Journal of Cellular Biochemistry 56:518–526 (1994)

Association of Hsp47, Grp78, and Grp94 With Procollagen Supports the Successive or Coupled Action of Molecular Chaperones

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Abstract Hsp47, Grp78, and Grp94 have been implicated with procollagen maturation events. In particular, Hsp47 has been shown to bind to nascent procollagen α1(I) chains in the course of synthesis and/or translocation into the endoplasmic reticulum (ER). Although, Hsp47 binding to gelatin and collagen has previously been suggested to be independent of ATP. Grp78 and Grp94 are known to dissociate from its substrates by an ATP-dependent release mechanism. The early association of Hsp47 with procollagen and its relatively late release suggested that other chaperones, Grp78 and Grp94, interact successively or concurrently with Hsp47. Herein, we examined how these events occur in cells metabolically stressed by depletion of ATP. In cells depleted of ATP, the release of Hsp47, Grp78, and Grp94 from maturing procollagen is delayed. Thus, in cells experiencing metabolic stress, newly synthesized procollagen unable to properly fold became stably bound to a complex of molecular chaperones. In that Hsp47, Grp78, and Grp94 could be recovered with nascent procollagen and as oligomers in ATP depleted cells suggests that these chaperones function in a series of coupled or successive reactions.

Key words: Hsp47, Grp78, Grp94, procollagen, molecular chaperones, metabolic stress

Until recently, it was generally felt that the amino-acid sequence contained all of the information necessary to specify the native conformation of procollagen. Thus, registration of the three pro-α-chains was believed to occur following release of the nascent chains into the cisterns of the endoplasmic reticulum (ER), by association, and disulfide bonding of the newly completed procollagen C-terminal extension propeptide regions. The growth of the triple-helix then proceeded from the C-propeptide site of association to the N-terminus of the molecule at a rate limited by *cis-trans* isomerization of the peptide bonds involving proline [Bachinger et al., 1980; Bruckner et al., 1981]. In spite of this orderly scheme, questions regarding the efficiency of chain selection, cotranslational and post-translational processing remain to be explained.

The discovery that the folding of certain newly synthesized proteins depends on molecular chaperones, or polypeptide chain binding proteins, provides a clue that would explain further how procollagen molecules are assembled [Ellis and van der Vies, 1991; Pelham, 1984]. In this regard, it appears that while molecular chaperones themselves do not directly convey information for folding or higher ordered assembly, they appear to facilitate these processes by reducing incorrect folding pathways and insure that such events occur rapidly with high fidelity [Beckmann et al., 1992]. Studies in the cellular functions of chaperone proteins have usually focused on one class of components; however, an integrated view of the roles of the different chaperone families in protein folding is now beginning to emerge [Langer et al., 1992; Hendrick et al., 1993].

Procollagen has been cross-linked with a number of potential chaperones that include Grp94, Grp78, and shown to bind Hsp47, a specific collagen binding protein [Nakai et al., 1992]. The early association of evolving nascent procollagen with Hsp47 indicates that Hsp47 may function as a primary molecular chaperone for α1(I) chains of procollagen. Recently, we have also shown that antisense "knock out" of Hsp47

Received February 23, 1994; accepted April 22, 1994.

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results in a slowing of the rate of translation of α1(I) chains [Sauk et al., 1994]. In this report, we go on to reveal that cells metabolically stressed by depletion of ATP, release of Hsp47, Grp78, Grp94, and the procollagen processing enzyme protein disulfide isomerase (PDI) from procollagen is delayed. Since the association of Hsp47 with procollagen is not ATP dependent, it appears that there is overlap in the successive action of chaperones and that cellular stress at one level of interaction produces a cascade effect on the entire integrated system of chaperones.

MATERIALS AND METHODS

Cell Culture and Metabolic Labeling

Mouse 3T6 cells obtained from the American Type Culture Collection were used in all experiments. The cells were grown and maintained in plastic flasks using Dulbecco's Modified Eagles Medium (DMEM), 1.16 g/l glutamine, 10% FBS, 10 μg/ml ascorbate, 100 units of penicillin, and 100 μg/ml streptomycin at 37°C. In those instances when it was necessary to label proteins, the medium was removed and replaced with fresh methionine-free DMEM containing ^{35}S-methionine (100 μCi/ml, New England Nuclear, Boston, MA) for varying periods (see figure legends).

For pulse-chase experiments, cells were incubated with ^{35}S-methionine in DMEM lacking methionine, either under normal conditions, during metabolic stress, or 43°C heat shock. After the pulse label, the medium containing the label was removed and the cells further chased in DMEM.

