HSP47 binds cooperatively to triple helical type I collagen, but has little effect on the thermal stability or the rate of refolding

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Summary

HSP47, a collagen specific molecular chaperone, interacts with unfolded and folded procollagens. Binding of chicken HSP47 to native bovine type I collagen was studied by fluorescence quenching and cooperative binding with a $K_{\text{half}}$ of $1.4 \times 10^{-7}$ M and a Hill coefficient of 4.3 was observed. Similar results are observed for the binding of mouse HSP47 recombinantly expressed in E. coli. Chicken HSP47 binds equally well to native type II and pN type III collagen, but binding to triple-helical collagen-like peptides is much weaker. Weak binding occurred to both hydroxylated and non-hydroxylated collagen-like peptides and a significant chain-length dependence was observed. Binding of HSP47 to native type I collagen had no effect on the thermal stability of the triple helix. Refolding of type I collagen in the presence of HSP47 showed minor changes, but these are probably not biologically significant. Binding of HSP47 to bovine pN type III collagen has only minor effects on the thermal stability of the triple helix and does not influence the refolding kinetics of the triple helix.
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Introduction

HSP47 (47 kDa heat shock protein) is a rough endoplasmic reticulum (RER) protein believed to function as a collagen specific molecular chaperone (1-3). It was shown that HSP47 (also known as colligin, J6, gp46 and CB48) is co-expressed with collagens in cells such as fibroblasts and chondrocytes (4,5). In mouse embryo development HSP47 is expressed mainly in mesoderm and mesoderm derived tissues and the expression correlates both temporally and spatially with that of type I and II collagen (6). HSP47 knockout mice show an embryonic lethal phenotype (7). HSP47 is easily isolated by affinity chromatography on gelatin sepharose (8,9) and was later shown to also interact with triple helical collagen types I through V (10). It was found that all these collagens have a similar affinity for HSP47 with dissociation constants of about $10^{-7}$ M (10). The interaction of HSP47 with both unfolded and folded procollagens is unusual and distinguishes HSP47 from other molecular chaperones.

A number of potential functions for HSP47 during procollagen biosynthesis have been described: HSP47 binds to nascent procollagen chains and therefore may help prevent premature association of procollagen chains and/or assists with the translocation of procollagen chains into the rough endoplasmic reticulum (11). HSP47 also interacts with procollagen chains in the RER when triple helix formation is inhibited by $\alpha,\alpha'$-dipyridyl, an inhibitor of prolyl-4-hydroxylase (9). A potential role in vesicular trafficking was explored with Brefeldin A and monensin (9,12,13). In Brefeldin A treated cells HSP47 remained associated with procollagen whereas in cells treated with monensin no HSP47 binding was detected. Together this shows that HSP47 seems to release procollagen on entry to the Golgi. Procollagens are not secreted as individual molecules, but rather are secreted in aggregates by a cisternal maturation pathway (14) and HSP47 could potentially be involved in this process.

Conflicting results were obtained with co-expression of HSP47 and procollagens. In human embryonic kidney cells 293 the secretion of type III procollagen was delayed when HSP47 was co-expressed (15), whereas an enhancement of secretion of human type I procollagen was found in mouse HSP47-expressing insect cells (16). The exact function of HSP47 during procollagen biosynthesis has not been established.

The structural requirements for HSP47 binding to procollagens have been studied recently using synthetic collagen-like peptides (17) and also in a yeast two-hybrid system (18).
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It was found that the collagen-like peptide (Gly-Pro-Pro)$_n$ possesses sufficient information to bind to HSP47 and that hydroxylation of this peptide (Gly-Pro-Hyp)$_n$ prevents this interaction (17). Using the two-hybrid system in yeast it was shown that the (Gly-Pro-Pro) tripeptide unit is essential to binding and that HSP47 preferentially recognized the triple helical conformation in these peptides (18). Another study suggest that HSP47 interacts with and stabilizes correctly folded procollagen (19).

In this report we show that HSP47 binds triple helical type I collagen cooperatively and with a much higher affinity than synthetic peptides. We also explore the effect of HSP47 on the thermal stability and refolding of type I and pN type III collagen.
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Experimental procedures

Purification of chicken HSP47. The following purification method was based upon previously published protocols for the purification of HSP47 (9,20), but has been modified to improve both the yield and purity of HSP47.

