HSP47 is an essential procollagen-specific molecular chaperone that resides in the endoplasmic reticulum of procollagen-producing cells. Recent advances have revealed that HSP47 recognizes the (Pro-Pro-Gly)$_n$ sequence but not (Pro-Hyp-Gly)$_n$, and that HSP47 recognizes the triple-helical conformation. In this study, to better understand the substrate recognition by HSP47, we synthesized various collagen model peptides and examined their interaction with HSP47 in vitro. We found that the Pro-Arg-Gly triplet forms an HSP47-binding site. The HSP47 binding was observed only when Arg residues were incorporated in the Xaa positions of the Xaa-Yaa-Gly triplets. Amino acids in the Xaa position did not largely affect the interaction. The recognition of the Arg residue by HSP47 was specific to its side-chain structure because replacement of the Arg residue by other basic amino acids decreased the affinity to HSP47. The significance of Arg residues in HSP47 binding was further confirmed by using residue-specific chemical modification of types I and III collagen. Our results demonstrate that Xaa-Arg-Gly sequences in the triple-helical procollagen molecule are dominant binding sites for HSP47 and enable us to predict HSP47-binding sites in homotrimeric procollagen molecules.

**EXPERIMENTAL PROCEDURES**

**Synthetic Peptides**—Peptide chains were constructed manually based on the standard FMOC (N-(9-fluorenyl)methoxycarbonyl)-based strategy on H-Gly-CTTrt-resin (Novabiochem). tert-butyl, tert-butoxycarbonyl, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, and triphenylmethyl (Trt) were used as side chain-protecting groups for hydroxyl, amino, guanidino, and thiol groups, respectively. Peptide cleavage from the solid support and simultaneous side-chain deprotection were performed by treatment with trifluoroacetic acid:m-cresol:thioanisole:water,1,2-ethanediol (82.5:5:5:2.5, v/v) at room temperature for 1 h. For the Arg-containing peptides, the treatment was prolonged to 3 h. Crude peptides were purified by reversed-phase HPLC with a linear gradient of acetonitrile in water, both containing 0.05% trifluoroacetic acid. Purity of the peptides was greater than 95% on analytical HPLC, and identity was confirmed by matrix-assisted laser desorption-ionization mass spectrometry (MALDI-TOF MS). The melting temperatures of refolded (Pro-Hyp-Gly)$_3$-Xaa-Yaa-Gly-(Pro-Hyp-Gly)$_3$ peptides in the binding buffer (10 mM HEPES-Na (pH 7.5), 3.7 mM EDTA, 150 mM NaCl) were measured by differential scanning calorimetry (details will be published elsewhere).
coli lysate containing the GST-HSP47 fusion protein (100 µl) was mixed with 100 µl of the binding buffer and a 30-µl bed of the affinity beads. The binding was carried out at 4 °C for 1 h. After washing the bed, proteins retained on the beads were eluted by adding Laemmli’s SDS sample buffer and separated by SDS-PAGE on 10% gels.

**Chemical Modification of Type I and III Collagen**—For Arg-specific chemical modification, a 80-µl bed of porcine type I or III collagen (Nitta Gelatin, Osaka, Japan) immobilized to NHS-activated Sepharose 4FF (Amersham Biosciences, Inc.) was treated with 0.2 M 2,3-butanedione in 160 mM sodium borate buffer (pH 8.3) containing 20% methanol at room temperature for 4 days. The Arg modification was confirmed by amino acid analysis of the corresponding 6 x HC3-hydrolysatess. To acetylate Lys residues, the collagen beads were treated with 0.8 M acetic anhydride in 1 M ethanolamine-HCl (pH 7.4) for 8 h to modify carboxyl groups. For modification of His and Met residues, the collagen beads were mixed with 150 mM iodoacetic acid in 0.2 M Tris-HCl (pH 7.5), and the suspension was incubated at room temperature for 4 h. These residue-specific modifications were confirmed by shifting the collagen bands on 7% SDS-polyacrylamide gels visualized by silver staining. Before the binding assay, all of the modified collagen beads were washed and equilibrated with the binding buffer.

