Specific Recognition of the Collagen Triple Helix by Chaperone HSP47

MINIMAL STRUCTURAL REQUIREMENT AND SPATIAL MOLECULAR ORIENTATION

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Takaki Koide, Shinichi Asada, Yoshifumi Takahara, Yoshimi Nishikawa, Kazuhiro Nagata, and Kouki Kitagawa

From the Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata 950-2081, Japan, the Faculty of Engineering, The University of Tokushima, Tokushima 770-8506, Japan, and the Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8397, Japan

The unique folding of procollagens in the endoplasmic reticulum is achieved with the assistance of procollagen-specific molecular chaperones. Heat-shock protein 47 (HSP47) is an endoplasmic reticulum-resident chaperone that plays an essential role in normal procollagen folding, although its molecular function has not yet been clarified. Recent advances in studies on the binding specificity of HSP47 have revealed that Arg residues at Yaa positions in collag- enous Gly-Xaa-Yaa repeats are critical for its interactions (Koide, T., Takahara, Y., Asada, S., and Nagata, K. (2002) J. Biol. Chem. 277, 6178–6182; Tasab, M., Jenkinson, L., and Bulleid, N. J. (2002) J. Biol. Chem. 277, 35007–35012). In the present study, we further examined the client recognition mechanism of HSP47 by taking advantage of systems employing engineered collagen model peptides. First, in vitro binding studies using conformationally constrained collagen-like peptides revealed that HSP47 only recognized correctly folded triple helices and that the interaction with the corresponding single-chain polypeptides was negligible. Second, a binding study using heterotrimeric model clients for HSP47 demonstrated a minimal requirement for the number of Arg residues in the triple helix. Finally, a cross-linking study using photoreactive collagenous peptides provided information about the spatial orientation of an HSP47 molecule in the chaperone-collagen complex. The obtained results led to the development of a new model of HSP47-collagen complexes that differs completely from the previously proposed “flying capstan model” (Dafforn, T. R., Della, M., and Miller, A. D. (2001) J. Biol. Chem. 276, 49310–49319).

Heat-shock protein 47 (HSP47) is a unique client-specific molecular chaperone that specifically interacts with procollagens during their folding process (1). This chaperone shows no homology to either other heat-shock proteins or other chaperones but belongs to the serine protease inhibitor (serpin) superfamily. HSP47 is the only heat-shock protein identified in the secretory pathway to date. Previous hsp47 gene disruption studies have revealed that HSP47 is indispensable for normal procollagen biosynthesis and therefore essential for normal embryogenesis in mice (2, 3). Although the molecular function of HSP47 remains unclear, recent progress in elucidating its procollagen binding mechanism has provided information about the collagen structures required for specific recognition by HSP47 (4–9). Using self-assembling homotrimeric collagen model peptides, HSP47 was shown to interact with Xaa-Arg-Gly sequences in collagen-like triple helices based on Gly-Pro-4-hydroxy-L-proline (Gly-Pro-Hyp) repeats (6). This conclusion was independently confirmed by using genetically engineered homotrimeric procollagens introduced into semipermeabilized cells (8). However, many types of collagen, such as types I, IV, and V, are comprised of different α-chains that form heterotrimeric triple helices, and HSP47 is also known to interact with these heterotrimeric collagens both in vitro (10) and in vivo (11, 12). The triple-helical (Gly-Xaa-Yaa)n region of type I collagen possesses 34 sites in which all three chains contain Arg residues at the same Yaa positions. There are also nine and eight additional heterotrimeric sites that contain one and two Arg residues, respectively. However, it has not yet been elucidated whether or not such regions with asymmetric Arg residues can serve as binding sites for HSP47. From the structural point of view, HSP47 binding should not require that all three Arg residues pointing out from the triple helix, since serpins (including HSP47) are unlikely to possess a trench that can accommodate all three polypeptide chains of a triple helix. Although HSP47 has been shown to interact with triple-helical clients, it remains unclear whether it solely recognizes the triple-helical conformation or whether it also recognizes single-chain clients as indicated in earlier reports (12, 13).

