Involvement of the Stress Protein HSP47 in Procollagen Processing in the Endoplasmic Reticulum

Akira Nakai, Mamoru Satoh, Kazunori Hirayoshi, and Kazuhiro Nagata
Department of Cell Biology, Chest Disease Research Institute, Kyoto University, Kyoto 606, Japan

Abstract. The 47,000-D collagen-binding glycoprotein, heat shock protein 47 (HSP47), is a stress-inducible protein localized in the ER of collagen-secreting cells. The location and collagen-binding activity of this protein led to speculation that HSP47 might participate in collagen processing. Chemical crosslinking studies were used to test this hypothesis both before and after the perturbation of procollagen processing. The association of procollagen with HSP47 was demonstrated using cleavable bifunctional crosslinking reagents. HSP47 and procollagen were shown to be coprecipitated by the treatment of intact cells with anti-HSP47 or with anticollagen antibodies. Furthermore, several proteins residing in the ER were noted to be crosslinked to and coprecipitated with HSP47, suggesting that these ER-resident proteins may form a large complex in the ER. When cells were heat shocked, or when stable triple-helix formation was inhibited by treatment with α,α′-dipyridyl, coprecipitation of procollagen with HSP47 was increased. This increase was due to the inhibition of procollagen secretion and to the accumulation of procollagen in the ER. Pulse label and chase experiments revealed that coprecipitated procollagen was detectable as long as procollagen was present in the endoplasmic reticulum of α,α′-dipyridyl-treated cells. Under normal growth conditions, coprecipitated procollagen was observed to decrease after a chase period of 10–15 min, whereas total procollagen decreased only after 20–25 min. 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 support a specific role for HSP47 in the intracellular processing of procollagen, and provide evidence of a new category of "molecular chaperones" in terms of its substrate specificity and the dissociation mechanism.

Membrane proteins as well as secretory and lysosomal proteins enter the ER where they are targeted for the secretory pathway. The ER membrane and the membrane enclosed lumen contain many resident proteins that are involved in the processing of secretory proteins. Among these are certain stress proteins. Glucose-regulated protein 78 (GRP78 or immunoglobulin-binding protein [BiP]), a member of the heat shock protein 70 (HSP70) family of proteins, acts like an ATP-dependent intracellular detergent (for review see Pelham, 1989; Rothman, 1989). Another glucose-regulated protein, GRP94, is similarly reported to be synthesized at a higher rate when secretory proteins increase or when aberrant proteins accumulate in the ER (Ozutsumi et al., 1988; Dorner et al., 1989). HSP47 is a novel 47,000-D heat shock protein which also exists in the ER of collagen-secreting cells (Nagata et al., 1986; 1988a; Saga et al., 1987). This protein binds specifically to collagen (type I and IV) in vitro (Nagata et al., 1986). While this binding is sufficiently strong to prevent secretion of these proteins (Haas and Wabl, 1983; Bole et al., 1986; Gething et al., 1986; Dorner et al., 1987; Kassenbrock et al., 1988; Flynn et al., 1989). Another glucose-regulated protein, GRP94, is similarly reported to be synthesized at a higher rate when secretory proteins increase or when aberrant proteins accumulate in the ER (Kozutsumi et al., 1988; Dorner et al., 1989).

HSP47 is a novel 47,000-D heat shock protein which also exists in the ER of collagen-secreting cells (Nagata et al., 1986; 1988a; Saga et al., 1987). This protein binds specifically to collagen (type I and IV) in vitro (Nagata et al., 1986). While this binding is sufficiently strong to prevent secretion even in the presence of 2 M NaCl, the collagen becomes completely dissociated from HSP47 at pH levels below 6.3 (Saga et al., 1987). The synthesis of HSP47 decreases after viral transformation (Nagata and Yamada, 1986; Cates et al., 1987; Nagata et al., 1988b) and drastically increases during the differentiation of mouse F9 teratocarcinoma cells (Kurkinen et al., 1984; Takechi et al., 1992). These changes in HSP47 expression are consistent with changes in the levels of collagen. Immunofluorescence study has demonstrated the colocalization of HSP47 with procollagen (Nakai et al., 1990). Furthermore, procollagen has been coprecipitated with HSP47 after immunoprecipita-

1. Abbreviations used in this paper: CCCP, carbonyl cyanide m-chlorophenylhydrazone; DSP, dithio bis(suc cinimidylpropionate); DSS, disuccinimidy1 suberate; DTSSP, 3,3′-dithiobis(sulfosuccinimidylpropionate); GRP, glucose-regulated protein; HSP, heat shock protein; PDI, protein disulfide isomerase; TCR, T cell antigen receptor; TRAP, T cell receptor-associated protein.

Akira Nakai's present address is Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL 60208.
tion with anti-HSP47 antibody (Nakai et al., 1990). Direct in vivo association of HSP47 with procollagen, however, has not been demonstrated. Similarly, the locus of procollagen association with HSP47, and the method of dissociation remain to be elucidated.

In this study, membrane-permeable crosslinking reagents were successfully used to demonstrate the association of HSP47 with procollagen in vivo. This association was observed to be transient under normal growth conditions, but more permanent following the prevention of procollagen secretion. Furthermore, the dissociation of procollagen from HSP47 appeared to be regulated by a change in pH.

