Mapping Hsp47 binding site(s) using CNBr peptides derived from type I and type II collagen

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(RECEIVED October 18, 2002; FINAL REVISION May 7, 2003; ACCEPTED May 19, 2003)

Abstract

As a crucial molecular chaperone in collagen biosynthesis, Hsp47 interacts with the nascent form as well as the mature triple-helical form of procollagen. The location(s) of Hsp47 binding sites on the collagen molecule are, as yet, unknown. We have examined the substrate specificity of Hsp47 in vitro using well-characterized CNBr peptide fragments of type I and type II collagen along with radiolabeled, recombinant Hsp47. Interaction of these peptides with Hsp47 bound to collagen-coated microtiter wells showed several binding sites for Hsp47 along the length of the $\alpha 1$ and $\alpha 2$ chains of type I collagen and the $\alpha 1$ chain of type II collagen, with the N-terminal regions showing the strongest affinities. The latter observation was also supported by the results of a ligand-blot assay. Except for two peptides in the $\alpha 2(I)$ chain, peptides that showed substantial binding to Hsp47 did so in their triple-helical and not random-coil form. Unlike earlier studies that used peptide models for collagen, the results obtained here on fragments of type I and type II collagen identify, for the first time, binding of Hsp47 to specific regions of the collagen molecule. They also point to additional structural requirements for Hsp47 binding besides the known preference for third-position Arg residues and the triple-helical conformation.

Keywords: Hsp47; molecular chaperone; CNBr peptides of collagen; radioligand competition assay

The endoplasmic reticulum (ER) is responsible for the folding and export of proteins destined for intracellular organelles and the cell surface. To accomplish this task, the ER contains several folding enzymes and molecular chaperones that assist in the proper folding of proteins and in the retention of misfolded proteins (Helenius et al. 1992). Hsp47 is one such ER-resident heat-shock protein that acts as a chaperone specific to collagen, the most abundant mammalian protein. The expression pattern of Hsp47 is highly correlated with that of collagen both in diseased and normal

states (Nagata 1996). In situ experiments have shown that Hsp47 binds to nascent procollagen chains and remains associated with them as they form the triple-helical structure, and are exported via the secretory pathway (Sauk et al. 1994; Smith et al. 1995). Once in the *cis*-Golgi compartment, Hsp47 releases the bound procollagen and returns to the ER via the ER-retention sequence found on its C terminus (Sato et al. 1996). The importance of Hsp47 in collagen biosynthesis is illustrated by the retention, in the ER, of abnormal procollagen molecules produced in the genetic disorder, osteogenesis imperfecta, through enhanced expression and colocalization of Hsp47 with procollagen in fibroblasts (Kojima et al. 1998). Furthermore, Hsp47 knock-out mice show embryonic lethality and defects in collagen biosynthesis (Nagai et al. 2000).

The promiscuous binding of Hsp47 to both the unfolded and fully folded procollagen chains makes it difficult to understand the exact stage(s) in collagen biosynthesis where this protein might carry out its chaperone function. In a

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Abbreviations: ER, endoplasmic reticulum; CD, circular dichroism; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Hyp or O, 4-hydroxyproline; BSA, bovine serum albumin.

Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.0236903>.

recent study, we have shown that Hsp47 prevents collagen fibril formation *in vitro* (Thomson and Ananthanarayanan 2000). The pH-dependence of this inhibition correlated with pH-induced conformational changes in Hsp47 (Thomson and Ananthanarayanan 2000). This led us to suggest that, *in vivo*, Hsp47 may keep procollagen from forming fibrils until it reaches the relatively acidic Golgi compartment where Hsp47 would dissociate from procollagen. This does not, however, rule out other possible roles for Hsp47, including its involvement in preventing aggregation of the procollagen chains before they form a triple helix. Conceivably, Hsp47 may have multiple roles similar to the ER chaperone BiP that is involved not only in translocation but also in folding and retention (Gething and Sambrook 1992).