Preparation of a crude microsomal extract. After removal of the heads, 6-dozen 15-17 day old chick embryos were homogenized in an equal volume of homogenization buffer (10 mM Tris/HCl, pH 7.5, containing 0.25 M sucrose, 5 mM EDTA, and 2 mM each phenylmethylsulfonylfluoride and N-ethylmaleimide and 1 µg/ml each leupeptin and pepstatin A) in an ice-cold Warring Blender at full speed for 2 minutes, in a 4 ºC cold room. All further steps were carried out at 4 ºC. Large tissue debris was removed by centrifugation of this homogenate at 5,000 x g in a H-6000A rotor (Sorval) for 15 minutes. The supernatant from this step was then centrifuged at 150,000 x g in a 45 Ti rotor (Beckman) for 1 to 2 hours. The pelleted material from this step was transferred to a plastic specimen container and frozen at -20 ºC for storage until later purification steps could be carried out. After 4 such microsomal pellets had been prepared and stored, they were thawed together in an equal volume of extraction buffer (50 mM Tris/HCl, pH 7.5, containing 0.2 M NaCl and 1% Triton X-100, and all the previously used protease inhibitors). After the pellets had completely thawed, 0.5-1.0 µl diisopropyl fluorophosphate/ml of extract was added, and this solution was stirred vigorously for 4 hours on ice. The final microsomal extract was obtained by centrifugation of this solution at 150,000 x g in a 45 Ti rotor (Beckman) for 1-2 hours.

Gelatin affinity chromatography of crude microsomal extract. Using a peristaltic pump, the crude microsomal extract (approximately 400 ml) was loaded at 0.5-1.0 ml/min onto a 200 ml column (XK50; 5.0 cm x 10.1 cm) of gelatin-Sepharose 4B (Pharmacia) equilibrated with gelatin-Sepharose buffer A (50 mM Tris/HCl, pH 7.5, containing 0.2 M NaCl, 0.1 % Tween 20, 0.2 mM phenylmethylsulfonylfluoride). After loading the extract, the column was washed with 200 ml gelatin-Sepharose buffer B (50 mM Tris/HCl, pH 7.5, containing 1.0 M NaCl, 0.1 % Tween 20, 0.2 mM phenylmethylsulfonylfluoride) and re-equilibrated with 400 ml of gelatin-Sepharose buffer A. Gelatin-binding proteins were eluted with a 200 ml pH gradient from 7.5 to 5.0 of gelatin-Sepharose Buffer A, followed by 200 ml of gelatin-Sepharose Buffer A, pH 7.5, and collected in 10 ml fractions. The protein containing fractions (identified by SDS-PAGE)
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**MonoQ anion-exchange chromatography.** The dialyzed gelatin-Sepharose pH-eluate was loaded into a 50 ml FPLC Superloop (Pharmacia), run over a monoQ HR 5/5 column, washed with 25 ml monoQ buffer A, and collected in 5 ml fractions. The flow-through fractions were highly enriched with the protein HSP47, but also contained some of the ER-resident cyclophilin B. Bound proteins were eluted with a 40 ml gradient of 0 to 100 % monoQ buffer B (20 mM triethanolamine/HCl, pH 7.0, containing 0.8 M NaCl), followed by 5 ml 100% monoQ buffer B, and collected in 1 ml fractions.

**Molecular-sieve chromatography of gelatin-binding proteins.** The monoQ flow-through fractions were pooled and concentrated to 6-8 ml using Centricon-10 concentrators (Amicon) according to manufactures recommendations. The HSP47 and cyclophilin B were separated in runs of 2 ml each by chromatography in gelatin-Sepharose buffer A over 2 tandemly arranged Superose 12 HR 16/50 columns (Pharmacia) and collected in 2 ml fractions. After SDS-PAGE, the peak fractions containing HSP47 were pooled, concentrated to about 1 mg/ml and stored on ice for later analysis.

**Other materials.** Type I and pN type III collagens were extracted from fetal bovine skin. Type II collagen was extracted from fetal bovine cartilage. (Pro-Pro-Gly)\(_{10}\) and (Pro-Hyp-Gly)\(_{10}\) was purchased from Peptide Institute, Inc. (Osaka, Japan). And (Pro-Gly-Pro)\(_n\) was from Sigma-Aldrich. (Pro-Gly-Pro)\(_n\) (average molecular weight of about 10 kDa, corresponding to an average chain length of \(n = 40\)) was fractionated on a Sephadex 75 column and divided into two pools with a \(K_{av}\) of 0.41 and 0.66 respectively.

