Differential interaction of molecular chaperones with procollagen I and type IV collagen in corneal endothelial cells

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Purpose: Procollagen I is synthesized and intracellularly degraded in corneal endothelial cells (CEC), whereas type IV and VIII collagens are secreted into Descemet’s membrane. In our previous study, we demonstrated that procollagen I synthesized by CEC is improperly folded and that the molecule was largely colocalized with protein disulfide isomerase (PDI) within the endoplasmic reticulum (ER). In the present study, we further investigated whether the α-subunit of prolyl 4-hydroxylase (P4Hα) and glucose regulatory protein/immunoglobulin heavy chain binding protein (Grp78/BiP) were also involved in ER retention of procollagen I in CEC.

Methods: Immunocytochemical analysis was performed to determine the colocalization of procollagen I with molecular chaperones. Protein synthesis was measured by immunoblot analysis and the association between proteins was determined by coimmunoprecipitation followed by immunoblot analysis. mRNA was quantitated using RT-PCR.

Results: To study the interaction of procollagen I with certain molecular chaperones involved in the collagen biosynthetic pathway, we determined whether procollagen I colocalized with P4Hα and Grp78/Bip, and then compared this molecular chaperone colocalization with their association with type IV collagen. Procollagen I was colocalized with either P4Hα or Grp78/Bip to a much lesser degree than type IV collagen was colocalized with these same ER proteins. Colocalization between the molecular chaperones demonstrated that P4Hα and Grp78/Bip were largely colocalized in the peripheral region of the ER, whereas colocalization of P4Hα and PDI was mostly limited to a small region of the ER. When cells were treated with α,α'-dipyridyl, the inhibitor did not affect the colocalization profiles of collagens with the molecular chaperones. However, the inhibitor markedly increased colocalization of P4Hα and PDI, but it significantly decreased colocalization between P4Hα and Grp78/Bip. When synthesis of the molecular chaperones was compared between CEC and corneal stromal fibroblasts (CSF), more Grp78/Bip and PDI were produced by CEC than by CSF. On the other hand, expression of Hsp47 was lower in CEC than it was in CSF. Coimmunoprecipitation was used to compare the association of P4Hα or Grp78/Bip with collagens in CEC and CSF. The association of collagens (regardless of type) with P4Hα was much higher in CEC than in CSF. When the association of collagen molecules with respective molecular chaperones was compared in CEC, the degree of association between Grp78/Bip and procollagen I was similar to that between the molecular chaperone and type IV collagen. On the other hand, the degree of association between P4Hα and type IV collagen was much higher than that between P4Hα and procollagen I.

Conclusions: These data suggest that procollagen I and type IV collagen may use different molecular chaperones in the ER, thus targeting their distinct destinations.

Corneal endothelium is a monolayer of endothelial cells that covers the posterior surface of the cornea. The physiologic collagen phenotypes in corneal endothelial cells (CEC) are collagen types IV and VIII, the basement membrane collagens [1-3]. Collagen expression in CEC is unique in that CEC synthesize procollagen I, which is then intracellularly degraded immediately after its synthesis [4,5]. Secretion of procollagen I into Descemet’s membrane would have an adverse affect on corneal function, including maintenance of transparency and transmittance of light. Thus, although CEC erroneously produces this undesirable procollagen I, they then block its secretion through intracellular degradation. This final stage of gene regulation acts as an important quality control mechanism, regulating the levels of many undesired proteins by blocking the secretion of mutant or malfolded proteins. It has been well documented that from 10% to 30% of newly synthesized procollagens are degraded intracellularly by a process termed basal degradation [6,7]; the degradation is markedly increased in those cells that synthesize procollagens with structurally abnormal triple helical domains [8,9]. Yet, the molecular basis of such intracellular degradation has not been fully defined. Furthermore, there are no studies concerned with how a single cell that produces a number of different collagen types recognizes a specific collagen type from its secreted counterparts and specifically degrades it, as is uniquely observed in CEC, under physiologic conditions.