Studies on the substrate specificity of Hsp47 would be useful in understanding the nature of Hsp47-collagen interaction, and could thereby provide clues to Hsp47 function, including its inhibitory effect on collagen fibril formation. An early study by Hu et al. (1995) suggested that the N-propeptide region of the pro- $\alpha 1(I)$ chain of collagen may be the predominant domain for binding Hsp47. This, however, does not account for the known binding of Hsp47 to processed collagen lacking the propeptide region. More recent studies have employed synthetic peptide models for collagen as well as peptide libraries to delineate Hsp47 binding sites in collagen. Nagata and coworkers found that the triple-helical conformation in the substrate has a much higher affinity for Hsp47 than the random-coil form (Koide et al. 1999, 2000, 2002). At the primary structural level, the presence of a Hyp residue was found not to be conducive to Hsp47 binding. Particularly strong binding to Hsp47 was displayed by the Xaa-Arg-Gly sequence embedded within a (Pro-Pro-Gly)_n peptide. In contrast, Sauk et al. (2000) have found Arg to be among amino acid residues that disfavor Hsp47 binding. These authors also found certain short, non-natural peptides to be very effective in binding Hsp47 (Hebert et al. 2001). So far, no data are available on Hsp47 binding to peptides containing naturally occurring collagen sequences.

In this study, we have examined the Hsp47-binding site(s) on collagen using well-characterized peptide fragments generated by CNBr treatment of type I and type II collagen. In conjunction with sensitive binding assays that employ radiolabeled recombinant Hsp47, we were able to identify regions towards the N terminus in the triple-helical portion of collagen as the dominant sites of interaction with Hsp47.

Results

Binding of Hsp47 to collagenase cleavage fragments

Using radiolabeled Hsp47, we have previously shown that this protein binds to both the $\alpha 1$ and the $\alpha 2$ chains of type

I collagen immobilized on nitrocellulose (Thomson and Ananthanarayanan 2001). To map the regions of collagen involved in Hsp47 binding, we first investigated the binding of Hsp47 to peptide fragments generated by digestion of type I collagen by vertebrate collagenase. The enzyme cleaves both the $\alpha 1$ and $\alpha 2$ chains at a site corresponding to Gly⁷⁷⁵-Xaa⁷⁷⁶ (Gross et al. 1974) to generate an N-terminal fragment A and a C-terminal fragment B of each chain (Fig. 1). Incubation of ³⁵S-Hsp47 with these fragments immobilized on nitrocellulose membrane after transfer from SDS-PAGE gels, revealed that Hsp47 was bound only to the A fragment of both the $\alpha 1$ and $\alpha 2$ chains of type I collagen (Fig. 2).

Use of the competition assay

To further map the binding site(s) of Hsp47 on collagen, we developed an assay that quantified the interaction of ³⁵S-Hsp47 with plastic microtiter wells coated with type I collagen. This assay allowed us to examine the ability of potential Hsp47 substrates to compete for Hsp47 binding to the wells. In a control experiment, ³⁵S-Hsp47 binding to the wells was examined in the presence of increasing amounts of gelatin. Gelatin was chosen as the competing ligand for the following reasons: (a) Hsp47 was originally identified as a gelatin-binding glycoprotein (Kurkinen et al. 1984); (b) gelatin-affinity chromatography is often being used as a means of isolating purified Hsp47 (Saga et al. 1987); (c) gelatin has been shown to bind Hsp47 with higher affinity than collagen types I, II or IV (Jain et al. 1994), and hence, will be able to effectively compete out collagen; (d) gelatin

Figure 1. Schematic diagram of the $\alpha 1$ and $\alpha 2$ chains of type I collagen and the $\alpha 1$ chain of type II collagen. The nomenclature and positions of the CNBr fragments are indicated. Also shown are the fragments generated by the cleavage of type I collagen by vertebrate collagenase.