**Fluorescence based collagen binding assays.** Measurement of fluorescence spectra was performed on an SLM8000C instrument (SLM Instruments Inc. Urbana, IL) using the software provided by the manufacturer. For most experiments the excitation wavelength was 275 nm (4 nm band width), but in some cases excitation was at 295 nm. Emission scans were obtained over the wavelength range 300 to 400 nm (2 nm band width) for excitation at 275 nm, or 310 to 410 nm for excitation at 295 nm. In most of the binding assays, stock solutions of bovine collagens (0.5 - 2.0 mg/ml) were added to a solution of tissue purified chicken HSP47 (0.05 - 0.2 mg/ml) in 50 mM Tris/HCl, pH 7.5, containing 0.2 M NaCl, in a 1 x 1 cm cell in a thermostatted block at
25 °C with constant stirring. Constant temperature was maintained by a circulating water bath (RCS, Lauda Division, Brinkmann Instruments). After each addition of substrate, samples were equilibrated for 2 min before emission spectra were acquired. In some experiments recombinant mouse HSP47 (rmHSP47) was used instead of chicken HSP47. Analysis of the binding data was done by subtracting the emission of the collagens (this is a very small correction) and correcting for dilution.

Collagen thermal stability and refolding monitored by optical rotary dispersion. The thermal stability and refolding of bovine type I and pN type III collagen was monitored at 365 nm using a 241MC polarimeter (Perkin-Elmer), using a 10 cm path length thermostatted cell. The temperature was controlled by two circulating water baths (RCS, Lauda Division, Brinkmann Instruments), and measured with a digital thermometer (Omega Engineering, Inc., Stamford, CT) and a thermistor inserted into the cell. Both the temperature and the ORD signals were recorded and digitized on an HP9070A measurement and plotting system (Hewlett-Packard) connected to an IBM-compatible computer. Protein concentrations were determined by amino acid analysis.
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Results

Chicken HSP47 was extracted and purified from chick embryos and mouse HSP47 was expressed in E. coli. Figure 1A shows a 5 to 20% SDS polyacrylamide gel of purified chicken HSP47. The final yield of highly purified HSP47 from 6 dozen chick embryos was 4 to 8 mg. Figure 1B shows a 10% SDS polyacrylamide gel of purified mouse HSP47 expressed in E. coli. The yield of purified and refolded mouse HSP47 was about 30 mg from a 500 ml culture.

Binding of HSP47 to type I collagen was measured by observing the tryptophane fluorescence of HSP47 that is quenched by the addition of type I collagen. These measurements are facilitated by the fact that type I collagen and collagen-like peptides do not contain tryptophane. Figure 2 shows the change in fluorescence as a function of added type I collagen. A sigmoidal curve is observed, indicating cooperative binding. The data were analyzed using the Hill equation. For the binding of chicken HSP47 to type I collagen a $K_{\text{half}}$ of $1.4 \times 10^{-7}$ M and a Hill coefficient $P$ of 4.3 were found (Figure 2A). For recombinantly expressed mouse HSP47 binding to type I collagen a $K_{\text{half}}$ of $1.1 \times 10^{-7}$ M and a Hill coefficient $P$ of 3.2 were determined (Figure 2B).

In order to determine the structural determinants of the HSP47 binding to the triple helix, binding studies using different collagens and collagen-like peptides were done using the same technique. Figure 3 shows the fluorescence quenching of chicken HSP47 by type I collagen, type II collagen, pN type III collagen. All three collagens seem to bind about equally to HSP47. However binding of collagen-like peptides is much weaker. Again, a cooperative effect is observed when the high and low molecular weight fractions of (Pro-Gly-Pro)$_n$ are compared, the higher molecular weight fraction shows better binding to HSP47. Binding of the classical collagen-like peptides (Gly-Pro-Pro)$_{10}$ and (Gly-Pro-Hyp)$_{10}$ to HSP47 is very weak and these peptides seem to be a poor choice for determining a change in binding between hydroxylated and non-hydroxylated triple helices.