Effects of ATP Depletion In Vivo

To assess the effects of ATP depletion, cells growing at 37°C were simultaneously treated with 20 μm of the mitochondrial uncoupler, carbonyl cyanide m-chlorophenylhydrazone (CCCP), and 12.5 mm 2-deoxyglucose (2DG) in glucose free DMEM [Beckmann et al., 1992]. In some cases, the cells were first treated with 100 μgm/ul cycloheximide before ATP depletion. After a 2 h incubation at 37°C, the medium was removed and the cells were washed with DMEM lacking methionine, labeled with ^{35}S-methionine for 2 h, and then harvested and analyzed. To examine the effects of ATP depletion on newly synthesized proteins, 3T6 cells growing at 37°C were pulsed for 20 min with ^{35}S-methionine. Afterwards, the label was removed and the cells further incubated in 20 μm CCCP and 12.5 mM 2DG in glucose free DMEM for either 30 or 60 min. The cells were then harvested in lysis buffer, clarified by centrifugation, and analyzed by native immunoprecipitation.

Gelatin Binding Assay

Cells labeled with ^{35}S-methionine were maintained in Dulbecco's modified medium as described above. After extraction with lysis buffer (1% Nonidet p40, 0.15 M NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 2 mM N-ethylmaleimide, and 2 mM phenylmethyl sulfonyl fluoride) on ice for 20 min, the samples were then centrifuged at 12,000*g* for 20 min and the supernatant mixed with 200 μl of a 50% (v/v) suspension of gelatin-Sepharose 4B (Pharmacia) as described by Nakai et al. [1989]. Some samples were supplemented with 50 μM ATP or ATPγS (Boehringer Mannheim, Indianapolis, IN) (50 mM Tris-HCl, pH 7.5, 50 mM $NaCl_2$, and 2 mM $MgCl_2$) for 2 h. The gelatin-Sepharose beads were then collected by centrifugation, washed twice with lysis buffer, and once with 10 mM Tris-HCl buffer (pH 6.8). Samples for gel electrophoresis were suspended in Laemmli SDS-PAGE buffer and heated at 90°C for 5 min before loading onto 4–20% polyacrylamide gradient slab gels after the method of Laemmli [1970]. The gels were fixed, dried, and autoradiographed using the method of Bonner and Laskey [1974].

Isolation of Intact Polysomes and Nascent Procollagen

Dense polysomes were prepared after a modified protocol of Kirk, Evans, and Veis [1987]. In essence, Mouse 3T6 cells were grown to near confluence as described above and protein synthesis was blocked by the addition of cycloheximide (100 μg/ml) for 10 min. In some instances cross-linking with DSP was used to determine the near neighbors of polysome associated proteins [Sauk et al., 1994]. The cells were suspended in buffer A (0.2 M Tris-HCl, pH 7.4, 0.24 M KCl, 0.0075 M $MgCl_2$, 0.1 mg/m; cycloheximide, 0.2 mg/ml heparin, 2 mM DTT, 0.05% Na deoxycholate, 0.16 mg/ml PMSF, and 0.78 mg/ml benzamidine) and Triton X-100 was then added to a final concentration of 2%. The cells were homogenized with a loose fitting Dounce homogenizer and maintained on ice for 10 min to ensure detergent lysis. The homogenate was centrifuged at 10,000*g* for 30 min to remove nuclei and cellular debris. The resulting super-

natant was collected and the volume adjusted to 5 ml with buffer A. The supernatant (2.5 ml) was then layered on top of 1 ml of 1 M sucrose layered over 1.5 ml of 2 M sucrose. The samples were centrifuged for 12 h in a Beckmann SW 55 Ti rotor at 100,000*g*. Polysomes were collected, washed with water suspended in Q-Sepharose buffer (0.02 M Tris-HCl, pH 7.4, 0.24 M KCl, and 0.0075 M $MgCl_2$), and applied to a 3 ml Q-Sepharose Fast Flow column (Pharmacia, Piscataway, NJ) as described by Bergman and Kuehl [1977] and modified by Kirk, Evans, and Veis [1987]. The resulting flow through fraction contained t-RNA-free nascent chains. The retained t-RNA bound material was eluted with 1.0 M NaCl.