Figure 2. Binding of Hsp47 to the $\alpha 1$ and $\alpha 2$ chains and to collagenase fragments of type I collagen. The collagenase-digested collagen fragments were run in SDS-PAGE, and were either stained with Coomassie Brilliant Blue (*left*) or transferred to nitrocellulose membrane and probed with ^{35}S -Hsp47 and autoradiographed (*right*).

is known to contain short triple-helical stretches amidst random-coil regions (Dolz and Engel 1990), thus mimicking collagen in both these conformations; and (e) gelatin is soluble in our buffer system. As illustrated in Figure 3, the ability of gelatin to compete for ^{35}S -Hsp47 binding to the wells increased with increasing amounts of gelatin and began to saturate at 0.1% gelatin. This saturating concentration of gelatin was subsequently used in determining the nonspecific binding of Hsp47 to the wells (see Materials and Methods).

Figure 3. Binding of ^{35}S -Hsp47 to collagen-coated microtiter wells in the presence of increasing amounts of gelatin. ^{35}S -Hsp47 concentration was 0.6 μM ; gelatin concentration is given in w/v percentage (0.005%–0.2%) in 20 mM sodium phosphate, 50 mM NaCl, pH 7.4. Data shown are the mean ($\pm\text{SE}$) of triplicate measurements.

Binding of Hsp47 to synthetic collagen model peptides

It has been previously shown that Hsp47 binds to synthetic peptides that mimic the collagen triple helix (Koide et al. 1999). Koide et al. (1999) showed that Hsp47 binds to triple-helical $(\text{PPG})_{10}$ but not to triple-helical $(\text{POG})_{10}$, whereas MacDonald and Bachinger (2001) have shown that Hsp47 binding by both these peptides is relatively very weak compared to collagen types I–III, and therefore, the data do not warrant a comparison between the two peptides. Our results with these peptides in their triple-helical conformation (as judged from their CD spectra) are shown in Figure 4. Both $(\text{POG})_{10}$ and $(\text{PPG})_{10}$ caused only a slight decrease in ^{35}S -Hsp47 binding to the type I collagen-coated microtiter wells, with $(\text{PPG})_{10}$ showing relatively more binding affinity, albeit still weak.

Binding of Hsp47 to CNBr peptides of type I collagen

The $\alpha 1$ and $\alpha 2$ chains of collagen can be cleaved with CNBr to generate discrete peptides representing various regions of the collagen molecule based on the distribution of Met residues (Fig. 1). The properties of these peptides have been investigated in earlier studies by one of the authors (Rossi et al. 1996; Consonni et al. 2000; Zanaboni et al. 2000; Tenni et al. 2002). Many, but not all, form soluble, triple-helical homotrimers in solution at room temperature as assessed from CD data. Their random-coil forms may be generated by heat denaturation as described in Materials and Methods. The interactions of Hsp47 with the CNBr peptides in both these conformations were investigated using our radioligand competition assay system.

CNBr-cleavage of the $\alpha 1$ chain of type I collagen results in seven peptides of varying lengths (Fig. 1; Rossi et al. 1996). The four large peptides, CB8, CB3, CB6, and CB7

Figure 4. Inhibition of ^{35}S -Hsp47 binding to collagen-coated microtiter wells by collagen model peptides. Binding of ^{35}S -Hsp47 (0.3 μM) to collagen-coated wells in the presence and absence of the synthetic collagen peptides $(\text{PPG})_{10}$ and $(\text{POG})_{10}$, at 240 μM in 20 mM sodium phosphate, 50 mM NaCl, pH 7.4. Data shown are the mean ($\pm\text{SE}$) of triplicate measurements.

all form triple-helical homotrimers in solution, whereas the smaller peptides, CB2, CB4, and CB5 adopt a random-coil configuration at room temperature (Rossi et al. 1996; Consonni et al. 2000), possibly due to their short chain lengths. Each of the peptides was added at low μM concentrations to microtiter wells coated with type I collagen, and their abilities to compete for ^{35}S -Hsp47 binding were examined. As shown in Figure 5, none of the CNBr peptides of the $\alpha 1$ chain had a significant effect on ^{35}S -Hsp47 binding to the collagen-coated wells when they were in the random-coil conformation. However, in the triple-helical conformation, the peptides CB8, CB3, CB6, and CB7 were able to compete for ^{35}S -Hsp47 binding to the collagen-coated well. Among them, CB8 was found to exhibit the strongest binding to Hsp47 (Fig. 5B).