Binding of HSP47 to collagen could influence the properties of the triple helix. The effect of HSP47 on the thermal stability of type I and pN type III collagen was studied. Because the interaction of HSP47 with collagen is abolished at pH 5, and collagens tend to form fibrils at neutral pH and elevated temperatures, measurements were done in 50 mM Tris/HCl buffer, pH 7.5, containing 0.4 M NaCl. Figure 4A shows, that the thermal stability of type I collagen is not effected by the presence of HSP47. There is a small effect on the melting behavior of pN type
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III collagen. We have previously observed that pN type III collagen has a tendency to form aggregates even in the high salt buffer. This is evident from a fraction of higher melting material. In the presence of HSP47, this aggregation is not observed and the melting curve is slightly shifted to lower temperatures (Figure 4B).

Because HSP47 interacts with both folded and unfolded triple helices, the refolding of type I collagen in Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl, was measured. The type I collagen was denatured for 15 minutes at 45 °C and refolding was observed at 25 °C. Type I has been previously shown to refold very poorly, and also in these experiments we only get less than 30% of the initial signal back after 90 minutes of refolding. In the presence of HSP47, the recovered signal is slightly higher (Figure 5A). More remarkable is the change from an exponential kinetic to a zero order kinetic typically observed for the refolding of type III collagen (21). However, these changes are quite small. Refolding of pN type III collagen proceeds with an initial zero order reaction and reaches at least 90% of the initial signal. The initial rate was independent of the presence or absence of HSP47 (Figure 5B).
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Discussion

Binding of HSP47 to native type I - V collagens was previously investigated by surface plasmon resonance measurements. A dissociation constant of $1.1 \times 10^{-6}$ M was determined for the binding of recombinantly expressed mouse HSP47 to porcine type I collagen (10). This dissociation constant results from a relatively high association rate of $2.08 \times 10^{4}$ M$^{-1}$s$^{-1}$ and a rapid dissociation rate of $2.36 \times 10^{-2}$ s$^{-1}$. No cooperativity in binding was reported. The $K_{\text{half}}$ determined here by fluorescence quenching is about an order of magnitude smaller ($1.4 \times 10^{-7}$) and binding is cooperative with a Hill coefficient of 4.3. Cooperative binding is consistent with the finding that a strong chain length dependence is observed for $(\text{Pro-Gly-Pro})_{n}$. The fraction with a $K_{\text{av}}$ of 0.41 corresponding to a greater chain length than the fraction with a $K_{\text{av}}$ of 0.66 binds HSP47 better. On a per tripeptide unit basis increased fluorescence quenching is observed going from $(\text{Pro-Pro-Gly})_{10}$ to larger $(\text{Pro-Gly-Pro})_{n}$ polypeptides. However, the native collagens type I and pN III bind HSP47 much stronger. Type I collagen $(\text{Gly-Xaa-Yaa})_{336}$ quenches the HSP47 fluorescence much more than even the longest collagen-like peptides measured. The reason for this can be that there is still a chain length effect or that HSP47 prefers other tripeptide units over $(\text{Gly-Pro-Pro})$. Binding of $(\text{Gly-Pro-Pro})_{10}$ and $(\text{Gly-Pro-Hyp})_{10}$ to HSP47 is weak and not much difference in fluorescence quenching is observed. It was proposed recently that HSP47 binds to $(\text{Pro-Pro-Gly})_{10}$ but not to $(\text{Pro-Hyp-Gly})_{10}$ and that this is a possible mechanism for the release of HSP47 from collagen (17). The collagens measured here are extracted from tissues and believed to be fully hydroxylated. Because HSP47 binds these collagens well, the proposed mechanism seems unlikely.

Because HSP47 binds to folded collagens there is the potential that HSP47 modifies the properties of the collagen triple helix. The stability of type I collagen in the presence and absence of HSP47 shows no differences. It was shown previously that the interaction of HSP47 with collagen is strongly pH dependent with binding being abolished below pH 6.3 (9). Measuring the stability of the collagen triple helix around neutral pH is difficult because collagens tend to form fibrils at that pH and elevated temperatures. Aggregation has been previously observed with pN type III collagen but these aggregates can be minimized by the addition of 0.4 M NaCl. Interestingly, during the measurement of the stability of pN type III collagen in the presence of HSP47 no aggregation was observed, while in the absence of HSP47,
some higher melting aggregates were found under the same conditions. Thus binding of HSP47 influences the aggregation behavior of collagens and this was shown in a recent paper (22).