Materials and Methods

Chemicals and Reagents

The chemical crosslinkers dithiobis (succinimidylpropionate) (DSP), di-succinimidyl suberate (DSS), and 3,3'-dithiobis (sulfosuccinimidylpropionate) (DTSSP) were purchased from Pierce Chemical Co. (Rockford, IL). Chromatographically purified bacterial collagens (form III) and endoglycosidase H (endo H) were obtained from Advance Biofactures Co. (Lynbrook, NY) and Genezyme (Boston, MA), respectively. α,α′-Dipyridyl was purchased from Nakai Tesque Inc. (Kyoto, Japan). Rabbit serum raised against rat type I collagen was obtained from Advanced Biofactures Co., (Tokyo, Japan). Rabbit polyclonal and rat monoclonal (1D10) IgG against chick HSP47 was generated previously (Saga et al., 1987), and used after affinity purification by chick HSP47-coupled Sepharose 4B column. Rabbit polyclonal IgG against protein disulfide isomerase was a kind gift from T. Yoshimori (Kansai Medical University, Osaka, Japan). [35S]Methionine was obtained from New England Nuclear (Boston, MA). Other reagents were purchased from Wako Junyalm (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), and were of the highest purity available from each company.

Cell Culture and Metabolic Labeling

Chick embryo fibroblasts were maintained in Vogt's GM medium (Vogt, 1969), and passed with 0.05 % trypsin-0.02 % EDTA (Gibco Laboratories). Experiments were conducted between passages three and eight. Metabolic labeling was carried out by plating 6 × 10^5 cells in 35-mm plastic tissue culture dishes and incubating at 37°C for 16-24 h in 5% CO2. Subconfluent cultures were rinsed with methionine-free medium, preincubated in this medium for 30 min, then labeled with 0.1 to 0.4 mCi/ml [35S]methionine and 5% dialyzed FCS for the times indicated.

To show the coprecipitation of HSP47 with procollagen more clearly (see Fig. 4 B), cells were labeled for 24 h with 0.1 mCi/ml [35S]methionine in the GM medium containing 1/5 fold of normal methionine concentration, and then chased for 1.5 h with excess methionine (chase medium). After treatment with or without α,α′-dipyridyl for 1.5 h in the chase medium, cells were washed with methionine-free medium and further incubated in the methionine-free medium for 30 min. The second labeling of the cells with 0.3 mCi/ml [35S]methionine was performed for 10 min as described above.

Crosslinking and Immunoprecipitation

Cells cultured in 35-mm plastic dishes were rinsed with Ca2+ and Mg2+-free PBS, and detached by incubation with 200 μl trypsin (0.025 %)-EDTA (0.02 %) at 37°C for 3 min. The cells were then treated with 800 μl medium containing 0.1% collagenase (WakoJunyaku) and 10% FCS at 37°C for 3 min to block the trypsin activity and to remove the extracellular collagen. After rinsing and resuspension in 100 μl PBS, the cells were combined with 2 μl of DSP (stored at a concentration of 0.1 M in DMSO), vortexed and plated on 3 cm for 5 min (Kreis and Lodish, 1986; Roth and Pearce, 1987). In some experiments, DSS (0.1 M in DMSO) or DTSSP (0.1 M in distilled water) were used in place of DSP. After 30 min, the cells were rinsed with 2 mM glycine in PBS to block the DSP activity and then with PBS. The cells were then lysed with lysis buffer (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1% NP-40, 5 mM EDTA, 1 mM PMSF, 1 mM N-ethylmaleimide, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) containing 0.5 mg/ml gelatin on ice for 15 min. The excess gelatin functions to compete with procollagen and prevents the binding of procollagen with HSP47 in the cell extract after cell lysis. After centrifugation at 12,000 g for 15 min, the supernatants were divided into two equal aliquots. Rabbit polyclonal IgG against HSP47 or rabbit serum raised against rat type I collagen was added to the samples. The cell extracts were incubated with antibodies for 2 h at 4°C, then precipitated by mixing with 20 μl protein A-Sepharose beads (Pharmacia Fine Chemicals; Piscataway, NJ) for 1 h. The precipitated immune complexes were washed three times with 1 ml of 50 mM Tris-HCl, pH 8.0, 0.4 M NaCl, 5 mM EDTA, and once with 1 ml of 10 mM Tris-HCl, pH 6.8. The samples were resuspended in Laemmli's sample buffer (Laemmli, 1970) containing DTT, boiled for 5 min and analyzed by SDS-PAGE as described previously (Nakai, 1990). The gels were exposed on Fuji HR-H film after enhancement.

For crosslinking in different pH's, cells were washed and suspended in KH2PO4/NaOH buffer at pH 6.3 or 7.5 (ionic strength was adjusted to 0.16) in the presence of 5 × 10^-6 M carbonyl cyanide m-chlorophenylhydrazine (CCCP). Incubation at 37°C for 30 min was followed by the addition of DSP (Heyler, 1963; Hayashi et al., 1975; Woods and Lazarides, 1985; Lippincott-Schwartz et al., 1988).

Immunoblot Analysis with Anti-HSP47 Antibody after Immunoprecipitation with Anticollagen Antibody

Cells were treated with α,α′-dipyridyl for indicated periods, crosslinked, and lysed as described above. Proteins immunoprecipitated with anticallogen antibody were applied to SDS-8% polyacrylamide slab gel, and blotted onto nitrocellulose membrane as described by Towbin et al. (1979). After incubation in a 5% skim milk solution (Yukijirushi Milk Co., Sapporo, Japan), the blotted membrane was cut at the position around 60 kD. The upper half and the lower half of the membrane were separately incubated with rabbit anti-rat type I collagen or with rat anti-HSP47 mAb (1D10), respectively. Peroxidase-conjugated goat antibody against rabbit or rat IgG (Cap pel, Organon Teknika Co., West Chester, PA) was used as the secondary antibody, and detected with ECL Western Blotting Detection Reagents (Amersham International plc., Amersham, UK).

