Direct in Vitro and in Vivo Evidence for Interaction between Hsp47 Protein and Collagen Triple Helix

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Background: Procollagen needs the collagen-specific molecular chaperone Hsp47 for correct folding.
Results: Hsp47 binds to the triple-helix form of collagen model peptides in vitro and in vivo, but not to the monomer form.
Conclusion: Hsp47 functions for triple-helix collagen as a molecular chaperone in the endoplasmic reticulum.
Significance: This study provides the basis of the functional mechanism of Hsp47 in collagen molecular maturation.

Hsp47 (heat shock protein 47), a collagen-specific molecular chaperone, is essential for the maturation of various types of procollagens. Previous studies have suggested that Hsp47 may preferentially recognize the triple-helix form of procollagen rather than unfolded procollagen chains in the endoplasmic reticulum. However, the underlying mechanism has remained unclear because of limitations in the available methods for detecting in vitro and in vivo interactions between Hsp47 and collagen. In this study, we established novel methods for this purpose by adopting a time-resolved FRET technique in vitro and a bimolecular fluorescence complementation technique in vivo. Using these methods, we provide direct evidence that Hsp47 binds to collagen triple helices but not to the monomer form in vitro. We also demonstrate that Hsp47 binds a collagen model peptide in the trimer conformation in vivo. Hsp47 did not bind collagen peptides that had been modified to block their ability to form triple helices in vivo. These results conclusively indicate that Hsp47 recognizes the triple-helix form of procollagen in vitro and in vivo.

Collagen is the most abundant protein in mammals and, as a major component of the extracellular matrix, plays a pivotal role in tissue architecture and robustness and in cell-cell interactions. Individual collagen polypeptide chains contain large numbers of repetitive amino acid sequences, most often Gly-Xaa-Yaa, where Xaa is often Pro and Yaa is often 4-hydroxy-L-proline, the latter amino acid being produced by hydroxylation of Pro following collagen polypeptide synthesis. All types of collagen have a characteristic triple-helical structure. The presence of Gly at every third residue and the high imino acid content allow the formation of a triple-helical structure in which the three helical chains are staggered by one residue and are supercoiled in a right-handed manner. Appropriate folding of collagen into its triple helix followed by processing of N- and C-propeptides is critical for the formation of the extracellular matrix. Because the extracellular matrix is required for the formation of bones and other tissues, collagen folding defects lead to severe bone fragility and deformities such as osteogenesis imperfecta (1–3).

Several molecular chaperones and folding enzymes are involved in the molecular maturation of various types of procollagen: BiP, Grp94, and Hsp47 (heat shock protein 47) as molecular chaperones and protein-disulfide isomerase (PDI),2 prolyl 3-hydroxylase, and prolyl 4-hydroxylase as folding enzymes (4–9). Hsp47 was first identified as a collagen-binding protein residing in the endoplasmic reticulum (ER) (10, 11) and functions as a collagen-specific molecular chaperone (12, 13). Disruption of both alleles of hsp47 in mice causes aberrant procollagen folding in the ER, which results in embryonic lethality by 11.5 days post-coitus due to defects in the formation of collagen fibers and basement membranes in the embryo (12, 14, 15). Triple-helix formation, secretion, and processing of the N-terminal propeptide of type I procollagen are impaired in hsp47-disrupted fibroblasts, resulting in a failure to accumulate collagen fibers in the extracellular matrix (15, 16). These results indicate that Hsp47 is essential for the maturation of collagen.

To elucidate the mechanism of client recognition by Hsp47, in vitro binding analysis using a synthetic collagen model peptide has been used to identify a specific Hsp47-binding sequence in collagen; Arg residues at the Yaa position of the collagen Gly-Xaa-Yaa repeats are a critical minimum for Hsp47 binding (17–19). Hsp47 appeared to preferentially recognize such sequences on the triple helices of procollagen rather than on the unfolded procollagen α-chains in the ER (18–20). However, because of a lack of direct mechanistic studies of the interaction between Hsp47 and procollagen, it remains controversial as to whether Hsp47 recognizes only the triple-helix conformation or whether it also recognizes the single-chain polypeptide.

