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Accumulation and Degradation in the Endoplasmic Reticulum of a Truncated ER-60 Devoid of C-Terminal Amino Acid Residues[1]

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The accumulation and degradation in the endoplasmic reticulum (ER) of a truncated ER-60 protease, from which the C-terminal 89 amino acid residues have been deleted (K 417 ochre), was examined. K 417 ochre overexpressed in COS-1 cells is not secreted into the medium, but accumulates as insoluble aggregates in non-ionic detergent without degradation in unusual clump membrane structures. K 417 ochre, stably expressed, forms soluble aggregates in non-ionic detergent and is distributed in the reticular structures of ER. Under these conditions, K 417 ochre is not secreted into the medium but is degraded with a half-life time of more than 8 h. Since K 417 ochre/C all S, in which all the Cys residues of K 417 ochre are replaced by Ser, also forms aggregates, an inter-disulfide bond appears unnecessary for aggregation. In both types of aggregates, Ig heavy chain binding protein, calnexin, glucose regulated protein 94, calreticulin, ERp72, and protein disulfide isomerase are scarcely found. Since degradation of the stably expressed K 417 ochre was not inhibited by lactacystin, leupeptin, NH_4Cl, or cytocharasin B, but was inhibited by *N*-acetyl-leucyl-leucyl-norleucinal, the self-aggregated abnormal protein in the lumen of ER is assumed to be degraded by an unknown protease system other than proteasome, lysosome or autophagy.

Key words: COS-1 cell, ER-60 protease, endoplasmic reticulum, misfolded protein aggregation, misfolded protein degradation.

Many proteins synthesized on polysomes of the rough endoplasmic reticulum (ER) interact with molecular chaperones to fold properly and assemble in the lumen of ER. Most proteins that fail to attain the correct conformation are retained and degraded in the ER. In many cases, the ER degradation process is found to be linked to the cytoplasmic proteasome (*1*). For instance, cystic fibrosis transmembrane conductance regulator, major histocompatibility complex class I molecules, the α-subunit of T cell receptor and ribophorin I have been reported to be degraded by the proteasome after their retrograde transport to the cytosol *via* Sec 61/63 (*2–11*). However, the proteasome-independent degradation of misfolded proteins has also been reported. The misfolded rat nerve growth factor receptor expressed in yeast is degraded in an energy-dependent but proteasome-independent manner (*12*). Mutant P-glycoprotein and the transmembrane domain of human amyloid precursor protein are degraded by protease(s) other than the proteasome (*13*, *14*). Furthermore, an unknown protease(s) localized in the ER may participate in the degradation of apolipoprotein B-100 (*15–17*), stearoyl-CoA desaturase (*18*), and 3-hydroxy-3-methylglutaryl-CoA reductase (*19*, *20*), the degradation of which is controlled by lipids. Molecular chaperones such as Ig heavy chain binding protein (BiP), glucose regulated protein 94 (Grp 94) and calnexin are known to play important roles in the retention of proteins to be degraded on the ER and the retrograde transport of these proteins from the ER to the cytosol (*11*, *21–27*). For recognition of these proteins by molecular chaperones, Asn-linked oligosaccharide modification (*28–30*) and Cys residue(s) of the proteins (*31*) are targeted, since most proteins synthesized in ER are *N*-glycosylated and constructed through disulfide bonds. However, the detailed mechanism underlying the interaction of molecular chaperones with the proteins to be degraded, and the quality control of proteins deficient in glycosylation remain unclear.

ER-60 protease (ER-60) is non-glycosylated and retained in the lumen of ER with a C-terminal retention signal. It has multiple functions including a proteolytic activity (*32–34*) and a protein disulfide isomerase (PDI) activity enhanced by calnexin (*35*, *36*). We report that a stably expressed or overexpressed C terminally truncated variant of ER-60 devoid of the retention signal is retained as an abnormal protein in the lumen of the ER or unusual mem-

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Abbreviations: ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; BFA, brefeldin A; BiP(Grp78), Ig heavy chain binding protein; β-ME, β-mercaptoethanol; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-propanesulfonic acid; DMEM, Dulbecco's minimum essential medium; DSP, 3,3′-dithio bis-succinimidyl propionate; Endo H, endoglycosidase H; ER, endoplasmic reticulum; ER-60, ER-60 protease; Grp 94, glucose regulated protein 94; PBS, phosphate-buffered saline; PDI, protein disulfide isomerase; PVDF, polyvinylidene difluoride.

brane structures, respectively, and that this stably expressed variant protein is degraded in the ER by some protease(s) other than the proteasome.

EXPERIMENTAL PROCEDURES

Materials—The EXPLE ^{35}S ^{35}S protein labeling mix (37.0 TBq/mmol) and Enlightning were purchased from Du Pont, NEN, MA. The Dye Termination Cycle Sequencing Kit was from Perkin Elmer, Applied Biosystems Division, CT. *Tli* DNA polymerase, goat HRP-anti-rabbit Ig serum and goat HRP-anti-mouse Ig serum were purchased from Promega, WI. Mouse anti-Grp 78 (BiP) monoclonal and rabbit anti-calnexin-C serum were obtained from Stressgen Biotechnologies, BC. Goat biotin-anti-rabbit Ig serum, goat biotin-anti-mouse Ig serum, tetramethylrhodamine-conjugated wheat germ agglutinin and fluorescein isothiocyanate–conjugated concanavalin A were from Vector, CA. Sheep Texas red-anti-mouse Ig serum and Cy5-streptavidin were obtained from Amersham Pharmacia Biotech., Uppsala. Goat colloidal gold-anti-rabbit Ig serum was purchased from British BioCell International Limited, London. Pansorbin and lactacystin were purchased from Calbiochem-Novabiochem, CA. Protein A–Sepharose CL-4B (binding capacity, approx. 20 mg human IgG/ml) was purchased from Sigma, MO. Polyvinylidene difluoride (PVDF) protein sequencing membranes and X-ray film, X-OMAT AR, were obtained from Bio-Rad, CA and Eastman Kodak, NY, respectively. BSA was purchased from Miles Laboratories, IL. Geneticin (G418) was from Gibco BRL, MD. Brefeldin A (BFA) and tunicamycin were obtained from Wako Pure Chemical Industries, Osaka. *N*-Acetyl-leucyl-leucyl-norleucinal (ALLN) and endoglycosidase H (Endo H) were purchased from Boehringer Mannheim, Mannheim. Leupeptin was obtained from the Peptide Institute, Osaka. Cytochalasin B was purchased from Aldrich Chem., WI. All other chemicals were of reagent grade.