Collagenase Treatment

Immune complexes precipitated with protein A-Sepharose (20 μl bed volume) were suspended in 33 μl of collagenase buffer (50 mM Tris, pH 7.5, 2 mM PMSF, 10 mM CaCl2) containing 20 μg/ml collagenase (Advance Biocare Co.), and incubated at 37°C for 30 min. The samples were then boiled for 5 min in 12 μl of 5% Laemmli's sample buffer and 3 μl of 2 M DTT, and applied to SDS-polyacrylamide gel.

Results

Coprecipitation of Procollagen with HSP47

Chick embryo fibroblasts were incubated for 1 h with [35S]methionine, and then lysed with lysis buffer. Aliquots of equal TCA-precipitable radioactivity were mixed with rabbit IgG against HSP47 and, then immunoprecipitated with anti-HSP47 antibody (Fig. 4, lanes 1 and 2). Complexes were analyzed on SDS-10% polyacrylamide gel. As reported previously in mouse BALB 3T3 cells (Nakai et al., 1990), two bands of α1 and α2 procollagen (Fig. 1, lane j), identified by digestion with collagenase (data not shown, see Nakai et al., 1990), were specifically coprecipitated with HSP47 even after washing with a high ionic strength buffer containing 0.4 M NaCl. Notably much more α1 procollagen was coprecipitated with anti-HSP47 antibody than α2 procollagen (Fig. 1, lane 3). Recently, HSP47 was found to bind preferentially to the αI(1)-specific portion in the N-propeptide of type I procollagen as well as the Gly-XY portion of the triple helix (Gavin, T. G., A. Veis, K. Nagata, M. J. Somerman, and J. J. Sauk, manuscript submitted for publication). This might be the reason why α1 procollagen was preferentially coprecipitated with HSP47 when the cell lysate was incubated with anti-HSP47 antibody.
Figure 1. Coprecipitation of procollagen with HSP47. Chick embryo fibroblasts were labeled for 1 h with 0.1 mCi/ml of [3S]methionine in methionine-free medium supplemented with dialyzed FCS. After lysing by treatment with lysis buffer, aliquots of cell extracts containing equal amounts of TCA-precipitable radioactivity were mixed with rabbit anti-HSP47 IgG (lanes 3 and 4) or with nonimmune serum (lanes 1 and 2). Immune complexes were precipitated by adding protein A-Sepharose, washed with lysis buffer containing 0.4 M NaCl (lanes 1 and 3) or pH 6.0 lysis buffer (lanes 2 and 4), and applied to SDS-10% polyacrylamide gel. Arrowheads on the right-hand margin indicate the position of α1(I) and α2(I) chains of procollagen, and of HSP47. The positions of prestained molecular mass markers are shown in the left-hand margin; 94k, phosphorylase b; 67k, BSA; 43k, ovalbumin, and 30k, carbonic anhydrase.

The effect of low pH on procollagen binding to HSP47 was analyzed by washing immunoprecipitates with pH 6.0 lysis buffer. The bands of procollagen diminished following washing with low pH buffer in both control (Fig. 1, lane 4) and heat-shocked cells (data not shown). These results are consistent with the observation that HSP47 dissociates from gelatin-Sepharose at pH ≤ 6.3 in vitro (Saga et al., 1987).

The high molecular weight bands which coprecipitated nonspecifically (Fig. 1, lanes 1–4) were identified as fibronectin by immunoblot analysis using antifibronectin antibody (data not shown). Faint 43-kD bands precipitated either by nonimmune serum or by anti-HSP47 antibody were identified as actin by two-dimensional SDS-PAGE.

Crosslinking under Normal Growth Condition

Coproccipitation experiments using anti-HSP47 shown in Fig. 1 cannot preclude the possibility that procollagen may bind to HSP47 in the cell extract after cell lysis. Accordingly, crosslinking was used to demonstrate directly the procollagen association with HSP47 in living cells. Cells were pulse labeled with [3S]methionine for 20 min, trypsinized, and then treated with collagenase to remove extracellular collage. Exposure to crosslinking reagents for 30 min on ice was followed by treatment with lysis buffer containing 1% NP-40 and excess gelatin (0.5 mg/ml) to prevent the binding of HSP47 to procollagen.

Three homo-bifunctional crosslinking reagents with similar structures, but different chemical properties, were used. DSP is permeable to membranes and can crosslink spatially-close intracellular polypeptides (Lomant and Fairbanks, 1976; Kreis and Lodish, 1986; Roth and Pierce, 1987). Internal disulfide bonds in this crosslinking reagent permit cleavage by reducing agent. DSS is similarly membrane permeable but not cleavable (Brenner et al., 1985; Fanger et al., 1986). The third reagent, DTSSP, is cleavable but not membrane permeable due to the presence of hydrophilic moieties (Lee and Conrad, 1985).

In the absence of crosslinking reagents, α1 and α2 procollagen were coprecipitated with HSP47 by anti-HSP47 antibody when the lysis buffer did not contain gelatin (Fig. 1 and Fig. 2 A, lane 4). The addition of excess gelatin to the lysis buffer led to the loss of banding, as gelatin competes with procollagen and binds to HSP47 in the cell lysate (Fig. 2 A, lane 5). When cells were pretreated with DSP, the bands of procollagen coprecipitated with HSP47 by anti-HSP47 reappeared (Fig. 2 A, lane 6). A higher amount of procollagen was coprecipitated in the presence of DSP compared with that in the absence of crosslinking reagent, and the band corresponding to α1 procollagen was again preferentially coprecipitated with HSP47 when the cells were pretreated with DSP as was seen without crosslinking (Fig. 2 A, lanes 4 and 6). When cells were pretreated with DSS, and anti-HSP47 antibody was used for immunoprecipitation, no α1 and α2 procollagen bands were detected, and the HSP47 band was notably diminished (Fig. 2 A, lane 7). A band appeared at the boundary between the stacking and the resolving gels, however, suggesting the presence of a high molecular weight complex comprised of crosslinked polypeptides, including procollagens and HSP47. When crosslinking was performed with a membrane impermeable crosslinker, DTSSP, procollagen bands were not coprecipitated (Fig. 2 A, lane 8) as observed in the absence of crosslinker (Fig. 2 A, lane 5). For control experiments, 2% DMSO was added to the cell lysate in the presence or absence of DTSSP (Fig. 2 A, lanes 9 and 10) to exclude a possible effect of DMSO, and the results were similar to the lane 8 in Fig. 2 A.