The secretion of procollagen molecules is first regulated by the biosynthetic events that take place within the endoplasmic reticulum (ER). These events include assembly of three individual prochains, the hydroxylation of prolyl and lysyl residues and the formation of the triple helix [10,11]. Numer-
ous studies further demonstrate that newly synthesized procollagens in the ER are associated with molecular chaperones, such as Hsp47, glucose regulatory protein 78/immunglobulin binding protein (Grp78/Bip), Grp94, and protein disulfide isomerase (PDI) [12-15]. This association suggests that these molecular chaperones assist in the procollagen folding process. In the ER, Grp78/Bip is known to be involved in retention of procollagen I that is synthesized by cells from osteogenesis imperfecta patients with mutations in the prot(1) chain carboxyl-terminal propeptide [15,16]. Prolyl 4-hydroxylase (P4H) is also reported to induce ER retention of procollagen, independent of the enzymatic activity [17]. In addition to its role as the β-subunit of P4H [18,19], PDI acts as a molecular chaperone during the assembly of procollagen [20]. In previous studies, we have also reported that procollagen I in CEC preferentially binds to PDI [21] and that the association of procollagen I with Hsp47 is maintained at a very low level in CEC [22], despite its well-known specific binding to any collagen, including type I [12,23,24]. Our previous studies to elucidate the molecular basis of specific degradation of procollagen I in CEC demonstrated that the newly synthesized procollagen I is not properly folded, as evidenced by its pepsin-susceptibility [21]. In vitro pulse chase experiments [5] showed that the malfolded molecules were completely degraded inside the cells within 2 h after synthesis. Type IV collagen, in contrast, is secreted and deposited into Descemet’s membrane [1].

The present study was undertaken to examine whether specific molecular chaperones within the ER regulate how the cell recognizes and retains unassembled or malfolded collagen. We compared the ER proteins bound to procollagen I and type IV collagen. From these ER proteins, we chose the two subunits of P4H for their critical roles as molecular chaperones and their role in the hydroxylation of procollagen [18-20,25]. We also chose Grp78/Bip for its binding capacity to procollagen molecules and the α-subunit of P4H (P4Hα) [15,16,26]. We examined the subcellular localization of procollagen I and type IV collagen, their respective colocalization profiles with the molecular chaperones, and the in vivo association profiles between collagen and these ER proteins. In addition, we compared expression levels of the ER proteins in CEC to their level in corneal stromal fibroblasts (CSF), which secrete type I collagen into the extracellular matrix (ECM). We have demonstrated that individual collagens may have preferential association potentials with the respective molecular chaperones in CEC. Procollagen I is largely associated with PDI, whereas type IV collagen is colocalized with P4Hα and Grp78/Bip.

**METHODS**

**Cell cultures:** Rabbit CEC were isolated and cultures were established as previously described [1]. Briefly, the Descemet’s membrane–corneal endothelium complex was treated with 0.2% collagenase and 0.05% hyaluronidase (Worthington Biochemical, Lakewood, NJ) for 60 min at 37 °C. Cultured cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA) and 50 µg/ml of gentamicin (DMEM-10) in a 5% CO2 incubator. First passage CEC were used for all experiments. For subculture, confluent cultures were treated with 0.2% trypsin and 5 mM EDTA for 3 to 5 min. To establish CSF, the corneas composed of stroma only were cut into small pieces and digested with collagenase cocktail for 3 to 4 h at 37 °C. The cells were resuspended in DMEM-10 and maintained and subcultured as described above. Third or fourth passage CSF were used for the study. In some experiments, cells were treated with 0.3 mM α,α’-dipyridyl for 2 h to inhibit the hydroxylation of collagens.

**Protein preparation and protein determination:** Cell cultures (90% confluent) were washed three times with phosphate-buffered saline (PBS). The cells were scraped in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 50 mM EDTA, 0.5 mM gelatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide, 1 µg/ml leupeptin, and 1 µg/ml aprotinin) and then sonicated on ice. Protein concentration of the resultant lysates was assessed with a Bradford protein assay system [27].

