Hsp47 and the Translation-Translocation Machinery Cooperate in the Production of \(\alpha_1(1)\) Chains of Type I Procollagen* 

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Hsp47, an endoplasmic reticulum resident protein, has gelatin-binding and procollagen-binding properties and has been hypothesized to function as a molecular chaperone in regulating procollagen folding and/or assembly. In this report, we further investigate the interaction of Hsp47 with polysome-associated \(\alpha_1(1)\) procollagen chains following antisense treatment of 3T3 cells. For these studies, we employed phosphorothioate oligodeoxynucleotides directed to the first five codons of Hsp47 that straddle the predicted translation initiation site of mouse Hsp47. Cells depleted of Hsp47 in this manner were observed to produce diminished amounts of fully elongated nascent \(\alpha_1(1)\) procollagen while accumulating shorter procollagen peptides associated with peptidyl-tRNA. Pulse-labeling of cells with \(^{35}\)Smethionine followed by treatment with puromycin and immunoprecipitation with anti-Hsp47 and anti-procollagen antibodies revealed that Hsp47 is associated with \(\alpha_1(1)\) procollagen at a very early point during translocation of the nascent procollagen chains. Although Hsp47 appears to possess properties similar to grp78/BiP, Hsp47 binding early during translocation favors a more specialized specific function relative to chain selection or completion of stable folding in type I procollagen. 

Most proteins are translocated across the mammalian endoplasmic reticulum (ER) membrane prior to being targeted for secretion. This process is initiated by binding of the signal sequence of the nascent chain to the signal recognition particle (1–4). Following association of this particle with its docking membrane receptor, the nascent protein is shifted into the membrane. Commencement of the channel to permit translocation entails conformational changes within a preassembled or newly formed complex that is activated upon development of the nascent ribosome-signal recognition particle complex to the ER (5–7). The subsequent molecular events associated with the translocation processes are just now being uncovered. For example, it has become apparent that in some cases translocation proceeds through a protein-conducting channel comprised of a complex of ER-associated proteins called translocons (6, 8–11). Furthermore, it appears that elements in the translocons, topogenic sequences, can regulate steps in translocation (5, 9, 12, 13).

Although it is generally believed that simple elongation of the nascent chain drives extrusion of the polypeptide into the ER (14, 15), recent studies suggest that the protein translocation channel may also exist as a passive pore without a directional preference for polypeptide movement (16). Consequently, the bias in random movement of a polypeptide that results in vectorial translocation is driven by nascent chain elongation and sustained by inclusion of a stop codon or post-translational events, i.e., glycosylation, folding, and/or binding to chaperones (16, 17).

While it is not established whether distinctive chaperones or translocons are utilized for specialized proteins, there are strong implications that some classes of proteins use distinctive machinery for their organization. For example, a T cell receptor antigen protein is an ER resident that is transiently associated with newly synthesized multiheteromeric CD3 chains (18). Likewise, monocistronic procollagen I mRNAs coding for pro-\(\alpha_1\) and pro-\(\alpha_2\) chains are brought to the same ER compartment to ensure the coordinate synthesis of pro-\(\alpha\) chains in each heterotrimeric molecule (19). To accommodate these requirements, unique translation/translocation-associated machinery are considered necessary for assembly of heterotrimeric type I collagen. Recently, we and others have demonstrated that a collagen-specific proposed molecular chaperone, Hsp47, was associated with regions of developing long bone and teeth forming heteropolymeric type I collagen (20, 21). These findings suggest that Hsp47 may comprise at least a portion of distinctive translation/translocation machinery complex required for the processing of heteropolymeric procollagen.

Hsp47/colligin was first described as a 47-kDa collagen binding glycoprotein (colligin) from murine parietal endodermal cells (22). Hsp47/colligin was found to bind types I and IV collagen as well as gelatin. Similar proteins, GP46, were identified in L6 rat myoblasts, and keratinocytes and Hsp47 were described in 3T3 fibroblasts and chick embryo fibroblasts (23, 24). Hsp47 has been shown to co-localize with type I procollagen in the ER (25, 26). Subsequently, chemical cross-linking studies revealed that Hsp47 was one of several proteins that were intimately associated with type I procollagen. In addition, the intracellular association between Hsp47 and procollagen was shown to be disrupted by a change in physiological pH, suggesting that the dissociation of procollagen from Hsp47 is pH-dependent. These findings sustain some role for Hsp47 in the intracellular processing of procollagen (27).

