Specific Recognition of the Collagen Triple Helix by Chaperone HSP47

II. THE HSP47-BINDING STRUCTURAL MOTIF IN COLLAGENS AND RELATED PROTEINS*

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The endoplasmic reticulum-resident chaperone heat-shock protein 47 (HSP47) plays an essential role in procollagen biosynthesis. The function of HSP47 relies on its specific interaction with correctly folded triple-helical regions comprised of Gly-Xaa-Yaa repeats, and Arg residues at Yaa positions have been shown to be important for this interaction. The amino acid at the Yaa position (Yaa3) in the N-terminal-adjoining triplet containing the critical Arg (defined as Arg0) was also suggested to be directly recognized by HSP47 (Koide, T., Asada, S., Takahara, Y., Nishikawa, Y., Nagata, K., and Kitagawa, K. (2006) J. Biol. Chem. 281, 3432–3438). Based on this finding, we examined the relationship between the structure of Yaa3 and HSP47 binding using synthetic collagenous peptides. The results obtained indicated that the structure of Yaa3 determined the binding affinity for HSP47. Maximal binding was observed when Yaa3 was Thr. Moreover, the required relative spatial arrangement of these key residues in the triple helix was analyzed by taking advantage of heterotrimeric collagen-model peptides, each of which contains one Thr3 and one Arg0. The results revealed that HSP47 recognizes the Yaa3 and Arg0 residues only when they are on the same peptide strand. Taken together, the data obtained led us to define the HSP47-binding structural epitope in the collagen triple helix and also define the HSP47-binding motif in the primary structure. A motif search against human protein database predicted candidate clients for this molecular chaperone. The search result indicated that not all collagen family proteins require the chaperonying by HSP47.

Heat-shock protein 47 (HSP47)3 is an essential molecular chaperone for normal procollagen biosynthesis in mammals. Disruption of both alleles of the hsp47 gene in mice causes abnormal procollagen folding in the endoplasmic reticulum (ER), resulting in an embryonic lethal phenotype (1, 2). Although the molecular function of HSP47 remains unclear, it has been suggested that HSP47 could stabilize correctly folded triple-helical intermediates of procollagen that are otherwise unstable at body temperature (3, 4). HSP47 has also been suggested to inhibit lateral associations that form insoluble aggregates in the ER (5). Efforts toward elucidating the collagen-recognition mechanism of HSP47 have been made by our group and others, and to date, Arg residues at Yaa positions in the collagenous Gly-Yaa-Yaa repeats have been shown to be necessary for interaction with HSP47 (6, 7). The importance of the triple-helical structure for HSP47 binding has also been strongly suggested, and it was finally proven in the preceding paper by using conformationally constrained collagenous peptides (8). The minimal number of Arg residues per triple helix required for the specific interaction was further determined to be one. In addition, the Yaa residue occupying position −3 (denoted as Yaa−3), and similar nomenclatures are used later), based on the essential Arg residue at a Yaa position (whose position is defined as position 0), has also been suggested to be important for the interaction, since replacement of the 4-hydroxy-L-proline (Hyp) residue at position −3 in (Pro-Hyp-Gly)4-Pro-Hyp-amide with p-benzoyl-phenylalanine (Bpa) abolished the binding, even though the peptide had a triple-helical structure and contained an Arg residue at a Yaa position. In the present study, we focused on the structure of Yaa−3, which was expected to be another structural determinant for HSP47 binding, together with the side chain structure of Arg3. A structure-activity relationship study was performed using collagen-like peptides with various Yaa−3 substitutions. Moreover, the three-dimensional arrangement of the Yaa−3 and Arg3 residues required for HSP47 binding was determined using heterotrimeric collagen-model peptides with different fixed chain arrangements.

To date, HSP47 has been shown to interact with at least the major types of collagen, including types I to V (9). However, it has not been elucidated whether all 27 types of mammalian collagens require the assistance of HSP47 during their folding processes. In addition, there is currently little available information regarding the interactions of HSP47 with other collagen-related proteins. In the final part of this article, we show the results of a motif search for HSP47-binding proteins against human protein data bases using an originally developed computational search program and predict possible clients of HSP47.

EXPERIMENTAL PROCEDURES

Synthetic Peptides—Peptide chains were constructed manually based on the N-(9-fluorenyl)methoxycarbonyl-based solid-phase method on
HSP47-binding Motif

Rink-amide resins (Novabiochem, Darmstadt, Germany). Heterotrimerization of the peptides were conducted according to the procedure described elsewhere (Ref. 10 and also see supplemental materials). The peptides were purified by reversed-phase high performance liquid chromatography and characterized by electron spray-ionization mass spectrometry or matrix-assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF-MS). Prior to use, all peptide solutions were kept at 4°C for at least 2 days to allow the formation of triple helices.