The identity of procollagen bands, following crosslinking with DSP, was determined by the digestion of immune complexes with collagenase (Fig. 2 B). Four bands specifically disappeared after collagenase digestion. The lower two bands, defined by pro α1(I) and pro α2(I), were identified as α1 and α2 procollagen, respectively. The upper two bands (defined by arrows) were similarly determined to represent collagenous proteins as evidenced by their sensitivity to collagenase treatment.

After crosslinking with DSP, proteins precipitated with anti-HSP47 were analyzed by SDS-PAGE, blotted onto nitrocellulose filters, and then immunostained with antifibronectin and anti-protein disulfide isomerase (PDI) anti-
Figure 2. Crosslinking of HSP47 with procollagens using various crosslinking reagents. (A) Chick embryo fibroblasts were labeled for 20 min with 0.2 mCi/ml of [35S]methionine at 37°C, trypsinized, and then treated with collagenase to remove cell surface collagen. Cells were incubated with or without the various crosslinking reagents indicated at the top of each lane for 30 min at 0°C, and extracted by the addition of lysis buffer with or without 0.5 mg/ml gelatin. For control experiments, cells were incubated with 2% DMSO in the presence (lanes 3 and 10) or absence (lanes 2 and 9) of DTSSP. Each extract was immunoprecipitated with rabbit anti-HSP47 IgG (lanes 4-10), or with nonimmune rabbit IgG (lanes 1-3). Immune complexes were applied to an SDS-8% polyacrylamide gel. (B) After crosslinking with 0.1 mM DSP, cell lysate was precipitated with anti-HSP47 as described in A. The immune complex was incubated with or without 20 U/ml collagenase for 30 min at 37°C, then applied to SDS-8% polyacrylamide gel. Pro α1 (I) and pro α2 (I) indicate the α1 and α2 chains of the procollagen, respectively. The two upper arrows indicate collagenase-sensitive bands. FN, fibronectin; PDI, protein disulfide isomerase; grp94, glucose-regulated protein 94; and grp78, glucose-regulated protein 78.

bodies. The fibronectin and PDI coprecipitated with HSP47 are identified by FN and PDI, respectively in the right margin of Fig. 2 B. HSP47 was similarly coprecipitated with PDI by rabbit anti-PDI antibody (data not shown). Fibronectin may be coprecipitated by anti-HSP47 forming a ternary complex with procollagen and HSP47 via its collagen-binding domain, because the coprecipitation was inhibited by the presence of gelatin in the lysis buffer when precipitated without crosslinker (Fig. 2 A, lanes 4 and 5). Total cell lysates pretreated with DSP, and the immune complexes coprecipitated with anti-HSP47 were analyzed by two-dimensional gel electrophoresis before and after treatment with tunicamycin. The acylation of the amino groups of proteins caused by crosslinking reagents (Lomant and Fairbanks, 1976) resulted in the streaking of spots in the basic regions. Nevertheless, many spots were identifiable on the two dimensional gels (data not shown). Two glucose-regulated proteins, GRP78 and GRP94, were identified in the immune complexes by comparison of the two dimensional patterns before and after tunicamycin treatment, and marked in the right margin of Fig. 2. These proteins, fibronectin, GRP94, GRP78, and PDI, which were coprecipitated with HSP47 after crosslinking with DSP (Fig. 2 A, lane 6) disappeared when cells were pretreated with DSS, and anti-HSP47 antibody was used for immunoprecipitation (Fig. 2 A, lane 7), indicating that these polypeptides were crosslinked directly to HSP47, or crosslinked indirectly by binding to procollagen. HSP47 and these coprecipitated proteins including GRP94, GRP78, and PDI were not precipitated by the incubation with preimmune serum as shown in lanes 1-3 of Fig. 2 A. These results suggest that ER-resident proteins, including HSP47, GRP78, GRP94 and PDI, may form a large complex in the ER.
Effects of Heat-Shock and \( \alpha, \alpha'- \)Dipyridyl

The effects of heat-shock and \( \alpha, \alpha'- \)dipyridyl on the binding of HSP47 to procollagen were analyzed. At elevated temperatures, procollagen is sensitive to protease in vitro (Miller, 1971), such that heat treatment can destabilize the triple helix of procollagen in the ER. \( \alpha, \alpha'- \)Dipyridyl, an iron-chelating reagent, inhibits the triple-helix formation of procollagen by preventing prolyl and lysyl hydroxylation and subsequent glycosylation (Juva et al., 1966; Margolis and Lukens, 1971). In both cases, the secretion of procollagen is inhibited by conformational changes in the procollagen molecule and/or direct inhibition of membrane traffic (Jimenez et al., 1973; Berg and Prockop, 1973; Jimenez and Yanowski, 1978).