**Peptide Binding Assays Based on Fluorescence Quenching**—Chick HSP47 was purified from 13-day-old chick embryos according to the protocol described by Saga et al. (10). Fluorescence spectra were recorded using a Hitachi F-3010 spectrophotometer using a 1-cm path length quartz cuvette. The excitation wavelength was 295 nm. Fluorescence quenching was monitored at the emission wavelength of 340 nm. In the binding assays, stock solutions of type I collagen or collagenous peptides were added to a solution of chick HSP47 (31 µg/ml) in 50 mM bis-Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA. After each addition, the sample solution was equilibrated for 2 min. To ensure the triple-helical conformation of collagenous peptides, all measurements were performed at 4 °C using an Eyela NCB-2200 circulating water bath. The spectra were corrected for dilution.

**RESULTS**

**Introduction of Arg to Yaa Positions of (Pro-Pro-Gly)ₙ Enhances the Binding of HSP47**—In the previous study (9), we identified dozens of HSP47-binding sequences from a random DNA library designed to encode Gly-Pro-Pro-(Gly-Pro-Yaa)ₙ-Pro-Gly-Pro in the yeast two-hybrid screening using HSP47 as a bait. At the Yaa positions in the selected peptides, Arg and Pro residues were highly enriched (6- and 5-fold enrichment, respectively) from the basal frequencies (9). To investigate the effect of Arg residues on the interaction with HSP47, we first analyzed the binding of the GST-HSP47 fusion protein produced in *E. coli* to synthetic Arg-containing collagenous peptides in vitro. Because only (Pro-Pro-Gly)ₙ-peptides are known to interact with HSP47 in the in vitro binding assay (7), sequences of the Arg-containing peptides to be tested were designed to be (Pro-Pro-Gly)ₙ-Pro-Arg-Gly-(Pro-Pro-Gly)ₙ (Fig. 1A). In accordance with the previous data (7), specific binding of GST-HSP47 to (Pro-Pro-Gly)ₙ was observed in a chain length-dependent manner (Fig. 1B). The minimal length required for the HSP47 binding was shortened to five triplets (15 amino acid residues) when one of the Pro residues at the Yaa positions was replaced by an Arg residue (Fig. 1B, lane 9, R8-(PPG)₅). The binding of HSP47 to the Arg-containing peptide was saturated at the 18-mer peptide R11-(PPG)₅ (lanes 10 and 11). Single substitution of the Pro₁₁ of (Pro-Pro-Gly)ₙ for an Arg residue markedly enhances the affinity to GST-HSP47 (lanes 7 and 11). Taken together, the Arg residue incorporated in the...
action with HSP47 using similar (Pro-Hyp-Gly)3-Xaa-Yaa-Gly-(Pro-Hyp-Gly)4 peptides. In the binding assay, four variations of the triplets, Pro-Hyp-Gly, Pro-Arg-Gly, Arg-Hyp-Gly, and Arg-Pro-Gly, were tested for GST-HSP47 binding. As shown in Fig. 3A, only the Pro-Arg-Gly triplet interacted with GST-HSP47, and peptides possessing Arg residues at Xaa positions did not show detectable interaction with GST-HSP47.

The possible contribution of side-chain basicity to the interaction with HSP47 was also examined by replacing the Arg residue with other basic amino acids. We prepared Cys-(Pro-Hyp-Gly)3-Pro-Yaa-Gly-(Pro-Hyp-Gly)4, to Yaa of which Arg, homoarginine, Lys, and Orn were incorporated, and these peptides were coupled to Sepharose beads through N-terminal disulfide bonds. The result of a similar solid-phase binding assay using these basic peptides is shown in Fig. 3B. Replacement of the Arg residue in the Pro-Arg-Gly triplet with a homoarginine residue resulted in a decrease of affinity to GST-HSP47 (Fig. 3B, lane 5). When the Arg was replaced with either a Lys or an Orn, the binding to GST-HSP47 was not detected (lanes 6 and 7).

These results indicate that the positive charge of the Yaa residue does not account for the specific binding to HSP47 and probably the guanidino group, and the length of the Arg side chain is a critical factor for the interaction. We concluded that HSP47 recognizes the Arg residue in a position-specific and side chain-specific fashion.