**Crosslinking:** Procedures used for protein crosslinking were previously reported [22]. Dithiothreitsylmethyl propionate (DSP), a substance that has been shown to cross cell membranes, was selected for this study. Cells were treated with 0.2% trypsin, containing 5 mM EDTA and 0.1% collagenase (Worthington Biochemical), to ensure removal of extracellular collagen. Cells were then combined with DSP (stored at a concentration of 0.1 M in dimethyl sulfoxide) to a final concentration of 2 mM, vortexed and placed on ice for 30 min. Following the reaction, cells were rinsed with 2 mM glycine in PBS to block the DSP activity and then with PBS alone. The cells were lysed with lysis buffer on ice for 15 min.

**SDS-polyacrylamide gel electrophoresis:** The conditions of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were as described by Laemmli, using the discontinuous Tris-Glycine buffer system [28].

**Immunoprecipitation and immunoblot analysis:** Cell lysates (5 mg) were precipitated with either anti-type I collagen antibody (3 µg) or anti-type IV collagen antibody (3 µg) for 18 h at 4 °C. The antigen–antibody complex was then precipitated with 50 µl of protein G-Sepharose beads (Sigma, St. Louis, MO) for 2 h at 4 °C. The precipitated immune complexes were washed three times with lysis buffer. The proteins bound to the Sepharose beads were eluted with Laemmli sample buffer [28], containing dithiothreitol, boiled for 5 min and applied to a 10% SDS-polyacrylamide gel for electrophoresis. The proteins separated by SDS-PAGE were transferred to 0.45 µm nitrocellulose membrane (Bio-Rad Lab, Hercules, CA) at 0.22 amper for 10 h in a semidry transfer system (transfer buffer: 0.1 M CAPS, pH 11). Immunoblot analysis was performed as described previously [21,22], using a commercial ABC Vectastain kit (Vector Laboratories, Burlingame, CA). All washes and incubations were carried out at room temperature in TTBS (0.9% NaCl, 100 mM Tris-HCl, pH 7.5, 0.1% Tween 20). Briefly, the nitrocellulose membrane was immediately placed in blocking buffer (10% nonfat milk in dil...
PCR product was run on a one-dimensional image analyzer (LKB Ultrascan XL; Pharmacia LKB Biotechnology, Pleasant Hill, CA). The relative density of the polypeptide bands detected on ECL film was estimated using the ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ) and exposed to ECL film. The relative density of the polypeptide bands detected on ECL film was estimated using the ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ) and exposed to ECL film. The relative density of the polypeptide bands detected on ECL film was estimated using the ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ) and exposed to ECL film.

Relative quantitative reverse polymerase chain reaction: Total cellular RNAs from CEC and CSF were isolated using a Qiagen RNeasy Mini kit (Qiagen Inc, Valencia, CA), as described in the protocol provided with the kit. Relative quantitative reverse polymerase chain reaction (RQ RT-PCR) was also performed using QuantumRNA™ Universal 18S Internal Standard (Ambion, Austin, TX), as described in the protocol provided by the manufacturer. Total RNA (2 µg), 5 µmole random primers for 18S internal standard [29], and 40 mM dNTP in nuclease-free water were heated to 85 °C for 3 min, then cooled on ice for 1 min. cDNA synthesis was initiated using 100 U of recombinant Moloney-murine leukemia virus reverse transcriptase at 42 °C for 60 min. The Grp78-specific primers were made from the cDNA sequences of human, rat, and hamster [30,31] to obtain the gene-specific primers to react with rabbit species. The sense primer was 5’-GACATCAAGTTCTTGCCGTT-3’ and the antisense primer was 5’-CTCATAACATTTAGGCCAGC-3’. Before performing RQ RT-PCR, we determined the linear range reaction for Grp78/Bip in order to have the maximum amplification efficiency for both Grp78/Bip and the 18S internal standard. PCR was performed using a RoboCycler (Stratagen, La Jolla, CA). RQ RT-PCR was performed as follows: hot start for 5 min at 95 °C, followed by denaturation for 1 min at 95 °C, annealing for 1 min at 56 °C, and elongation for 1 min at 72 °C for a total of 28 cycles with 18S internal standards and competitors (4:6). The reaction mixtures were separated on a 2% agarose gel and stained with ethidium bromide to analyze the RQ RT-PCR product.