CD Spectrometry—To characterize the conformational states of the synthetic collagen-like peptides, CD spectra of the refolded trimers (1 mg/ml in 20 mM bis-Tris-HCl (pH 7.4), 1.5 M NaCl) were recorded on a Jasco J-820 spectropolarimeter as described previously (10). Thermal transition curves of the triple helices were determined by monitoring the changes in the molar ellipticity at 225 nm at a heating rate of 0.3 °C/min. All the peptides were triple-helical in the buffer used for the experiments at the temperature at which the binding assays were conducted.

Solid-phase Peptide Binding (Peptide Pull-down) Assay—Solid-phase binding assays to examine HSP47 binding to peptide-immobilized Sepharose beads were performed as reported previously (6). Briefly, Escherichia coli lysates containing either recombinant mouse (11) or human (12) HSP47 protein (70 μl) were mixed with 130 μl of binding buffer (50 mM HEPES-Na (pH 7.5), 150 mM NaCl, 1 mM EDTA) and 25-μl bed of the affinity beads. The binding was carried out at 4°C for 1 h with gentle rotation. After washing, the proteins retained on the beads were eluted with 2× Laemml’s SDS sample buffer (50 mM Tris-HCl (pH 6.7), 2% SDS, 10% glycerol, 0.002% bromphenol blue) and separated by SDS-PAGE in 12% gels. The gels were stained with Coomassie Brilliant Blue R-250 (CBB).

Purification of HSP47—Recombinant mouse HSP47 was purified from E.coli lysates by gelatin-affinity chromatography essentially as described previously (8, 13).

Competition Assay—Competitive binding assays were conducted at 25°C on a surface plasmon resonance (SPR) biosensor (BIACORE X; Biacore AB, Uppsala, Sweden). The R/R/R peptide (10) was immobilized on the surface of a CM5 sensor chip to 495.2 resonance units using standard amine coupling at pH 5.6, according to the manufacturer’s protocol. The binding reaction was performed in 50 mM bis-Tris-HCl (pH 7.4) containing 0.4 M NaCl, 1 mM EDTA, and 0.005% Tween 20. Purified HSP47 was preincubated with various concentrations of the competitor peptides for 10 min at 25°C. Each analyte solution was run over the sensor chip three times at a flow rate of 40 μl/min. The bulk shift due to changes in the refractive index measured using a mock-coupled surface was subtracted from the binding signal under each condition to obtain the specific signals. The surface was regenerated by washing with 5 μl of 100 mM HCl.

Computational HSP47-binding Protein Search—Search programs were written in Perl CGI scripts (Perl 5.8.1) and run on an Apache 1.3.33 HTML server preinstalled on a local Apple Macintosh Darwin Core system. Collections of FASTA-formatted amino acid sequence files from sequenced Homo sapiens (human), Mus musculus (mouse), and Rattus norvegicus (rat) genes were downloaded from the Kyoto Encyclopedia of Genes and Genomes anonymous FTP site to the local host. The total numbers of H. sapiens, M. musculus, and R. norvegicus genes registered in each database were 23,476, 25,371, and 21,879, respectively. Briefly, HSP47-binding proteins were extracted from the database by following steps: 1) extraction of proteins containing at least 6 Xaa-Yaa-Gly repeats with two (or more) Pro in its Xaa- or Yaa positions; 2) elimination of proteins whose Xaa-Yaa-Gly repeated region was compatible with 9 repeats of Gly or three repeats of Gly-Xaa-Gly, thereby resulting in the selection of collagenous proteins; and 3) extraction of proteins with Yaa-Gly-Xaa-Arg in their triple-helical region to obtain putative HSP47-binding proteins. The binding sites were classified into “high affinity” (Yaa³ is Thr or Pro), medium affinity (Yaa³ is Ser, Val, or Ala) and low affinity (Yaa³ is Ile, Leu, Asn, Met, His, Phe, or Tyr) sites, according to the results of the competitive binding assays (see supplemental materials).

Cell Culture—HepG2 (human hepatoma cell line, JCRB1054) and THP-1 (human monocyte leukemia cell line, JCRB0112.1) cells were obtained from Health Science Research Resources Bank (Osaka, Japan). The Chinese Hamster ovary cell line stably expressing human CL-P1 (CL-P1/CHO-IldA7) was established as described previously (14). Cells were grown for 2 days post confluence with their optimal medium containing 50 μg/ml sodium ascorbate.

