Substrate Recognition of Collagen-specific Molecular Chaperone HSP47

STRUCTURAL REQUIREMENTS AND BINDING REGULATION*

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Prior to secretion, procollagen molecules are correctly folded to triple helices in the endoplasmic reticulum (ER). HSP47 specifically associates with procollagen in the ER during its folding and/or modification processes and is thought to function as a collagen-specific molecular chaperone (Nagata, K. (1996) Trends Biochem. Sci. 21, 23–26). However, structural requirements for substrate recognition and regulation of the binding have not yet been elucidated. Here, we show that a typical collagen model sequence, (Pro-Pro-Gly)10, possesses sufficient structural information required for recognition by HSP47. A structure-activity relationship study using synthetic analogs of (Pro-Pro-Gly)10 revealed the requirements in both chain length and primary structure for the interaction. The substrate recognition of HSP47 has also been shown to be similar but distinct from that of prolyl 4-hydroxylase, an ER resident enzyme. Further, it has been shown that the interaction of HSP47 with the substrate peptides is abolished by prolyl 4-hydroxylation of the second Pro residues in Pro-Pro-Gly triplets and that the fully prolyl 4-hydroxylated peptide, (Pro-Hyp-Gly)10, does not interact with HSP47. We thus have proposed a model in which HSP47 dissociates from procollagen during the process of prolyl 4-hydroxylation in the ER.

Collagen is the most abundant protein in vertebrate bodies and is folded as procollagen in the endoplasmic reticulum (ER). The folding of procollagen in cells is unique, as is the final structure itself. In the folding of type I collagen, for instance, three α chains (two α1(I) and one α2(I)) first associate at C-terminal propeptides, and then approximately 340 repeats of the Xaa-Yaa-Gly tripeptide form the 300-nm length triple helix.

In the triple helix, three left-handed polyproline type II (PP-II) helices are supercoiled. The helix-forming process is believed to be coupled with prolyl 4-hydroxylation at Yaa positions. HSP47 transiently interacts with procollagen molecules in the ER in the process of folding and/or modification. HSP47 not secreted with procollagen from the cells, because it possesses the ER retention signal, Arg-Asp-Glu-Leu, at the carboxy end. The expression of HSP47 is well correlated with that of various types of collagen in cultured cells, in various tissues during development, and in pathophysiological conditions. Thus HSP47 is assumed to be a collagen-specific molecular chaperone. However, the binding sites or motif in the procollagen molecule have not been well elucidated. The molecular basis of HSP47 function, including the regulation of the substrate association and dissociation, also remains an enigma.

Here, we studied substrate recognition of HSP47 using the recombinant chaperone protein and synthetic collagen model peptides and found that a very simple collagen model peptide mimics the native substrate of HSP47. The difference in substrate recognition between HSP47 and prolyl 4-hydroxylase (P4-H) was discussed in both primary and secondary structural aspects. We also investigated the effects of prolyl 4-hydroxylation, a characteristic post-translational modification for procollagen, on HSP47 interaction.

EXPERIMENTAL PROCEDURES

Peptides—(Pro-Pro-Gly)10, (Pro-Hyp-Gly)10, (Pro-Pro-Gly)10, and (Pro-Hyp-Gly)10 were purchased from Peptide Institute Inc. (Minoh, Japan). Polyglycine (molecular mass = 12–13.3 kDa) and poly-L-proline (molecular mass = 5.2 kDa) were purchased from Sigma. Other peptides were synthesized on a PE Biosystems Model 433 A synthesizer using the 9-fluorenlymethoxycarbonyl/tertiary butyl protecting strategy. After deprotection/cleavage, the crude products were purified by reversed-phase high performance liquid chromatography (RP-HPLC). All peptides were identified by ion spray mass spectrometry on a Sciex API IIIE mass spectrometer.

Purification of P4-H—P4-H was obtained from 13-day-old chick embryos as described elsewhere (14) based on the procedures of Kedersha and Berg (15).