Immunofluorescent staining: CEC (3 x 10⁴/chamber) were seeded on 4-well chamber slides and maintained in culture until they reached 50% confluence. Cells were washed with PBS and fixed for 10 min with ice-cold methanol or 1% acetic acid in 95% ethanol, depending on the primary antibodies used. Cells were permeabilized and blocked with 0.1% Triton X-100 and 1% BSA in PBS for 15 min at room temperature. After washing, cells were simultaneously incubated with both primary antibodies prepared in PBS. The cells were incubated overnight at 4 °C or 1 h at room temperature and then washed with PBS. Cells were then simultaneously incubated with FITC-conjugated secondary antibody (1:100 dilution) and rhodamine-conjugated secondary antibody (1:200 dilution) for 30 min at 37 °C in the dark. After extensive washing with PBS, the slides were mounted in a drop of Vectashield mounting medium (Vector Laboratories) to reduce photobleaching. Control experiments were performed in parallel with the omission of one of the secondary antibodies. For double-staining experiments in which both primary antibodies were produced in the same species (mouse), the previously reported experimental procedures were used with a slight modification [21]. Cells were blocked with 5% normal goat serum after fixing and then incubated with the first primary monoclonal antibody at 37 °C for 1 h. Cells were then rinsed in PBS and incubated at 37 °C for 1 h with an excess of the unconjugated rabbit Fab antibody directed against mouse IgG (20 µg/ml dilution, Jackson Immunoresearch Laboratories, West Grove, PA). After extensive washing, cells were incubated with rhodamine-conjugated goat anti-rabbit IgG antibody (1:200 dilution) at 37 °C for 30 min in the dark. Following extensive washing in PBS, the second monoclonal antibody was incubated at 37 °C for 1 h and cells were rinsed in PBS. The corre-

Figure 1. Subcellular localization of P4Hα, PDI and Grp78/Bip. CEC cells were fixed, permeabilized, and stained with antibodies as described in the methods. A: Cells stained for P4Hα; B: Cells stained for PDI; C: Cells stained for Grp78/Bip. Bar represents 10 µm. The data represent three independent experiments.

Figure 2. Subcellular localization of procollagen I and type IV collagen with P4Hα. CEC cells were fixed, permeabilized, and stained with antibodies as described in the methods. Some cells were treated with 0.3 mM α,α’-dipyridyl for 2 h prior to fixation and staining (C and D). A and C: Cells were stained for procollagen I (red) and P4Hα (green). B and D: Cells were stained for type IV collagen (green) and P4Hα (red). Bar represents 10 µm. The data represent four independent experiments.
sponding secondary FITC-conjugated anti-mouse IgG (1:100 dilution) was incubated for 30 min at room temperature in the dark. Control experiments were performed in parallel with the omission of one of the primary antibodies. The staining profiles of the control experiments were examined exclusively and the data were not shown in the text.

**Confocal microscopy and image analysis:** Antibody labeling was examined using a Zeiss LSM-510 laser scanning confocal microscope. Optical slices (1.8 µM) were taken perpendicular to the cell monolayer (apical to basal orientation). A 488-nm Argon laser was used in combination with a 499/505-530 excitation/emission filter set for fluorescein examination. For rhodamine, the 543-nm Helium neon laser was used with a 543 excitation filter and 560 emission filter. Simultaneous images of FITC or rhodamine were captured from the same optical section. The captured images were then pseudocolored: red for rhodamine and green for FITC. Regions of colocalization appear in yellow, reflecting the additive effect of superimposing green and red pixels. Image analysis was performed using the standard system operating software provided with the Zeiss LSM-510 series microscope.