CNBr-cleavage of the $\alpha 2$ chain of type I collagen yields three peptides of varying lengths, CB4, CB2, and CB3,5. Of these, only CB4 is able to form the triple-helical homotrimer

in solution as reported earlier (Rossi et al. 1996) and as verified by our CD measurements (data not shown). Each of these peptides was assayed for its ability to bind ^{35}S -Hsp47 (Fig. 5C). As with the shorter fragments of the $\alpha 1$ chain, Hsp47 binding to CB2 was insignificant. Maximum binding was seen with CB4 in the triple-helical conformation. This peptide showed reduced, but significant, binding to Hsp47 even in its random-coil form. Moderately strong binding was observed with the CB3,5 peptide which, as reported earlier (Rossi et al. 1996), could not be obtained in the triple-helical conformation at room temperature.

The above results demonstrate that while Hsp47 binds to the CNBr peptides in solution when they adopt the triple-helix conformation, some peptides bind relatively more strongly than others. This was further tested by using the nitrocellulose transfer assay used earlier for the collagenase cleavage fragments. This assay singled out those peptides that possessed a relatively high binding affinity to Hsp47 so

Figure 5. Inhibition of ^{35}S -Hsp47 binding to collagen microtiter wells by CNBr fragments of type I collagen. (A) Competition of ^{35}S -Hsp47 (0.1 μM) binding to collagen-coated wells by CNBr peptides (6 μM) generated from the N-terminal 120 residues of the $\alpha 1$ chain of type I collagen. The peptides were in a random-coil conformation as determined by CD. (B) Competition of ^{35}S -Hsp47 (0.6 μM) binding to collagen wells by CNBr peptides (12 μM) generated from the rest of the $\alpha 1$ chain of type I collagen. The triple helical conformation of the peptides was determined by CD and the random conformation was generated as described in Materials and Methods. (C) Competition of ^{35}S -Hsp47 (0.6 μM) binding to collagen wells by CNBr peptides (12 μM) generated from the $\alpha 2$ chain of type I collagen. Peptides were in either a random and/or triple helical conformation as determined by CD. Data shown are the mean ($\pm$ SE) of triplicate measurements.

that some of the peptides identified in the radioligand competition assay were not picked up by this assay. As shown in Figure 6, labeled Hsp47 bound to the CB8 peptide of the $\alpha 1$ chain and CB4 peptide of the $\alpha 2$ chain. The bound Hsp47 could be released by the addition of 1% gelatin to the binding buffer (data not shown). The results of this assay (Fig. 6) combined with those obtained by the collagen competition assay (Fig. 5) show that, although Hsp47 is capable of binding to several regions on the type I collagen molecule, the tightest binding occurs towards the N terminus represented by the regions covered by CB8($\alpha 1$) and CB4($\alpha 2$), when these are in the triple-helical conformation.

Binding of Hsp47 to CNBr peptides of type II collagen

Type II collagen is a homotrimer. CNBr cleavage of the $\alpha 1$ chain of this collagen yielded six peptides of varying chain lengths (Fig. 1; Tenni et al. 2002). The CD spectral properties of CB6, CB12, CB11, CB8, and CB10 have been examined earlier (Tenni et al. 2002) and verified by us (data not shown). CB11, CB8, and CB10 are able to form triple-helical homotrimers in solution at room temperature. The

Figure 6. Binding of ^{35}S -Hsp47 to representative CNBr fragments of type I collagen. CNBr peptides from the $\alpha 1$ (lanes 1–4) and $\alpha 2$ chains (lanes 5 and 6) of type I collagen were run in SDS-PAGE and were either stained with Coomassie Brilliant Blue (top) or transferred to nitrocellulose membrane and probed with ^{35}S -Hsp47 (bottom). Lane 1, CB8; lane 2, CB3; lane 3, CB7; lane 4, CB6; lane 5, CB4; lane 6, CB3,5. The bottom panel shows an autoradiograph of ^{35}S -Hsp47 binding to CB8($\alpha 1$) and CB4($\alpha 2$). Arrows indicate correspondence between the respective bands in the two panels.