This report further defines the role of Hsp47 in type I procollagen processing in mouse 3T6 cells. We find that Hsp47 is associated with the translating membrane-bound polysomes. This organization is not completely disrupted by the drug puromycin, except at high salt concentrations. Also, we show that phosphorothioate antisense oligodeoxynucleotides to Hsp47 inhibit both Hsp47 production and consequently diminish the production of type I procollagen \(\alpha_1(1)\) chains. We conclude that Hsp47 is an important molecular chaperone for \(\alpha_1(1)\) procollagen synthesis. Moreover, we suggest that the function of Hsp47...
in translation/translocation may be to aid in the organization of the translation machinery and then to assist in passage of nascent procollagen through the ribosome channel into the ER.

**MATERIALS AND METHODS**

**Cell Culture**—Mouse 3T6 cells were obtained from the American Type Culture Collection and grown to confluence in 150-cm² plastic flasks using Dulbecco’s modified Eagle’s medium, 1.16 g/liter glutamine, 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C. In those instances when it was deemed necessary to radiolabel proteins, the medium was removed and replaced with fresh methionine-free Dulbecco’s modified Eagle’s medium containing methionine (100 µCi/ml, DuPont NEN) for varying periods (see figure legends).

**Distribution of Hsp47 in Free, Cytoskeletal, and Membrane-bound Polysomes**—The distribution of Hsp47 among cellular polysomes utilized the methods of Veder et al. (28). In essence, harvested cells were collected by centrifugation at 1000 × g for 5 min. Cells were lysed for 10 min on ice in a low salt buffer (0.25% sucrose, 25 mM KCl, 5 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM triethanolamine (pH 7.4) containing 1% Nonidet P-40 and then centrifuged at 3,000 × g for 10 min. This resulted in a supernatant containing free ribosomes and a pellet consisting of insolubilized membranes, nuclei, and polymerized cytoskeletal components. The pellet was washed in low salt buffer and then resuspended in buffer containing 500 mM KCl and were extracted by collagenase buffer (130 mM KCl, 0.25% sucrose, 5 mM MgCl₂, 0.1 mM PMSF, and 10 mM triethanolamine (pH 7.4)) at room temperature for 30 min. The suspension was centrifuged at 3,000 × g for 10 min, and the resulting supernatant containing cytoskeletal-bound polysomes was collected. The cytoskeletal polysomes were washed once in 50 mM KCl buffer, and the membrane-bound polysomes were released in KCl buffer containing 0.5% Triton X-100 and 0.5% deoxycholate.

**Isolation of Intact Polysomes and Nascent Procollagen**—Dense polysomes were also prepared after a modified protocol of Kirk et al. (29). In essence, mouse 3T6 cells were grown to near-confluence as described above, and protein synthesis was blocked by the addition of cycloheximide. The retained tRNA-bound material was eluted with phosphate-buffered saline-azide. The samples were then incubated at 4 °C with constant shaking and then centrifuged at 10,000 × g for 10 min. The resulting immunoprecipitates were then washed twice with phosphate-buffered saline-azide. The final pellets were suspended in 2 x gel electrophoresis sample buffer, heated for 10 min at 90 °C, and then centrifuged to remove GammaBind G-agarose. Samples of the supernatant were counted in a scintillation counter, and another sample was analyzed by PAGE and autoradiography as described above.

**Antisense Oligodeoxyribonucleotide to Hsp47 mRNA**—Antisense phosphorothioate oligodeoxynucleotides to the first five codons of Hsp47 that straddled the predicted translation-initiation site of mouse Hsp47 mRNA were synthesized and the sense version of these regions were synthesized and obtained from Oligos Etc. (Wilsonville, OR). A computer-assisted search of the GenBank data base for mammalian sequences complementary to this antisense oligonucleotide revealed little homology with other genes. To eliminate nonspecific cytotoxic activity, the oligodeoxynucleotides were evaporated to dryness, reconstituted at 1-2 µm in sterile Dulbecco’s phosphate-buffered saline (Life Technologies, Inc.), and stored at -20 °C. Toxicity was monitored by trypan blue dye exclusion, cell counts, and assessment of cellular actin, a protein not directly targeted for manipulation.