**Antibodies:** Mouse anti-P4Hα antibody was purchased from ICN. Mouse monoclonal anti-PDI antibody and rabbit anti-Grp78 polyclonal antibody for immunoblotting analysis were purchased from Stressgen Biotechnologies Corp. (Victoria, BC, Canada), while mouse anti-Grp78 monoclonal antibody for immunofluorescence staining was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-type IV collagen antibody was a gift from Dr. Nirmala SundarRaj (University of Pittsburgh, Pittsburgh, PA), and goat anti-type I collagen was purchased from Chemicon International, Inc. (Temecula, CA). FITC-conjugated goat antibody against mouse IgG, FITC-conjugated rabbit antibody against goat IgG, rhodamine-conjugated horse antibody against mouse IgG, and rhodamine-conjugated rabbit antibody against goat IgG were purchased from Vector Laboratories Inc. Goat serum, Fab fragment anti-mouse IgG (H+L), and rhodamine-conjugated goat anti-rabbit IgG were purchased from Jackson Immunoresearch Laboratories. Primary antibodies were used for immunofluorescent staining in the following dilutions: anti-type I collagen (1:50); anti-type IV collagen (1:200); anti-P4Hα (1:200); anti-Grp78 (1:400).

**RESULTS**

**Subcellular localization:** In the previous study, we examined the subcellular localization of P4H containing both subunits, and PDI (the β subunit). The subcellular localization of the α-subunit of P4H was not determined. Therefore, for the present study, we examined the subcellular localization of P4Hα along with PDI and of Grp78/Bip, the key molecular chaperones during procollagen biosynthetic events. Figure 1 shows an even distribution of P4Hα throughout the ER (Figure 1A) and a diffuse distribution of PDI, with some areas lacking the protein (Figure 1B). Distribution of Grp78/Bip was largely localized toward the peripheral region of the ER, while there was much less perinuclear staining (Figure 1C). When CEC were

Figure 3. Subcellular localization of procollagen I and type IV collagen with Grp78/Bip. CEC cells were fixed, permeabilized, and stained with antibodies as described in the methods. Some cells were treated with 0.3 mM α,α’-dipyridyl for 2 h prior to fixing and staining (D and H). A: Stained for Grp78/Bip alone. B: Stained for procollagen I alone. C: Merged image of A (red) and B (green). D: Merged image of Grp78/Bip (red) and procollagen I (green) in α,α’-dipyridyl treated cells. E: Stained for Grp78/Bip alone. F: Stained for type IV collagen alone. G: Merged image of E (red) and F (green). H: Merged image of Grp78/Bip (red) and type IV collagen (green) in α,α’-dipyridyl treated cells. Bar represents 10 µm. The data represent four independent experiments.
double-stained with antibodies against type I collagen and P4H\(\alpha\), a minor fraction of the two proteins was colocalized at the perinuclear ER, however, the major portion of the two proteins was not colocalized (Figure 2A). In contrast, type IV collagen and P4H\(\alpha\) were largely codistributed throughout the ER (Figure 2B). When cells were treated with \(\alpha,\alpha'-\)dipyridyl, an ion chelator that inhibits hydroxylation of prolyl residues in procollagen chains, colocalization profiles of procollagen I and P4H\(\alpha\) was not altered from those of the untreated cells (Figure 2C). On the other hand, the unhydroxylated type IV collagen showed a large degree of colocalization with P4H\(\alpha\) throughout the ER (Figure 2D), similar to that observed in untreated cells.

Grp78/BiP is known to stably bind malfolded procollagen I that has mutation in the carboxy-terminal propeptide in fibroblasts from patients with osteogenesis imperfecta [15,16]. We examined whether procollagen I synthesized in CEC was associated with Grp78/Bip. When cells were double-stained with antibodies against type I collagen and Grp78/Bip, the colocalization of the two proteins was shown in the restricted areas of the ER if only Grp78/Bip was present (Figure 3A,C). Type IV collagen and Grp78/Bip were largely colocalized at the peripheral region of the ER, where the molecular chaperone was predominantly present (Figure 3E,G). Of interest is that procollagen I was distributed throughout the ER (Figure 3B), whereas a more restricted distribution of type IV collagen was observed in the ER of CEC (Figure 3F). When collagen molecules were unhydroxylated with the treatment of \(\alpha,\alpha'-\)dipyridyl, the colocalization profile of procollagen I and Grp78/Bip was unaltered compared to that of the untreated cells (Figure 3D). However, \(\alpha,\alpha'-\)dipyridyl promotes the colocalization of type IV collagen and Grp78/Bip throughout the ER and even at perinuclear region of the ER (Figure 3H).