Immunoprecipitation and Analysis by Gel Electrophoresis

For immunoprecipitation, samples were suspended in an equal volume of 2× immunoprecipitation buffer [0.2 m Tris-HCl, 0.3 M NaCl, 2% Triton X-100, 2% deoxycholate, and 0.2% SDS, pH 7.2, containing apyrase (Sigma Chemical Co., St. Louis, MO) to deplete ATP]. The samples were centrifuged for 5 min at 10,000*g* in an Eppendorf centrifuge and a 50 μl sample of the radiolabeled supernatant was added to a mixture of Protein A-Sepharose and antibody (polyclonal anti-Hsp47 [Sauk et al., 1992]; monoclonal anti-Grp78, and anti-Grp94, Affinity BioReagents, Neshanic Station, NJ) in PBS-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 PBS-azide. The final pellets were suspended in 2× gel electrophoresis sample buffer, heated for 10 min at 90°C, and then centrifuged to remove Protein A-Sepharose. Samples of the supernatants were counted in a scintillation counter and another sample analyzed by PAGE and autoradiography as described above.

RESULTS

We and others have shown that Hsp47 tenaciously binds gelatin-Sepharose, even in the presence of high ionic concentrations of salt, and that Hsp47 only dissociated from this matrix by lowering the pH of elution buffers below pH <6.3 [Saga et al., 1987; Shroff et al., 1993]. Although, the association of Hsp47 with collagen is suggested to be unaffected by the addition of ATP [Nakai et al., 1992]. Nevertheless, it still is not clear whether Hsp47 is dependent on the energy from ATP to effect any of its chaperone functions. To begin to address this question the effect of ATP depletion and supplementation was investigated utilizing gelatin-Sepharose binding. These studies showed that when mouse 3T6 fibroblast cell lysates were either depleted of ATP with apyrase (Sigma Chemical Co., St. Louis, MO), or supplemented with exogenous ATP or ATPγS, the degree of Hsp47 associated with gelatin-Sepharose remained unchanged (Fig. 1).

Next, we examined the effects of intracellular ATP depletion on the association of Hsp47 and other chaperones with procollagen. To accomplish this, mouse 3T6 cells were pulse-labeled with ^{35}S-methionine and one group of plates was immediately harvested. In the remaining plates, the label was removed and the cells incubated for periods up to 60 min at 37°C, either in the presence or absence of CCCP and 2DG. After pulse-chase labeling, the cells were harvested and the lysates subjected to immunoprecipitation using anti-Hsp47 and anti-procollagen antibodies. In control cells labeled for 20 min at

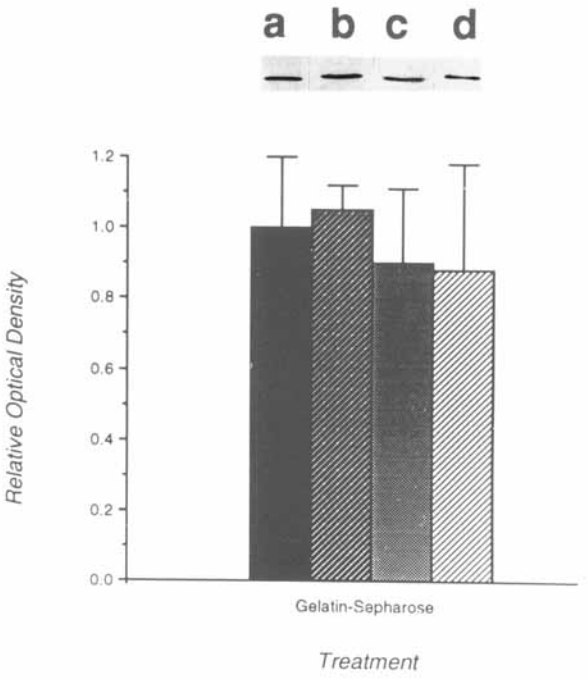

Fig. 1. Binding of Hsp47 to gelatin-Sepharose-4B beads. ^{35}S-methionine-labeled mouse 3T6 cells were lysed and incubated with gelatin-Sepharose [Nakai et al., 1989]. After washing samples were treated by the following: Lane a, 50 μM of ATP (50 mM Tris-HCl, pH 7.5, 50 mM NaCl); lane b, 50 μM of ATPγS (50 mM Tris-HCl, pH 7.5, 50 mM NaCl); lane c, Apyrase (50 mM Tris-HCl, pH 7.5, 50 mM NaCl; lane d, control (50 mM Tris-HCl, pH 7.5, 50 mM NaCl). Mean relative optical density of three separate experiments ± standard deviation of mean.