In the native collagen sequences, various residues are found in the Xaa positions. Among Xaa-Arg-Gly sequences, Gly-Glu-Arg (2.7%), Gly-Pro-Arg (2.6%), Gly-Asp-Arg (1.2%), and Gly-Ala-Arg (1.1%) are prominent, and other combinations are at less than 1% frequency each (12). To examine the effect of Xaa residues on the interaction with HSP47, we synthesized peptides containing an Xaa-Arg-Gly triplet and afforded to the GST-HSP47 binding assay. All of the tested triplets, Pro-Arg-Gly, Ala-Arg-Gly, Asp-Arg-Gly, and Glu-Arg-Gly, showed significant binding to GST-HSP47, suggesting that Xaa amino acids does not largely contribute to the binding to HSP47 (Fig. 3C).

**Importance of Arg Residues of Native Collagens for the Interaction with HSP47**—All of the data presented above strongly suggest that Xaa-Arg-Gly triplets in the triple helix form binding sites for HSP47 in collagen. To confirm this suggestion, homotrimeric porcine type III collagen was chemically modified by several residue-specific modification reagents, and the binding of GST-HSP47 to the modified collagens were examined. When type III collagen was modified with 2,3-butanedione to block the guanidino group of Arg residues, the binding of GST-HSP47 was abolished (Fig. 4, upper panel, lane 4). Modification of other amino acid side chains including Lys (lane 6), His/Met (lane 8), and Lys/Asp/Glu (lane 10) did not largely affect the GST-HSP47 binding. This result indicates that Arg residues are important for type III collagen to be recognized by HSP47.
Fig. 4. Effect of chemical modification of collagen on HSP47 binding. The interaction of GST-HSP47 with immobilized type III (upper panel) and type I collagen (lower panel) with various side chain modifications was analyzed in a manner similar to that shown in panel B of Fig. 1. Modified residues are indicated on the top of the lanes. Lanes for mock-modified (treated only with the buffers) collagens are indicated by minus (−). An arrowhead indicates the band position of GST-HSP47. Molecular sizes are shown in kilodaltons.

Relative binding affinity of chick HSP47 to type I collagen and synthetic substrates was further estimated by means of fluorescence quenching of intrinsic Trp residues of HSP47 (13). As shown in Fig. 5, specific interaction of HSP47 with type I collagen, Arg11-(Pro-Hyp-Gly)_{10} and (Pro-Pro-Gly)_{10}, whose binding was detectable in the previous solid-phase binding assay, was also detected in this spectrometric assay. The non-binder, (Pro-Hyp-Gly)_{10}, did not quench the fluorescence of HSP47. Comparison of the triple-helical triplet-based affinity revealed that the affinity of a Pro-Gly triplet in (Pro-Pro-Gly)_{10} was weaker than that of the mean Xaa-Yaa-Gly triplet in type I collagen. When the concentration of type I collagen was converted to that of the Xaa-Arg-Gly triplet unit (34 homotrimeric Xaa-Arg-Gly triplets in type I collagen), the affinity was comparable with that of a Pro-Gly triplet in Arg^{11i}- (Pro-Hyp-Gly)$_n$. This result also indicates that the interaction of HSP47 with type I collagen is attributed to the binding to the triple-helical Xaa-Arg-Gly triplets.