In the present paper, we address the following three questions regarding the client recognition mechanism of this procollagen-specific molecular chaperone. First, does HSP47 only recognize triple-helical clients, or does it also interact with single-stranded nascent prochains? Second, what is the minimal structure of collagen required for HSP47 binding? Third, what is the shape and size of the HSP47-collagen binding interface? To answer these questions, we designed and used various synthetic collagen-like peptides rather than purified native collagen, which is a huge molecule with a lower solubility and a tendency to aggregate. The information obtained led to the development of a new model of HSP47-collagen complexes that differs completely from the previously proposed “flying capstan model” (14).

EXPERIMENTAL PROCEDURES

Peptides—Peptide chains were constructed manually based on the standard N-(9-fluorenylmethoxycarbonyl (Fmoc)-based strategy on Rink-amide resins (Novabiochem, Darmstadt, Germany). For p-ben-
zoyl-L-phenylalanine (Bpa)-containing peptides, Fmoc-Bpa-OH (Watanabe Chemical Co. Ltd., Hiroshima, Japan) was used as a precursor of Bpa residues. All Bpa-containing materials were handled in the dark. Peptide cleavage/deprotection was performed by treatment with a standard trifluoroacetic acid-scavenger mixture. For biotinylation, the synthetic peptides were treated with 4.5 equivalents of a Sulfo-NHS-S-ethylmaleimide (NEM). The lysates were loaded onto a gelatin-Sepharose column. After washing with phosphate-buffered saline and then incubated with an antiserum raised in rabbits against a synthetic collagen-like peptide, Fmoc-Bpa-OH (Bio-Rad) as appropriate.

**Surface Plasmon Resonance (SPR) Measurement**—Binding of recombinant human HSP47 to collagenous peptides was measured at 25 °C using an SPR biosensor (BIACORE 3000; Biacore, Uppsala, Sweden). The peptides were covalently immobilized to a CM5 biosensor chip through their α-amino groups at pH 6.5 following the manufacturer’s protocol. A surface concentration of about 300 pg/mm², calculated using 1 resonance unit = 1 pg/mm², was obtained. Resonance was measured in 50 mM bis-Tris-HCl (pH 7.4) containing 150 mM NaCl and 1 mM EDTA as a running buffer at a flow rate of 20 µl/min. After each cycle, the chip was regenerated with a 15-s pulse of 0.1 M HCl. The response at equilibrium was corrected for bulk refractive index errors using a mock-coupled flow cell blocked with ethanolamine as a reference flow cell.

For competition assays, a solution of HSP47 (final concentration, 2 µM) was premixed with varying concentrations of the competing peptides. The mixtures were injected over immobilized R/R/R peptide (Fig. 2A) until binding equilibrium was achieved. Inhibition of binding was measured as a decrease in the response as a function of the peptide concentration.

**UV-induced Cross-linking**—Recombinant mouse HSP47 (final concentration, 3.4 µM) was mixed with refolded Bpa peptides (final concentration, 17 µM) in 50 mM bis-Tris-HCl (pH 7.4) containing 0.4 M NaCl and 1 mM EDTA. The cross-linking experiments were carried out in 96-well microtiter plates in a final volume of 50 µl. The mixtures were UV-irradiated at 365 nm at a distance of 10 cm for 30 s on ice using a UV irradiator (model L2859-01; Hamamatsu Photonics, Hamamatsu, Japan). Following the cross-linking reaction, 10 µl of 5× Laemmli’s SDS-sample buffer was added to 40 µl of the mixture and heated at 95 °C for 3 min.