**Cross-linking in Living Cells**—DSP was selected for cross-linking in that this compound has been shown to cross cell membranes and be internal disulfide bonds in this cross-linking in living cell reagent allow cleavage by reducing agents (39-41). For these studies, cells were first labeled with [35S]methionine for a specified time period (see figure legends), trypsinized, and treated with collagenase to ensure removal of extracellular collagen. Cells were then exposed to DSP for 30 min on ice prior to lysis (27). The lysates were then immunoprecipitated using anti-Hsp47 or anticollegen antibodies.

**RESULTS AND DISCUSSION**

The sequential treatment of mouse 3T6 cells with Nonidet P-40 at low salt, 130 mM salt, and Triton X-100/deoxycholate made it possible to distinguish cytoskeletal and membrane-bound polysomes similar to those which had been described previously for 3T3 cells and Krebs II ascites cells (28). Western blot analysis of these individual fractions revealed that this compound has been shown to cross cell membranes and be internal disulfide bonds in this cross-linking in living cell reagent allow cleavage by reducing agents (39-41). For these studies, cells were first labeled with [35S]methionine for a specified time period (see figure legends), trypsinized, and treated with collagenase to ensure removal of extracellular collagen. Cells were then exposed to DSP for 30 min on ice prior to lysis (27). The lysates were then immunoprecipitated using anti-Hsp47 or anticollegen antibodies.

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Fibroblasts were labeled with [35S]methionine, and procollagen was immunoprecipitated with α(I) procollagen antibodies. Co-immunoprecipitate with nascent α(I) procollagen chains, associated chaperones, and collagen processing enzyme (PDI). Coincident with the diminishment of Hsp47, the amount of polysome-associated pro-α(I) chains (Fig. 3) were decreased by ~90% compared to untreated controls or cells exposed to Hsp47 sense oligonucleotides. The Q-Sepharose-retained pool of collagen was then examined by Western blot using anti-N-propeptide procollagen antibodies. Since the synthesis of the pro-α chains of type I collagen does not appear to proceed at a uniform rate, there are accumulations of nascent peptides at particular chain lengths with a major portion of completely elongated but not completely hydroxylated chains associated with the polysomes (28). Accordingly, these data revealed that there

![Immunoprecipitation of [35S]methionine-labeled nascent procollagen and Hsp47 from the flow-through fraction of Q-Sepharose-fractionated polysomes treated for 4 days with antisense and sense oligomers to Hsp47. Mouse 3T6 cells were labeled for 12 h with [35S]methionine following treatment with either sense or antisense oligomers to Hsp47. Lane A, pro-α(I) chains and pN-α(I) chains following immunoprecipitation with anti-Hsp47 polyclonal antibodies from 3T6 cells treated with 25 μM antisense phosphorothioate oligomers to Hsp47. Lane B, pro-α(I) and pN-α(I) chains derived from cells treated with 25 μM of the sense version of Hsp47 oligomers. Lane C, supernatant derived from lane A immunoprecipitated with α(I) procollagen antibodies. Lane D, supernatant derived from lane B, immunoprecipitated with α(1) procollagen antibodies.](image)

**Fig. 1.** Immunoprecipitation of [35S]methionine-labeled nascent procollagen and Hsp47 from the flow-through fraction of Q-Sepharose-fractionated polysomes treated for 4 days with antisense and sense oligomers to Hsp47. Mouse 3T6 cells were labeled for 12 h with [35S]methionine following treatment with either sense or antisense oligomers to Hsp47. Lane A, pro-α(I) chains and pN-α(I) chains following immunoprecipitation with anti-Hsp47 polyclonal antibodies from 3T6 cells treated with 25 μM antisense phosphorothioate oligomers to Hsp47. Lane B, pro-α(I) and pN-α(I) chains derived from cells treated with 25 μM of the sense version of Hsp47 oligomers. Lane C, supernatant derived from lane A immunoprecipitated with α(I) procollagen antibodies. Lane D, supernatant derived from lane B, immunoprecipitated with α(1) procollagen antibodies.