Glutathione S-Transferase (GST)-HSP47 Binding Assay (GST Pull-down Assay)—Human serum was obtained from one of the authors of this article. Cell lysates were obtained by resuspension of collected cells into lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40), followed by the removal of insoluble materials by centrifugation at 13,000×g for 10 min. Three-hundred μg of lysate or 100 μl of human serum was mixed with a 30-μl bed volume of GST- or GST-HSP47 fusion protein-bound glutathione-Sepharose 4B beads (Amersham Biosciences, Uppsala, Sweden). After washing the beads with TBST buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.005% Tween 20) three times, proteins retained on the beads were separated by SDS-PAGE using 4–20% gradient gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% (w/v) skim milk in TBST and incubated with anti-complement 1q (Clq) antibody (sc-27658, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Macrophage scavenger receptor (0100-0404, Biogenesis, Poole, UK), rabbit anti-CL-L1 polyclonal serum, rabbit anti-CL-P1 polyclonal serum (14), anti-MBL (sc-25615, Santa Cruz) or anti-HSP47 (SPA-470, StressGen Biotechnologies, San Diego, CA). Horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibody was used and the immunoreactive bands were visualized with a peroxidase staining kit.
RESULTS

Yaa<sup>−3</sup> and Arg<sup>0</sup> in the Collagen Triple Helix Are Key Residues for the Interaction with HSP47—HSP47 has been shown to interact with Xaa-Arg-Gly (where Xaa is any amino acid) sequences in triple-helical regions of collagen (6, 7). As shown in previous binding assays, replacement of the Hyp<sup>−3</sup> residue of (Pro-Hyp-Gly)<sub>2</sub>-Pro-Hyp<sup>−3</sup>-Gly-Pro-Arg<sup>0</sup>-Gly-(Pro-Hyp-Gly)<sub>4</sub>-Pro-Hyp-amide with a Bpa residue results in complete loss of HSP47 binding, while similar substitutions of other positions have little effect on the binding (8). These observations indicate that the amino acid Yaa<sup>−3</sup> is another key residue for recognition by HSP47. Based on this finding, the relationship between the structure of the Yaa<sup>−3</sup> residue and HSP47 binding was first analyzed using (Pro-Hyp-Gly)<sub>3</sub>-Pro-Yaa<sup>−3</sup>-Gly-Pro-Arg<sup>0</sup>-Gly-(Pro-Hyp-Gly)<sub>4</sub>-amide with various substitutions in the Yaa<sup>−3</sup> residue. The peptides synthesized and used are shown in Fig. 1. Gly, Trp, and Cys residues, which are rarely found in Yaa positions of native collagens (15), were omitted. All the peptides formed triple-helical structures at 25 °C in the buffer used for the experiments, as judged by CD spectroscopic analyses (data not shown). The peptides were immobilized onto N-hydroxysuccinimide (NHS)-activated beads, and their interactions with recombinant HSP47 were examined by peptide-pull-down assays. The Lys-containing col.KR peptide was omitted from these assays because its ε-amino group would react with the NHS group. As shown in Fig. 2A, the amounts of HSP47 retained on the beads appeared to differ depending on the structure of the Yaa<sup>−3</sup> residue. The bindings with col.TR and col.PR were the most prominent, followed by those with col.SR and col.OR. When Asp, Asn, Glu, and Gln were incorporated at the Yaa<sup>−3</sup> position, the protein band corresponding to bound HSP47 was hardly detected on the gel, although they all contained Arg residues at Yaa-positions.

## TABLE 1

| Peptide | IC<sub>50</sub> | T<sub>m</sub> |
|---------|----------------|-------------|
| col.TR  | 0.69 ± 0.068   | 45.5        |
| col.PR  | 0.94 ± 0.095   | 51.3        |
| col.SR  | 4.0 ± 0.73     | 42.5        |
| col.OR  | 4.6 ± 0.81     | 45.2        |
| col.VR  | 8.4 ± 0.64     | 47.8        |
| col.AR  | 9.3 ± 1.1      | 47.0        |
| col.IR  | 19 ± 7.6       | 47.8        |
| col.LR  | 37 ± 0.84      | 39.5        |
| col.NR  | 41 ± 5.6       | 34.3        |
| col.MR  | 43 ± 3.3       | 49.5        |
| col.HR  | 64 ± 12        | 39.0        |
| col.FR  | 79 ± 13        | 32.9        |
| col.YR  | 95 ± 9.8       | 33.0        |
| col.KR  | >100           | 43.5        |
| col.QR  | >100           | 47.4        |
| (POG)6  | >100           | 47.0        |
| col.ER  | >100           | 44.0        |
| col.DR  | >100           | 34.5        |
| col.RR  | >100           | 37.5        |

*IC<sub>50</sub> values represent the means ± S.D. obtained from three independent determinations. Molar concentrations per trimer unit was used. T<sub>m</sub> values represent the temperature at which a half of a triple helix is denatured.