**RESULTS**

**Oligomeric States of Purified Recombinant HSP47**—Recombinant human or mouse HSP47 was readily purified from lysates of *E. coli* harboring the corresponding expression plasmids by gelatin (or collagen) affinity chromatography essentially as reported previously (17). When the protein was prepared under non-reducing conditions, a slowly migrating band of HSP47 (~130 kDa) was observed in SDS-PAGE gels in addition to the major band corresponding to the monomeric protein (Fig. 1A, lanes 4 and 8), consistent with previous reports (9, 14). On the other hand, the larger band was not observed when NEM, an alkylating reagent for thiol groups, was added to the lysis buffer (lanes 3 and 7) or the SDS samples were reduced with dithiothreitol (DTT) (lanes 2 and 6). In the case of chick HSP47, which does not contain any cysteine residues, the oligomeric form was never observed in similar SDS-PAGE analyses (data not shown). These results clearly indicate that the larger...
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FIGURE 1. Characterization of recombinant HSP47. A, SDS-PAGE of recombinant HSP47 purified in the presence or absence of NEM. The SDS-PAGE samples were prepared in the presence or absence of 20 mM DTT. Proteins were visualized by either CBB staining (left panel) or Western blotting with an anti-HSP47 antibody (right panel). Arrowheads and asterisks indicate monomeric and oligomeric HSP47, respectively. B, size exclusion chromatography of recombinant HSP47 purified in the presence (right panel) or absence (left panel) of NEM. The arrow indicates the peak position of oligomeric HSP47. Vo denotes the void volume.

band represents a disulfide-linked oligomer. Although the estimated size of the disulfide-linked oligomer (~130 kDa) implies the formation of a trimer of the recombinant HSP47 (molecular mass, 45 kDa), human, and mouse HSP47 only contain one cysteine residue and therefore can only form dimers. The origin of the strange mobility in the gel currently remains unclear.

The oligomeric states of recombinant HSP47 under non-denaturing conditions were also analyzed by size-exclusion chromatography. In the chromatographic profile of HSP47 purified in the absence of NEM, the oligomer corresponding to the slowly migrating band on the SDS-PAGE gel (Fig. 1A, lanes 4 and 8) appeared as a shoulder peak (Fig. 1B, left panel). This population was also sensitive to subsequent DTT treatment under non-denaturing conditions (data not shown). Consistently, when the sample was prepared in the presence of NEM, most of the HSP47 population appeared in the monomeric fraction and the oligomeric population was negligible (Fig. 1B, right panel). In the following experiments, we used monomeric HSP47 prepared in the presence of NEM.

HSP47 Binds to Triple-helical, Not Single-chain, Collagen—A previous study involving immunoprecipitation of HSP47 complexed with genetically engineered procollagen molecules from semipermeabilized cells demonstrated that HSP47 interacts preferentially with triple-helical procollagen molecules (7). This conformational preference was also supported by results obtained from two-hybrid selection of HSP47 binding collagenous peptides (5). However, the difference in the HSP47 binding affinities between triple-helical and random coil clients has not yet been directly compared with date.

To elucidate the HSP47 binding conformation of collagen, we used trimeric collagen models and one of their single-chain components (Fig. 2A). Since (Pro-Pro-Gly)2-Pro-Arg-Gly-(Pro-Pro-Gly)2 was reported to be the shortest collagen model that binds to HSP47 in solid-phase binding assays (6), each peptide chain was designed to contain five repeats of the Gly-Xaa-Yaa triplet. Only the N-terminal α-amino groups in the bis-cysteinyl chains were left free, to allow immobilization on solid supports in defined molecular orientations. The heterotrimetric peptides were synthesized by a combination of the standard solid-phase method and subsequent regioselective disulfide-bond formation (15). All the trimeric collagen models were triple-helical at 4 °C as assessed by CD spectrometry, and their melting temperatures (the temperature at which half the triple helix is unfolded) were estimated to be in the range of 37.5–47.2 °C (15). Since N-terminally introduced cysteine knots largely contribute to stabilization of the triple-helical conformation, the monomeric counterpart of R/R/R (R-monomer) does not completely fold into the triple-helical conformation even at 4 °C (15).