Solid-Phase Binding Assay—Peptides and porcine type I collagen (Nitta Gelatin, Osaka, Japan) were immobilized onto cyanogen bromide (CNBr)-activated-Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's protocol. To introduce an N-terminal C9 spacer, N-hydroxysuccinimide-activated-Sepharose 4FF (Amersham Pharmacia Biotech) was used instead of CNBr-activated beads. Overnight culture of E. coli harboring expression plasmid for mouse recombinant HSP47 (rHSP47) (16) or GST-HSP47 fusion protein (pYL43 (14)) in LB medium was diluted 5-fold and continued in culture for 1.5 h. Protein synthesis was induced by raising the temperature to 40.5 °C or by adding isopropyl-β-D-thiogalactopyranoside (0.5 mM) for rHSP47 or GST-HSP47, respectively. The induction was terminated at 10 min. Cell lysates were prepared by treatment with lysozyme (0.2 mg/ml) in lysis buffer (50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 30% glycerol) followed by the addition of protease inhibitors and Nonidet P-40 (0.2%). The lysate (100 μl) was mixed with 100 μl of binding buffer (10 mM HEPES-Na (pH 7.5), 3.7 mM EDTA, 0.4 mM NaCl, 0.005% Tween 20) and a 40-μl bed of the affinity beads. The binding reaction was carried out at 4 °C for 1 h with gentle mixing. The supernatant was discarded, and the beads were washed twice with 0.4 ml of binding buffer. Proteins retained on the beads were eluted by adding 2 × Laemmli sample buffer (40 μl), separated by 10% SDS-PAGE, and visualized by CBB-staining or Western blotting using an anti-HSP47 antibody (SPA470, Stressgen, Victoria, Canada).
The triple helical region of collagen is composed of the characteristic repeats of Xaa-Yaa-Gly. Here, Xaa and Yaa are any amino acid residues, but Gly, Cys, and aromatic amino acid residues are unfavorable in native collagen sequences. Pro and 4-hydroxyproline (Hyp) residues are most frequently found at the Xaa and Yaa positions, respectively. Previous studies showed that HSP47 binds to gelatin and various types of collagen without propeptides (16), indicating the importance of the triple helical regions for the interaction. To elucidate the structural requirements of collagen for interaction with HSP47, we first performed a solid-phase pull down assay using immobilized commercially available peptides that could mimic the collagen-like structure. Poly-L-proline, polyglycine, (Pro-Pro-Gly)$_n$, and (Pro-Pro-Gly)$_{10}$ (17, 18) were used for the initial assay. The E. coli-harborizing expression plasmid encoding rHSP47 was cultured, and the protein expression was induced moderately so as not to accumulate too much rHSP47 as compared with other endogenous proteins. The cell lysate was mixed with the beads on which type I collagen or the peptides were covalently coupled, and the proteins retained on the beads were assessed by SDS-PAGE followed by CBB staining or Western blotting (Fig. 1A and B). Recombinant HSP47 was purified by the immobilized (Pro-Pro-Gly)$_{10}$ with high specificity. No major proteins other than rHSP47 were detected on a CBB-stained gel (Fig. 1A, lane 7). On the other hand, rHSP47 did not interact with polyglycine, poly-L-proline, and (Pro-Pro-Gly)$_5$ (Fig. 1, A and B, lanes 4–6). This result indicates that a simple collagen model peptide, (Pro-Pro-Gly)$_{10}$, possesses sufficient structural information for specific binding to HSP47. HSP47 does not require amino acid residues with any charged side-chains for this specific interaction. It was also reported that the interaction of chick HSP47 with gelatin was not prevented even under high salt condition in vitro (19). Hydrophobic interaction is suggested as mainly contributing to the binding of HSP47 to its substrates.