In this study, we provide direct observational evidence that Hsp47 interacts with triple-helix collagen but not with its monomer. This result was achieved using self-assembling

2 The abbreviations used are: PDI, protein-disulfide isomerase; ER, endoplasmic reticulum; TR, time-resolved; BIFC, bimolecular fluorescence complementation; mKG, monomeric Kusabira-Green; SA, streptavidin; Eu-K, europium cryptate; GFC, gel filtration chromatography.
homotrimeric collagen model peptides, separated by gel filtration chromatography, in a novel binding assay based on a time-resolved (TR) FRET technique. We also developed a versatile visualization system for detecting the interaction between Hsp47 and a collagen model peptide fused to foldon, which is derived from the C-terminal domain of T4 fibrin and is known to facilitate trimer conformation (21–23). This assay used a bimolecular fluorescence complementation (BiFC) technique (24) in living cells based on the reconstitution of two split fragments of monomeric Kusabira-Green (mKG) as a fluorescent protein (25).

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were purchased from Hokkaido System Science Co., Ltd. (Ibaraki, Japan). Synthetic peptides were purchased from TORAY Research Center, Inc. (Kanagawa, Japan). Streptavidin (SA)-XL665 and anti-GST-europium cryptate (Eu-K) antibody were purchased from Cisbio International (Bagnols-sur-Cèze, France).

Plasmid Construction—To express target proteins in the ER, we modified the expression vectors in the CoralHue Fluor-chase kit (Amalgaam, Tokyo, Japan). A cDNA fragment containing a Kozak sequence and a sequence encoding the ER signal sequence derived from human Hsp47 (MRSLLLASFCL-LEAAL) was subcloned into the NheI site of the phmKGN-MC vectors, respectively. The resulting constructs were designated pmKGN-CP2 and pmKGC-PPG vectors, respectively. The stop codon of the CAYA mutant of human Hsp47 was subcloned into a bacterial expression vector based on pET-32a (Novagen, Madison, WI), which was modified to allow the fusion of the target protein N-terminal to a polyhistidine tag and GST and to incorporate a recognition sequence for PreScission protease (GE Healthcare). Escherichia coli strain BL21(DE3) cells, expressing the rare Arg-tRNA that recognizes the similarly rare AGA codons (26), were transformed with the expression vector encoding His/GST-Hsp47 and cultured in ZB medium (1% N-2-Acetamidyl glucose (Wako, Tokyo, Japan), 0.5% NaCl) containing 100 µg/ml carbenicillin and 30 µg/ml kanamycin at 30 °C.

Expression of Hsp47 was induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside and culturing at 20 °C for 16–20 h. Cells were collected by centrifugation and resuspended in PBS containing 0.5% Nonidet P-40 (Nacalai Tesque, Kyoto, Japan), 1 mM phenylmethylsulfonyl fluoride, and 0.5% protease inhibitor mixture (P8849, Sigma). Lysozyme (Wako) was added to a final concentration of 0.5 mg/ml, and the mixture was incubated for 10 min on ice. The lysate was frozen in liquid nitrogen and stored at −80 °C. After thawing, the lysate was sonicated and cleared by centrifugation at 16,000 × g for 30 min at 4 °C. Soluble proteins were purified with glutathione-Sepharose 4B (GE Healthcare) according to the manufacturer’s batch protocol. GST-fused proteins were eluted with elution buffer (10 mM glutathione and 50 mM Tris-HCl, pH 8.8). The total protein concentration was determined using a Coomassie Plus protein assay reagent kit (Pierce) according to the manufacturer’s instructions with BSA as a standard.