The conformational preference of HSP47-collagen interactions was analyzed by competition assays using an SPR biosensor. The SPR signal was observed when recombinant HSP47 was injected over a sensor chip containing immobilized R/R/R peptide. The binding was effectively competed by the addition of soluble R/R/R peptide in a dose-dependent manner. On the other hand, no obvious competition was observed when the R-monomer was used as a competitor (Fig. 2B). Since the competition assays were carried out at 25 °C, a temperature at which the R/R/R-trimer is fully triple-helical and the R-monomer is almost a random coil (15), the results reveal that HSP47 mainly recognizes triple-helical collagen, while its binding to the single-chain form is negligible. This conclusion was further confirmed by direct binding assays utilizing the fluorescent quenching of intrinsic Trp residues in the chaperone (6, 18) (Fig. 2C).
Interaction of HSP47 with Heterotrimeric Collagen Peptides—To elucidate the minimal requirement for the number of Arg residues at Yaa positions in a collagen triple helix, the interactions of HSP47 with trim-}

FIGURE 3. Interaction of HSP47 with heterotrimeric peptides. A, binding of recombinant HSP47 to heterotrimeric collagen peptides. A lysate of E. coli expressing HSP47 was mixed with affinity beads to which the indicated peptides were immobilized. After washing, the proteins retained on the beads were separated in a 12% SDS-polyacrylamide gel and visualized by CBB staining. B, competitive SPR analysis was performed in a similar manner as described in Fig. 2B. R/R/R (circles), R/R/O (diamonds), R/O/O (squares), and O/O/O (triangles) were used as competitors.

FIGURE 4. Design of photoreactive clients. A, the proposed mechanism of the UV-induced cross-linking reaction. The absorption of UV by the benzophenone moiety of a Bpa residue results in the formation of the highly reactive diradicaloid. The diradicaloid covalently captures a closely placed amino acid side chain of HSP47. B, structures of the peptides synthesized for the UV-induced cross-linking. The position of the Arg residue was defined as 0. All peptides were triple helical at 4 °C.

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UV-induced Cross-linking of HSP47 to Bpa-containing Collagen Peptides—To obtain information regarding the binding interface in HSP47-client complexes, we performed photoaffinity cross-linking utilizing photoreactive Bpa-containing clients (Ref. 19 and Fig. 4A). We synthesized self-assembling open-chain collagen peptides that contained Bpa residues for these UV-induced cross-linking experiments (Fig. 4B). Except for the POG peptide used as a negative control (4), all the peptides were designed to contain one Pro-Arg-Gly tripeptide unit per molecule, shown to be essential for recognition by HSP47. In peptides B\(^{-1}\) to B\(^{14}\), Bpa residues were introduced at various Xaa and Yaa positions. An N-terminally biotinylated version of B\(^{-1}\) (Bio-B\(^{-1}\)) was also prepared to facilitate detection of HSP47-peptide complexes by Western blotting. CD spectrometric analyses of the peptides in aqueous solutions demonstrated that all of them spontaneously associated to form triple helices at 4 °C, the temperature at which all the experiments were performed (data not shown).

In a previous study (6), we reported that an amino acid residue in the Baa position of a Xaa-Arg-Gly sequence does not largely contribute to recognition by HSP47. Therefore, we first examined the specificity of HSP47 binding and UV-induced cross-linking using the B\(^{-1}\) peptide containing a Bpa-Arg-Gly sequence. As shown in Fig. 5A, recombinant HSP47 was effectively purified from a crude E. coli lysate using B\(^{-1}\)-immobilized affinity beads (lane 3) as well as using ARG-immobilized beads (lane 4). Photo-induced cross-linking of biotinylated B\(^{-1}\) (Bio-B\(^{-1}\)) to HSP47 was demonstrated by SDS-PAGE of UV-irradiated samples (Fig. 5B). Slowly migrating cross-linking bands were only detected on a CBB-stained gel when the HSP47 solution was UV-irradiated in the presence of Bio-B\(^{-1}\) (Fig. 5B, upper panel, lane 4). These bands were characterized to be covalently cross-linked HSP47-peptide complexes, since they were recognized by both SA-HRP and an anti-HSP47 antibody (Fig. 5B, middle and lower panels). The cross-linking reaction appeared to produce several products, and the differences in the migration of these bands in the gel would be caused by differences in the cross-linking sites on an HSP47 molecule. The specificity of the cross-linking was further confirmed by competition assays. As shown in Fig. 5C, production of cross-linked products was competed out in the presence of 15 equivalents or more of the ARG peptide, a specific competitor. On the other hand, even in the presence of 15 equivalents of the POG peptide, which is not recognized by HSP47, little competition in the UV-induced cross-linking was observed. These results indicate that this photoaffinity cross-linking system is effective for investigating specific interactions between HSP47 and collagen-like peptides.