This result brought us some clues to the understanding of substrate recognition of HSP47. First, the minimal number of Pro-Pro-Gly triplet repeats required for the interaction is between 5 and 10. Second, substrate recognition of HSP47 is different from that of P4-H, because poly-L-proline, which is known to interact with P4-H (20), did not bind to HSP47. Finally, HSP47 does not require prolyl 4-hydroxylation at the Yaa positions of Xaa-Yaa-Gly repeats for the interaction. In the following experiments, we did precise analyses to clarify these issues.

**Structural Requirements for the Interaction with HSP47—**

The minimal requirements in substrate structure for the interaction of HSP47 was next examined using synthetic tandem repeats of a Pro-Pro-Gly triplet with various lengths by similar solid-phase binding assays using the GST-HSP47 fusion protein hereafter. Significant binding of GST-HSP47 was observed for peptides with as few as seven repeats (21-mer) of the triplet, and the binding increased with increases in chain length (Fig. 2, A–C). As the collagen triple helix has a rigid rod-like shape with the increase/residue of 2.9 Å, the length of this minimal peptide was estimated to be about 60 Å (2). This value is comparable to the long axis length of HSP47 (approximately 65 Å as estimated by molecular modeling (21)). To eliminate the possible steric hindrance at the N-terminal linkage site of the peptide, we performed a similar binding assay introducing an N-terminal flexible spacer of nine carbons. In the presence of the flexible linker, we obtained similar results to that obtained in the absence of the linker. The minimal length was the same regardless of the existence of the linker (data not shown). We concluded that N-terminal steric hindrance was not a major reason for the relatively long length requirement for the substrate recognition of HSP47.

Because the sequence of (Pro-Pro-Gly)$_n$ is also known to be a substrate of P4-H (22), the interaction of purified chick P4-H with the same set of immobilized peptides was subsequently investigated (Fig. 2, D and E). The binding of P4-H to immobilized (Pro-Pro-Gly)$_n$ peptides was observed from $n = 5$ to 10. In contrast to GST-HSP47 binding, an increase in chain length had essentially an inverse effect on the interaction with P4-H.

This observation indicates that HSP47 and P4-H have different conformational requirements in peptides with the same primary sequence. The former prefers longer chains, which have more tendency to form triple helices, compared with the latter. P4-H is most likely to preferentially interact with a single chain PP-II helix as reported in previous studies (23–25).

The structural features of substrate peptides requisite for the interaction with HSP47 were further studied using analogues of (Pro-Pro-Gly)$_n$ with different Pro and Gly arrangements including polyglycine, (Pro-Gly-Gly)$_n$, (Pro-Gly)$_{10}$, (Pro-Pro-Gly)$_{10}$, and poly-L-proline (Fig. 3A). Among the peptides tested, only (Pro-Pro-Gly)$_n$ interacted with GST-HSP47 (Fig. 3B, lane 7). On the other hand, P4-H bound to (Pro-Pro-Gly)$_n$, (Pro-Pro-Gly)$_{10}$, and poly-L-proline (Fig. 3C, lanes 6–8). These P4-H-binding peptides showed a tendency to form PP-II helices in solution when analyzed by circular dichroism spectroscopy (data not shown). These results demonstrated that Gly residues at every third residue are important for substrate recognition. It should be noted that the Gly residues in collagen sequences are important for stabilization of the triple-helical conformation through the formation of interchain hydrogen bridges (2). The PP-II helix-forming tendency, which correlated with P4-H binding, is not by itself important for the interaction with HSP47.
Effects of Prolyl 4-Hydroxylation on HSP47 Interaction—The most important post-translational modification during procollagen folding is prolyl 4-hydroxylation at the Yaa positions of Xaa-Yaa-Gly sequences catalyzed by P4-H. Most of the susceptible Pro residues are converted to Hyp residues in the final forms of various types of procollagen (26). The effect of prolyl 4-hydroxylation of procollagen on interaction with HSP47 was investigated using immobilized synthetic peptides; the various positions of which were substituted with Hyp residues instead of Pro residues (Fig. 4A). Comparing (Pro-Pro-Gly)$_{10}$ and (Pro-Hyp-Gly)$_{10}$, prolyl 4-hydroxylation of all susceptible Pro residues abolished the binding of GST-HSP47 (Fig. 4B, lanes 3 and 4). The effect of increasing the degree of prolyl 4-hydroxylation was also examined by comparing non-, mono-, tetra-, and fully (octa-) hydroxylated versions of the (Pro-Pro-Gly)$_{8}$ sequence (lanes 5–8). Immobilized monohydroxylated peptide bound half as much GST-HSP47 as (Pro-Pro-Gly)$_{8}$ beads did (lane 6). Introduction of Hyp residues at four of eight possible sites resulted in the loss of most of the binding affinity to GST-HSP47 (lane 7), and a fully hydroxylated peptide (Pro-Hyp-Gly)$_{8}$ did not show any affinity (lane 8). The effect of prolyl hydroxylation on interaction with P4-H was also examined (Fig. 4C). The results were parallel to that of GST-HSP47 as P4-H also prefers less hydroxylated peptides as its substrates. This result implies that both P4-H and HSP47 dissociate from the substrate during the progression of prolyl 4-hydroxylation of procollagen.