Antibodies—Mouse monoclonal antibodies against Hsp47 (Stressgen, Victoria, Canada) and mKG_C (MBL, Nagoya, Japan) and rabbit polyclonal antibodies against PDI (Stressgen) were obtained from the indicated sources. HRP-conjugated anti-mouse or rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA), Alexa Fluor 555-conjugated anti-mouse IgG (Invitrogen), and Alexa Fluor 488-conjugated anti-rabbit IgG (Invitrogen) were used as secondary antibodies.

Western Blot Analysis—Proteins were separated by SDS-PAGE using 10–20% gradient gels and transferred to either a polyvinylidene difluoride or nitrocellulose membrane. The membranes were blocked with PBS containing 2.5% skim milk, and specific binding of the antibody was detected using a chemiluminescence detection kit (ECL Plus Western blotting detection system, GE Healthcare).

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Gel Filtration Chromatography—Gel filtration chromatography (GFC) was carried out using Superdex Peptide PE 7.5/300 (GE Healthcare) on an ÄKTApexsor 10 S system (GE Healthcare) at room temperature. Aliquots (100 µl) of biotinylated collagen model peptide were loaded and separated at a flow rate of 0.4 ml/min using a Superose 12 gel filtration column. The case-encoding the ER signal sequence, pER-mKGC-h47wt, pER-mKGN-h47CAYA, pER-mKGC-PPG×3F, and pER-mKGC-CP2×9F were digested with Nhel and self-ligated. The resulting constructs were designated pmKGN-h47wt, pmKGN-h47CAYA, pmKGC-h47wt, pmKGC-h47CAYA, pmKGC-PPG×3F, and pmKGC-CP2×9F. All constructs described above were confirmed by DNA sequencing using an ABI Prism 3130xl DNA sequencer (Applied Biosystems, Foster City, CA).
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of 0.28 ml/min using PBS, and 0.2-ml fractions were collected. Peptide elution was monitored by absorbance at 215 nm.

In Vitro TR-FRET Binding Assay—The assay was performed in a 384-well plate in 20 μl of binding buffer containing 10 nM recombinant GST-Hsp47 protein, 300 nM biotinylated collagen model peptide, 83.4 nM SA-XL665, 0.37 nM anti-GST-Eu-K antibody, 50 mM HEPES/NaOH, 150 mM NaCl, 1 mM EDTA, 0.01% Nonidet P-40, 0.1% BSA, and 100 mM KF. All reaction mixtures were incubated for 2 h at room temperature. Emission signals at 665 and 620 nm were measured simultaneously on an Analyst GT multimode plate reader (Molecular Devices, Sunnyvale, CA) using a 50-μs delay following an excitation pulse at 337 nm.

The 665:620 nm ratio was calculated as follows: ratio = (emission at 665 nm/emission at 620 nm) × 10³. Values are means ± S.D. (n = 3).

Cell Culture and Transfection—HeLa cells, which are a human cervical epithelial carcinoma cell line, were obtained from American Type Culture Collection and maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were cultured at 37 °C in an atmosphere containing 5% CO₂ and transfected with each vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Immunofluorescence Microscopy—Cells were washed in PBS and fixed with 4% (w/v) paraformaldehyde in PBS for 20 min. After washing three times, fixed cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 5 min. Nonspecific protein binding in permeabilized cells was blocked by incubation in PBS containing 2% BSA for 30 min. After incubation with specific antibodies in PBS containing 1% BSA, cells were incubated with Alexa Fluor-conjugated anti-mouse or anti-rabbit IgG and 1 μg/ml Hoechst 33342 (Invitrogen) in PBS containing 1% BSA. Fluorescent images were collected using an automated image acquisition system (IN Cell Analyzer 1000, GE Healthcare).

BiFC Binding Assay in Mammalian Cells—Twenty-four hours after transfection, the HeLa cells were subcultured onto BD Falcon Optilux 96-well microplates (353948, BD Biosciences), and the culture medium was replaced after 48 h with Opti-MEM I reduced serum medium (Invitrogen) containing 1 ng/ml Hoechst 33342 at room temperature. Values are means ± S.D. (n = 3).