Study the role of Hsp47 in procollagen processing, 3T6 mouse fibroblasts were labeled with [35S]methionine, and procollagen polysomes were prepared after a modified method of Kirk et al. (29). The resulting polysomes were then fractionated using Q-Sepharose Fast Flow chromatography. As previously reported (29), two major pools of procollagen are obtained by this procedure. One pool consisted of elongating procollagen bound to peptidyl-tRNA that was initially retained and eluted with high salt. The flow-through fraction consisted of recently completed nascent chains still polysome-associated and nearly completed ρN α(I) chains that were disrupted during column fractionation. The fractions were then immunoprecipitated with anti-Hsp47 and anti-procollagen antibodies. Hsp47 was noted to co-immunoprecipitate with nascent α(I) procollagen chains recovered in the flow-through fraction (Fig. 1).

To further investigate the purpose of Hsp47 in procollagen I processing, cells were depleted of Hsp47 by treating the cells with antisense phosphorothioate oligodeoxynucleotides against Hsp47 mRNA. Sulfur-containing oligodeoxynucleotides were selected over phosphodiester because of their high potency and because these compounds exert a prolonged effect in culture that lasted up to 7 days in spite of having a delayed onset (37, 38). The specificity of the inhibition that we observed with phosphorothioate oligodeoxynucleotides demonstrated that these compounds had a minimal effect on cell proliferation, viability, and general protein synthesis. As visualized by Western blot analysis, these antisense oligomers selectively impaired Hsp47 production after 4 days of treatment (Fig. 2).

![Western blot anti-Hsp47](image)

**Fig. 2.** Top panel, anti-Hsp47 Western blot of 3T6 cells treated with antisense phosphorothioate oligodeoxynucleotides to Hsp47 mRNA. American culture 3T6 cells were grown as described under "Materials and Methods" and treated with sense or antisense oligomers to Hsp47 mRNA. Cells were harvested and lysed, and proteins were separated by SDS-PAGE followed by Western blot staining using anti-Hsp47 antibodies. Densitometric scanning reveals that ~50% of day 1 Hsp47 can be detected by day 3 and that on day 4 only a trace of Hsp47 was detected. Bottom panel, anti-Hsp47 and anti-actin Western blot of 3T6 cells treated with phosphorothioate oligodeoxyribonucleotides to Hsp47 mRNA. 3T6 cells grown as described under "Materials and Methods" and treated with sense and antisense oligomers to Hsp47 mRNA. Densitometric analysis reveals that ~50% of Hsp47 is produced following 3 days of treatment, while actin levels remain relatively constant.

To ensure that procollagen binding to Hsp47 did not result as a consequence of cell lysis, cross-linking was used to demonstrate this relationship in living cells. These studies revealed that procollagen processing enzymes as well as other molecular chaperones are closely associated with α(I) procollagen chains (Fig. 3); thus, suggesting that a number of chaperones and processing enzymes may be successively integrated in procollagen production. Consequently, reduction of Hsp47 using antisense strategies also reduced the levels of α(I) procollagen chains, associated chaperones, and collagen processing enzyme (PDI).
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Fig. 3. Immunoprecipitation of 3T6 cell lysates following 4 days of treatment with phosphorothioate oligoribonucleotides to Hsp47. To ensure that procollagen binding to Hsp47 did not result as a consequence of cell lysis, cross-linking was used to demonstrate this relationship in living cells. American type 3T6 cells were grown to confluence as described under “Materials and Methods” and labeled with [35S]methionine for 12 h. Cells were harvested, cross-linked with DSP prior to lysing, as described under “Materials and Methods,” and immunoprecipitated with polyclonal anti-Hsp47 antibodies. Arrows indicate the Mr for various collagen processing proteins, chaperones, α1(I) procollagen, and Hsp47.

was a preponderance of low molecular weight partially elongated pro-α1(I) chains following antisense treatment compared to control cells treated with sense version oligomers (Fig. 4, top). Thus, Hsp47’s association with procollagen appears to influence vectorial transport into the ER lumen. This influence may occur through direct interaction with procollagen or possibly through additional interactions with integral translocation channel proteins.