Figure 7. Inhibition of ^{35}S -Hsp47 binding to collagen-coated microtiter wells by CNBr fragments of type II collagen. Competition of ^{35}S -Hsp47 (0.3 μM) binding to collagen-coated wells by CNBr peptides (6 μM) generated from the $\alpha 1$ chain of type II collagen. Peptides used were in either a random and/or triple-helical configuration as determined by CD. Data shown are the mean ($\pm\text{SE}$) of triplicate measurements.

random-coil CB6 peptide did not show any significant binding to Hsp47 (Fig. 7). Due to the limited supply of these peptides, experiments with CB11, CB8, and CB10 were performed only in their triple-helical and not random-coil conformation. When added to the type I collagen-coated microtiter wells, all the triple-helical peptides competed for ^{35}S -Hsp47 binding with CB11 showing the strongest binding followed by CB10 and CB8 (Fig. 7). Significant binding was also seen with random-coil CB12.

Binding of Hsp47 to chemically modified CB4 peptide of $\alpha 2(1)$

To understand the possible involvement of lysine and hydroxylysine in Hsp47 binding, competition studies were conducted with chemically modified CB4 peptide of the $\alpha 2$ chain of type I collagen. The derivatized peptides were generated by N-methylation and N-acetylation of the primary amino group of lysine and hydroxylysine side chains under mild conditions as described previously (Tenni et al. 2002). Figure 8 shows that neither of these derivatives significantly affected CB4's ability to inhibit ^{35}S -Hsp47 binding to the wells. It may therefore be concluded that the absence of positive charges on lysine and hydroxylysine or the addition of a methyl group to these residues is not important for Hsp47 binding to collagen.

Discussion

Hsp47 has been known to be intimately associated with one or more steps in the elaboration of nascent procollagen chains into the mature, functional collagen molecule. Knowledge of the structural details of the interaction of this

Figure 8. Inhibition of ^{35}S -Hsp47 binding to collagen-coated microtiter wells by derivatized CB4 ($\alpha 2$) peptide of type I collagen. Competition of ^{35}S -Hsp47 (0.3 μM) binding to collagen-coated wells by CB4 ($\alpha 2$) peptide modified by N-methylation and N-acetylation of its primary amino groups on lysine and hydroxylysine side chains. All peptides (3 μM) were in a triple helical conformation as determined by CD. Data shown are the mean ($\pm\text{SE}$) of triplicate measurements.

chaperone protein with its substrate is, therefore, of importance from the basic and clinical points of view. In addition to its association with procollagen in the nascent, nontriple-helical form (Sato et al. 1996), Hsp47 also accompanies the triple-helical procollagen to the *cis*-Golgi compartment (Sato et al. 1996). In vitro, Hsp47 binds to mature collagen type I–V with similar affinities ($\sim 10^{-7} \text{ M}^{-1}$; Natsume et al. 1994). In an earlier study, we showed that Hsp47 inhibits fibril formation among the triple-helical collagen monomer units in vitro (Thomson and Ananthanarayanan 2000). It would be interesting to know the site(s) on collagen where Hsp47 binds and exerts its fibril inhibition effect. Several recent studies have sought to understand the intriguing collagen-binding behavior of Hsp47 and clarify the primary structural and conformational requirement for the binding. Using collagen model peptides in vitro (Koide et al. 1999) and a yeast two-hybrid system (Koide et al. 2000), Nagata and coworkers found that Hsp47 did not interact with the peptides in the single-stranded polyproline-II helix conformation and that the Gly-X-Y repeat sequence in the triple-helical, but not in the unfolded, conformation contained the required information for Hsp47 binding. With the significant exception of hydroxyproline (Hyp), residues in the Y position of the tripeptide repeat that enhance triple-helix stability were seen to also enhance Hsp47 binding. In particular, Arg and Pro residues were predominant in the peptides that showed enhanced Hsp47 binding. In contrast, Sauk et al. (2000), who performed panning experiments with bacteriophage-peptides, found enriched binding of Hsp47 to sequences containing Trp, Leu, Val, and Ala.

