each point represents the average value for at least two experiments.

C406A, also exhibited activity (data not shown). These results suggest the N-terminal cysteine residues of the CGHC motifs are not responsible for the protease activity. Next, the C-terminal cysteine residues of the CGHC motifs were replaced by serine residues. The double-mutated enzyme with Cys-60 and Cys-409 both modified to serine (C60S/C409S) degraded more PDI than the wild-type enzyme (Fig. 3B, lane 5). The replacement of Cys-60 and Cys409 by serine residues may have changed the substrate specificity. The amino acid sequence of the 54-kDa product of the C60S/C409S enzyme was determined to be AP-DEEVHVLV, which was the same as that of the wild-type enzyme. Hence, the difference in the cleavage site(s) could not be identified between the wild-type and C60S/C409S enzymes. It is noted that the activity of the C60S/C409S enzyme was not inhibited by *p*CMB but was by diisopropyl fluorophosphate (DFP) (Fig. 3B, lanes 6 and 7), indicating that the cysteine protease was changed into a serine protease. It was confirmed that the mutant enzyme, C57A/C406A, was not inhibited by DFP (Fig. 3B, lane 3). Hence, it seems likely that Cys-60 and Cys-409 are included in the active site of the ER-60 protease. To determine whether Cys-60, Cys-409, or both are responsible for the activity, we mutated Cys-60 and Cys-409 separately to alanine. However, neither intact mutant enzyme, C60A or C409A, could be obtained from the expression system of *E. coli*.

C-Terminal QEDL Sequence as the Retention Signal in ER—ER-60 protease is localized in ER in a soluble form but does not contain a C-terminal KDEL sequence, the well-known retention signal in ER. However, ER-60 protease