37°C, pro-α1(I) chains were observed to coprecipitate with Hsp47. Upon allowing the 37°C pulse-labeled cells a cold chase period, significantly less pro-α1(I) chains were observed to coprecipitate indicating that there is a transient association of newly synthesized procollagen with Hsp47 with pro α1(I) chains (Fig. 2). However, different results were observed in cells depleted of ATP. First, when cell lysates were immunoprecipitated with anti-Hsp47 or procollagen antibodies, a number of newly synthesized proteins including procollagen were found to coprecipitate with Hsp47. These proteins included PDI, Grp78, and Grp94. However, in contrast to the situation with normal cells, the proteins synthesized were still found associated with Hsp47 after 60 min chase period (Fig. 2 a,b).

Previously it had been noted that under conditions where Hsp70 proteins become stably complexed to their targets, the corresponding reduction in the available levels of free Hsp70s induced a stress response [Beckmann et al., 1992]. Accordingly, we examined whether such treatment would constitute a general response and include Hsp47. For these experiments, mouse 3T6 cells were incubated with CCCP + 2DG for 2 h at 37°C. To some cells cycloheximide was added either concurrently, or 2 h before the addition of the ATP depleting agents. The rationale here was to inhibit the levels of newly synthesized proteins before the ATP depletion. Thus, there would be few pro-α1(I) chains available, subsequent depletion of ATP might not result in any significant reduction of free Hsp47, and the cells might not respond by induction of a stress response. After appropriate incubation to deplete intracellular ATP, the culture medium containing the various agents was removed, the cells extensively washed with DMEM, and then subjected to a metabolic labeling with ^{35}S-methionine. After 2 h labeling period, the cells were harvested and the radiolabeled proteins analyzed by immunoprecipitation followed by SDS-PAGE.

These studies revealed that in the presence of CCCP + 2DG, cells immunoprecipitated with anti-Hsp47 yielded immune precipitates that contained Hsp47, PDI, Grp78, Grp94, and procollagen (Fig. 3a). When these lysates were immunoprecipitated with anti-Grp78 antibodies, Hsp47, and procollagen were similarly recovered in the immunoprecipitates (Fig. 3b). However, the levels of procollagen represented only a small fraction of the procollagen precipitated with anti-Hsp47. Also, when cycloheximide and the ATP-depleting agents were added concurrently, the levels of Hsp47, Grp78, Grp94, and

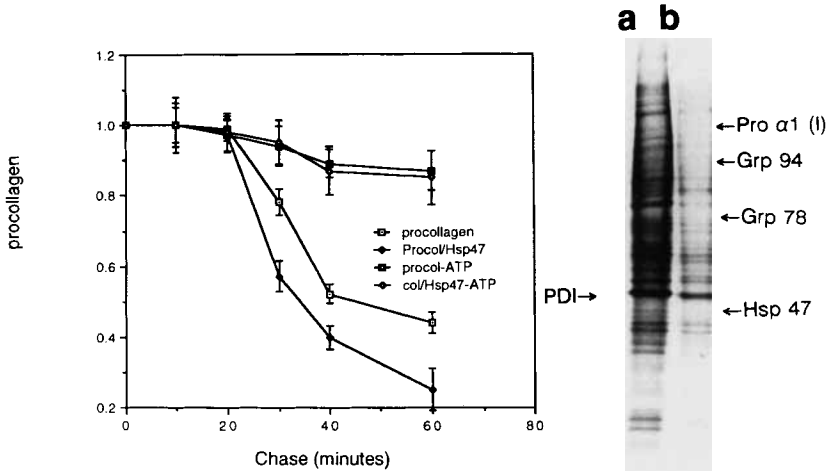

Fig. 2. Total cellular procollagen and procollagen immunoprecipitated with Hsp47. 3T6 cells were pulse labeled with ^{35}S-methionine. The samples were chased in DMEM cold medium in the presence and absence of CCCP + 2DG for up to 60 min. At each time point cells were harvested, lysed, and immunoprecipitated with anti-Hsp47 antibodies and anti-procollagen antibodies. The resulting immune precipitates were analyzed by SDS-PAGE followed by autofluorography. The relative optical density of the bands corresponding to α1(I) procollagen were estimated using a densitometer. The results represent the mean values and standard deviations from three experiments. [▣] indicate α1(I) procollagen immunoprecipitated with anti-procollagen I antibodies; [●] indicate α1(I) chains immunoprecipitated with anti-Hsp47 antibodies; [■] α1(I) chains immunoprecipitated with procollagen antibodies, cells treated with CCCP + 2DG; [◉] α1(I) procollagen immunoprecipitated with anti-Hsp47, cells treated with CCCP + 2DG. Lane a, cell homogenate derived from cells treated with CCCP + 2DG, precipitated at 60 min with anti-Hsp47 antibodies; Lane b, control cells immunoprecipitated after 60 min with anti-Hsp47 antibodies.