The above structural studies using synthetic peptides have not been able to provide any clue about the location(s) in the triple-helical collagen molecule where Hsp47 is likely

to bind. Koide et al. (2002) recently used residue-specific chemical modification to show that Hsp47 requires the presence of Arg to bind collagen types I and III. This observation is supported by that of Tasab et al. (2002), who showed that at least one Gly-X-Arg triple within collagen is required for Hsp47 binding. However, this would imply an unrealistically large number of Arg-containing binding sites for Hsp47 along the collagen molecule, because X-Arg-Gly occurs with a high frequency (13%) in collagen (Yang et al. 1997). No indication of the relative affinities of putative Arg binding sites is obtainable from these studies. In the present investigation, we have addressed this problem by examining discrete regions in the primary structure of collagen. This was made possible by the use of peptides generated by CNBr cleavage of the α chains of type I and type II collagens. These CNBr peptides have been well characterized for their primary structures and conformations in the laboratory of one of us (Rossi et al. 1996; Consonni et al. 2000; Zamboni et al. 2000; Tenni et al. 2002). CNBr peptides derived from type I collagen would be expected form a homotrimer in their triple conformation. Our use of these peptides, in Hsp47 binding assays, to represent the chain configuration of the heterotrimeric type I collagen would be justified by the following considerations: (a) The sequences of CB8 in the $\alpha 1$ chain and CB4 in the $\alpha 2$ chain of type I collagen, both of which are strong Hsp47 binders, overlap significantly in the heterotrimeric type I collagen; and (b) the $\alpha 1$ chain of type I collagen has been found to be a homotrimer in vivo and may be potentially bound by Hsp47 (Moro and Smith 1977).

To assess the binding of these peptides, we made use of the facile radiolabeling of the C terminus of Hsp47 with ^{35}S during the purification of this protein using the Intein-based overexpression procedure (Evans and Ming-Qun 2000) and developed a relatively simple binding assay. This competition assay is sensitive so that low μM amounts of the peptides were sufficient to compete with collagen bound to the microtiter wells. This is in contrast to other binding studies (Koide et al. 1999; MacDonald and Bachinger 2001), which necessitate the use of unrealistically high amounts of peptides. The competition assay used by us yielded a ranking of the peptides by their relative strengths of Hsp47 binding. An alternate binding assay based on the visualization of ^{35}S -Hsp47 bound to peptides immobilized on nitrocellulose membrane was also used to examine binding of these as well as the collagenase cleavage fragments to Hsp47. This ligand blot assay was used as a verification of the results obtained from the competition assay on Hsp47 binding of the CNBr peptides. The ligand blot assay has been used in several other studies to identify ligand-binding sites in proteins (Kleinschmidt and Seiter 1988; Kouklis et al. 1994). It is likely that the proteins would refold during the blocking stage after their transfer from the SDS-PAGE gel to the nitrocellulose membrane (Edmondson and Dent 2001).

Thus, the CNBr peptides may be presumed to exist in the triple-helical conformation during their interaction with Hsp47 on nitrocellulose. The results from this assay showed that both of the $\alpha 1$ and $\alpha 2$ chains as well as the N-terminal fragment A obtained by collagenase cleavage bound Hsp47 (Fig. 2). The absence of Hsp47 binding by the C-terminal fragment B would imply that the inhibition of collagen fibril formation by Hsp47 is mechanistically different from fibril inhibition by the peptides (at relatively high concentrations) used by Prockop and Fertala (1998), who found the region in fragment B between residues 776–796 of the $\alpha 1(I)$ chain to be important for fibril assembly.