Chick embryo fibroblasts were labeled with \([35S]\)methionine for 8 h, and incubated at 45°C for the last 4 h of the labeling period. The cells were treated with \( \alpha, \alpha'- \)dipyridyl during the labeling period. After heat shock, HSP47 synthesis was increased as reported previously (Fig. 3, lane 7, and Nagata et al., 1986). Newly synthesized procollagen, which was detected by immunoprecipitation with anticolagen antibody, was similarly increased by heat shock treatment (Fig. 3, lane 11). This increase was attributed to the inhibition of procollagen secretion. In the heat-shocked cells, the procollagens coprecipitated with HSP47 by treatment with anti-HSP47 antibody also increased (Fig. 3, lane 7). Similar results were obtained by treatment with \( \alpha, \alpha'- \)dipyridyl (Fig. 3, lanes 8 and 12). When cells were treated with \( \alpha, \alpha'- \)dipyridyl, apparent molecular sizes of \( \alpha_1 \) and \( \alpha_2 \) procollagen which were immunoprecipitated using anticolagen antibody (Fig. 3, lane 12), and coprecipitated with HSP47 by anti-HSP47 (Fig. 3, lane 8) were lowered compared with those without the treatment. This decrease in apparent molecular size was due to the inhibition of glycosylation of procollagen molecules by the treatment with \( \alpha, \alpha'- \)dipyridyl.

A band of \( M_r, 70,000 \), observed in the immune complexes only after heat shock, was identified as HSP70. Binding was determined to be nonspecific as this band was also observed in nonimmune control lanes (Fig. 3, lanes 3, 7, and 11). Since the half-life of HSP47 is long (at least 24 h; our unpublished observation and Nagata and Yamada, 1986), the labeling efficiency of HSP47 with \([35S]\)methionine was much less compared to the secretory proteins including collagen and fibronectin, and HSP47 coprecipitated with procollagen by anticolagen antibody was hardly observed unless X-ray film was overexposed (Fig. 3, lanes 9–12). As shown in Figs. 1 and 2, procollagen was observed to be coprecipitated with HSP47 by ati-HSP47 even without cross-linking (Fig. 1, lane 3 and Fig. 2, lane 4). Coprecipitated procollagen bands were, however, not observed in Fig. 3 (lane 5). This apparent discrepancy was due to the differences in the labeling period with \([35S]\)methionine. Since HSP47 was not sufficiently labeled after a pulse label (60 min in Fig. 1 and 20 min in Fig. 2), X-ray film was necessary to be overexposed to detect HSP47 band, and thus procollagen coprecipitated was observed in these experiments. In the experiment shown in Fig. 3, cells were labeled for a longer period (8 h), and the HSP47 was sufficiently labeled. Short exposure period of X-ray film was enough to observe the HSP47 band, which caused the apparent failure in the detection of procollagen coprecipitated without crosslinking (Fig. 3, lane 5).

To confirm the coprecipitation of HSP47 with procollagen, cells were treated with \( \alpha, \alpha'- \)dipyridyl for various periods, crosslinked with DSP, and immunoprecipitated using anticolagen antibody. The immune complexes were then electrophoretically separated, blotted onto nitrocellulose membranes, and the upper half and the lower half of the membrane were separately immunostained using anticolagen...
body, goat anti-rat IgG, used for immunoblot analysis had weak cross-reactivity with rabbit IgG used for immunoprecipitation.

Figure 4. Detection of coprecipitated HSP47 with procollagen after immunoprecipitation by anticollegen antibody. (A) Chick embryo fibroblasts were treated with α,α'-dipyridyl for 0 min (lanes 1 and 3), 30 min (lane 4), 60 min (lane 5), and 90 min (lanes 2 and 6), then crosslinked with DSP and extracted as described in Fig. 2. Each extract was incubated for 2 h at 4°C with rabbit anticollegen antibody (lanes 3–6) or with rabbit nonimmune serum (lanes 1 and 2), and the immune complexes were precipitated with protein A-Sepharose beads. The precipitated materials were applied to an SDS-8% polyacrylamide gel and blotted onto nitrocellulose membrane as described in Materials and Methods. After the membrane was cut at a position around 60 kD which was determined by prestained molecular mass markers, the upper half and the lower half of the membrane were separately incubated with anticollegen and with anti-HSP47 antibodies, respectively. Incubation with the secondary antibodies and the detection of procollagen and HSP47 were performed as described in Materials and Methods. The second antibody, goat anti-rat IgG, used for immunoblot analysis had weak cross-reactivity with rabbit IgG used for immunoprecipitation. Arrow indicates the position of rabbit IgG detected by the crossreaction. (B) After labeled with 0.1 mCi/ml [35S]methionine for 24 h in the medium containing one-fifth of the normal concentration of methionine, cells were chased for 2 h in the medium (chase medium) containing excess methionine. Cells were then treated for 2 h in the chase medium in the presence (lane 2) or absence (lane 1) of 0.3 mM, α,α'-dipyridyl, followed by the pulse label with 0.3 mCi/ml [35S]methionine for 10 min. Cells were crosslinked with DSP, lysed with NP-40, and immunoprecipitated with anticollegen antibody.

Quantification of HSP47-associated Procollagen

The kinetics of newly synthesized procollagen were analyzed by pulse and chase experiments combined with crosslinking and immunoprecipitation using anti-HSP47 and anticollegen antibodies. Cells were labeled for 10 min with [35S]methionine, chased with excess methionine for various periods, and then treated with the crosslinker, DSP. Cell lysates were mixed with sufficient quantities of each antibody, and the resulting immune complexes were incubated with or without endoglycosidase H and analyzed on SDS-PAGE (Fig. 5). HSP47 was sensitive to digestion with endoglycosidase H as shown in Fig. 5 A, in agreement with the previous report that HSP47 resides in the ER (Saga et al., 1987; Hughes et al., 1989). The bands of procollagen precipitated using anticollegen (Fig. 5 B) and procollagen coprecipitated with HSP47 by anti-HSP47 antibody (Fig. 5 A) were both sensitive to digestion with endoglycosidase H, indicating that the dissociation of procollagen from HSP47 necessarily occurs in the pre-Golgi compartments.