The unassembled \(\alpha\)-subunits of P4H are associated with Grp78/Bip, leading to an assembly-competent form in the ER [26]. Therefore, we determined whether P4H\(\alpha\) in CEC was colocalized with Grp78/Bip. When cells were double-stained with antibodies against P4H\(\alpha\) and Grp78/Bip, the two proteins were largely colocalized in the peripheral region of the ER (Figure 4A). But when PDI and P4H\(\alpha\) were analyzed for codistribution, the two subunits of the P4H enzyme were not largely colocalized (Figure 4B). Under the stress condition mediated by \(\alpha,\alpha'-\)dipyridyl, the colocalization profile of P4H\(\alpha\)and Grp78/Bip in the treated cells (Figure 4C) was less than that in the untreated cells (Figure 4A). On the other hand, P4H\(\alpha\) and PDI showed complete colocalization in the cells treated with \(\alpha,\alpha'-\)dipyridyl (Figure 4D), suggesting that the two subunits of the enzyme were preferentially associated with each other when underhydroxylated procollagen molecules prevailed.

Expression and association of chaperones with collagen: Our previous study demonstrated that the expression level of Hsp47 in CEC was much less than that of CSF at both the protein and mRNA levels [22], whereas CEC contained much higher amounts of PDI than did CSF [21]. Since these measurements were performed separately, we determined the expression level of PDI, Hsp47, and Grp78/Bip simultaneously. The relative expression of these three molecular chaperones in CEC was compared to that of the CSF that served as control cells secreting type I collagen into the ECM. Three differ-

![Figure 4](image-url) Figure 4. Subcellular localization of P4H\(\alpha\) with PDI and Grp78/Bip. CEC cells were fixed, permeabilized, and stained with antibodies as described in the methods. Some cells were treated with 0.3 mM \(\alpha,\alpha'-\)dipyridyl for 2 h prior to fixing and staining (Figures A and C). A and C: Cells were stained for Grp78 (green) and P4H\(\alpha\) (red). B and D: Cells were stained for PDI (green) and P4H\(\alpha\) (red). Bar represents 10 \(\mu\)m. The data represent four independent experiments.

![Figure 5](image-url) Figure 5. Immunoblot analysis of Grp78/Bip, PDI and Hsp47. Cell lysates prepared from CEC and CSF were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane followed by immunoblotting with mouse anti-PDI, goat anti-Grp78 or mouse anti-Hsp47 antibodies. The relative density of the polypeptide bands was estimated using a one-dimensional image analyzer. Abbreviations: C, CEC; S, CSF; Conc., serial concentration of cell lysates. The data represent two independent experiments.
ent concentrations were used to semi-quantitatively determine the expression levels of the ER proteins. The amount of Grp78/ BiP and PDI in CEC were higher than the corresponding amounts in CSF, but the amount of Hsp47 was much lower in CEC (Figure 5). When the expression levels of these proteins were compared within the same cells, the levels of PDI and Hsp47 in CEC were similar, but the expression level of Grp78/ BiP was low. In CSF, on the other hand, the expression level of Hsp47 was much greater than those of PDI and Grp78/Bip. When the relative density of the respective peptide bands was estimated, there was an approximate 50% increase in Grp78/ BiP levels, an approximate two-fold increase in PDI, and a 25% decrease in Hsp47 in CEC compared to that in CSF (Figure 5). The steady state level of Grp78/Bip mRNA in CEC was examined using RQ RT-PCR analysis (Figure 6). The primers used for the analysis of Grp78/Bip generated a product of 260 bp, whereas the 18S rRNA used as the endogenous control generated a product of 310 bp. The steady state level of Grp78/Bip mRNA was much higher in CEC than in CSF, while the levels of 18S internal standard were similar in both cells.