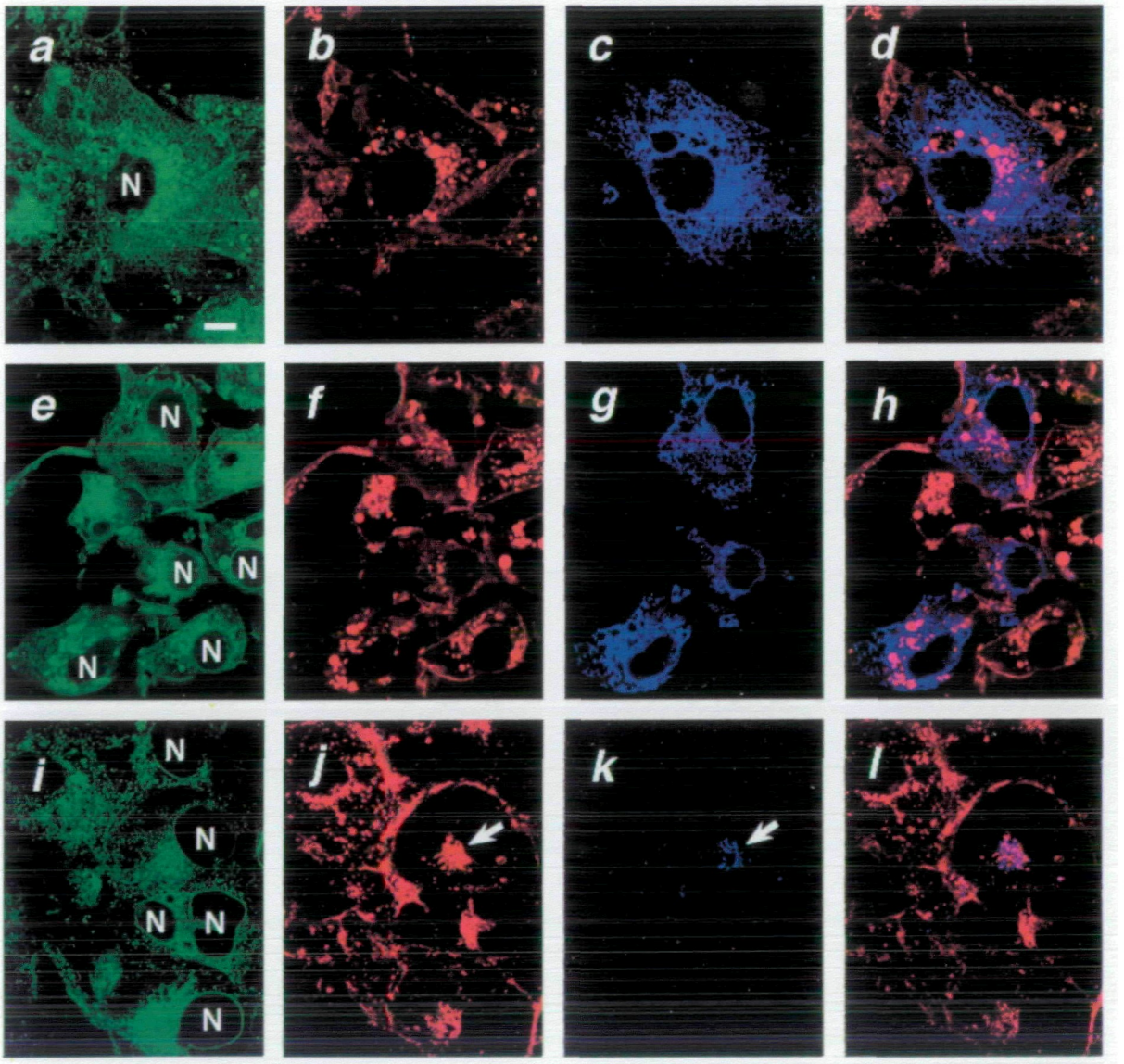

Fig. 5. **Immunofluorescence staining of the wild-type or C-terminal tetrapeptide mutants of ER-60 protease expressed in COS cells.** COS cells transfected with rat ER-60 protease expression plasmids were triple-stained with anti-rat ER60F serum (c, g, k), concanavalin A (a, e, i), and wheat germ agglutinin (b, f, j), as described under "EXPERIMENTAL PROCEDURES." The wild-type (a-d) and KDEL mutant (e-h) ER-60 proteases are retained in ER, whereas the QEDL deletion mutant enzyme (i-l) is mostly colocalized with the Golgi marker proteins (arrow). (d), (h), and (l) are merged images of (b) and (c), (f) and (g), or (j) and (k), respectively. N, nucleus. Bar, 10 μm.

has the unique tetrapeptide, QEDL, at its C-terminal position (Fig. 1) (*15*). To determine the function of the C-terminal QEDL sequence, we expressed the wild-type and mutant rat ER-60 proteases in COS cells, and observed the localization of the protease proteins in the cells by immunostaining. Anti-rat ER60F serum (*6*), which specifically crossreacts with the rat ER-60 protease, did not crossreact with the COS-cell ER-60 protease, but did with the wild-type and mutant rat ER-60 proteases expressed in COS cells (Fig. 4A). On immunostaining with this anti-rat ER-60F serum, untransfected COS cells were not stained (data not shown), but the COS cells transiently expressing the wild type rat ER-60 protease were stained, showing a typical ER profile (Fig. 5c). The mutant ER-60 protease, of which the QEDL sequence was deleted (Fig. 5k) or replaced by a non-functional tetrapeptide, AAGL (data not shown), were colocalized with the Golgi marker proteins, which were stained with tetramethylrhodamine-conjugated wheat germ agglutinin (Fig. 5, j and l). The ER of cells expressing the mutant ER-60 protease with KDEL substituted for QEDL was stained (Fig. 5g), as in the case of the cells expressing the wild-type ER-60 protease. The ER-60 proteases of the wild type and the KDEL-substituted mutant were not colocalized with the Golgi marker proteins (Fig. 5, d and h).