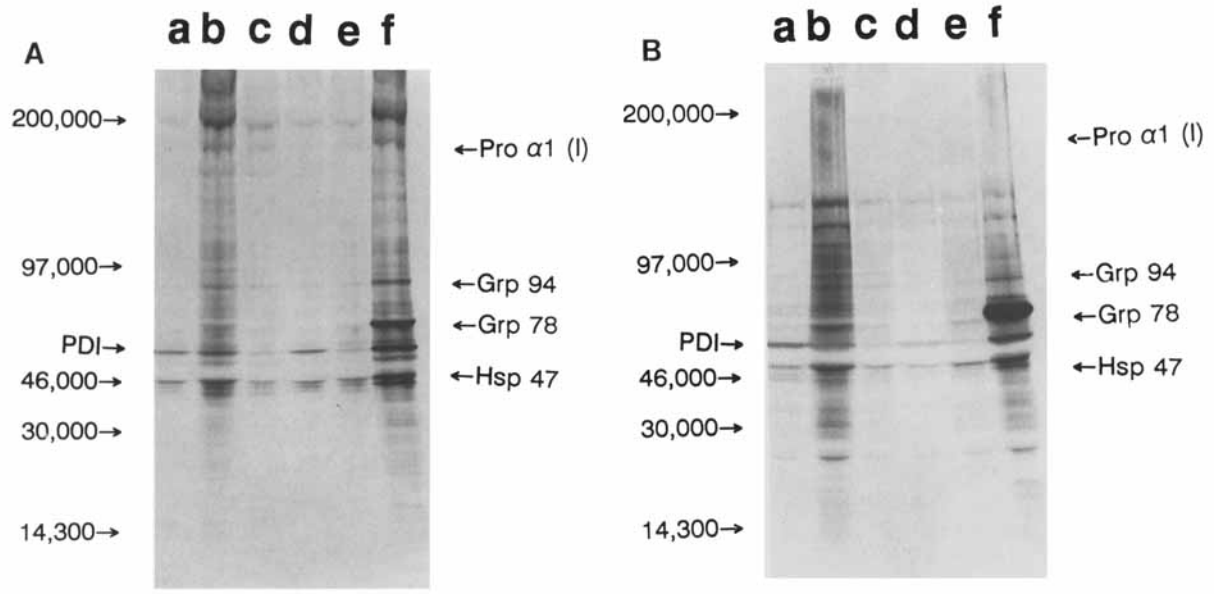

Fig. 3 A,B: Mouse 3T6 cells were incubated in the presence of 20 μM CCCP and 12.5 mM 2DG in glucose free medium for 2 h to deplete intracellular ATP. To some cells cycloheximide was added either before ATP depletion or alternatively added concurrently with CCCP + 2DG. One set of samples was subjected to a 43°C/90 min heat-shock in the absence of other treatments, labeled with ^{35}S-methionine for 2 h at 37°C. The cells were then harvested, lysed, and immunoprecipitated with anti-Hsp47 or anti-Grp78 antibodies. Lane designations are the same in A and B. Lane a, 37°C control; lane b, cells treated with CCCP + 2DG for 2 h; lane c, cells incubated with cycloheximide for 2 h; lane d, cells treated with cycloheximide for 4 h; lane e, cells treated with CCCP + 2DG and cycloheximide added concurrently; lane f, cells heat-shocked 43°C/90 min.

PDI were diminished along with procollagen (Fig. 3). Thus, treatment with cycloheximide before ATP depletion resulted in reduction in procollagen production and an absence of a stress response with Hsp47. Interestingly, when the ATP depleted supernatants from anti-Hsp47 immunoprecipitations were precipitated with anti-grp78 antibodies, additional Hsp47 and Grp94 were recovered with Grp78 (Fig. 4).

Next, cells were treated with α,α-dipyridyl to inhibit prolyl and lysyl hydroxylation resulting in conformational changes that result in the retention of procollagen in the ER. Some cells were then treated with CCCP + 2DG to deplete the intracellular stores of ATP. The cells were then lysed and immunoprecipitated with anti-Hsp47 or procollagen I antibodies. These studies showed increased quantities of Hsp47 and procollagen co-precipitating in α,α-dipyridyl treated cells. However, when the cells were depleted of ATP with CCCP + 2DG for 1 h following labeling, a number of additional proteins including Grp78, and Grp94 were co-precipitated with Hsp47 and procollagen (Fig. 5).