We then investigated the association of procollagen I and type IV collagen with the molecular chaperones. The membrane permeable homobifunctional cleavable cross-linking reagent DSP was used before cell lysis to probe the in vivo association of chaperones with collagen molecules. A fraction of the cell lysates was immunoprecipitated with collagen antibodies, then immunoblotted with anti-Grp78 antibody or anti-P4Hα antibody (Figure 7). The association profiles between collagen molecules with molecular chaperones were compared within the same cells. The immune complexes precipitated with either anti-type I collagen antibody or anti-type IV collagen antibody showed that the association of Grp78/Bip with collagen molecules, regardless of collagen types, was far greater in CEC than in CSF. A similar finding was observed in the association of collagen molecules with P4Hα; more collagen molecules were associated with P4Hα in CEC than in CSF. When the association of collagen molecules with respective molecular chaperones was compared in CEC, the degree of association between Grp78/Bip and procollagen I was similar to that between the molecular chaperone and type IV collagen. On the other hand, the degree of association between P4Hα and type IV collagen was much higher than that between P4Hα and procollagen I. This confirms the finding that colocalization of type IV collagen with P4Hα was much greater than the colocalization of procollagen I and P4Hα (Figure 2A,B).

**DISCUSSION**

The study reported here grew out of an attempt to investigate the expression and subsequent intracellular degradation of procollagen I in CEC. CEC synthesize not only types IV and VIII collagen, but procollagen I as well. Upon secretion, types IV and VIII collagen are deposited into the network-forming basement membrane [1-3], whereas procollagen I is intracellularly degraded, as evidenced by in vitro pulse-chase experiments and in vivo staining of the corneal tissues [5]. Selective intracellular degradation is the major quality control mechanism preventing the secretion of unassembled and improperly folded proteins [11,32,33]. Our previous studies of the intracellular degradation of procollagen I in CEC demonstrate that intracellular procollagen I with a correct composition of two protα(I) and one protα(II) chains is pepsin-sensitive. This suggests that the molecule has an unstable triple helical conformation [21,22]. We also showed that this improperly folded procollagen I is preferentially bound to PDI (the β subunit of P4H) throughout the ER. Since CEC routinely operate the post-translational modification enzyme systems for types IV and VIII collagen, procollagen I can also be an equally specific substrate to the modification enzyme P4H. However, our previous study shows that procollagen I may not use such a unique modification step for procollagen biosynthetic events; instead, procollagen I preferentially binds to PDI. This extensive association of procollagen I and type IV collagen with Grp78/Bip and P4Hα. Cells were cross-linked with 2 mM DSP prior to lysis. Cell lysates (2 mg) obtained from CEC or CSF were immunoprecipitated with anti-type I collagen or anti-type IV collagen antibodies. The immune complexes were electrophoresed on a 10% gel followed by immunoblotting analysis with anti-Grp78 or anti-P4Hα antibodies. Abbreviations: C, CEC; S, CSF; IP, immunoprecipitation; IB, immunoblot analysis. The data represent five independent experiments.
sociation with PDI may prohibit the binding of the molecule to the active P4H enzyme complex.

In the present study, we further examined differences in the behavior of procollagen I from that of type IV collagen in the ER before they follow their respective destined pathways; one is targeted to the cytosolic degradation site, the other is guided to the normal secretory pathway via the Golgi apparatus. From the ER resident proteins, we chose two subunits of P4H for their critical role in hydroxylation of procollagen chains and their additional roles as molecular chaperones, and we chose Grp78/Bip for its binding capacity to procollagen molecules and the α-subunit of P4H. Our data demonstrate that, under normal conditions, P4Hα appears to be associated with Grp78/Bip more than it binds with its β-subunit (PDI) in CEC. This finding is in agreement with the previous report in which the unassembled P4Hα forms complexes with Grp78/Bip until the P4Hα possesses an adequate secondary structure to prevent aggregation [26]. Note that under the stress condition induced by treating the cells with α,α’-dipyridyl, P4Hα is readily dissociated from Grp78/Bip and makes active enzyme complexes with PDI. It is interesting that the stress condition facilitates the dissociation of P4Hα from Grp78/Bip and promotes formation of the active enzyme complex between the two subunits. However, the underlying mechanism for this event is not known.