RESULTS

Development of Novel Hsp47 Binding Assay in Vitro—Although some in vitro methods for detecting the binding of Hsp47 to native collagen or collagen model peptides have been reported (27–29), no simple “mix and read” method has been described. To facilitate the identification of the conformation of collagen binding to Hsp47, we attempted to develop a novel binding assay using TR-FRET (Fig. 1A). GST-tagged recombinant Hsp47 proteins were readily purified by glutathione affinity chromatography from the lysates of E. coli cells harboring the corresponding expression plasmids (supplemental Fig. 1).

For use as substrates, we prepared two biotinylated collagen model peptides, Bio-CP1 and Bio-CP2, which differ in the positions of key Thr and Arg residues (Fig. 1B, boldface), which are required for the interaction with Hsp47 (17, 30). As shown in Fig. 1C, an increase in FRET signals was observed when wild-type chicken Hsp47 was incubated with each of the biotinylated collagen model peptides. FRET signals were much lower for the interaction of human Hsp47 and collagen model peptides compared with that of chicken Hsp47, possibly due to the decreased protein stability of human Hsp47 (Fig. 1, C and D). On the other hand, no obvious increase in the signal was observed when the human Hsp47 CAYA mutant, in which Cys-139 and Tyr-366 were replaced by Ala, was used as a negative control. Cys-139 is
interaction of two target proteins.

The increase in FRET signals was greater for Bio-CP2 than for Bio-CP1 (Fig. 1C). The difference in signal intensities between the two peptides would be caused by the difference in the distance between the Hsp47-binding motif (GPTGPR) and labeled biotin/SA-XL665 (Fig. 1B). Thus, we used Bio-CP2 in the following experiments. Concentration-dependent increases in the FRET signal were observed with Bio-CP2 (Fig. 1D). These results demonstrate that the binding assay based on TR-FRET technology provides a useful quantitative measurement of the binding of Hsp47 to collagen model peptides in vitro.

Hsp47 Specifically Binds to Trimer Conformation of Collagen Model Peptide in Vitro—To elucidate the conformational requirements for the interaction of Hsp47 with collagen using the TR-FRET system, we separated Bio-CP2 using GFC. In the chromatographic profile of Bio-CP2, the trimer appeared as the first peak, and the monomer appeared as the second peak (Fig. 2A). The third peak was due to the presence of Nonidet P-40 in the buffer for the collagen model peptide. After collecting the first and second peaks, the binding of Hsp47 to the collagen model peptide in each fraction was analyzed. A dose-dependent increase in the FRET signal was observed for the trimer form of the collagen peptide (Fig. 2C), whereas no increase was observed for the monomer (Fig. 2B). These results conclusively demonstrate that Hsp47 interacts with the trimer (but not monomer) form of collagen peptides.

Next, we examined the reversibility of conformational changes in the collagen model peptides from trimer to monomer and the influence of these changes on binding to Hsp47. When Bio-CP2 was heated at 37 °C for 2 h, the trimer fraction disappeared, with a corresponding increase in the monomer peak and a complete loss of binding capacity for Hsp47 (Fig. 3, A and B). When samples heated at 37 °C were cooled to 4 °C for 4 days, the trimer peak reappeared in GFC analysis (Fig. 3F), suggesting that the conformational shift between trimer and monomer is reversible (Fig. 3, F and B). The ability to bind Hsp47 was almost completely restored in the cooled samples (Fig. 3, C and E). These results indicate that the trimer form of collagen model peptides can reversibly convert to the monomer form in response to changes in temperature and that Hsp47 binds only to the trimer form, most possibly to the triple-helix form.

Visualization of Interaction between Hsp47 and Collagen Model Peptides Fused to Foldon in Living Mammalian Cells—To examine the interaction of Hsp47 with the collagen model peptide in living mammalian cells, we developed a novel visualization system using a BiFC technique (24) based on the reconstitution of two split fragments of the mKG fluorescent protein. When two proteins harboring different mKG fragments interact with each other, the mKG fragments (mKG_N and mKG_C) are drawn into close proximity with one another, resulting in the emission of chromophore fluorescence (Fig. 4B). The single mKG fluorescent protein fragments do not emit fluorescence unless both fragments are brought together by the interaction of two target proteins.