In order to ascertain whether these molecular chaperones were associated with nascent α1(I) chains, dense polysomes were fractioned and subjected to Q-Sepharose Fast Flow chromatography. This resulted in two major pools of procollagen. One pool consisted of elongating procollagen bound to peptidyl t-RNA that was initially retained and a second pool recovered as the flow through (FT). The FT pool consisted of recently

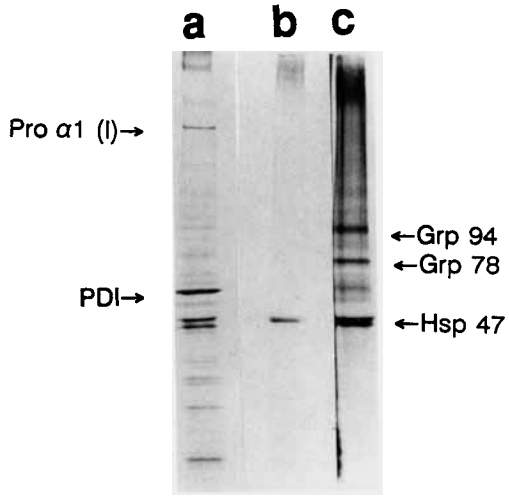

Fig. 4. 3T6 cells treated with CCCP + 2DG for 2 h and then labeled for 2 h with ^{35}S-methionine. Lane a, sample immunoprecipitated with anti-Hsp47 antibodies; lane b, ^{35}S-methionine Hsp47 eluted from gelatin-Sepharose as a molecular marker; lane c, supernatant from lane a immunoprecipitated with anti-Grp78 antibodies contain oligomers of Grp78/Grp94/Hsp47 that are separated by SDS-PAGE.

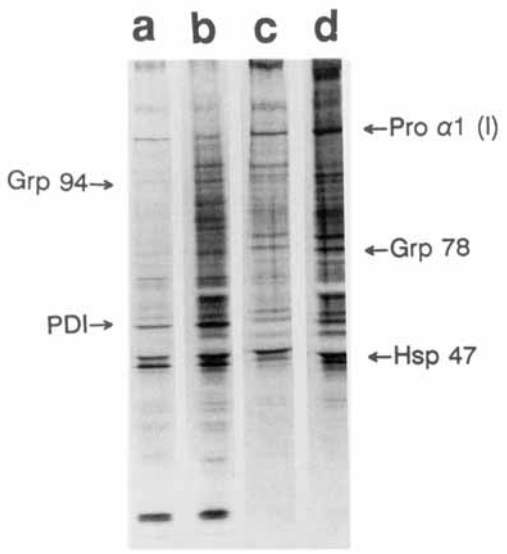

Fig. 5. 3T6 cells labeled with ^{35}S-methionine in the presence and absence of α,α-dipyridyl, and CCCp + 2DG. The samples were then immunoprecipitated with anti-Hsp47 antibodies. Lane a, control cells; lane b, cells treated with CCCP + 2DG; lane c, α,α-dipyridyl treated cells; and lane d, α,α-dipyridyl treated cells depleted with CCCP + 2DG. Proteins adjacent to arrows were verified by Western blots.

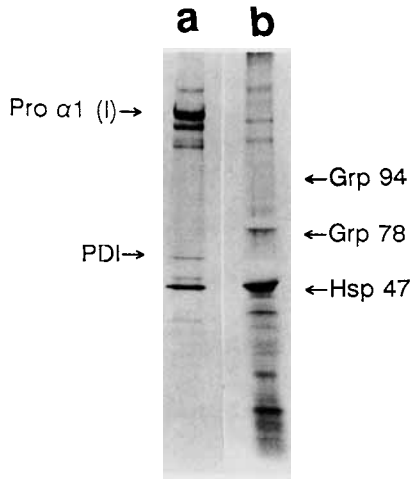

Fig. 6. Chaperone and procollagen associated with dense polysomes in 3T6 cells. Cells were labeled with ^{35}S-methionine for 20 min and protein synthesis blocked by the addition of cycloheximide for 10 min. The cells were immediately cross-linked with DSP and polysomes prepared after the method of Kirk and Veis [1987]. Lane a is the flow through fraction from Q-Sepharose fractionation of the polysomes immunoprecipitated with anti-procollagen antibodies. Lane b is the retained fraction that is eluated with 1 M NaCl, dialyzed and immunoprecipitated with anti-procollagen antibodies.

completed nascent chains and nearly completed pN α1(I) chains disrupted during column fractionation. Immunoprecipitation with anti-procollagen antibodies revealed that Hsp47 co-immunoprecipitated with PDI and α1(I) procollagen chains in the FT fraction. Meanwhile, Grp78 and Grp94 were associated with peptidyl t-RNA associated procollagen following cross-linking with DSP (Fig. 6).