Expression Plasmids—pcDNA3-rER60 and pcDNA3-Q 502 ochre were the plasmids used for the expression of the wild type and deletion mutant lacking the C-terminal tetrapeptide of rat ER-60 in mammalian cells (*34*). For the generation of an ER-60 protease C-terminal mutant, K 417 ochre (Fig. 1A), PCR was performed with pcDNA3-rER60 as a template, a forward primer corresponding to nucleotide sequence 967–991 of rat ER-60 cDNA (*37*), and reverse primers containing the mutation and a *Xho*I sites. The PCR fragments were digested with *Nhe*I and *Xho*I, and then ligated between the *Nhe*I and *Xho*I restriction sites of pcDNA3-rER60. For the generation of a Cys mutant (K 417 ochre/C all S) (Fig. 4A), PCR was carried out using K 417 ochre as a template and primers designed to change Cys-57, Cys-60, Cys-85, Cys-92, Cys-244, Cys-406, and Cys-409 to Ser. The obtained PCR fragments were digested with *Eco*RI and *Xho*I, and then ligated between the *Eco*RI and *Xho*I restriction sites of pcDNA3. The nucleotide sequences of all mutants were confirmed by the fluorescence dideoxy chain termination method (Perkin Elmer, Applied Biosystems).

Expression of Recombinant Rat ER-60 Proteases in COS-1 Cells—COS-1 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 24-h incubation, the cells were transfected with expression plasmids (25 μg/100-mm petri dish) for 20 h under a 2% CO_2 atmosphere by the method of Chen and Okayama (*38*, *39*). After 24-h incubation in 10% serum/DMEM under a 5% CO_2 atmosphere, the cells were used in experiments. Transfectant clones were selected in DMEM containing 400 μg/ml G418 and 10% serum for 3 weeks, and confirmed to express the mutant rat ER-60 by Western blotting with anti–rat ER-60F serum, which crossreacts with rat ER-60 but not with the endogenous monkey ER-60 of COS-1 cells (*32*, *34*). One of the several transfectants obtained, which expressed the largest quantity of K 417 ochre or K 417 ochre/C all S, was plated at 5.3×10^4 cells/cm^2 in 10% serum/DMEM, incubated for 24 h, and used in experiments.

Metabolic Labeling and Immunoprecipitation—Cells were grown in a 12-well plate. The transfected cells were washed with serum-free DMEM, and then preincubated for 1 h in Met- and Cys-free DMEM containing 5% dialyzed serum. The preincubated cells were metabolically labeled with 0.4 ml of DMEM containing 40 μCi each of [^{35}S]Met and Cys and 5% dialyzed serum for 30 min at 37°C, after which the radioactive medium was replaced with 0.4 ml of complete medium containing Met, Cys, and 10% serum, and the labeled cells were incubated for the indicated times. BFA (5 μg/ml), ALLN (150 μg/ml), lactacystin (40 μM), leupeptin (100 μM), or NH_4Cl (30 μM) was added to the medium 1 h prior to preincubation and supplemented until the end of the chase. NaN_3 (2.5 mM) was added to the chase medium. Cytochalasin B (70 μM) was added to the labeling and chase media. After removal of the medium, the cells were washed three times with ice-cold phosphate-buffered saline (PBS), suspended in 0.1 ml of lysis solution comprising 1% SDS and 50 mM DTT, and then boiled for 5 min. The medium (0.4 ml) was also boiled in 1% SDS and 50 mM DTT. The boiled cell lysate and medium were diluted with 1.9 or 7.6 ml of lysis buffer comprising 1% Nonidet P-40, 150 mM NaCl, and 50 mM Tris/HCl buffer, pH 7.5, respectively. In other experiments, cells were dissolved in 1% Nonidet P-40, 0.05% SDS, 150 mM NaCl, and 50 mM Tris/HCl buffer, pH 7.5, for 30 min at 4°C. The cell lysate and medium were incubated with Pansorbin for 20 min on ice, and then centrifuged at 9,000 ×*g* for 10 min at 4°C. The precleared cell lysate or medium was supplemented with anti-rat ER-60F serum and BSA (3%), and then incubated at for 16 h 4°C. The immunocomplexes were collected on Protein A–Sepharose (50% suspension in PBS) with shaking for 1 h, and then washed four times with 0.05% Tween 20 in PBS. The immunocomplexes were analyzed by 10% SDS-PAGE (*40*). The gels were fixed, equilibrated in a mixture of Enlightning, ethanol, and distilled water (5:2:3), and dried before fluorography.

Sucrose Density Gradient Centrifugation—Cells were grown in 100-mm petri dishes. The transfected cells were washed with PBS, collected and then allowed to stand in PBS in the presence or absence of 1 mM 3,3'-dithio bis-succinimidyl propionate (DSP) for 30 min at 4°C. DSP was then inactivated by the addition of glycine (50 mM). The cells were lysed in 1 ml of 2% 3-[(3-cholamidopropyl)dimethyl-ammonio]propanesulfonic acid (CHAPS)/0.2 M NaCl/50 mM HEPES buffer, pH 7.5, containing 0.2 mg/ml soybean trypsin inhibitor and leupeptin for 30 min at 4°C, and then centrifuged at 572 ×*g* for 5 min. The cell extract was overlayed on a 12 ml 5–25% sucrose density gradient containing 0.5% CHAPS, 0.2 M NaCl, and 50 mM HEPES

buffer, pH 7.4, and centrifuged at 100,000 ×*g* for 20 h at 4°C. Twelve fractions were collected from the bottom of the gradient. The proteins in each fraction were precipitated with 10% trichloroacetic acid for 30 min at 4°C, separated by SDS-PAGE under reducing conditions, and then blotted onto a PVDF membrane. The blotted proteins were immunostained with anti–rat ER-60F serum, anti–human ER-60 serum, which crossreacts with both rat and monkey ER-60 (*34*), anti–BiP monoclonal antibodies, which crossreact with BiP and Grp 94, or anti–calnexin-C serum, using Renaissance Chemiluminescence Reagent (Du Pont NEN).

Detection of Intermolecular Disulfide Bonds—Cells were grown in 100-mm petri dishes. The transfected cells were washed with PBS and then treated with 20 mM *N*-ethylmaleimide/PBS for 10 min at 4°C to block free sulfhydryl groups in the proteins. Sample preparation and 10% SDS-PAGE were performed in the presence or absence of β-mercaptoethanol (β-ME) as a reductant. The proteins separated on the gel were blotted onto a PVDF membrane, and then immunostained with anti–ER-60F serum, using Renaissance Chemiluminescence Reagent.

Endo H Treatment—Cells were grown in 100-mm petri dishes. The transfected cells were washed with PBS and then solubilized by boiling in 0.2% SDS/0.1 M acetate buffer, pH 5.5, for 5 min. The lysate was diluted with a fourfold volume of 0.1 M acetate buffer, pH 5.5, and then incubated in the presence of 20 mU of Endo H at 37°C overnight.

Laser Scanning Immunofluorescence Microscopy—COS-1 cells were grown on polylysine-coated cover glasses. The transfected cells were fixed with 2% formaldehyde and 0.1% glutalaldehyde for 15 min, permeabilized with 1% Triton X-100 in PBS for 10 min, and then treated with 1 mg/ml $NaBH_4$. The cells were then triple-stained with rabbit anti–rat ER-60F serum, biotin-anti–rabbit Ig serum and Cy 5-streptavidin for the rat ER-60 protein, tetramethylrhodamine-conjugated wheat germ agglutinin for the Golgi apparatus, and fluorescein isothiocyanate-conjugated concanavalin A for ER. In other experiments, the fixed cells were triple-stained with anti–rat ER-60F serum, biotin-anti-rabbit Ig serum and Cy 5-streptavidin, fluorescein isothiocyanate-conjugated concanavalin A, and anti-BiP monoclonal antibodies and sheep Texas red-anti–mouse Ig serum. The specimens were examined with a MRC-1024 laser scanning confocal imaging system (Bio-Rad).