![Image](attachment:image.png)
The relative HSP47 binding affinities of these peptides were further determined by competition assays on an SPR biosensor. Purified HSP47 protein was mixed with varying concentrations of the competitor peptides and injected over a sensor chip on which an Arg-containing homotrimeric peptide, R/R/R (10), was immobilized. In these assays, the col.RR peptide was designed as (Pro-Hyp-Gly)3-Pro-Glu -Gly-Pro-Arg -Gly-Arg-Pro-Hyp-Gly) to discriminate between the two Arg residues at positions Yaa 0 and Yaa -3 by placing a disfavored Glu residue at position Yaa -3 (Figs. 1 and 2A). The inhibition curves obtained by plotting the bound HSP47 signals (Fig. 2B) were used to estimate the 50% inhibitory concentrations (IC50 values) (Table 1). The results were consistent with those obtained by the peptide pull-down assay (Fig. 2A). The IC50 values were used to range from 1.3 to 100 μM. Regarding the Yaa -3 residue, small hydrophobic and hydroxyl-containing amino acids were preferred by HSP47, while amino acids with long or charged side chains were disfavored. The pair of Thr and Arg was revealed to serve as the best client structure for HSP47. A Pro residue at the Yaa -3 position was also found to contribute to the high affinity binding. However, it should be noted that in the folding pathway of procollagen, most of the Pro residues in Yaa positions are enzymatically converted to Hyp residues, prior to the triple helix formation. In addition, no correlation between the IC50 values and the Tm (melting temperature) values of the triple helices were observed (Table 1).

**Determination of the Three-dimensional Epitope for HSP47 Binding**

The analyses using self-assembling homotrimeric collagen-like peptides indicated that the Yaa -3 residue and the Arg residue at Yaa 0 form an HSP47-binding epitope in the collagen triple helix. The collagen triple helix is a supercoil of three polyproline II-like helices with a one-residue stagger relative to each other (16). Thus, five different spatial arrangements for the combination of these two key residues are possible (Fig. 3A). Here, we used cystine knots to tether the three peptide strands, since cystine knots were reported to be structurally compatible with the collagen triple helix and a one-residue stagger relative to each other (16). Thus, five different spatial arrangements for the combination of these two key residues are possible (Fig. 3A). Therefore, we next investigated which arrangement is recognized by HSP47 by employing a system utilizing heterotrimeric collagen-like peptides. Covalently linked trimeric peptides 1–5 were designed to possess fixed arrangements of the two important residues (Fig. 3A). Here, we used cystine knots to tether the three peptide strands, since cystine knots were reported to be structurally compatible with the collagen triple helix and a one-residue stagger between the adjacent chains can be unambiguously introduced (17). Only one of the three N-terminal amino groups was left free to enable immobilization onto amine-reactive Sepharose beads in a fixed molecular orientation. In these heterotrimers, the position of the Arg residue was fixed to a Yaa 0-position, and one Thr and two Glu residues were introduced at the three corresponding Yaa -3-positions in five different combinations (Fig. 3A). In these peptides, Thr and Glu residues were chosen as representatives of the favored and disfavored amino acids for HSP47 binding, respectively (Fig. 2 and Table 1). These heterotrimeric peptides were triple-helical at the assay temperature, showing similar positive Ω225 values to one another on CD measurements (Fig. 3B). The Tm values for the triple helices of these peptides were also similar to one another (around 45 °C). After coupling to Sepharose beads, the interactions of these trimeric peptides with HSP47 were examined by solid-phase pull-down assays. As shown in Fig. 3C, HSP47 only specifically interacted with Trimer 3, in which the Thr residue and Arg residue are present in the same peptide

**FIGURE 3. Spatial arrangement of Yaa -3 and Arg 0 on the triple helix required for recognition by HSP47. A, structure of the synthetic heterotrimeric peptides (left). Spatial arrangements of the Thr -3 (blue) and Arg 0 (red) residues in the triple-helical structure of (Pro-Hyp-Gly)3-Gly-Pro-Arg -Gly-Pro-Hyp-Gly)3-amide to discriminate between the two Arg residues at positions Yaa 0 and Yaa -3 (Figs. 1 and 2A). The inhibition curves obtained by plotting the bound HSP47 signals (Fig. 2B) were used to estimate the 50% inhibitory concentrations (IC50 values) (Table 1). The results were consistent with those obtained by the peptide pull-down assay (Fig. 2A). The IC50 values were found to range from 1.3 to 100 μM. Regarding the Yaa -3 residue, small hydrophobic and hydroxyl-containing amino acids were preferred by HSP47, while amino acids with long or charged side chains were disfavored. The pair of Thr -3 and Arg 0 was revealed to serve as the best client structure for HSP47. A Pro residue at the Yaa -3 position was also found to contribute to the high affinity binding. However, it should be noted that in the folding pathway of procollagen, most of the Pro residues in Yaa positions are enzymatically converted to Hyp residues, prior to the triple helix formation. In addition, no correlation between the IC50 values and the Tm (melting temperature) values of the triple helices were observed (Table 1).