DISCUSSION

Generally, constitutively expressed Hsps, particularly members of the Hsp70 family, interact transiently with other proteins during their translation and translocation [Beckmann et al., 1990]. Indirect evidence indicates that such interactions are sensitive to ATP and likely occur as the polypeptide is being synthesized [Beckmann et al., 1990, 1992]. In that Hsp47, Grp78, and Grp94 are associated with peptidyl t-RNA procollagen, a similar relationship between procollagen and its molecular chaperones appears to exist.

However, Hsp47 is unique in that it continues to bind to procollagen even in the presence of ATP. Also, Hsp47 binds mature collagen as effectively as it binds denatured collagen [Nakai, 1989, 1992] and its release can apparently only be effected by lowering of pH [Nakai et al., 1992]. This has lead to the speculation that the dissociation mechanism of Hsp47 from procollagen is mediated in an intermediate compartment between the ER and Golgi. Support for this is based on a) the demonstration that procollagen and Hsp47 distribution in odontoblasts has localized a fraction of Hsp47 to vesicles between the ER and Golgi [Shroff et al., 1993], b) that Hsp47 has been shown to possess an ER-retention signal [RDEL] at the COOH terminus that salvages Hsp47 following its dissociation from procollagen [Hirayoshi et al., 1991; Takechi et al., 1992; Hosokawa et al., 1993], and c) treatment by Brefeldin A inhibits the dissociation of Hsp47 from procollagen [Ripley et al., 1993; Sauk et al., 1990].

Although Hsp47 is tightly associated with procollagen, the use of chemical crosslinkers has revealed other ER resident proteins as near neighbors of procollagen and Hsp47. These ER residents have included protein disulfide isomerase (PDI), Grp78, and Grp94 [Nakai et al., 1992]. In the studies reported here, mouse 3T6 cells pulse labeled for 20 min then chased in the presence of CCCP + 2DG revealed that the interaction of newly synthesized proteins with Hsp47 was less transient following ATP depletion than reported previously [Nakai et al., 1992]. Furthermore, Grp78, Grp94, and PDI were com-

monly collected in the immune complexes following immunoprecipitation, without employing chemical cross-linking. These data support the idea that under conditions of stress that deplete the cell of its energy, newly synthesized proteins are unable to assume their properly folded state and remain bound to their chaperones and some of their processing enzymes.

The studies examining the effects of the protein synthesis inhibitor cycloheximide, in both normal cells and cells depleted of ATP, indicated that treatment with cycloheximide for 2 h, and the drug then removed, reduced de novo expression of Hsp47, Grp78, and Grp94. We speculate that in the absence of newly synthesized procollagen, the levels of available chaperone probably reach maximal levels such that the cell now no longer requires new expression of the chaperone. Whereas, in cells lacking cycloheximide the levels of chaperones persist and are immunoprecipitated with procollagen.

Our studies examining the effects of ATP depletion verify that in vivo the release of Grp78 and Grp94 proteins from their protein targets, including procollagen, requires ATP [reviewed in Welch, 1990]. However, since the release of Hsp47 from newly synthesized procollagen was also effectively blocked by depletion of intracellular ATP levels. These data further suggest that Hsp47, along with Grp78 and Grp94 and possibly other molecular chaperones, forms complexes or close associations that facilitate the maturation of procollagen by a series of coupled or adjacent reactions. Furthermore, in that Hsp47 is associated early in the translation of procollagen and is released as a late event in the export from the ER, this indicates that there is probably an overlap in the action of these chaperones.

Constitutive Hsp70 has been shown to exist in oligomeric forms [reviewed in Kim et al., 1992], and the amount of these oligomers increased by depleting the ATP content of the protein solution and dissociated to monomers in the presence of ATP [Kim et al., 1992]. Carlino et al. [1992] similarly demonstrated that Grp78 dissociated to form monomers in the presence of ATP. Our data indicates that with ATP depletion oligomerization of a number of molecular chaperones, including Hsp47, Grp78, and Grp94, occurs. Furthermore, it appears that these oligomeric forms are probably induced by ATP-driven conformational change in these proteins.