Total amounts of α1 procollagen and coprecipitated α1 procollagen were determined by densitometric analysis (Fig. 6). The amounts of total procollagen, as detected by precipitation using anticollegen antibody, decreased after a chase period of 20–25 min, whereas procollagen coprecipitated with HSP47 began to decrease after a chase period of 10–15 min. The differences between two curves showing total α1 procollagen and coprecipitated α1 procollagen were revealed to be statistically significant (P < 0.01 by two-way ANOVA, Tukey’s procedure).

HSP70 family proteins are known to associate with proteins of abnormal conformation (Gething et al., 1986; Dorner et al., 1987; Kassenbrock et al., 1988; Hurtley et al., 1989). Accordingly, the kinetics of procollagen disappearance in α,α'-dipyridyl-treated cells were analyzed to examine the possibility that HSP47 prevents the secretion of procollagen lacking triple helix formation, by binding it continuously. Cells were pulse labeled and chased with or without α,α'-dipyridyl for various periods at 37°C. After crosslinking with DSP, immunoprecipitation was performed with anti-HSP47 and anticollegen antibodies (Fig. 7. A and B). Without α,α'-dipyridyl, most procollagen disappeared within 120 min (Fig. 7 B) and procollagen coprecipitated with HSP47 disappeared within 60 min (Fig. 7 A). In contrast, total procollagen precipitated with anticollegen antibody remained in the cells for up to 120 min of the chase period when the cells were treated with α,α'-dipyridyl (Fig. 7 A). Procollagen in the cells treated with α,α'-dipyridyl was observed to be bound to HSP47 for at least 60 min, and a con-
Figure 5. Pulse and chase analysis of total procollagen and HSP47-bound procollagen. Chick embryo fibroblasts were pulse labeled for 10 min with 0.3 mCi/ml of [35S]methionine and chased for various times (0–40 min as indicated at the top of each lane) in the presence of excess methionine. After crosslinking with DSP, immunoprecipitates using antibodies against HSP47 (A) or type I collagen (B) were treated with or without 55 U/ml of endoglycosidase H (endo H) at 37°C for 12 h, and applied to SDS-8% polyacrylamide gels as shown in Fig. 2.

Figure 6. Kinetics of total cellular procollagen and procollagen co-precipitated with HSP47. Pulse and chase experiment was performed without endoglycosidase H treatment as described in Fig. 5. After the gels were exposed on Fuji HR-H film, the relative density of the bands corresponding to cd(I) procollagen was estimated using a densitometer (model PAN-802; JOOKKO Co., Ltd., Japan). The value of the relative density before chasing was designated as one unit. The mean values and the standard deviations from three independent experiments were plotted. Open circles indicate α(I) procollagen immunoprecipitated with rabbit anti-type I collagen serum, and closed circles indicate α(I) procollagen co-precipitated with HSP47 using rabbit anti-HSP47 IgG.

Dissociation of Procollagen from HSP47 at Low pH

Procollagen must dissociate from HSP47 to be secreted, since the latter protein is retained in the ER (Saga et al., 1986). When the cells were pulse labeled at 37°C and chased at 15°C to examine the effect of temperature, a significant amount of procollagen was observed to be bound to HSP47 for up to 120 min of the chase period, while almost all procollagen was dissociated from HSP47 after 60 min of the chase period at 37°C (Fig. 8 A). The amount of total procollagen, as detected by precipitation using anticollagen antibody, showed the similar kinetics (Fig. 8 B), indicating the inhibition of procollagen secretion at the temperature below 15°C. Chase experiment at 25°C revealed the intermediate profiles between those at 37°C and at 15°C.

Monensin was reported to cause the neutralization of the content in acidic organelles, resulting in inhibition of lysosomal degradation and ligand-receptor dissociation in endosomes (Tartakoff, 1983). When cells were pulse labeled and chased in the presence of 10 μg/ml monensin, dissociation of HSP47 from procollagen was inhibited (data not shown). While this result apparently indicated that the inhibition of the acidification in intracellular compartments caused the
inhibition of the dissociation of procollagen from HSP47, monensin is, on the other hand, reported to cause the inhibition of ER-Golgi transport of the secretory proteins. Accordingly, the effect of low intracellular pHs on the dissociation of procollagen from HSP47 was examined using a proton ionophore CCCP.

Cells were suspended in low pH buffer in the presence or absence of CCCP before crosslinking, and followed to immunoprecipitation with anti-HSP47 antibody (Woods and Lazarides, 1985; Lippincott-Schwartz et al., 1988). In the absence of CCCP, external buffer pH did not affect the coprecipitation of α1 procollagen with HSP47 either at pH 7.5 or at 6.3 (Fig. 9, lanes 2 and 3). In contrast, the α1 band clearly decreased in the immune complex when the pH of the external buffer was lowered to 6.3 in the presence of CCCP (Fig. 9, lane 5). CCCP itself did not affect the binding at pH 7.5 (Fig. 9, lane 4). The identity of the α1 band was confirmed by collagenase treatment of the immune complex (Fig. 9, lane 7). Coprecipitated GRP78 and GRP94, however, failed to disappear even in the presence of CCCP at low pH, indicating that low intracellular pH does not affect the efficacy of immunoprecipitation or the crosslinking of HSP47 with other ER-resident proteins. Thus, the specific dissociation of procollagen from HSP47 is apparently caused by a change in physiological pH.