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HSP47-binding Motif

TABLE 2

| HSP47-binding motif | IC50 | Yaa in Yaa-Gly-Pro-Arg-Gly |
|----------------------|------|---------------------------|
| High affinity        | IC50 < 1 | Thr, Pro                 |
| Medium affinity      | 1 ≤ IC50 < 10 | Ser, Hyp, Val, Ala     |
| Low affinity         | 10 ≤ IC50 < 100 | Ile, Leu, Asn, Met, His, Phe, Tyr |

To confirm this prediction, five collagen-related proteins were examined in their binding ability to GST–HSP47 immobilized to Sepharose beads. C1q, which has one medium affinity and one low affinity HSP47-binding motif (OGXR and LGXR) in its A-chain (entry 48), was shown to specifically interact with GST–HSP47 (Fig. 5A, C1QA). Macrophage scavenger receptor 1 (MSR1, entry 46) with two medium affinity sites, collectin liver 1 (CL-L1/COLEC10, entry 63) with one low affinity site, and collectin placenta 1 (CL-P1/ COLEC12, entry 59) with four medium affinity sites also showed specific binding to GST–HSP47 (Fig. 5A, MSR1, CL-L1, and CL-P1). On the other hand, mannose-binding lectin (MRL2), which has no HSP47-binding site (data not shown), did not show any detectable binding (Fig. 5A, MBL2). These results obtained by the in vitro binding assay proved the validity of our in silico search.

If the biosynthesis of a collagen-related protein requires the assistance of HSP47, its HSP47 binding property is expected to be conserved among animal species. Since the HSP47 binding properties of ficolins, Emillin and Multimerin (EMI) domain–containing 1 (entry 51) and C1q, and tumor necrosis factor-related protein 7 (entry 56) were not found to search for proteins that could interact with HSP47. By using a newly developed computational search program, we selected candidate HSP47-binding proteins from human, rat, and mouse genome databases. The search results derived from the human genome data base are shown in Table 3. Among 102 putative collagenous proteins possessing at least six repeats of the Xaa–Yaa–Gly triplet, 80 proteins contained sequences matching the HSP47-binding motif. All the genes encoding precursors of the α-chains of all types of collagen (types I to XXVII, Table 3, entries 1–40) were revealed to contain multiple HSP47-binding sites, regardless of the subclassifications based on their supramolecular structures, and no significant differences in the frequencies of occurrence were found among the collagen types. These results therefore imply that all collagen precursors are clients of HSP47. In addition to the collagen family, a number of collagen-related proteins, such as pulmonary-surfactant proteins (entries 41–43), ficolins (entries 44 and 45), macrophage scavenger receptors (entries 46 and 47), C1q (entries 48 and 49), C1q-related proteins (entries 52–57), asymmetric acylcholinesterase (ColQ, entry 50), and some collectins (entries 59 and 63) were also selected as possible HSP47-interacting proteins.

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| Entry | Code | High-affinity | Medium-affinity | Low-affinity | Total | Amino acids in triple helix | Name/Description |
|-------|------|---------------|----------------|--------------|-------|---------------------------|-----------------|
| 1 | COL1A1 | 2 11 | 4 1 7 | 2 1 6 | 27 | 1062 Type I, alpha 1 |
| 2 | COL1A2 | 3 12 | 1 12 1 2 7 | 3 2 | 32 | 1062 Type I, alpha 2 |
| 3 | COL2A1 | 1 14 | 2 1 6 | 3 2 | 27 | 1062 Type II, alpha 1 |
| 4 | COL3A1 | 2 14 | 3 7 | 2 1 1 | 28 | 1062 Type III, alpha 1 |
| 5 | COL5A1 | 1 14 | 1 1 | 1 1 | 17 | 1065 Type V, alpha 1 |
| 6 | COL5A2 | 3 17 | 4 2 1 2 1 1 | 1 1 | 31 | 1068 Type V, alpha 2 |
| 7 | COL5A3 | 9 | 1 1 2 | 1 1 | 15 | 1062 Type V, alpha 3 |
| 8 | COL11A1 | 1 11 | 1 2 | 3 1 1 1 | 18 | 1065 Type XI, alpha 1 |
| 9 | COL11A2 | 1 11 | 2 2 | 1 1 | 19 | 1065 Type XI, alpha 2 |
| 10 | COL24A1 | 2 12 | 1 2 | 1 2 | 21 | 981 Type XXV, alpha 1 |
| 11 | COL27A1 | 1 13 | 1 2 | 1 1 | 20 | 990 Type XXVII, alpha 1 |