Electron Microscopy—Cells were grown in 100-mm petri dishes. The transfected cells were collected and fixed with 2% glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.4, for 1.5 h at 4°C. The samples were postfixed with 1% OsO_4/50 mM sucrose/50 mM sodium phosphate buffer, pH 7.4, dehydrated, and then embedded in resin. Ultrathin sections were stained with uranyl acetate followed by lead citrate. The specimens were viewed under an electron microscope (model H-700H, Hitachi, Tokyo).

Protein A–Immunogold Electron Microscopy—A cell pellet was fixed with 1% glutaraldehyde/0.1 M sodium phosphate buffer, pH 7.4, for 30 min at 4°C, and frozen ultramicrotomy was performed as described by Tokuyasu (*41*). The sections were blocked with 1% BSA in 0.1 M sodium phosphate buffer, pH 7.4 (BSA solution), for 10 min and then reacted with anti–rat ER-60F serum diluted with the BSA solution for 30 min. The sections were washed three times with the BSA solution and then reacted with colloidal gold-anti-rabbit Ig serum (10 nm in diameter) for 30 min. After washing with 0.1 M sodium phosphate buffer, the sections were stained with uranyl acetate and viewed.

RESULTS

Accumulation and Degradation of a C-Terminally Truncated Variant of ER-60 (K 417 Ochre)—The wild type and C-terminally truncated rat ER-60 proteins (Fig. 1A) were

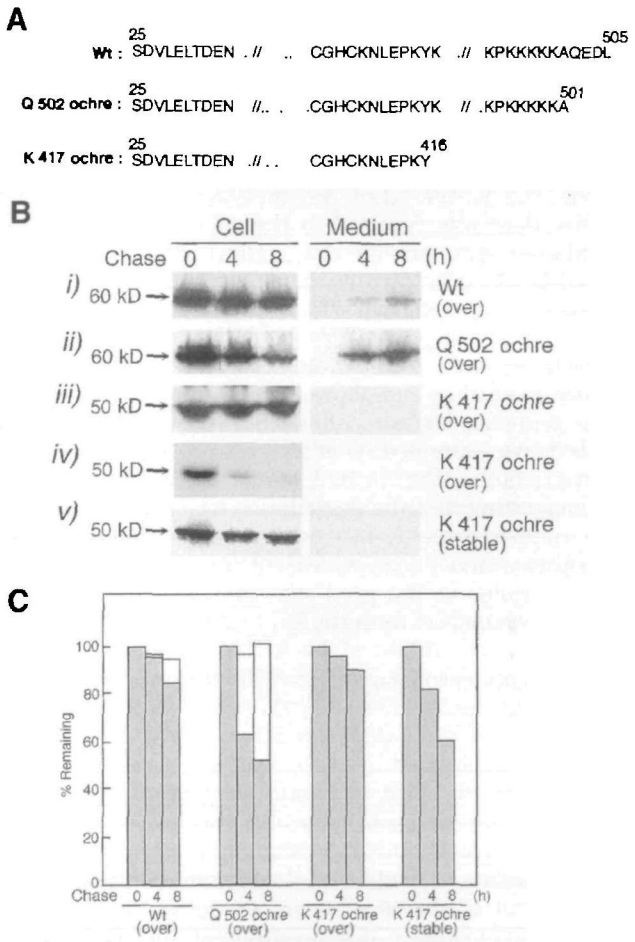

Fig. 1. **Effects of C-terminal truncation on the secretion and retention of ER-60. (A)** Wild type rat ER-60 (Wt) after removal of the N-terminal signal peptide (amino acids 1–24) is composed of 481 amino acid residues. Q 502 ochre is a mutant ER-60 from which the C-terminal retention signal, QEDL, has been deleted. K 417 ochre is a mutant ER-60, from which the C-terminal 89 amino acid residues have been deleted. **(B)** COS-1 cells were transfected with expression plasmids. The cells (over) that overexpressed wild type or mutant rat ER-60, and the cloned cells (stable) that were stably transfected with the expression plasmid for K 417 ochre, were labeled with [^{35}S]Met and Cys for 30 min, and then chased as described under "EXPERIMENTAL PROCEDURES." Cells were boiled in 1% SDS and 50 mM DTT for 5 min (i, ii, iii, and v) or dissolved in 1% Nonidet P-40 buffer for 30 min at 4°C (iv). The cell lysate and medium were immunoprecipitated with anti-rat ER-60F serum, and then subjected to SDS-PAGE and fluorography. **(C)** The amounts of radiolabeled wild type or mutant rat ER-60 recovered in the cell lysate (gray columns) and medium (white columns), were calculated from the values obtained by scanning the fluorogram (i, ii, iii, v) shown in (B) with a densitometer, AE-6920 (ATTO, Tokyo), as follows: [ER-60 in cell or medium at chase time 4 or 8 h/ER-60 in cells at 0 time] × 100.

overexpressed in COS-1 cells. After a pulse-chase, the proteins were immunoprecipitated with anti–rat ER-60F serum, which can crossreact with rat ER-60 but not with monkey ER-60 from COS-1 cells (*34*), and then analyzed by SDS-PAGE and fluorography. Q 502 ochre, from which only the C-terminal retention signal (*34*), QEDL, is deleted, was rapidly secreted into the medium (Fig. 1B, ii). About 45% of the Q 502 ochre synthesized during the pulse time was secreted within 8 h (Fig. 1C). However, K 417 ochre, from which the C-terminal 89 amino acid residues are deleted, was not secreted but retained in cells, although QEDL was absent (Fig. 1B, iii, and C). Overexpressed K 417 ochre could not be solubilized with a non-ionic detergent, Nonidet P-40, 4 h after the chase, but was solubilized by boiling with SDS (Fig. 1B, iii and iv), in contrast with overexpressed wild type rat ER-60, which could be solubilized with Nonidet P-40 anytime after the chase (data not shown). The K 417 ochre resistant to solubilization with Nonidet P-40 was retained in the cells thereby escaping degradation after an 8-h chase. However, the stably expressed K 417 ochre in the transfectant clone cells, whose expression in a single cell was about 0.5% that of the overexpressed form, as judged by calculating densities on Western blots, was not secreted, but could be solubilized with Nonidet P-40 (data not shown). In addition, about 40% of the protein was degraded after a 8-h chase (Fig. 1B, v, and C). Next, we examined whether protease inhibitors affect this degradation (Fig. 2). BFA did not inhibit the degradation, suggesting that the degradation may occur in the pre-Golgi compartment. It has been reported that misfolded polypeptides and unassembled subunits are degraded by the proteasome in the pre-Golgi compartment after their retrograde transport from the ER to the cytosol (*1–11*). The degradation was inhibited by ALLN which is known to inhibit the proteasome and intracellular cysteine proteases. However, the degradation of K 417 ochre was not inhibited by lactacystin, a proteasome-specific inhibitor. The degradation was not inhibited by NaN_3 suggesting the reaction is energy-independent. In addition, cytochalasin B, an autophagy inhibitor (*42*), and leupeptin and NH_4Cl, lysosomal inhibitors, did not inhibit the degradation of K 417 ochre. Hence, it seems unlikely that the degradation was caused by lysosomal proteases after autophagy of the ER. From these lines of evidence, it was considered that K 417 ochre might be degraded through a new degradation pathway, including an ALLN-sensitive protease(s).