The secretion into the medium of the rat recombinant ER-60 protease pulse-labeled with [^{35}S]methionine and -cysteine in transient transfectants of COS cells was determined by immunoprecipitation with the anti-rat ER60F serum (Fig. 4B). The wild-type enzyme mostly remained in cells after a 5-h chase. Only 13% of the total enzyme was secreted into the medium (Fig. 4C). About 45% of the total mutant enzyme, of which the QEDL sequence was deleted or replaced by an AAGL sequence, was secreted into the medium after a 5-h chase (Fig. 4C). The radioactivity of QEDL-deleted or AAGL mutant protein recovered as the immune precipitates from cell and medium was scarcely decreased during a 5-h chase. This suggests that these mutant proteins were not degraded and distributed in the Golgi body and medium. It is likely that the C-terminal QEDL sequence of ER-60 protease may function as a retention signal in ER. The substitution of KDEL for QEDL could also keep the ER-60 protease in ER.

DISCUSSION

The proteolytic activity of the highly purified recombinant human ER-60 protease was inhibited by *p*CMB, like that of the rat liver microsomal ER-60 protease (*6*). The sensitivity of the ER-60 protease to cysteine protease inhibitors suggests that the active site includes a cysteine residue(s). ER-60 protease has seven cysteine residues, four of which constitute two CGHC motifs. The amino acid sequences around these cysteine residues exhibit little homology to the consensus sequence around the active center cysteine residues of well-known cysteine proteases such as members of the papain family (*16*). Therefore, ER-60 protease was assumed to have a different active center from those of the papain family. ER-72 protease, another microsomal cysteine protease, has no cysteine residue other than those constituting three CGHC motifs (*18*). These facts suggest that a cysteine residue(s) of the CGHC motifs functions as the active site. Among the mutated ER-60 proteases, the C60S/C409S enzyme, in which the C-terminal cysteine residues of the two CGHC motifs are changed to serine residues, showed proteolytic activity sensitive to serine protease inhibitors, unlike the wild-type enzyme, which is sensitive to cysteine protease inhibitors. Hence, it seems likely that Cys-60 or Cys-409 is the active site cysteine residue, which can be replaced by a serine residue to yield serine protease activity. Replacement of the active center cysteine of a cysteine protease with serine was reported to change the cysteine protease into a serine protease in the case of grapevine fanleaf nepovirus cysteine protease (*31*) and picornavirus 3c proteinase (*32, 33*). Unfortunately, we could not determine whether Cys-60, Cys-409, or both residues of the CGHC motifs are responsible for the proteolytic activity by determining the loss of activity after changing a cysteine residue to an alanine residue, since neither the C60A nor the C409A enzyme could be obtained. Intact C60A or C409A ER-60 protease was not expressed in *E. coli*, but small fragments immunoreactive to the anti-ER-60 protease serum were obtained. The intact proteins are probably unstable and rapidly degraded in this expression system. Preparation of an intact C60A or C409A enzyme will be necessary to elucidate the reaction mechanism.

The mouse ER-60 showed the proteolytic activity (*34*) as well as the rat ER-60 protease, when PDI was used as the substrate. However, the recombinant human ER-60 protein from a baculovirus expression system did not significantly degrade PDI (*29*), unlike the recombinant enzyme from the *E. coli* expression system. The reason for this discrepancy is unclear. However, the possibility cannot be excluded that the activity from the baculovirus expression system was modified or inactivated during the purification procedures, where protease inhibitors were not included.

The CGHC motif appears to be multifunctional. It was reported that cysteine residues in CGHC motifs are responsible for a thiol-dependent reductase activity of ER-60 (*12*). This was shown by substituting serine residues for all of the cysteine residues in both motifs. However, the role of each cysteine residue (Cys57, 406, 60, and 409) remains unknown.