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Values are means ± S.D. (n = 3).
To express both Hsp47 and the collagen model peptide in the ER, we inserted the ER signal sequence from human Hsp47 into the mKG expression vectors (Fig. 4A). We first attempted to detect the interaction between the collagen model peptide and Hsp47 in this system by transfecting mKG_N-fused wild-type Hsp47 (N-Hsp47 wt) and mKG_C-fused model peptide (C-CP2/H11003) into the cells. However, no significant interaction between the collagen peptide and Hsp47 was detected (Fig. 5B, lower row), which may be due to a failure to achieve the correct triple-helix conformation in the collagen model peptide.

Foldon, derived from the C-terminal domain of T4 fibritin, is known to participate in the formation of trimeric proteins (21–23, 31). To promote trimer formation, we incorporated foldon at the C terminus of the collagen model peptide (Fig. 4A). The localization of ER-mKG_C-Hsp47wt (C-Hsp47 wt) and ER-mKG_C-CP2×9 fused to foldon (C-CP2×9) in the ER was confirmed by immunofluorescence staining using an antibody specific for mKG_C together with co-staining with the ER marker PDI (Fig. 5A). C–F, cooling of heat-treated trimer fractions. TR-FRET binding assays (C and E) and GFC (D and F), as described above, were performed using untreated (C and D) or heat-treated (E and F) trimer fractions of Bio-CP2 after cooling for 4 days at 4 °C. In B, D, and F, we applied the peptides at a concentration of 3.25 μM as the bases of trimer.

**FIGURE 3. Temperature-dependent reversibility of collagen triple-helix formation.** A, binding of recombinant Hsp47 to Bio-CP2 following heat treatment. A Bio-CP2 mixture (3 μM), before separation by GFC, was heated for 2 h at 37 °C. The TR-FRET binding assays were performed as described in the legend to Fig. 2, except that the reaction mixtures were incubated for 2 h at 37 °C. B, heat treatment of the trimer fraction. The trimer fractions of the Bio-CP2 mixture was obtained by GFC and heated for 2 h at 37 °C. Using the trimer fraction, GFC analysis was performed as described in the legend to Fig. 2A. C–F, cooling of heat-treated trimer fractions. TR-FRET binding assays (C and E) and GFC (D and F), as described above, were performed using untreated (C and D) or heat-treated (E and F) trimer fractions of Bio-CP2 after cooling for 4 days at 4 °C. In B, D, and F, we applied the peptides at a concentration of 3.25 μM as the bases of trimer.
model peptide fused to mKG in cells. These observations also clearly indicated that Hsp47 could interact with the collagen model peptide only in its trimer form.

To confirm that the fluorescence of reconstituted mKG observed in Fig. 5 was due to a specific interaction between Hsp47 and the collagen model peptide, we tested the activity of an Hsp47 mutant and model peptides with GPP repeats of varying length in the experimental system. When the mKG_N-Hsp47_CAYA mutant and mKG_C-CP2x9 peptide were cotransfected, no reconstituted mKG fluorescence was observed (Fig. 6A and supplemental Fig. 6A), even though these proteins were expressed at the same level as the wild-type proteins (Fig. 6, B and C). This result is consistent with our in vitro data (Fig. 3). Cotransfection of mKG_N-Hsp47 wt and mKG_C-CP2GAx9, which has only three PPG repeats, also did not result in mKG fluorescence (Fig. 6A and supplemental Fig. 6A). The same result was obtained using the PPG×5 peptide, with five PPG repeats (data not shown). Considering that these peptides had foldon at the C terminus, the failure to reconstitute mKG might be due to a failure to achieve triple-helix conformation because of the short GPP repeat region, which is consistent with a previous report on the interaction of recombinant Hsp47 with collagen model peptides with various lengths of PPG repeats in vitro (29). Similarly, no specific reconstitution of mKG was observed following the cotransfection of mKG_N-Hsp47 wt and mKG_C-CP2GAx9, in which all Gly residues in CP2×9 were replaced by Ala (see Fig. 4A), leading to a failure to form a triple helix (Fig. 6A and supplemental Fig. 6A). These results clearly establish that Hsp47 recognizes the triple-helix form of collagen not only in vitro but also in vivo.