Aggregation of K 417 Ochre—It has been suggested that K 417 ochre is an abnormal protein that forms aggregates in cells, since this protein is retained in cells despite its lack of a retention signal. Then, the size of K 417 ochre was determined by comparing it with wild type ER-60 by the sucrose density gradient centrifugation method. The endogenous monkey ER-60 in sham-transfected cells, which was detected by immunostaining with anti–human ER-60 serum (*34*), was present as a monomer in fractions 1–6 (Fig. 3A, i). The overexpressed rat wild type ER-60 showed a profile similar to that of monkey ER-60 (Fig. 3A, iii). Only part of the monkey and rat wild type ER-60 sedimented as a pellet, even after crosslinking by DSP treatment (Fig. 3A, ii and iv). However, a notable amount of overexpressed K 417 ochre was recovered as a pellet without DSP treatment (Fig. 3A, v). In cells overexpressing K 417 ochre, a large amount of the protein produced may form insoluble aggregates. Thus, 79% of the overexpressed K 417 ochre extracted with 2% CHAPS was recovered in the pellet after centrifugation at 100,000 ×*g* for 30 min. With DSP treatment, the amount of K 417 ochre recovered in the pellet fraction increased (Fig. 3A, vi). This suggests that overexpressed K 417 ochre basically exists as aggregates in cells. The aggregates are formed either through a hydrophobic interaction, since 2% CHAPS solubilizes part of the aggregates to yield monomers (Fig. 3A, v), or through disulfide bonds since oligomers can be detected on SDS-PAGE under non-reducing conditions (Fig. 3C, lane 4). Oligomers with inter disulfide bonds were mostly detected in the pellet fraction from the sucrose density gradient (data not shown). Since the overexpressed wild type ER-60 was present in the monomer region on SDS-PAGE under non-reducing conditions (Fig. 3C, lane 2), an inter disulfide bond appears not to be formed in the wild type ER-60. These findings suggest that it is not the overexpression itself but the unfolding of K 417 ochre that causes the formation of inter molecular disulfide bonds. Eighty-one percent of the stably expressed K 417 ochre extracted with 2% CHAPS was recovered in the supernatant after centrifugation at 100,000 ×*g* for 30 min. In addition, on the sucrose density gradient, most of the stably expressed K 417 ochre was recovered as a monomer, but shifted to fractions 7–10 following DSP treatment (Fig. 3A, vii and viii). Thus, it was assumed that most K 417 ochre may form soluble aggregates with non-covalent bonds in the case of low level expression, in contrast with in the case of overexpression as described above. In addition, part of the stably expressed K 417 ochre may form aggregates with inter molecular disulfide bonds, since oligomers were detected on SDS-PAGE under non-reducing conditions (Fig. 3C, lane 6). These oligomers were recovered in the pellet fraction from the sucrose density gradient (data not shown). The difference in the ratios of monomer and oligo-

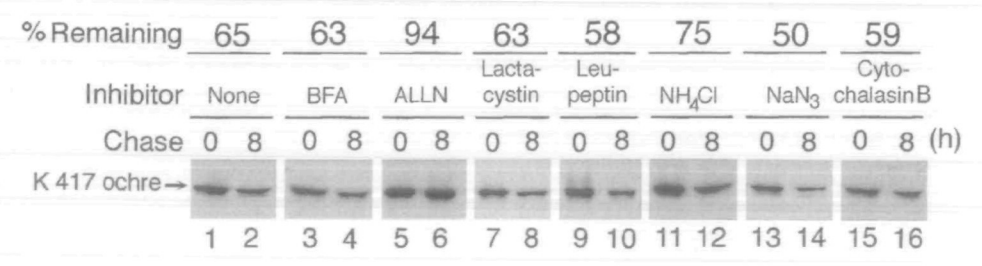

Fig. 2. **Effects of inhibitors on the degradation of stably expressed K 417 ochre in cells.** Cells stably transfected with the expression vector for K 417 ochre were labeled with [^{35}S]Met and Cys for 30 min and then chased for 8 h in the absence (lanes 1 and 2) or presence of inhibitors (lanes 3–16). Cells were boiled in 1% SDS and 50 mM DTT for 5 min, immunoprecipitated with anti–rat ER-60F serum, and then subjected to SDS-PAGE and fluorography as described under "EXPERIMENTAL PROCEDURES." The percentages of radioactive K 417 ochre remaining in the cells after an 8-h chase were calculated from the values obtained by scanning the fluorogram, as described in the legend to Fig. 1 and are shown at the top of the figure.

mers between the sucrose density gradient and non-reducing SDS-PAGE may be due to inaccuracy in mesasuring the intensity of the bands obtained by Western blotting using the chemiluminescence immunodetection reagents when high protein concentrations were blotted on a PVDF membrane as shown in Fig. 3C.

It is well known that the aggregates of unfolded polypeptides associate with BiP, Grp 94 or calnexin in ER (*11*, *21–27*). However, BiP, Grp 94, or calnexin did not concentrate in the pellets of the overexpressed K 417 ochre (Fig. 3B). Furthermore, calreticulin, PDI, and ERp72 were scarcely detected in the pellets of the overexpressed K 417 ochre (data not shown). A shift of BiP, Grp 94, calnexin, calreticulin, PDI, and ERp72 to fractions 7–10, in which soluble aggregates of stably expressed K 417 ochre are present, was not observed (data not shown). Probably, K 417 ochre may form soluble or insoluble aggregates alone without molecular chaperones.