**Network-forming collagens**

| Entry | Code | High-affinity | Medium-affinity | Low-affinity | Total | Amino acids in triple helix | Name/Description |
|-------|------|---------------|----------------|--------------|-------|---------------------------|-----------------|
| 12 | ULNA1 | 1 6 | 1 2 2 | 1 1 | 8 | 1311 Type IV, alpha 1 |
| 13 | COL4A2 | 12 | 1 2 2 | 1 2 | 17 | 1263 Type IV, alpha 2 |
| 14 | COL4A3 | 1 13 | 1 2 | 1 2 | 19 | 1287 Type IV, alpha 3 |
| 15 | COL4A4 | 1 18 | 1 2 | 1 2 | 20 | 1281 Type IV, alpha 4 |
| 16 | COL5A5 | 7 | 1 2 | 1 2 | 12 | 1284 Type IV, alpha 5 |
| 17 | COL6A8 | 9 | 1 | 1 | 3 | 438 Type VIII, alpha 1 |
| 18 | COL8A1 | 1 1 | 1 | 1 | 11 | 444 Type VIII, alpha 2 |
| 19 | COL8A2, PECAD | 10 | 1 | 1 | 9 | 447 Type X, alpha 1 |
| 20 | COL10A1 | 7 | 2 | 1 | 9 | 447 Type X, alpha 1 |

**FACL collagens**

| Entry | Code | High-affinity | Medium-affinity | Low-affinity | Total | Amino acids in triple helix | Name/Description |
|-------|------|---------------|----------------|--------------|-------|---------------------------|-----------------|
| 21 | COL9A1 | 1 7 | 1 1 | 1 1 | 11 | 585 Type IX, alpha 1 |
| 22 | COL9A2 | 1 5 | 1 2 | 1 | 9 | 585 Type IX, alpha 2 |
| 23 | COL5A3 | 1 7 | 1 1 2 | 1 | 13 | 585 Type IX, alpha 3 |
| 24 | COL12A | 1 1 | 1 | 1 | 7 | 246 Type XII, alpha 1 |
| 25 | COL14A1 | 1 4 | 1 1 | 1 | 4 | 249 Type XIV, alpha 1 |
| 26 | COL16A1 | 2 5 | 3 | 1 | 10 | 936 Type XVI, alpha 1 |
| 27 | COL19A1 | 4 | 1 | 1 | 6 | 669 Type XIX, alpha 1 |
| 28 | COL21A1 | 3 | 1 | 1 | 3 | 447 Type XXI, alpha 1 |
| 29 | COL22A1 | 12 2 | 1 | 15 | 1020 Type XXII, alpha 1 |

**Transmembrane collagens**

| Entry | Code | High-affinity | Medium-affinity | Low-affinity | Total | Amino acids in triple helix | Name/Description |
|-------|------|---------------|----------------|--------------|-------|---------------------------|-----------------|
| 30 | COL13A1 | 1 1 | 1 | 2 | 2 | 492 Type XIII, alpha 1 |
| 31 | COL17A1, BPAK2 | 5 | 1 | 1 | 7 | 432 Type XVIII, alpha 1 |
| 32 | COL23A1 | 5 | 1 | 1 | 9 | 366 Type XXIII, alpha 1 |
| 33 | COL25A1 | 4 | 1 | 1 | 6 | 441 Type XXV, alpha 1 |