There have been conflicting reports concerning the function of the C-terminal QEDL sequence (*11, 12*). Thus, it is uncertain whether QEDL acts as an ER-retention signal for ER-60 protease. This uncertainty may be due to the poor kinetics of the secretion of ER-60 protease into the medium. In this study, it was shown that the C-terminal tetrapeptide of ER-60 protease, QEDL, functions as a retention signal in ER. However, on replacing KDEL of PDI and KEEL of ERp72, QEDL failed to act as a ER-retention signal for these proteins (*21*). Thus, QEDL was assumed to be a non-functional tetrapeptide for PDI and ERp72, but to be functional for ER-60 protease. The possibility cannot be excluded that the QEDL sequence changed protein molecular structures of PDI and ERp72, and thereby interfered with the association of the QEDL sequence with the retention machinery. Because QEDL could not act as an ER-retention signal for PDI and ERp72, the complementary function of a region(s) other than the C-terminal tetrapeptide may be responsible for the retention of ER-60 protease in ER. Hence, it seems likely that the QEDL sequence serves as an ER-retention signal in cooperation with other amino acid residue(s) in ER-60 protease.

Finally, microsomal ER-60 protease was shown to be a cysteine protease with characteristic functional CGHC and QEDL motifs.

REFERENCES

1. Hurtley, S.M. and Helenius, A. (1989) Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol.* **5**, 277-307
2. Rothman, J.E. (1989) Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. *Cell* **59**, 591-601
3. Gething, M.-J. and Sambrook, J. (1992) Protein folding in the cell. *Nature* **355**, 33-45
4. Klausner, R.D. and Sitia, R. (1990) Protein degradation in the endoplasmic reticulum. *Cell* **62**, 611-614
5. Bonifacino, J.S. and Lippincott-Schwartz, J. (1991) Degradation of proteins within the endoplasmic reticulum. *Curr. Opin. Cell Biol.* **3**, 592-600
6. Urade, R., Nasu, M., Moriyama, T., Wada, K., and Kito, M. (1992) Protein degradation by the phosphoinositide-specific phospholipase C-α family from rat liver endoplasmic reticulum. *J. Biol. Chem.* **267**, 15152-15159
7. Urade, R. and Kito, M. (1992) Inhibition by acidic phospholipids of protein degradation by ER-60 protease, a novel cysteine protease, of endoplasmic reticulum. *FEBS Lett.* **312**, 83-86
8. Lewis, M.J., Mazzarella, R.A., and Green, M. (1986) Structure and assembly of the endoplasmic reticulum: Biosynthesis and intracellular sorting of ERp61, ERp59, and ERp49, three protein components of murine endoplasmic reticulum. *Arch. Biochem. Biophys.* **245**, 389-403
9. Mobbs, C.V., Fink, G., and Pfaff, D.W. (1990) HIP-70: An isoform of phosphoinositol-specific phospholipase C-α. *Science* **249**, 566-567
10. Srivastava, S.P., Chen, N., Liu, Y., and Holtzman, J.L. (1991) Purification and characterization of a new isozyme of thiol: protein-disulfide oxidoreductase from rat hepatic microsomes. *J. Biol. Chem.* **266**, 20337-20344
11. Mazzarella, R.A., Marcus, N., Haugejorden, S.M., Balcarek, J.M., Baldassare, J.J., Roy, B., Li, L., Lee, A.S., and Green, M. (1994) ERp61 is GRP58, a stress-inducible luminal endoplasmic reticulum protein, but is devoid of phosphatidylinositide-specific phospholipase C activity. *Arch. Biochem. Biophys.* **308**, 454-460
12. Hirano, N., Shibasaki, F., Kato, H., Sakai, R., Tanaka, T., Nishida, J., Yazaki, Y., Takenawa, T., and Hirai, H. (1995) Molecular cloning of the human glucose-regulated protein ERp57/GRP58, a thiol-dependent reductase. *Eur. J. Biochem.* **234**, 336-342
13. Oliver, J.D., van der Wal, F.J., Bulleid, N.J., and High, S. (1997) Interaction of the thiol-dependent reductase ERp57 with nascent glycoproteins. *Science* **275**, 86-88