Examination of Hsp47 binding region(s) using the collagen competition assay showed that Hsp47 interacts weakly with (Pro-Pro-Gly)₁₀ and (Pro-Hyp-Gly)₁₀. This is in line with others' data (MacDonald and Bachinger 2001). Also, as observed in the studies on synthetic peptides (Koide et al. 1999), the triple-helical conformation in the CNBr peptides was found to be more conducive for Hsp47 binding than the random-coil form (Figs. 5, 7). A range of affinities towards Hsp47 was found among the peptides binding Hsp47 in the triple-helical form. When comparing the inhibition of Hsp47 binding to the collagen-coated wells by the peptides at a peptide:Hsp47 molar ratio of 20:1, by far the strongest inhibition (96%) was exhibited by the $\alpha 2(I)$ CB4 peptide (Fig. 5C). Very strong inhibition (84%–87%) of Hsp47 binding was seen with $\alpha 1(I)$ CB8 and $\alpha 1(II)$ CB11 (Figs. 5B, 7), and moderately strong inhibition (68%–75%) was found with the $\alpha 1(II)$ CB8 and CB10 peptides (Fig. 7). Relatively weak inhibition was seen in the case of CB3, CB7, and CB6 peptides of the $\alpha 1(I)$ chain (Fig. 5B). Intriguingly, unlike the peptides derived from the $\alpha 1(I)$ chain (Fig. 5B), the peptides CB3,5 and CB4 of the $\alpha 2(I)$ chain showed substantial inhibition even in their random-coil forms (Fig. 5C). The case of the CB3,5 peptide is particularly noteworthy because, despite its spanning nearly two-thirds of the $\alpha 2(I)$ chain, it showed no significant triple-helical structure at room temperature (Rossi et al. 1996). It is very likely that, while refolding from the denatured state, this peptide may get trapped in metastable intermediate state(s). As such, it may still possess short triple-helical regions as may be expected to be present amidst the predominant disordered regions in gelatin. However, unlike gelatin, the CD spectra of CB3,5 does not reveal the presence of ordered regions in this peptide.

Taken together, the Hsp47 binding data on the CNBr peptides reinforce the idea that Hsp47 is capable of binding at several sites, possessing different affinities, along the collagen molecule, and that some regions are able to bind the protein even in an apparently random-coil form. Under similar experimental conditions, certain specific regions such as those represented by CB8 in the $\alpha 1(I)$ chain, by CB4 in the $\alpha 2(I)$ chain and by CB11 in the $\alpha 1(II)$ chain form the strongest binding sites while they are in the triple-helical

state. The former two peptides were also identified as strong binders in the nitrocellulose transfer assay (Fig. 6). Interestingly, all the three peptides lie towards the N-terminal of the respective collagens. It should, however, be pointed out that while our results highlight Hsp47 binding sites on mature collagen that match those in procollagen, other sites may well be found when procollagen is used in a similar study.

In a recent study on the thermal stability of type I collagen, Leikina et al. (2002) have suggested that collagen chaperone proteins such as Hsp47 may bind and stabilize regions in collagen that are relatively weak with respect to triple-helix stability. These "microunfolded regions" have been predicted to be important in collagen fibril formation (Kadler et al. 1988). Regions with relatively low triple-helix stability have earlier been identified in the $\alpha 1(I)$ and $\alpha 2(I)$ chains (Bachinger and Davis 1991). These may well be candidates for microunfolded. However, it is unclear if Hsp47 would bind to these regions in light of the observed requirement for triple-helix for Hsp47 binding in this and earlier studies. Additional experiments using smaller fragments of the collagen chains with well-defined conformations are necessary to identify the optimal chain length, primary structure, and tertiary structure required for Hsp47 binding. This will also help us understand the mechanism by which Hsp47 might prevent collagen fibril formation and design potent inhibitors that bind to Hsp47 in vitro and modify collagen biosynthesis under disease conditions such as fibrosis.