**Other collagens**

| Entry | Code | High-affinity | Medium-affinity | Low-affinity | Total | Amino acids in triple helix | Name/Description |
|-------|------|---------------|----------------|--------------|-------|---------------------------|-----------------|
| 34 | COL6A1 | 1 1 | 1 | 1 | 7 | 327 Type VI, alpha 1 |
| 35 | COL6A2 | 9 | 1 | 1 | 10 | 330 Type VI, alpha 2 |
| 36 | COL6A3 | 1 1 | 1 4 | 1 3 | 9 | 330 Type VI, alpha 3 |
| 37 | COL17A1 | 1 2 3 | 1 1 | 1 | 2 35 | 1392 Type VII, alpha 1 |
| 38 | COL17A1 | 2 1 1 | 4 | 3 | 2 | 363 Type XV, alpha 1 |
| 39 | COL18A1 | 2 1 | 1 | 1 | 4 | 507 Type XVIII, alpha 1 |
| 40 | EMD2, COL26A1 | 3 | 1 | 4 | 123 | Type XXVI, alpha 1 (EMD domain-containing 2) |
| Entry | Code | HSP47-binding motif | Name/Description |
|-------|------|---------------------|------------------|
| 41    | SFTPA1, SFTPA2 | 1 | 68 Surfactant, pulmonary-associated protein A1 |
| 42    | SFTPA1, SFTPA2 | 1 | 69 Surfactant, pulmonary-associated protein A2 |
| 43    | SFTPA1, SFTPA2 | 2 | 177 Surfactant, pulmonary-associated protein U |
| 44    | FON1<sup>a</sup> | 1 | 57 Ficolin (collagen/fibrinogen domain-containing) |
| 45    | FGN2<sup>a</sup> | 1 | 45 Ficolin (collagen/fibrinogen domain-containing lectin) 2 |
| 46    | MSR1 | 1 | 68 Mesothelin receptor |
| 47    | SCARA3 | 2 | 147 Scavenger receptor class A, member 3 |
| 48    | C1Q | 1 | 78 Complement component 1, q subcomponent, alpha polypeptide |
| 49    | C1QG | 1 | 81 Complement component 1, q subcomponent, gamma polypeptide |
| 50    | COLQ | 1 | 174 Collagen-like tail subunit of asymmetric acetylcholinesterase |
| 51    | EMR1<sup>b</sup> | 1 | 141 ERM domain-containing 1 |
| 52    | C1QTNF1 | 1 | 121 C1q and tumor necrosis factor-related protein 1 |
| 53    | C1QTNF2 | 1 | 102 C1q and tumor necrosis factor-related protein 2 |
| 54    | C1QTNF3 | 2 | 63 C1q and tumor necrosis factor-related protein 3 |
| 55    | C1QTNF5 | 2 | 68 C1q and tumor necrosis factor-related protein 5 |
| 56    | C1QTNF8<sup>d</sup> | 1 | 102 C1q and tumor necrosis factor-related protein 7 |
| 57    | C1QTNF8 | 1 | 42 C1q and tumor necrosis factor-related protein 8 |
| 58    | KIAA1510 | 1 | 138 KIAA1510, KIAA1510 protein |
| 59    | CLEC12 | 1 | 147 Collectin subfamily member 12 (CL-P1) |
| 60    | CFA81A | 1 | 24 Cysteine-rich protein 1 |
| 61    | CFA81A, CFA81B, CFA81G | 1 | 24 Cysteine-rich protein 1 |
| 62    | CFA82 | 1 | 24 Cysteine-rich protein 1B |
| 63    | COL10 | 2 | 111 Colecin |
| 64    | EWSR1<sup>d</sup> | 1 | 57 Colecin subfamily member 10 (C-type lectin, CL-L1) |
| 65    | COLM<sup>d</sup> | 1 | 22 Colecin |
| 66    | C1QL1<sup>d</sup> | 1 | 24 Colecin subfamily member 2 |
| 67    | C1QL3<sup>d</sup> | 1 | 24 Colecin subfamily member 3 |
| 68    | CDBE1<sup>d</sup> | 1 | 24 Colecin subfamily member 4 |
| 69    | MGC15476<sup>d</sup> | 1 | 24 Colecin subfamily member 5 |
| 70    | LOC331149<sup>f</sup> | 1 | 18 Colecin |
| 71    | LOC331873<sup>f</sup> | 1 | 20 Colecin |
| 72    | LOC625929<sup>f</sup> | 1 | 20 Colecin |
| 73    | LOC38767<sup>f</sup> | 1 | 20 Colecin |
| 74    | LOC3800267<sup>f</sup> | 1 | 20 Colecin |
| 75    | LOC387971<sup>f</sup> | 1 | 20 Colecin |
| 76    | LOC440376<sup>f</sup> | 1 | 20 Colecin |
| 77    | LOC440424<sup>f</sup> | 1 | 20 Colecin |
| 78    | LOC442598<sup>f</sup> | 1 | 20 Colecin |
| 79    | MGC45785<sup>f</sup> | 2 | 20 Colecin |
| 80    | MGC46915<sup>f</sup> | 1 | 20 Colecin |

<sup>a</sup> Most of the Pro residues at Yaa positions would be converted to Hyp prior to triple helix formation.
<sup>b</sup> The existence of a HSP47-binding motif is not conserved among species.
<sup>c</sup> Cytosolic or nuclear proteins.
<sup>d</sup> Only DNA sequences are available.
be conserved among the corresponding homologues in humans, mice, and rats, their folding processes are not likely to require the chaperone function of HSP47. It should also be mentioned that a true client protein during its folding pathway must co-exist with the chaperone. MSR1 is a type II membrane protein whose collagen-like domain is directed to the ER lumen (19), and the endogenous HSP47 proteins are expressed in the same cells (HepG2 cell, Fig. 5B). Therefore, the relationship between the folding pathways of the collagen-related proteins and the function of HSP47 are suggested. Similarly, since HSP47 was reported to be expressed in type II pneumocytes producing pulmonary surfactant protein A (20), there may be a relationship between the folding pathways of pulmonary surfactant proteins and HSP47 function.