Although the exact sequence of interactions has yet to be established, clues as to the organization of these ER residents and their interaction with procollagen can be gleaned from polysome associated nascent procollagen chains and by examining the fate of specific mutant procollagens. For example, when polysomes isolated from 3T6 cells were fractionated by Q-Sepharose chromatography, immunoprecipitation of the FT fraction with anti-procollagen antibodies or anti-Hsp47 antibodies yields proα(I) chains, PDI and Hsp47. In contrast, the retained fraction (RT) similarly treated produced elongating nascent chains of procollagen crosslinked with Grp78, Grp94, and Hsp47. Also, mutations in the genes that encode the chains of type I procollagen and result in the production of abnormal molecules that cause various forms of osteogenesis imperfecta have been informative. In particular, examination of the procollagen produced by fibroblasts harboring a 4.5 kilobase deletion in an allele of COL1A2 has revealed that type I molecules were produced that assumed triple helical conformations more slowly and possessed amino-terminal ends lacking protease resistance. These abnormal molecules were stably associated with PDI. Also, although not identified, the published autoradiograms contained a ~47 k Mr protein that might represent Hsp47 [Chessler and Byers, 1992]. The association of PDI here was believed to do so in its role as a subunit of proyl-4-hydroxylase [Bassuk and Berg, 1989; Kao et al., 1979). Thus, it appears that PDI binds to the triple helical domain of procollagen before the formation of the triple helix and has low affinity for the molecule after helix formation. Interestingly, neither BiP (Grp78) nor Grp94 was stably associated with this mutant form of procollagen. In contrast, three strains of osteogenesis imperfecta fibroblasts with unique mutations in the carboxyl-terminal of proα1(I) that impair proα chain association were noted to become stably associated with BiP and Grp94 [Chessler and Byers, 1993]. These data suggested that BiP synthesis increased in response to the synthesis of proα1(I) chains with a defect that impairs, but does not totally disable, chain association. Interestingly, carboxyl-terminal mutations that completely prevent incorporation of the affected chains into trimers or result in rapid turnover of the affected chains did result in increased BiP accumulation [Chessler and Byers, 1993]. In that BiP binds poorly to peptides that contain proline and

lysine [Flynn et al., 1991], this chaperone appears to interact with procollagen at C-terminal subunit interfaces similarly to its role in immunoglobulin [Gething and Sambrook, 1990].

Thus, we speculate that among the ER resident proteins, Hsp47 has a primary role as a procollagen chaperone. As we have suggested previously, Hsp47 probably interacts with luminal extensions of ER membrane proteins and procollagen near or at the lumen channel orifice. 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, Hsp47 may also prove to interact with integral membrane proteins during translocation to activate the translocation apparatus [Sauk et al., in press]. PDI may function either as an oligosaccharyl transferase [Bassuk and Berg, 1989; Geetha-Habib et al., 1988; Freedman, 1989] and a component of the translation/translocation machinery [reviewed in Gilmore, 1993], and then as an ingrdient of prolyl hydroxylase [Bassuk and Berg, 1989; Kao et al., 1979; Chessler and Byers, 1993]. Furthermore, it appears that Grp78 (BiP) and Grp94 interacts with procollagen towards the end of chain elongation probably at or near the C-terminal interfaces.

The idea that a successive series of chaperones is necessary for protein processing, folding, and polymer assembly is not unique to procollagen and integrated views of the roles of different chaperone families in protein folding is beginning to surface. For example, Bip and Grp94 have been localized in the same complex with immunoglobulin chains [Melnick et al., 1992]. Also, in *Escherichia coli* DnaK (Hsp70) recognizes the folding polypeptides as an extended chain and cooperates with DnaJ in stabilizing an intermediate conformational state lacking ordered tertiary structure. Dependent on GrpE and ATP hydrolysis, the protein is then transferred to GroEL (Hsp60) which acts catalytically in the production of the native state [Langer et al., 1992]. Recently, the control of folding and membrane translocation by binding of the chaperone DnaJ to nascent polypeptides in eukaryotes has also been shown to cooperate closely possibly successively with Hsp70 [Hendrick et al., 1993]. Although the destiny of many procollagen mutations is reflected in the phenotypes of collagen conditions such as osteogenesis imperfecta. The molecular fate of cellular stresses produced by gene mutations or metabolic insult and the successive events surrounding procollagen maturation pathways remain to be determined.

ACKNOWLEDGMENTS

This work has been supported by NIH Grants DE08648, AR41572. We thank Ms. JoAnn Walker for help in preparing the manuscript.

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