Mapping of the HSP47 Binding Interface in the Collagen Triple Helix—
Binding and UV-induced cross-linking between recombinant HSP47 and various Bpa-containing peptides were examined to map the HSP47 binding interface in a collagen triple helix. In solid-phase pull-down assays, all the peptides, except for B\(^{-3}\), appeared to specifically interact with HSP47 (Fig. 6A, upper panel). Slowly migrating cross-linked products were only found in the cases of B\(^{-10}\), B\(^{-9}\), B\(^{-7}\), B\(^{-6}\), B\(^{-4}\), B\(^{-1}\), B\(^{2}\), B\(^{3}\), B\(^{5}\), B\(^{6}\), and B\(^{8}\) after UV irradiation (Fig. 6A, lower panel, lanes 4–15). These results indicate that Bpa residues between positions –10 and 8 are placed very close to the surface of HSP47 molecules in the complex.

The specificity of the cross-linking products between the lanes on the gel would be caused by differences in the positions of the cross-linking sites on the HSP47 molecule.
the cross-linkable amino acid residues were mapped on the crystal structure of a synthetic model peptide for type III collagen, T3-785 (20). In this triple-helical model, the length of the cross-linkable region (positions −10 to 8) was estimated to be 5.3 nm (Fig. 6B, middle). When the longitudinal axis of a serpin molecule was allocated parallel to the common axis of the triple helix, the length of the cross-linkable region matched very well with the length of the serpin (Fig. 6B, left). If the serpin was arranged perpendicular to the triple helix, the length of the binding interface was too short and the curvature of the protein surface would be too large to explain the results of the cross-linking experiments (Fig. 6B, right). Therefore, we strongly suggest that the longer axis of HSP47 is oriented along the helical axis of the collagen triple helix in the chaperone complex.

Among the amino acid positions included in the cross-linkable region, only substitution of Hyp at position 3 with a Bpa residue resulted in complete loss of the HSP47 binding (Fig. 6A, upper panel, lane 9). This observation indicates that the side chain structure of the amino acid at position −3 is also directly recognized by an HSP47 molecule, in addition to the critical Arg residue at position 0. Moreover, HSP47 was shown to discriminate the direction of the triple helix, since a similar replacement of position 3, the symmetrical position of position −3 to the critical Arg residue, did not show any loss of the interaction (Fig. 6A, upper panel, lane 12).

DISCUSSION

Procollagen biosynthesis includes the unique process of triple helix formation, which is not found in other globular proteins. This unique step, which occurs in the endoplasmic reticulum, involves the successive actions of specific chaperones and modifying enzymes. To date, at least three classes of chaperones/enzymes are known to transiently interact with the triple helix-forming region comprised of Gly-Xaa-Yaa repeats in procollagens (1). Prolyl 4-hydroxylase (P4-H) interacts with these Gly-Xaa-Yaa repeats before triple helix formation. P4-H not only acts as the principal post-translational modification enzyme for procollagens (21), but also functions as a chaperone through its interaction with single-stranded procollagens. The chaperone function of P4-H has been suggested to participate in the quality control mechanism of procollagen biosynthesis by retaining premature or misfolded procollagen molecules in the endoplasmic reticulum (22). Peptidyl prolyl cis-trans-isomerases also recognize single-stranded polypeptides of procollagens and catalyze the isomerization of X-Pro or X-Hyp peptide bonds to accelerate propagation of the triple helix (23, 24). The function of HSP47 is the least elucidated among the procollagen-interacting chaperones, although it was revealed to be indispensable by gene knock-out experiments (2). It is particularly important to determine the stage of procollagen biosynthesis that involves the interaction with HSP47. Accumulating evidence has indicated that HSP47 recognizes triple-helical clients, and it has been suggested to be involved in the later stage of procollagen folding (4–8, 18). However, previous papers have reported that HSP47 also interacts with nascent single-chain polypeptides of procollagens (12, 13). In the present study, we directly compared the binding affinities of HSP47 to triple-helical clients and a corresponding single-chain peptide by utilizing conformationally constrained collagen models (Fig. 2). The results clearly demonstrated that HSP47 has a strong conformational preference for triple-helical clients. This finding indicates that HSP47 is unlikely to be involved in events preceding the triple helix formation.