Although CL-L1 was shown to interact with HSP47 in vitro (Fig. 5A), it is localized in the cytosol (21). This indicates that CL-L1 is very unlikely to be a client for the HSP47. C1q also was shown to interact with HSP47 in vitro (Fig. 5A), but the major source of C1q was reported to be macrophages (22) whose ER contains little HSP47. In addition, some collagen-like proteins, such as MBL2, MARCO (macrophage receptor with collagenous structure), and adiponectin, do not possess HSP47-binding motifs (data not shown). It was therefore revealed that not all biosynthetic pathways of collagen-related proteins require the assistance of HSP47.

**DISCUSSION**

To clarify the molecular basis of the function of a chaperone, it is particularly important to determine the structural requirements of its clients. For example, the studies in which HSP70 family proteins were revealed to recognize short hydrophobic sequences in unfolded structures contributed to a better understanding of their molecular functions (23–25). Unlike other chaperones, HSP47 specifically interacts with correctly folded, that is triple-helical, clients (8). In the present paper, we elucidated that the side chain structure of the Yaa-3 residue is a structural determinant for specific interaction with HSP47, in addition to the previously reported Arg residue at Yaa3 (6, 7). It was also shown that HSP47 recognizes Yaa-3 residues with relatively broad specificity and that the structure of Yaa-3 determines the affinity for HSP47 (Fig. 2). Moreover, binding assays involving heterotrimeric triple-helical peptides with fixed chain arrangements resulted in identification of the structural epitope in the triple helix. In this epitope, the side chains of Arg3 and Yaa-3 must radiate out from the same polypeptide (Fig. 3). From the structural point of view, this side chain arrangement supplies the flattest HSP47-binding interface among the five possible chain arrangements (Fig. 3A). This dual-site recognition pattern seems to be the most compatible with the recently determined molecular orientation of HSP47 in chaperone-client complexes (8).

Currently, HSP47 is thought to facilitate the propagation of procollagen triple helices by inhibiting thermal denaturation of partly folded triple-helical intermediates (3, 4). This scenario was supported by a previous report showing that the thermal stability of type I collagen is below body temperature (26), although the feasibility of this hypothesis is still under debate (27). Alternatively, HSP47 was suggested to function as an inhibitor of aggregate formation by procollagens in the ER, based on in vitro experiments (5). Thus, the question arises as to which function represents the major role of HSP47 in vivo. Our results appear to support the former, although we cannot exclude the latter. In the present study, most collagens were shown to possess a number of HSP47-binding sites with varying affinities depending on the structures of their Yaa-3 residues (Fig. 4 and Table 3). This finding implies the following scenario concerning the role of HSP47 as a heat-inducible protein. Under heat-stressed conditions, productive folding of procollagens would require more triple helix stabilizers. Such an enhanced stabilization effect could be achieved by an increase in the amount of HSP47 in the ER. The binding of HSP47 to affinity sites, those not utilized under normal conditions, could serve this function.

It has been an important issue whether all collagens and other proteins containing collagenous triple helices require the assistance of HSP47 during their biosynthesis. We successfully identified HSP47-binding motifs that are describable in the primary structure, and subsequent database searches selected a number of possible clients for HSP47. In humans, all types of collagen precursors are predicted to interact with HSP47, and the HSP47 binding properties were conserved in their mouse and rat homologues (data not shown). It is thus strongly suggested that all types of collagen precursors are clients of HSP47 if they co-localize in cells.

It has been little information available regarding the interactions of HSP47 with other collagen-related proteins. Despite the relatively broad binding specificity of HSP47, not all collagen related-proteins were retrieved by the search for the HSP47 clients. Furthermore, subcellular localizations and tissue distributions of a client protein must match with that of the chaperone. The relationship between HSP47-function and the biosynthesis of individual candidate clients should be carefully examined.

Inhibition of excessive collagen synthesis by blocking HSP47 function...
is expected to be a promising mechanism for the treatment of fibrotic diseases, since the progression of fibrosis in various tissues and organs has been shown to be associated with increases in HSP47 (28–30). To date, the administration of antisense oligonucleotides against HSP47 was reported to suppress the accumulation of collagen in experimental glomerulonephritis (31) and peritoneal fibrosis (32). Recently, several low molecular weight compounds were also found to inhibit HSP47 function in vitro (33). In the present study, we found that an HSP47-binding site containing the pairing of a Thr at Yaa and an Arg at Yaa exhibits the maximum binding to HSP47. The structural basis of the HSP47-collagen interactions elucidated here should provide useful structural information for the lead generation of novel antifibrotic drugs. Our ongoing structure-activity relationship study including non-natural amino acid residues would elucidate a putative pharmacophore structure of an HSP47 inhibitor.

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