There is a case in which a specific Cys residue(s) is used to regulate the retention and degradation of newly synthe-

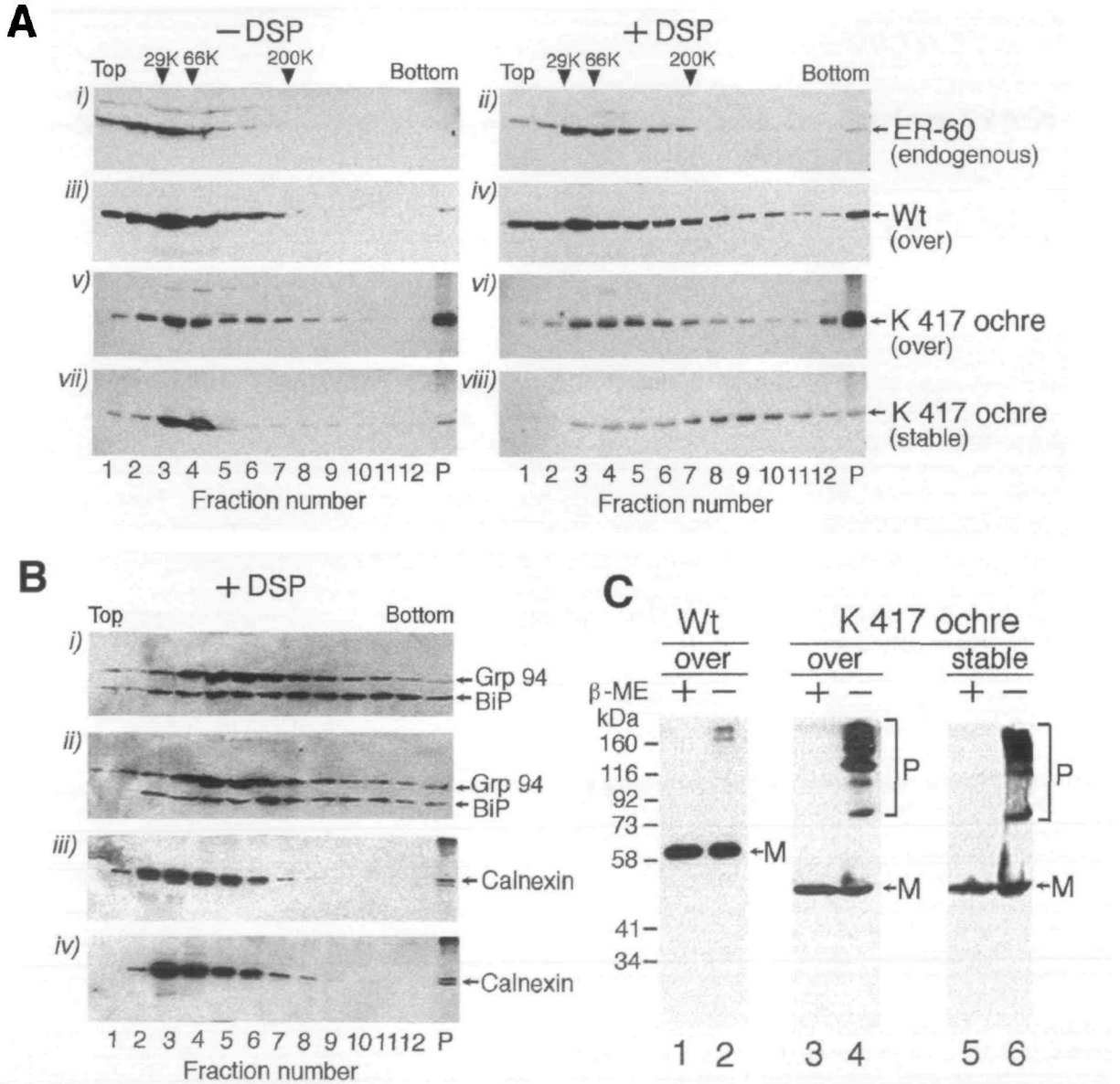

Fig. 3. **Aggregation of K 417 ochre in the cells.** (A) COS-1 cells were transfected with vector plasmids, pcDNA3 (i and ii), or the expression plasmids for wild type rat ER-60 (iii and iv) and K 417 ochre (v and vi). These cells and the stably transfected clone cells with the expression plasmid for K 417 ochre cells (vii and viii) were collected, treated with or without DSP, and dissolved in 2% CHAPS buffer. The cell lysate was fractionated by sucrose density gradient centrifugation and analyzed by Western blotting with anti-human ER-60 serum (i and ii) or anti-rat ER-60F serum (iii–viii) as described under "EXPERIMENTAL PROCEDURES." The positions of the molecular size markers, carbonic anhydrase (29 K), BSA (66 K) and β-amylase (200 K), are shown at the top of (i) (arrowheads). P, pellet. (B) Samples (iv) and (vi) in panel (A) were analyzed by Western blotting with anti-BiP monoclonal antibodies (i and ii) or anti-calnexin-C serum (iii and iv). (i) and (iii), cells overexpressing wild type rat ER-60. (ii) and (iv), cells overexpressing K 417 ochre. (C) Cells, overexpressing wild type rat ER-60 (lanes 1 and 2) or K417 ochre (lanes 3 and 4), or stably expressing K417 ochre (lanes 5 and 6), were subjected to SDS-PAGE in the presence (lanes 1, 3, 5) or absence of β-ME (lanes 2, 4, 6), and then analyzed by Western blotting with anti-rat ER-60F serum. P, polymerized K 417 ochre. M, monomer of wild type ER-60 or K 417 ochre.

sized polypeptides in the ER (*31*). Thus, the function of Cys residues in the retention and degradation of K 417 ochre in the ER was examined. An expression plasmid in which all the Cys residues of K 417 ochre were replaced by Ser residues (K 417 ochre/C all S) was prepared and expressed in COS-1 cells (Fig. 4A). K 417 ochre/C all S behaved as a monomer on SDS-PAGE, as expected, under non-reducing conditions (Fig. 4B, lane 1). However, the mobility of K 417 ochre/C all S was slower than that of K 417 ochre (Fig. 4B, lanes 2 and 3). This was due to the *N*-glycosylation of Asn90 in a consensus sequence for *N*-glycosylation newly formed by the replacement of Cys92 with a Ser residue (Fig. 4A). The size of the expressed K 417 ochre/C all S was reduced to that of the unmodified K 417 ochre by Endo H treatment (Fig. 4B, lanes 5 and 6). Thus, it appears that K 417 ochre/C all S was not transported to the Golgi apparatus, since the oligosaccharide was sensitive to Endo H. In addition, the size of K 417 ochre/C all S expressed in tunicamycin-treated cells was the same as that of K 417 ochre (Fig. 4B, lane 8). Most overexpressed K 417 ochre/C all S in tunicamycin-treated cells formed insoluble aggregates (Fig. 4C, ii). Similarly, overexpressed K 417 ochre/C all S in the absence of tunicamycin formed insoluble aggregates (Fig. 4C, i). In the case of stably transfected cells, part of the sta-