Our data further demonstrate that a minor fraction of procollagen I was colocalized with P4Hα at the perinuclear region of the ER and that the majority of the two proteins were not colocalized. On the other hand, subcellular localization of type IV collagen and P4Hα is largely coincidental throughout the ER. Placing the cells under stress conditions by treating them with α,α’-dipyridyl does not alter the unique subcellular localization of procollagen I and type IV collagen with P4Hα. These data suggest that neither procollagen I nor type IV collagen associated with P4Hα is hydroxylated under physiological conditions. This is consistent with the fact that the substrate of P4H is unhydroxylated collagen molecules. Colocalization of collagen molecules with Grp78/Bip also demonstrates that procollagen I shows much less colocalization with this ER resident protein than type IV collagen does with Grp78/Bip. Interestingly, the degree of association of procollagen I with Grp78/Bip at the protein level is similar to that between the molecular chaperone and type IV collagen. This observation, however, does not influence our interpretation of the immunohistochemical analysis because CEC contain much less Grp78/Bip than they do PDI or Hsp47 (Figure 5). The present findings and our previous data taken together show that procollagen I is largely colocalized with PDI in the ER rather than with P4Hα, Grp78/Bip, or Hsp47. On the other hand, type IV collagen is largely colocalized with P4Hα and Grp78/Bip. These data thus suggest that individual collagens may have preferential association potentials with their respective molecular chaperones. It is likely that the extensive binding of PDI to procollagen I inhibits the association of procollagen I with its active enzyme complex, P4H, thus leading to unhydroxylated molecules. Unhydroxylated procollagen I subsequently forms an unstable triple helix, resulting in the improperly folded molecule. Finally, this improperly folded procollagen I is recognized as a bad quality protein by the quality control system operating in the ER. This scenario may be further supported by the in vivo binding data which shows that P4Hα binds much less to procollagen I than to type IV in CEC. On the other hand, type IV collagen in CEC binds equally well to both subunits of P4H, thus, it is properly hydroxylated and folded. As a consequence, type IV collagen is transported out of the ER to the Golgi apparatus and secreted into the ECM. A recent study demonstrated that the C-propeptide from the protα2(I) chain is retained within the cell, where it forms a complex with PDI [34]. Although this study suggests that PDI binds to C-propeptide of prot chains, it is unknown whether PDI is associated with procollagen I via the individual C-propeptide chain of prot1(I) or prot2(I), or the trimeric C-propeptide in CEC. Furthermore, whether PDI interacts with the (Gly-X-Y) repeat domain as well as with the C-propeptide domain is yet to be determined.

Another interesting finding from the present study and our previous study [21] is the differential amounts of respective molecular chaperones in the respective cell lines. CEC produce more PDI, P4Hα, and Grp78/Bip than do CSF. On the other hand, CEC produce less Hsp47 than do CSF. The excess amount of PDI in CEC may be required for the ER retention of procollagen I, whereas the higher level of Grp78/Bip in CEC than in CSF may be caused by the presence of unfolded procollagen I, as reported in other systems [35]. It is also likely that CEC, containing less but sufficient Hsp47, need this particular molecular chaperone for their physiologic collagens, type IV and VIII. This is supported by the report that Hsp47 interacts with and stabilizes correctly folded collagens [36]. In CEC, types IV and VIII collagen are the binding partners of Hsp47, as they meet the requirement of the molecular chaperone. Our previous report that procollagen I and Hsp47 have much less in vivo association in CEC than in CSF [22] further suggests that procollagen I may not be correctly folded in CEC.

The data presented in this study may suggest that procollagen I, preferentially bound to PDI, is directed to the degradation site, whereas type IV collagen, bound to the normal molecular chaperones and the modifying enzymes, is guided to complete the full pathway for secretion. It is not known whether the excessive binding of PDI to procollagen I causes improper folding of procollagen I and the subsequent ER retention, or whether the improperly folded procollagen I is protected by the prolonged association with PDI before the molecule is targeted to the degradation site. Although several cellular compartments have been identified as sites for degradation, including the lysosomes, a post-Golgi non-lysosomal compartment, the ER, and the proteasome system [37-39], it is unknown precisely where procollagen I is degraded in CEC.

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