Discussion

HSP47 was previously found to be a major collagen-binding glycoprotein and HSP47 synthesis was observed to be substantially enhanced at elevated temperatures (Nagata et al., 1986). The expression of HSP47 was detected only in

Figure 7. Effect of α,α'-dipyridyl on the kinetics of total procollagen and procollagen coprecipitated with HSP47. Cells were pulse labeled for 10 min with 0.3 mCi/ml [35S]methionine and chased in the medium containing excess methionine in the presence (indicated on the top of the panel as α,α') or absence (indicated as no) of α,α'-dipyridyl for various periods. Crosslinking and immunoprecipitation experiments were performed as described in Fig. 5. Immune complexes precipitated with anti-HSP47 antibody (A) or anticollagen serum (B) were applied to SDS-8% polyacrylamide gel and the gels were exposed on FUJI HR-H film. The numbers on the top of each lane show the chase periods in min.
Figure 8. Effect of the temperatures on the kinetics of total cellular procollagen and procollagen coprecipitated with HSP47. Pulse labeling, crosslinking, and immunoprecipitation with anti-HSP47 and with anticollagen antibody were performed as described in Fig. 5. After pulse labeling, cells were chased at 37, 24, and 15°C for various periods. Immune complexes precipitated with anti-HSP47 antibody (A) were applied to SDS-8% polyacrylamide gel and the gels were exposed on FUJI HR-H film. Immune complexes precipitated with anticollagen antibody (B) were treated with or without endoglycosidase H (indicated as endo H) as described in Fig. 5 before the application to SDS-PAGE.

collagen-secreting cells. HSP47, for example, was detected immunohistochemically in the fibrocytes and smooth muscle cells in chick liver, but not in the hepatocyte (Saga et al., 1987). Among established cell lines, the fibroblast cell line (BALB/3T3) synthesizes HSP47, but a neuronal cell line (PC12) and a myeloid leukemic cell line (M1) do not (Satoh, M., A. Nakai, and K. Nagata, unpublished observation). This relationship led us to speculate that HSP47 was associated with the metabolism or processing of procollagen, and that the localization of HSP47 in the ER (Saga et al., 1987; Hughes et al., 1987) was related to an association with ER procollagen.

Subsequently, HSP47 was found to bind procollagen as effectively as it binds collagen and gelatin (Fig. 1 and see Nagata and Yamada, 1986). Concern that the binding of procollagen to HSP47 might be an artifact of homogenization prompted the initiation of chemical crosslinking studies before cell lysis. The procedure involves the use of lysis buffer containing excess gelatin as a competitor to avoid artificial binding of HSP47 to procollagen after the cells had been lysed. After crosslinking, procollagen bands were coprecipitated by immunoprecipitation of HSP47 even in the presence of excess gelatin (Fig. 2). These observations suggest that HSP47 is bound to procollagen within the cell or more probably in the ER. The amount of procollagen coprecipitated with HSP47 was determined to decrease earlier than the amount of total intracellular procollagen precipitated with anticollagen antibody (Fig. 6). Procollagen bands coprecipitated with anti-HSP47 antibody were sensitive to endoglycosidase H treatment, confirming that dissociation must occur before procollagen enters the medial-Golgi compartment. Gavin et al. (Gavin, T. G., A. Veis, K. Nagata, M. M. Somerman, and J. J. Sauk, manuscript submitted for publication) recently found that HSP47 binds to the α(I)-specific portion in the N-propeptide of procollagen as well as the Gly-X-Y portion of the triple helix (manuscript submitted for publication). This may explain preferential coprecipitation of the pro α(I) chain after immunoprecipitation with anti-HSP47 antibody (Figs. 1 and 2). All these results suggest that HSP47 may bind to N-propeptide immediately after the procollagen is transported into the ER, then bind to the triple helix region, and finally dissociate from it before the transport of procollagen into Golgi apparatus.

We previously reported that HSP47 could be dissociated from gelatin-Sepharose at low pH (pH < 6.3) (Saga et al., 1987), and that without crosslinking, procollagen coprecipitated with HSP47 decreased in pH 6.0 buffer (Nakai et al., 1990). In this study, we confirmed that procollagen coprecipitated with HSP47 was dissociated from HSP47 after washing the immune complex with low pH buffer (Fig. 1), and that procollagen was not crosslinked in vivo at low intracellular pH (pH ~ 6.3) which was achieved by treating the cells with CCCP (Fig. 9). Although GRP78 binds to un-
Dissociation of procollagen from HSP47 within the cells at low pH. Cells were labeled for 20 min with 0.3 mCi/ml [3S]methionine, trypsinized, and then treated with collagenase. Cells were suspended in KI-I2POJNaOH buffers at pH 7.5 (lanes 1, 2, and 4) or pH ~3 (lanes 3 and 5) in the presence (lanes 4 and 5) or absence (lanes 1-3) of CCCP at a final concentration of 5 x 10^-6 M. The cells were incubated for 30 min on ice, and then incubated with DSP for an additional 30 min on ice. Immunoprecipitation was performed as described previously using anti-HSP47 IgG, and the immune complexes were applied to an 8% polyacrylamide gel containing SDS. An immune complex was treated with 20 U/ml collagenase for 30 min at 37°C before the application to SDS-PAGE (lane 1).

folded or misfolded proteins and releases them in the presence of ATP (Gething et al., 1986; Munro and Pelham, 1986; Dorner et al., 1987; Kassenbrock et al., 1988; Flynn et al., 1989), HSP47 continues to bind to procollagen even in the presence of ATP. Furthermore, HSP47 binds mature collagen as effectively as it binds gelatin (denatured type I and III collagen) (Nagata and Yamada, 1986). We therefore speculate that the dissociation mechanism may involve a lowering of pH (acidification). The interior of endocytic vesicles, lysosomes, portion of trans-Golgi apparatus, and certain secretory vesicles is known to be acidic. This acidification is attributed to the function of the vesicular proton ATPase (for review see Anderson and Orci, 1988). Although the biological significances of low vesicular pH is not entirely understood, vesicular compartments have reportedly been involved in receptor recycling during receptor-mediated endocytosis (Gruenberg and Howell, 1989), degradation of proteins in the lysosomes (Bainton, 1981), and proteolytic processing of prohormones such as proinsulin (Orci et al., 1986). The interaction of the murine T cell antigen receptor (TCR) with an ER resident protein (TRAP) was also reported to be disrupted at acidic pHs (Bonifacino et al., 1988). The dissociation of TRAP from TCR is blocked by manipulations that inhibit ER to Golgi transport or with agents that inhibit organelle acidification. The dissociation kinetics of TRAP are similar to those of HSP47. However, the intracellular pH required for the dissociation of TRAP is more acidic than that required for HSP47 dissociation. Acidification in the compartments may, therefore, regulate the dissociation of procollagen from HSP47, resulting in the secretion of procollagen out of the ER.