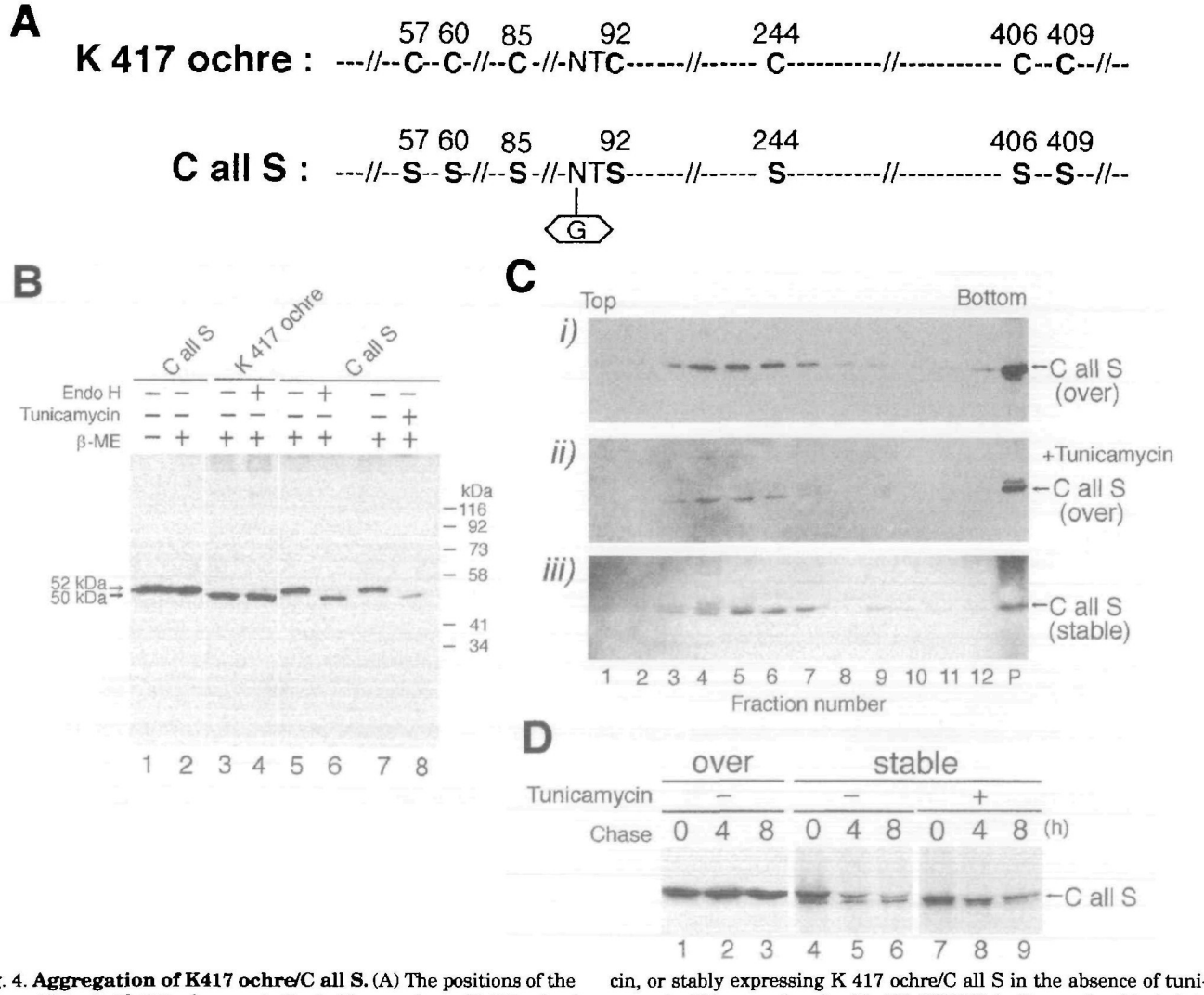

Fig. 4. **Aggregation of K417 ochre/C all S.** (A) The positions of the Cys residues in K 417 ochre are indicated by numbers. K 417 ochre/C all S (C all S) is a mutant ER-60 in which the seven Cys residues of K 417 ochre have been replaced with Ser. G indicates an *N*-linked oligosaccharide. (B) Cells were transfected with the expression plasmid for K 417 ochre (lanes 3 and 4) or K 417 ochre/C all S (lanes 1, 2, and 5–8) for 20 h, and then incubated in the presence (lane 8) or absence of 1 μg/ml tunicamycin (lanes 1–7) in DMEM for 24 h at 37°C. Cellular proteins treated (lanes 4 and 6) or non-treated (lanes 1–3, 5, 7, 8) with Endo H were separated by SDS-PAGE in the presence (lanes 2-8) or absence (lane 1) of β-ME, and then analyzed by Western blotting with anti–rat ER-60F serum as described under "EXPERIMENTAL PROCEDURES." (C) Cells overexpressing K 417 ochre/C all S in the presence (ii) or absence (i) of 1 μg/ml tunicamycin, or stably expressing K 417 ochre/C all S in the absence of tunicamycin (iii), were lysed with 2% CHAPS buffer, and subjected to sucrose density gradient centrifugation as described under "EXPERIMENTAL PROCEDURES." Fractions were analyzed by Western blotting with anti-rat ER-60F serum. P, pellet. (D) Cells, overexpressing K 417 ochre/C all S in the absence of tunicamycin (lanes 1–3) or stably expressing K 417 ochre/C all S in the presence (lanes 7–9) or absence (lanes 4–6) of tunicamycin, were labeled with [^{35}S]Met and Cys for 30 min, and then chased as described under "EXPERIMENTAL PROCEDURES." Cells were boiled in 1% SDS and 50 mM DTT for 5 min. The cell lysates were immunoprecipitated with anti–rat ER-60F serum, and then subjected to SDS-PAGE and fluorography.

bly expressed K 417 ochre/C all S was recovered in the pellet (Fig. 4C, iii). These results suggest that the aggregation of K 417 ochre/C all S is independent of *N*-glycosylation, and that Cys residues are not necessary for the formation of aggregates. No concentration of molecular chaperons such as BiP, calnexin, Grp 94, calreticulin, PDI and ERp72 in the aggregates of K 417 ochre/C all S was observed (data not shown), suggesting that the self-aggregation of K 417 ochre/C all S occurs similar to the case of K 417 ochre. The stably expressed K 417 ochre/C all S was degraded independent of glycosylation (Fig. 4D, lanes 4–9), whereas the overexpressed form was not degraded in the cells (lanes 1–3). No secretion of either the stably expressed or overexpressed K 417 ochre/C all S into the medium was detected (data not shown).

Localization of K 417 Ochre in Cells—The overexpressed K 417 ochre was localized in clump structures adjacent to the nucleus (Fig. 5E). These clump structures were stained