Materials and methods

Purification of Hsp47 and radiolabeling with ³⁵S-cysteine

Hsp47 was purified as described previously using the IMPACTTM T7 system (New England Biolabs) followed by hydroxylapatite chromatography (Thomson and Ananthanarayanan 2000, 2001). [³⁵S]-cysteine labeling of Hsp47 was performed as described elsewhere (Thomson and Ananthanarayanan 2001). It involved the incubation of 1.8 mL of purified Hsp47 (~150 μ g/mL) with 5 μ L of L-[³⁵S]-cysteine (1 μ Ci; (NEN Life Science Products) at 4°C for 8 h. The ³⁵S-Hsp47 was separated from free [³⁵S]-cysteine using a PD-10 desalting column (Amersham Pharmacia Biotech).

CNBr peptides

The CNBr peptides used in the competition experiments and ligand blots were generated and characterized in acidic and neutral conditions as described elsewhere (Rossi et al. 1996; Consonni et al. 2000; Zanaboni et al. 2000; Tenni et al. 2002). The nomenclature of these peptides and their positions along the collagen molecule are shown in Figure 1. Each peptide was dissolved in a phosphate buffer (buffer A; 20 mM sodium phosphate, 50 mM NaCl, pH 7.4) and its concentration verified by amino acid analysis (HSC/Pharmacia Biotechnology Service Center). Molar concentrations are expressed in terms of the monomeric forms of the

peptides. Random-coil forms of the CNBr peptides were generated immediately prior to experiments by incubation of the peptides at 50°C for 3 min.

CD spectroscopy

CD spectra used to determine the secondary structural characteristics of the peptides were obtained with a Jasco J-600 spectropolarimeter (JASCO Inc.) using a 1-mm pathlength cell. Peptide concentrations were in the low μ M range.

Collagenase cleavage

To generate type I collagen fragments by digestion with vertebrate collagenase, 60 μ g of acid-soluble calf skin collagen (Worthington Biochemical Corporation) was incubated with 30 μ L of a 38 μ g/mL solution of the enzyme (MMP-1; Sigma Chemical Co.) to a final volume of 305 μ L in 100 mM Tris, pH 7.4, 20 mM CaCl_2 , 100 mM NaCl. The cleavage reaction was allowed to proceed at room temperature overnight. The reaction products were separated under reducing conditions in 10% SDS-PAGE.

Ligand blot assay

Collagenase fragments and CNBr peptides were separated using 10% and 12.5% SDS-PAGE respectively, and were either stained with Coomassie Brilliant Blue or transferred to nitrocellulose at 100 V for 4 h. The membrane was blocked with 1% BSA (Sigma), 1% skim milk powder, 0.05% Tween-20 (Sigma) in buffer A. The blot was rinsed for 5 min with the buffer and then incubated for 1 h at room temperature in the same buffer containing 1 mL of ^{35}S -Hsp47. After binding, the excess ^{35}S -Hsp47 was decanted and the blot washed twice for 5 min in buffer A. The blot was then dried and exposed to an X-ray imaging film placed inside an intensifying screen (Kodak Laboratories).

Competition assay

Forty-eight-well plastic microtiter plates coated with rat tail collagen type I (BD Biosciences) were used to examine the interaction of Hsp47 with collagen mimetics. Individual wells were incubated in a constant final volume of 300 μ L of buffer A containing the competing substrates. ^{35}S -Hsp47 was added at room temperature ($23 \pm 2^\circ\text{C}$) and allowed to bind for 20 min. The binding solution was decanted and the wells washed twice with buffer A for 5 min. A 2% SDS solution was added to the wells, which were then left overnight with gentle agitation to dissociate the bound protein. The SDS solution was subsequently added to scintillation fluid for counting. Nonspecific binding was determined by measuring ^{35}S -Hsp47 binding to the wells in the presence and absence of 0.1% gelatin (BIO-RAD) in buffer A. The nonspecific binding found in the presence of 0.1% gelatin was subtracted from the binding values obtained to generate specific binding. Normalized values were used to plot the histograms.

Acknowledgments

This work was supported by a grant to V.S.A. from the Heart and Stroke Foundation of Ontario. C.A.T. was a recipient of a Research Traineeship from the Heart and Stroke Foundation of Canada.

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