While no low pH compartment has been found in the secretory pathway between the ER and the medial-Golgi apparatus, the presence of intermediate compartments between the ER and the Golgi apparatus has been recently established (Lippincott-Schwartz et al., 1990). These compartments, termed “salvage compartments,” are assumed to move between the ER and the Golgi apparatus, acting as carrier vesicles for secretory proteins. Furthermore, the receptor for the KDEL (Lys-Asp-Glu-Leu) signal (Munro and Pelham, 1987), by which several ER-resident proteins are retained in the ER, has been identified and determined to be localized in the compartments between the ER and the Golgi stack (Vaux et al., 1990; Lewis and Pelham, 1990). Recently, we cloned a cDNA encoding chick HSP47 (Hirayoshi et al., 1991) and mouse HSP47 (Takechi et al., 1992). Chick and mouse HSP47 contains a signal sequence at the NH2 terminus and a RDEL (Arg-Asp-Glu-Leu) sequence, ER-retention signal (Andres et al., 1990), by which several ER-resident proteins are retained in the ER (Saga et al., 1987). There are at least three stress proteins localized in the ER: GRP78, GRP94, and HSP47. GRP78, a member of the HSP70 family, is found to associate with many unfolded or misfolded proteins as well as with immunoglobulin heavy chains (Bole et al., 1986; Gething et al., 1986; Dorner et al., 1987; Kassenbrock et al., 1988; Flynn et al., 1989), HSP47 continues to bind to procollagen even in the presence of ATP. Furthermore, HSP47 binds mature collagen as effectively as it binds gelatin (denatured type I and III collagen) (Nagata and Yamada, 1986). We therefore speculate that the dissociation mechanism may involve a lowering of pH (acidification). The interior of endocytic vesicles, lysosomes, portion of trans-Golgi apparatus, and certain secretory vesicles is known to be acidic. This acidification is attributed to the function of the vesicular proton ATPase (for review see Anderson and Orci, 1988). Although the biological significances of low vesicular pH is not entirely understood, vesicular compartments have reportedly been involved in receptor recycling during receptor-mediated endocytosis (Gruenberg and Howell, 1989), degradation of proteins in the lysosomes (Bainton, 1981), and proteolytic processing of prohormones such as proinsulin (Orci et al., 1986). The interaction of the murine T cell antigen receptor (TCR) with an ER resident protein (TRAP) was also reported to be disrupted at acidic pHs (Bonifacino et al., 1988). The dissociation of TRAP from TCR is blocked by manipulations that inhibit ER to Golgi transport or with agents that inhibit organelle acidification. The dissociation kinetics of TRAP are similar to those of HSP47. However, the intracellular pH required for the dissociation of TRAP is more acidic than that required for HSP47 dissociation. Acidification in the compartments may, therefore, regulate the dissociation of procollagen from HSP47, resulting in the secretion of procollagen out of the ER.

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suggests that these proteins, including HSP47, may form complexes or close associations, and that secretory proteins may be modified by a series of coupled or adjacent reactions. Fibronectin was similarly shown to bind to HSP47. Although HSP47 did not bind to plasma fibronectin in vitro (Nakai et al., 1989), an association with the cellular form of fibronectin appears possible. Indirect binding of fibronectin to HSP47 via procollagen was also conceivable since fibronectin has a collagen-binding domain. However, we cannot exclude the possibility that HSP47 can bind to other minor cellular and secretory proteins in the ER, which was not detected in our present experimental conditions.

When cells are treated with α,α′-dipryridyl, an iron chelating reagent, prolyl and lysyl hydroxylation is blocked, and procollagen peptides cannot form a stable triple-helix (Jimenez et al., 1973; Berg and Prockop, 1973). The secretion of the non-helical procollagen chains is inhibited and they accumulate in the ER (Jimenez and Yankowsky, 1978). Under conditions preventing stable triple helix formation, procollagen crosslinking to HSP47 was enhanced (Figs. 3 and 7). Accordingly, HSP47 may function to prevent the secretion of immature or malformed procollagen and may facilitate triple-helix formation in procollagen.

Although the folding of subunits in many oligomeric proteins can occur independently, the maturation of procollagen is known to be complex and well regulated. The formation of this hetero-trimer would require a control or surveillance mechanism to regulate correct oligomerization of the individual chains. These specific requirements for procollagen maturation may in turn necessitate a specific chaperone protein such as HSP47. Recently, a novel 88-kD protein and TRAP have been reported to be involved in the biogenesis of murine class I histocompatibility molecules and of murine T cell receptor/CD3 complex, respectively (Degen and Williams, 1991; Bonifacino et al., 1988). These proteins including HSP47 are thought to be "specific chaperones" in terms of its binding specificity, whereas other well-known stress proteins like HSPT0 and GroEL family proteins are to be considered "general chaperones" which bind to many proteins in cytoplasm and in intracellular organelles. It would be necessary for establishing the concept of specific chaperone to accumulate increasing numbers of examples of such proteins and to analyze their functions in detail.

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