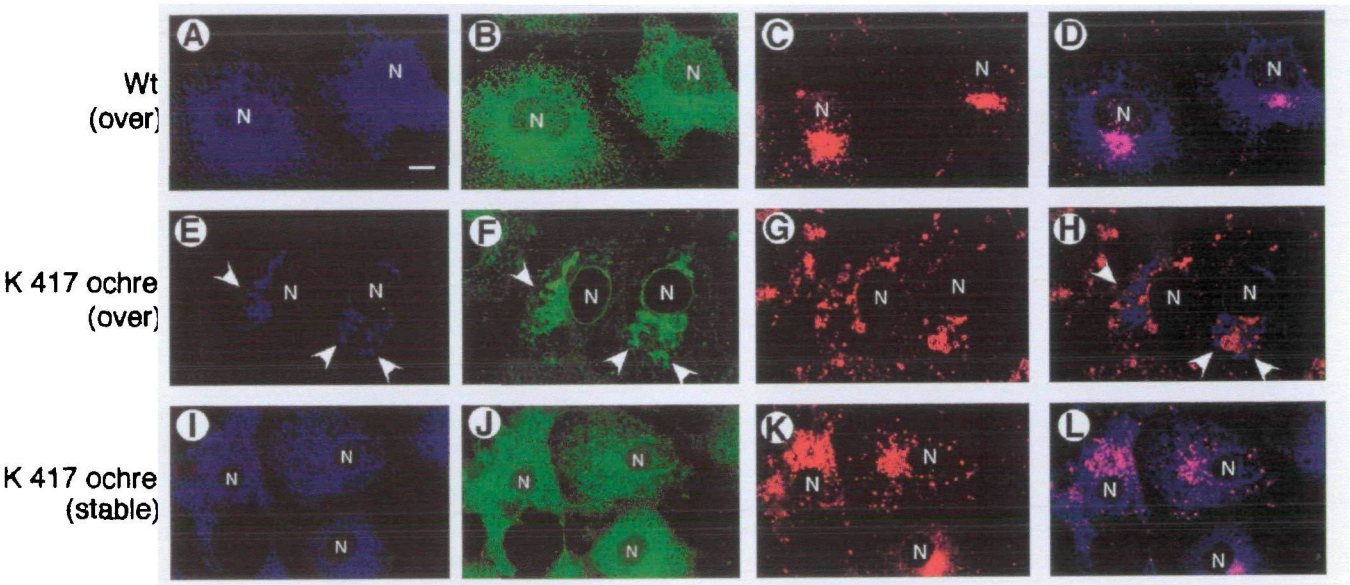

Fig. 5. **Immunofluorescence staining of wild type ER-60 or K 417 ochre.** Cells overexpressing wild type rat ER-60 (A–D) or K 417 ochre (E–H), or stably expressing K 417 ochre (I–L), were triple-stained with anti–rat ER-60F serum (A, E, I), fluorescein isothiocyanate-conjugated concanavalin A (B, F, J), and tetramethylrhodamine-conjugated wheat germ agglutinin (C, G, K), as described under "EXPERIMENTAL PROCEDURES." Overexpressed K 417 ochre was localized in unusual clump structures (arrowheads). (D), (H), and (L) are merged images of (A) and (C), (E) and (G), and (I) and (K), respectively. N, nucleus. Bar, 10 μm.

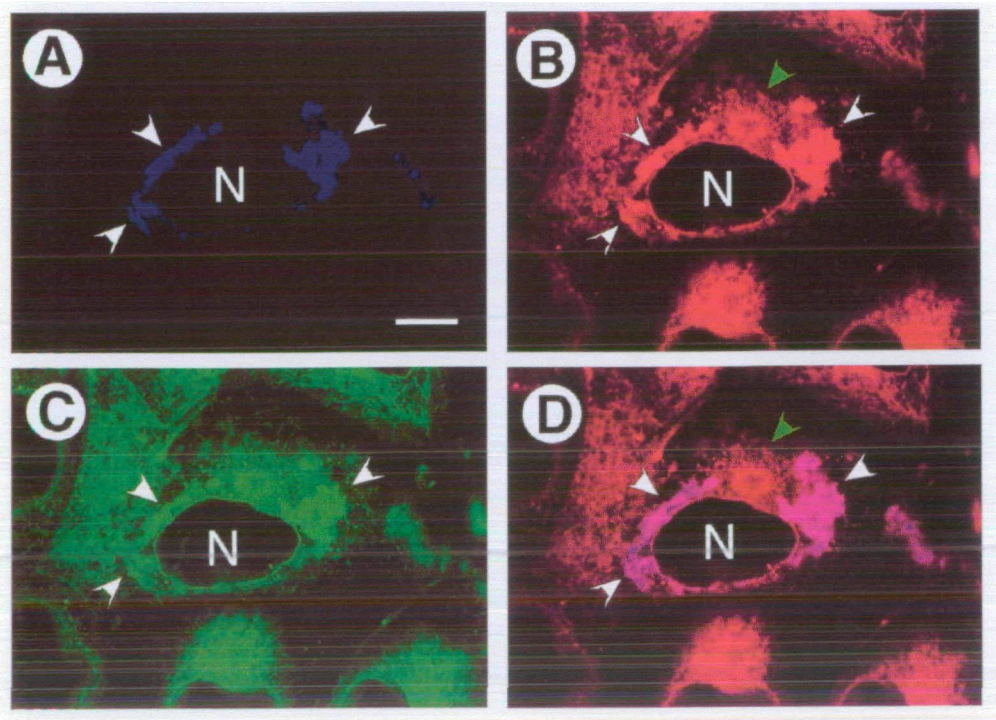

Fig. 6. **Localization of K 417 ochre in unusual membrane structures in the cells.** COS-1 cells that overexpressed K417 ochre were triple-stained with anti–rat ER-60F serum (A), anti-BiP monoclonal antibodies that could crossreact with BiP and Grp 94 (B), and fluorescein isothiocyanate-conjugated concanavalin A (C) as described under "EXPERIMENTAL PROCEDURES." K 417 ochre was localized only in unusual clump structures (white arrowheads). BiP and/or Grp 94 were present not only in the unusual clump structures, but also in the reticular structures of ER membranes (green arrowhead). (D) is a merged image of (A) and (B). N, nucleus. Bar, 10 μm.

with concanavalin A (ER marker) (Fig. 5F), but not with wheat germ agglutinin (Golgi apparatus marker) (Fig. 5G). BiP and/or Grp 94 were localized in a manner similar to that of overexpressed K 417 ochre in the clump structures