14. Otsu, M., Urade, R., Kito, M., Omura, F., and Kikuchi, M. (1995) A possible role of ER-60 protease in the degradation of misfolded proteins in the endoplasmic reticulum. *J. Biol. Chem.* **270**, 14958-14961
15. Kito, M. and Urade, R. (1995) Role of novel microsomal cysteine proteases. *Proc. Jpn. Acad.* **71B**, 189-192
16. Rawlings, N.D. and Barrett, A.J. (1994) Families of cysteine peptidase. *Methods Enzymol.* **244**, 461-486
17. Urade, R., Takenaka, Y., and Kito, M. (1995) Protein degradation by ERp72 from rat and mouse liver endoplasmic reticulum. *J. Biol. Chem.* **268**, 22004-22009
18. Mazzarella, R.A., Srinivasan, M., Haugejorden, S.M., and Green, M. (1990) ERp72, an abundant luminal endoplasmic reticulum protein, contains three copies of the active site sequence of protein disulfide isomerase. *J. Biol. Chem.* **265**, 1094-1101
19. Munro, S. and Pelham, H.R.B. (1986) An Hsp70-like protein in the ER: Identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell* **46**, 291-300
20. Munro, S. and Pelham, H.R.B. (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48**, 899-907
21. Haugejorden, S.M., Srinivasan, M., and Green, M. (1991) Analysis of the retention signals of two resident luminal endoplasmic reticulum proteins by *in vitro* mutagenesis. *J. Biol. Chem.* **266**, 6015-6018
22. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
23. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
24. Ito, W., Ishiguro, H., and Kurosawa, Y. (1991) A general method for introducing a series of mutations into cloned DNA using the polymerase chain reaction. *Gene* **102**, 67-70
25. Chen, C. and Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**, 2745-2752
26. Chen, C.A. and Okayama, H. (1988) Calcium phosphate-mediated gene transfer: A highly efficient transfection system for stably transforming cells with plasmid DNA. *BioTechniques* **6**, 632-638
27. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
28. O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007-4021
29. Bourdi, M., Demady, D., Martin, J.L., Jabbour, S.K., Martin, B.M., George, J.W., and Pohl, L.R. (1995) cDNA cloning and baculovirus expression of the human liver endoplasmic reticulum p58: Characterization as a protein disulfide isomerase, but not as a protease or a carnitine acyltransferase. *Arch. Biochem. Biophys.* **323**, 397-403
30. Charnock-Jones, D., Day, K., and Smith, S.K. (1996) Cloning, expression and genomic organization of human placental protein disulfide isomerase. *Int. J. Biochem. Cell Biol.* **28**, 811-889
31. Margis, R. and Pinck, L. (1992) Effects of site-directed mutagenesis on the presumed catalytic triad and substrate-binding pocket of grapevine fanleaf nepovirus 24-kDa proteinase. *Virology* **190**, 884-888
32. Kean, K.M., Howell, M.T., Grunert, S., Girard, M., and Jackson, R.L. (1993) Substitution mutations at the putative catalytic triad of the poliovirus 3C protease have differential effects on cleavage at different sites. *Virology* **194**, 360-364
33. Miyashita, K., Kusumi, M., Utsumi, R., Katayama, S., Noda, M., Komano, T., and Satoh, N. (1993) Site-directed mutagenesis of the putative active site residues of 3C proteinase of coxsackievirus B3: Evidence of a functional relationship with trypsin-like serine proteinase. *Protein Eng.* **6**, 189-193
34. Kozaki, K., Miyaishi, O., Asai, N., Iida, K., Sakata, K., Hayashi, M., Nishida, T., Matsuyama, M., Shimizu, S., Kaneda, T., and Saga, S. (1994) Tissue distribution of ERp61 and association of its increased expression with IgG production in hybridoma cells. *Exp. Cell Res.* **213**, 348-358