**DISCUSSION**

We have developed an in vitro novel binding assay in which the interaction between purified recombinant Hsp47 and synthetic collagen model peptides was analyzed by TR-FRET signals, without the need for washing. This technique provides a
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A. Immunofluorescence staining of HeLa cells transfected with ER-mKG_C-Hsp47wt (C-Hsp47 wt; upper row) and ER-mKG_C-CP2x9 fused to foldon (C-CP2x9; lower row). Three days after transfection, the cells were fixed, stained with an anti-mKG_C monoclonal antibody and an anti-PDI polyclonal antibody, and visualized with Alexa Fluor 555-conjugated anti-mouse IgG and Alexa Fluor 488-conjugated anti-rabbit IgG. The nuclei were stained with Hoechst 33342. All images were acquired using the IN Cell Analyzer 1000 system. Scale bar = 10 μm.

B. Immunofluorescence staining of HeLa cells transfected with ER-mKG_C-Hsp47 wt and N-CP2x9. Three days after transfection, the cells were fixed, stained with an anti-mKG_C monoclonal antibody and an anti-PDI polyclonal antibody, and visualized with Alexa Fluor 555-conjugated anti-mouse IgG and Alexa Fluor 488-conjugated anti-rabbit IgG. The nuclei were stained with Hoechst 33342. All images were acquired using the IN Cell Analyzer 1000 system. Scale bar = 10 μm.

C. Immunofluorescence staining of HeLa cells transfected with ER-mKG_C-Hsp47 wt and N-CP2x9. Three days after transfection, the cells were fixed, stained with an anti-mKG_C monoclonal antibody and an anti-PDI polyclonal antibody, and visualized with Alexa Fluor 555-conjugated anti-mouse IgG and Alexa Fluor 488-conjugated anti-rabbit IgG. The nuclei were stained with Hoechst 33342. All images were acquired using the IN Cell Analyzer 1000 system. Scale bar = 10 μm.

Additional advantages of our new technique include the shorter overall incubation time, greater throughput capacity, stability of the readout signal, and ease of automation for high-throughput screening. Recently, Okano-Kosugi et al. (32) reported a turbidimetric assay to screen inhibitors of collagen-binding proteins, including Hsp47. The assay is based on changes in absorbance at 313 nm due to collagen fibril formation in vitro. However, this interesting method is not suitable for drug screening because of the large number of compounds with absorption close to 313 nm, which could cause false positive or negative signals. Meanwhile, the TR-FRET technique that we have employed in this assay may be an effective tool for drug screening due to the large Stokes shift of the assay and the long lifetime of the emitted light.

Several lines of circumstantial evidence suggest that Hsp47 recognizes triple helices of collagen preferentially rather than unfolded collagen chains in the ER (18–20). Using a novel TR-FRET assay, we have now conclusively demonstrated in vitro that Hsp47 can interact with the trimer form of a collagen model peptide containing GPP triplets and GPTGPR, although it cannot interact with the monomer form (Fig. 2). Collagen model peptides with GPP triplet repeats form a triple helix at 25 °C when the number of repeats is greater than nine, which was judged by CD spectroscopic analysis (30, 33), and our results therefore strongly suggest that Hsp47 binds to the triple-helix form of collagen.