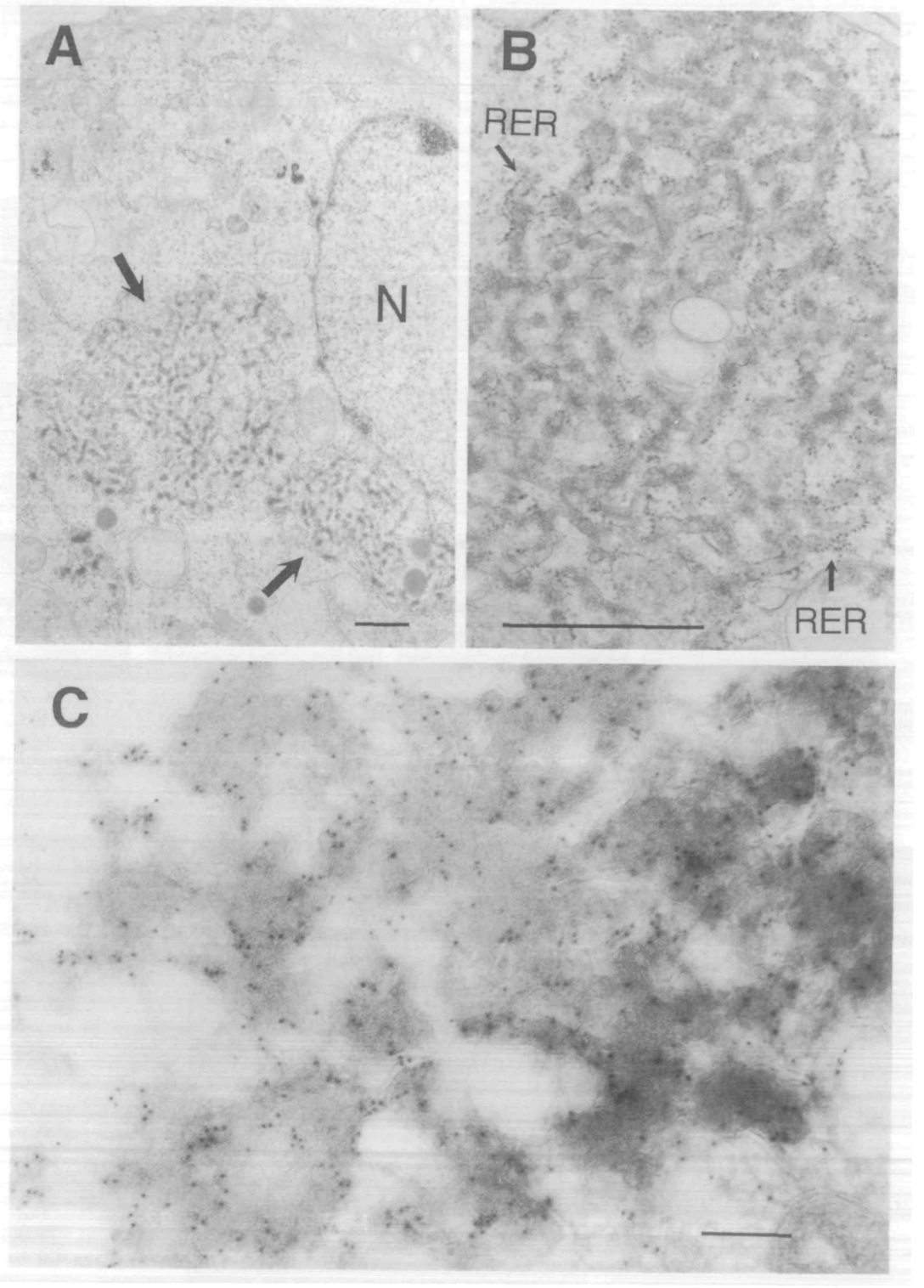

Fig. 7. **Electron micrographs of COS-1 cells overexpressing K 417 ochre.** COS-1 cells that overexpressed K 417 ochre were prepared for transmission electron microscopy (A, B) or Protein A–immunogold electron microscopy (C) as described under "EXPERIMENTAL PROCEDURES." (A) Clumps of the unusual membrane structures (arrows) adjacent to the nucleus (N) in a cell overexpressing K 417 ochre. Bar, 1 μm. (B) Higher magnification of a clump of an unusual membrane structure (B) shows dense materials accumulated in anastomosing complex tubular networks in continuity with the rough ER cisterna (RER). Bar, 1 μm. (C) A view of an immunogold-labeled ultrathin cryosection with anti–rat ER-60F serum shows the high density of 10 nm gold particles associated with dense materials accumulated in the anastomosing complex tubular network. Bar, 0.2 μm.

(Fig. 6). However, K 417 ochre was only localized in the clump structures (Fig. 6A), whereas BiP and/or Grp 94 were present in both the clump structures and the reticular structures of the ER (Fig. 6B). It is interesting that the clump structures were only observed in the K 418 ochre–overexpressing cells. In contrast with overexpressed K 417 ochre, the stably expressed K 417 ochre was localized in the typical reticular structures of the ER (Fig. 5I) similar to the overexpressed wild type ER-60 (Fig. 5A). No clump structures were seen in either cells stably expressing K 417 ochre, or those overexpressing wild type ER-60. On electron microscopic analysis, the clump structure was found to be a tubular smooth membrane network with a diameter of 110–180 nm linked to the rough ER (Fig. 7, A and B). The inside of the tubular network was filled with electron dense materials that were immunogold-labeled with anti–ER-60F serum (Fig. 7C). From these findings, it is assumed that the insoluble aggregates, as they accumulate, may expand a subcompartment of the ER linked to the rough ER to yield an abnormal membrane network in cells overexpressing K 417 ochre.

DISCUSSION

The abnormal protein, K 417 ochre, from which the C-terminal 89 amino acid residues are deleted, is not secreted but accumulates in cells. The fate of K 417 ochre depends on the degree of expression. The overexpressed K 417 ochre is hardly degraded, while the stably expressed form is degraded with a half-life time of more than 8 h by a protease system different from the proteasome or lysosomal system. The overexpressed K 417 ochre accumulates in clumps of the unusual membrane structure in which BiP and/or Grp 94 are localized, whereas the stably expressed K 417 ochre is present in the reticular ER. It seems likely that the clumps of unusual membrane structure are derived from the subcompartment of ER linked to the rough ER. Another putative organella that may give rise to the clumps of the unusual membrane structure may be the ER-Golgi intermediate compartment in which Rubella virus E1 glycoprotein and misfolded major histocompatibility class I molecules are present (*43*, *44*). It has been reported that BiP is present in a compartment close to or in continuity with rough ER (*43*, *44*). Taken together, it is assumed that a large amount of K 417 ochre synthesized on the rough ER is transported to a subcompartment of the ER or an ER-Golgi intermediate compartment and forms insoluble aggregates *in situ*, which may cause the expansion of the compartment as they accumulate. On the other hand, where protein production is low, as in the case of the stably expressed K 417 ochre, the protein forms small diffusible aggregates that could be distributed over the entire reticular ER, where it is degraded by the quality control system of the ER. It remains unknown whether the resistance to degradation of the insoluble aggregates of K 417 ochre is due to the absence of a protease system in the compartment where it accumulates. Probably, the resistance to a protease system may be caused by the inclusion body–like properties (*45*) of the insoluble aggregates of K 417 ochre. Denatured Ig M has been shown to become resistant to degradation by forming insoluble aggregates of Russel bodies in the pre-Golgi compartment (*46*). Part of α-1-antitrypsin is sedimented to yield inclusion bodies that cannot be degraded (*47*).

K 417 ochre does not associate with molecular chaperones such as BiP, calnexin, Grp 94, calreticulin, PDI, and ERp72 regardless of the degree of expression. The self-aggregation of K 417 ochre without any molecular chaperone may be the reason that it escapes the proteasome system, which requires the formation of aggregates associated with molecular chaperones before retrograde transport to the cytosol. There have been several reports that proteins are degraded by protease(s) other than the proteasome (*12–20),* suggesting that quality control in the ER is carried out by plural protease systems. Polypeptides, such as K 417 ochre, that do not associate with molecular chaperones or miss the chance to associate with molecular chaperones, may be degraded in the ER by a protease system other than the proteasome. It is interesting that ER-60 and ERp72, ER lumen proteins, have been shown to have ALLN-sensitive protease activities (*32*, *33*, *48*). However, participation of these enzymes in the degradation of K 417 ochre remains obscure.

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