In a previous paper, Miller and co-workers (14) proposed a model of HSP47-collagen complexes, designated the flying capstan model, in which each peptide binding site pointing out from a cyclic HSP47 trimer accommodates a single-stranded collagen polypeptide. They further speculated that HSP47 would facilitate triple helix formation by inducing proline-11 helix formation of each strand. However, none of the data obtained in the present study support this previous model. First, HSP47 is monomeric, and the oligomer is a disulfide-linked product rather than a β-sheet insertion (14). Even though the trimer exists and is functional, its population is very minor (Fig. 1). Second, HSP47 is not expected to recognize single-stranded collagen peptides (Fig. 2). Third, the length of the R/R/R trimer, which binds to HSP47 (Fig. 3), is shorter than the length of the longer axis of an HSP47 molecule (Fig. 6). Therefore, it is structurally impossible to create their model. Collectively, we conclude that the flying capstan model is unlikely to be correct.

Although x-ray crystallographic analysis is the most straightforward way to solve the structure of HSP47-peptide complexes, the physical characteristics of HSP47 have prevented crystallization in our trial studies. Instead, we have used biochemical analyses to collect information about HSP47-collagen interactions in the present study. Heterotrimeric collagen model peptides tethered by cystine knots have been already applied to analyses of substrate recognition by matrix metalloproteinases (25) and α1β1 integrin binding (26–28). Taking advantage of this peptide-based system, we were able to address the question of the minimal requirement for the number of Arg residues at Yaa positions and found that only one Arg residue incorporated in a Yaa position of a Pro-Hyp-Gly repeat provided sufficient structural information for recognition by HSP47 (Fig. 3). Photoaffinity cross-linking is another powerful strategy that is informative for investigating protein-small molecule binding (29) or protein-protein binding (30) interfaces. We successfully applied this system to investigate the interface of HSP47-client complexes and determined the orientation of an HSP47 molecule bound to a collagen triple helix (Fig. 6). This analysis also revealed that HSP47 discriminates the direction of the collagen triple helix. Moreover, the results indicated that the side chain structure of the amino acid residue at position −3 is another structural determinant for specific interaction with HSP47, in addition to the critical Arg residue at position 0 (Fig. 6). This dual-site recognition mechanism of HSP47 will be further investigated in the subsequent paper.

The obtained results for HSP47-client interactions have led to the development of an alternative model for the chaperone complex. When it is assumed that an HSP47 molecule recognizes an Arg residue (as evidenced by the experiment shown in Fig. 3), a homotrimeric binding site possessing three Arg residues could be surrounded by up to three molecules of HSP47. This model is structurally possible only when the longer axis of each HSP47 molecule is allocated along the common axis of the triple helix (Fig. 6B). The cooperative binding produces a Hill coefficient of about 3 (18), thereby implying the feasibility of this multiple binding model. Our preliminary stoichiometric analysis by fitting the equilibrium HSP47 binding curves for the heterotrimeric peptides also supported this model.4 To create a model for the chaperone complex with higher resolution, it is important to determine the collagen binding site of HSP47 at the amino acid level. For this purpose, we are currently mapping the sites to which cross-links were introduced in the photo cross-linking experiments (Fig. 6A).

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