The trimer fraction of the collagen model peptide was converted to the monomer form by heat treatment at 37 °C (Fig. 3B) but reverted to the trimer form after cooling at 4 °C (Fig. 3F). By referring to supplemental Fig. 2A, the transition from trimer to monomer did not occur during gel filtration. Interestingly, the monomer form separated by GFC did not revert to the trimer after cooling at 4 °C (supplemental Fig. 5F). The data presented in supplemental Fig. 3 suggest that the formation of the triple helix is concentration-dependent. The concentration of the monomer fraction separated by GFC was 4.9 μM, which was substantially higher than the concentration of 1 μM at which trimer formation can take place (supplemental Fig. 3A). Some unidentified difference might cause such a difference in the triple-helix formation.

We also successfully developed a novel visualization system for detecting the interaction of Hsp47 with collagen model peptides within the ER of mammalian cells using a BiFC technique (24) based on the reconstitution of two split fragments of the fluorescent protein mKG (25). We first attempted to detect the interaction of Hsp47 with the collagen model peptide fused to the C terminus of mKG together with the ER signal sequence. However, no significant interaction was detected (Fig. 5B, lower row). Detection of the interacting signals was dramatically improved by incorporating the foldon sequence into the collagen model peptide, suggesting that collagen model peptides do not form trimers without foldon and that Hsp47 cannot interact with non-trimeric model peptides within the cells. Collagen model peptides without foldon may be unable to form trimers because of conformational restrictions or poor prolyl hydroxylation by prolyl 4-hydroxylase due to the fusion with mKG or to decreased triple-helix formation at the incubation temperature of 37 °C, as described above. Du et al. (31) reported that the thermostability of recombinant collagen-like proteins was significantly improved when fused with the foldon sequence.
Thus, the promotion of trimer formation in the collagen model peptides by foldon was necessary for the interaction with Hsp47 in this in vivo visualization system.

No significant interaction was detected after the cotransfection of Hsp47 with (i) the PPG/H11003 peptide, which has only three Pro-Pro-Gly triplet repeats (Fig. 6A and supplemental Fig. 6A); (ii) the PPG×5 peptide, which has five Pro-Pro-Gly triplet repeats (data not shown); and (iii) the CP2GA×9 peptide, in which all of the Gly residues in CP2/H11003 were replaced by Ala (Fig. 6A and supplemental Fig. 6A). These results are attributed to the failure to form triple helices because of the small number of the triplet repeats (i and ii) or steric hindrance due to the substitution of Gly with Ala at the innermost position of the triple-helical structure (iii). Notably, the data using PPG×3 and PPG×5 peptides correlate well with previous work by Koide et al. (29), in which Hsp47 did not interact with collagen model peptides containing fewer than six GPP triplet repeats. From these results, we conclude that the novel visualization system described here provides an efficient and specific methodology for detecting interactions between collagen-binding proteins and collagen model peptides within the ER of mammalian cells.

In summary, we have conclusively demonstrated that Hsp47 recognizes and binds the triple-helix form (but not the monomer form) of collagen model peptides in vitro and in vivo using two newly developed methods. These findings suggest an important role for Hsp47 as a collagen-specific molecular chaperone in stabilizing the triple helices of procollagen in the ER. Our newly developed in vitro and in vivo analysis systems may also provide useful tools for screening small molecule inhibitors of the interaction between Hsp47 and collagen.

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FIGURE 6. Specific interaction of Hsp47 with triple helices of collagen model peptides in vivo. A, fluorescent images of HeLa cells coexpressing (i) various collagen model peptides fused to foldon and mKG_C and (ii) wild-type or CAYA mutant Hsp47 fused to mKG_N. All constructs also had an ER signal sequence. Three days after transfection, fluorescence from reconstituted mKG, together with Hoechst 33342 staining, was measured using the IN Cell Analyzer 1000 system. Scale bar = 10 μm. B and C, expression levels of Hsp47 and foldon-fused collagen model peptides in transfected HeLa cells, respectively. Whole cell lysates were extracted and analyzed by Western blotting using specific antibodies against Hsp47 (B) and the mKG_C fragment (C). The asterisk indicates a nonspecific band. The molecular sizes are shown in kilodaltons.

w, wild-type; C, CAYA mutant; Hsp47 (endo), endogenous Hsp47.
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