We further performed competition assays using fully hydroxylated and nonhydroxylated peptides as competitors. The unhydroxylated peptide, (Pro-Pro-Gly)$_{10}$, has an ability to elute GST-HSP47 from a complex of GST-HSP47 and (Pro-Pro-Gly)$_{10}$ immobilized on beads in a dose-dependent manner (Fig. 5A, lanes 3–5). In contrast, the corresponding fully hydroxylated peptide (Pro-Hyp-Gly)$_{10}$ did not compete with the peptide immobilized on the beads (lanes 6–8). These results coincide with those obtained in a solid-phase binding assay (Fig. 4B). In addition, the unhydroxylated peptide also competed with the interaction between GST-HSP47 and immobilized type I collagen (Fig. 5B, lanes 3–5), but the hydroxylated peptide did not (lanes 6–8). This observation confirmed that model peptides with a (Pro-Pro-Gly)$_{n}$ sequence act as a true substrate of HSP47 by mimicking the structure of the native substrate, procollagen.

Conclusion—In the present paper, we demonstrated for the first time that the simplest collagen model peptide (Pro-Pro-Gly)$_{n}$ could be used as a binding substrate for HSP47. The discovery of peptidic substrates enabled us to precisely analyze the molecular parameter required for the specific interaction.
Substrate Recognition of HSP47

Molecular chaperones must possess a mechanism to release the substrates. The interaction of HSP70 family proteins with their substrates is regulated by conformational changes in the chaperone itself between ATP- and ADP-bound forms (27, 28). Association and dissociation of calnexin and calreticulin, ER resident chaperone proteins, with folding intermediates of glycoproteins are regulated by cleavage and the re-addition of a terminal glucose on the N-linked oligosaccharides (29, 30). However, HSP47 has neither ATP binding nor lectin-like activities. Results obtained in the present study suggest a third possible regulatory mechanism for the interaction of a chaperone protein with its substrate. We propose a model in which HSP47 associates with immature (unhydroxylated) portions of the triple-helical regions of procollagen and that dissociation of HSP47 is regulated by prolyl 4-hydroxylation of the Yaa position from that of P4-H though they showed overlapped substrate specificity in primary structures. In addition, we have shown that both HSP47 and P4-H predominantly interact with less prolyl 4-hydroxylated substrates or immature-type collagen sequences.

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Fig. 4. Effect of prolyl 4-hydroxylation on interaction with GST-HSP47 fusion protein and with P4-H. A, a synthetic peptides with replacement of various Pro residues at the Yaa position with Hyp residues. B, binding of GST-HSP47 fusion protein to the prolyl-hydroxylated peptides on the beads. Proteins were visualized by CBB staining. C, interaction of P4-H with the same sets of peptides was similarly examined.
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