**DISCUSSION**

The indispensable role of HSP47 in collagen biosynthesis was recently demonstrated by the analysis of embryonic lethal hsp47$^{-/-}$ mice (6). In the mouse embryos, various collagen-based tissue structures were severely disrupted, and cells derived from the mice produced abnormal molecules of at least types I and IV collagen. In addition, two-hybrid screening of cDNA using HSP47 as bait yielded cDNA sequences encoding proteins containing collagenous Xaa-Yaa-Gly repeats. These findings were in good agreement with the fact that HSP47 interacts with various types of collagen (at least type I-V) (3). Taking this into consideration, HSP47 is expected to function through a specific interaction with common sequences in the domain(s) found in various types of collagen. In other words, HSP47-binding sites should exist in the triple helix-forming region of collagens. In our (7) previous search of HSP47-binding sequences, we identified the HSP47-binding sequence (Pro-Pro-Gly)$_n$. The triple-helical conformation was also revealed to be important in recognition by HSP47 (8, 9). However, in the biosynthetic pathway of procollagen, most of the Pro-Pro-Gly sequences are converted to Pro-Hyp-Gly, to which HSP47 does not bind, by the action of prolyl 4-hydroxylase before triple helix formation. This discrepancy led us to further search for HSP47-binding sequences other than (Pro-Pro-Gly)$_n$. Here, we identified Xaa-Arg-Gly triplets as the HSP47-binding sequences by using various synthetic collagen model peptides (Figs. 2 and 3), and the dominance of this sequence in HSP47 binding to collagen was demonstrated utilizing a residue-specific chemical modification technique (Fig. 4). The affinity of HSP47 to the Pro-Arg-Gly triplet was also shown to be much higher than that to the previously identified (Pro-Pro-Gly)$_n$ sequence (Fig. 2). In the fluorescent quenching-based binding assay, the affinity of HSP47 binding to a synthetic Pro-Arg-Gly triplet was shown to be comparable with that to an Xaa-Arg-Gly triplet in type I collagen when other triplets were assumed to have negligible affinity (Fig. 5). This result emphasizes our conclusion that Xaa-Arg-Gly triplets in procollagen are dominant binding sites for HSP47. In addition, interactions of HSP47 with Pro-Pro-Gly triplets in (Pro-Pro-Gly)$_{10}$ are also detectable in the spectrometric assay performed at 4 °C consistently with the previous data (7). However, the similar binding assay at 25 °C failed to detect significant binding between HSP47 and (Pro-Pro-Gly)$_{10}$ as reported by Macdonald and Bächinger (13). This temperature dependence in the HSP47 binding would be further evidence for the importance of triple-helical conformation of the substrates (8, 9).

The information derived from the experiments shown in this study has enabled us to estimate the HSP47-binding sites in native homotrimeric collagens. For instance, HSP47-binding sites in the triple-helical region of bovine type III collagen can

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2 Y. Watanabe, K. Sato, T. Yorihuzi, and K. Nagata, unpublished results.
be estimated as shown in Fig. 6. Type III collagen possesses 32 possible binding sites for HSP47 having the identified sequences of Xaa-Arg-Gly (Xaa = Glu, Pro, Asp, or Ala). An Xaa-Arg-Gly triplet containing another Xaa amino acid is indicated. N- and C-propeptides are shown in italics.

An Arg residue at the Yaa position in the Xaa-Yaa-Gly triplets is known to enhance the thermal stability of triple-helical conformation in a similar magnitude to a Hyp residue. This stabilizing effect was suggested to be attributed to hydrogen bond formation between the guanidino group and a backbone carbonyl group (11). We demonstrate that the basicity of the Arg side chain did not largely contribute to the interaction with HSP47 (Fig. 3B) and that high ionic strength buffer did not disrupt the binding of HSP47 to the Pro-Arg-Gly triplet (data not shown). This result coincides with the previous suggestion that a hydrophobic interaction would mainly contribute to the HSP47-collagen interaction as shown in this study. The 

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REFERENCES
1. Prokop, D. J., Kivirikko, K. I., Tuderman, L., and Guzman, N. A. (1979) Cell. 15, 13–23.
2. Nakai, A., Satoh, M., Hirasawa, K., and Nagata, K. (1992) J. Cell Biol. 117, 903–914.
3. Nakai, A., Satoh, M., Hirasawa, K., and Nagata, K. (1997) J. Biol. Chem. 269, 1183, 2488–2483.
4. Koide, T., Asada, S., and Nagata, K. (1999) J. Biol. Chem. 274, 34523–34526.
5. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
6. Koide, T., Asada, S., and Nagata, K. (1999) J. Biol. Chem. 274, 34523–34526.
7. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
8. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
9. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
10. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
11. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
12. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
13. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
14. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
15. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
16. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
17. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
18. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
19. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
20. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
21. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Biol. Chem. 262, 3481, 3488–3494.
Xaa-Arg-Gly Triplets in the Collagen Triple Helix Are Dominant Binding Sites for the Molecular Chaperone HSP47
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