To further examine this relationship, cells were treated with puromycin to terminate chain elongation and release proteins from peptidyl-tRNA. The resulting cell homogenates were, as before, applied to Q-Sepharose. Again, both the flow-through fraction and the retained fraction were noted to contain Hsp47. Furthermore, if the labeled polysomes were immunoprecipitated with anti-Hsp47 or anti-procollagen antibodies, Hsp47 was found to co-immunoprecipitate from the Q-Sepharose-retained fraction with elongating α-chains (Fig. 4, bottom). We have previously found that Hsp47 has a strong specific binding to the pro-α1(I)-N-propeptide in the SP-1 region and weaker binding to G-X-Y sequence domains in fully completed pro-α1(I) chains. However, the polysome-associated low molecular weight collagenase-digestible peptides precipitating with anti-Hsp47 reported here indicate Hsp47’s involvement in procollagen processing occurs as an earlier event than the fully translocated pro-α1(I) chain association initially demonstrated between Hsp47 and procollagen (27).

To confirm that polysome-associated procollagen binding to Hsp47 did not result as a effect of cell lysis, cross-linking with DSP was again used to demonstrate this relationship in living cells. Polysomes were isolated by Q-Sepharose as described under “Materials and Methods,” and the resulting tRNA fraction was eluted with high salt. Western blot analysis subsequently demonstrated the presence of Hsp47, and immunoprecipitation with anti-procollagen I antibodies revealed that Hsp47 was eluted with polysome-associated procollagen pep-

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tides (Fig. 5). It is noteworthy that this early interaction between a proposed molecular chaperone and evolving peptides is not a unique feature of Hsp47-procollagen. Beckman et al. (42, 43) showed that a significant number of newly synthesized proteins could be isolated in an apparent complex with members of the Hsp70 family of chaperones. Furthermore, indirect evidence revealed that such interactions are probably co-translational, occurring as the polypeptide is being synthesized. These interactions between chaperone and polypeptide were suggested to serve to prevent the premature or incorrect folding of the incoming protein until the translocation event had been completed. Support for this idea was provided by examining temperature-sensitive mutants, where in the absence of functional chaperone, proteins entering into the ER or mitochondria exhibited an arrest of translocation (44, 45).

In order to determine if Hsp47 association with puromycin-treated polysemes was contingent on ribosome binding, puromycin-treated polysemes were treated with 1 M NaCl and re-applied to the sucore gradient. These studies revealed that the bulk of the Hsp47 and procollagen was localized at the top of the gradient and none was found in the membrane pellet. Conversely, the release of peptidyl-tRNA-associated procollagen, following treatment with puromycin, appears to still occupy the top of the gradient, whereas treatment of polysemes with both puromycin and high salt leads to dissociation of the ribosomes into their subunits, closing the channel and release of nascent proteins and Hsp47. These data are similar to those published by Görlich et al. (13) which demonstrate that release of the nascent chain from the ribosome is insufficient for the detachment of Sec61p, an ER channel translocation protein. The basis for these findings is that although nascent chains released by puromycin at physiological salt concentration are translocated across the membrane (46), ribosomes remain bound and translocation is not terminated. However, if the puromycin reaction is carried out at high salt concentrations, the ribosomes dissociate into their respective subunits and associations with translocons are greatly weakened.

The exact basis for the association between Hsp47, procollagen, and the translocation channel remains to be determined. Candidates would include components of a ribosome binding site or translocons, i.e. the mammalian homologs of Sec61p, Sec62p, Sec63p proteins (47), or the 34K translocating chain-associating membrane protein, TRAM (48). Since Hsp47 is a soluble ER luminal protein and since anti-Hsp47 will immunoprecipitate evolving nascent low molecular weight procollagen peptides, Hsp47 might interact with luminal extensions of ER membrane proteins and procollagen near to or at the ER lumen channel orifice. Consequently, we envision that Hsp47 may function in a manner analogous to BiP/Kar2p in yeast (47). Accordingly, Hsp47 may be an acceptor for nascent translocating procollagen, maintaining the emerging peptide in an unfolded conformation until polymer assembly is completed. This would prevent aberrant or premature folding from occurring until polymer assembly is concluded. Alternatively, by analogy to Kar2p/BiP and Sec63p (49), Hsp47 may also prove to interact with integral membrane proteins during translocation to activate the translocation apparatus. However, since procollagen has also been shown to concurrently interact with grp78/BiP (27), Hsp47 and N-propeptides of procollagen may even have a more specialized specific function relative to chain selection or completion of stable folding in type I procollagen.

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