Because HSP47 binds both unfolded and folded collagens, we tested the effect on the rate of refolding of the triple helix of type I and pN type III collagen. The folding mechanism for pN type III collagen has been described (21) and HSP47 does not have an effect on the rate of folding. In refolding experiments with type I collagen, HSP47 binding seems to provide a nucleation site for refolding. However the increase in refolding is probably not biologically relevant.

From these studies we conclude that HSP47 preferentially binds to folded triple helices, but does not influence the stability and folding of the collagen triple helix. Therefore, it seems likely that HSP47 is involved in the segregation of procollagen to the cisternal maturation pathway.
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Footnotes

Abbreviations used: pN type III collagen, type III procollagen without the carboxyterminal propeptide; Hyp, 4-hydroxy-L-proline;

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Figure legends

Figure 1. **SDS polyacrylamide gels of purified HSP47.** The final purity of HSP47 was assessed by SDS polyacrylamide gel electrophoresis. A: The purity of chicken HSP47 is shown by electrophoresis on a 5 to 20 % SDS polyacrylamide gel in lane 1. Lane 2 shows molecular weight markers, whose molecular weights (in kDa) are indicated on the right. B: The purity of recombinant mouse HSP47 is shown by electrophoresis on a 10 % SDS polyacrylamide gel in lane 1. Lane 2 shows molecular weight markers, whose molecular weights (in kDa) are shown on the right.

Figure 2. **Binding of HSP47 to native type I collagen.** The fluorescence of HSP47 was monitored at 340 nm and the quenching observed by binding to type I collagen is plotted against the type I collagen concentration. A: binding of chicken HSP47. B: binding of recombinant mouse HSP47. The curves represent best fits to the equation $F = F_{\text{max}} C^p / (K_{\text{half}}^p + C^p)$, where $F$ is the fluorescence of HSP47 at concentration $C$ of type I collagen, $F_{\text{max}}$ is the fluorescence of HSP47 at maximum quenching, $K_{\text{half}}$ is the collagen concentration at half saturation, and $P$ is the Hill cooperativity parameter.

Figure 3. **Binding of chicken HSP47 to various collagens and collagen-like peptides.** The quenching of the fluorescence of HSP47 is shown as a function of the concentration of (Gly-Xaa-Yaa) tripeptide units in various triple helical molecules. Type I collagen (blue open circles), type II collagen (green open up triangles), and pN type III collagen (black open down triangles) show a much stronger binding than (Pro-Gly-Pro)$_n$ high molecular weight fraction (red open squares), (Pro-Gly-Pro)$_n$ low molecular weight fraction (green solid squares), (Pro-Hyp-Gly)$_{10}$ (black solid up triangles), and (Pro-Pro-Gly)$_{10}$ (blue solid circles).

Figure 4. **Melting curves of type I and pN type III collagen in the presence and absence of chicken HSP47.** Optical rotatory dispersion was measured at 365 nm and the temperature was increased at a rate of 10 °C/h. A: type I collagen (0.08 µM) in 50 mM Tris/HCl buffer, pH 7.5, containing 0.4 M NaCl, was measured in the absence (red curve) and presence (blue curve) of

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chicken HSP47 (0.85 µM). B: pN type III collagen (0.1 µM) was measured in the absence (red curve) and presence (blue curve) of chicken HSP47 (1.4 µM) in the same buffer.

Figure 5. **Refolding of type I collagen and pN type III collagen in the presence and absence of chicken HSP47.** Optical rotatory dispersion was measured at 365 nm. The signal from the native molecule was observed for about 10 minutes at 25 °C, then the temperature was raised to 45 °C for 15 minutes to unfold the triple helix. Refolding was started by lowering the temperature back to 25 °C. A: Refolding of type I collagen (0.22 µM) in the absence (red curve) and presence (blue curve) of chicken HSP47 (4.4 µM). B: Refolding of pN type III collagen (0.2 µM) in the absence (red curves, two experiments shown) and presence (blue curve) of chicken HSP47 (4.0 µM).
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A

![Graph A](image)

B

![Graph B](image)
Cooperative binding of HSP47 to type I collagen

![Graph showing relative fluorescence versus [GXY] (M) for different samples](image-url)
Cooperative binding of HSP47 to type I collagen

A

B
Cooperative binding of HSP47 to type I collagen

A

![Graph A with two lines representing type I collagen and type I collagen with HSP47.](image)

B

![Graph B with two lines representing pN type III collagen and pN type III collagen with HSP47.](image)
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