

# Intracellular Interaction of Collagen-specific Stress Protein HSP47 with Newly Synthesized Procollagen

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**Abstract.** Heat shock protein 47 (HSP47), a collagen-specific stress protein, has been postulated to be a collagen-specific molecular chaperone localized in the ER. We previously demonstrated that HSP47 transiently associated with newly synthesized procollagen in the ER (Nakai, A., M. Satoh, K. Hirayoshi, and K. Nagata. 1992. *J. Cell Biol.* 117:903–914). In the present work, we examined the location where HSP47 binds to and dissociates from newly synthesized procollagen within the cells, and whether HSP47 associates with nascent single procollagen polypeptide chains and/or with mature triple-helix procollagen. This was accomplished by biochemical coprecipitation with anti-HSP47 and anticollagen antibodies, combined with pulse-label and chase experiments in the presence or absence of various inhibitors for protein secretion, as well as by confocal laser microscopic observation of the cells double stained with both antibodies. We further examined whether the RDEL (Arg-Asp-Glu-Leu) sequence at the COOH terminus of HSP47 can act as an ER-retention signal, as the KDEL sequence does.

When the secretion of procollagen was inhibited by the presence of  $\alpha$ ,  $\alpha'$ -dipyridyl, an iron chelator that in-

hibits procollagen triple-helix formation, or by the presence of brefeldin A, which inhibits protein transport between the ER and the Golgi apparatus, procollagen was found to be bound to HSP47 during the chase period in the intermediate compartment. In contrast, the dissociation of procollagen chains from HSP47 was not inhibited when procollagen secretion was inhibited by monensin or bafilomycin A1, both of which are known to be inhibitors of post-*cis*-Golgi transport. These findings suggest that HSP47 and procollagen dissociated between the post-ER and the *cis*-Golgi compartments. HSP47 was shown to bind to nascent, single-polypeptide chains of newly synthesized procollagen, as well as to the mature triple-helix form of procollagen. HSP47 with the RDEL sequence deleted was secreted out of the cells, which suggests that the RDEL sequence actually acts as an ER-retention signal, as the KDEL sequence does. This secreted HSP47 did not acquire endoglycosidase H resistance. The biological significance of the interaction between HSP47 and procollagen in the central secretory pathway, as well as possible mechanisms for this pathway, will be discussed.

**N**EWLY synthesized membrane and secretory proteins are translocated across the ER membrane and are then transported to the Golgi apparatus for subsequent distribution to the cell surface, lysosomes, and secretory vesicles. Many of these proteins are not exported from the ER until they are folded and assembled correctly (Hurtley and Helenius, 1989). The ER contains several ER-resident proteins that are termed “molecular chaperones” or “folding enzymes.” These molecular chaperones are involved in the processing and maturation of secretory and membrane proteins; they are thought to associate with folding intermediates or misfolded proteins to

prevent nonproductive side reactions such as irreversible aggregation, and they may accelerate the slower steps in the folding process (for reviews see Hurtley and Helenius, 1989; De Silva et al., 1990, 1993; Gething and Sambrook, 1992). Of these proteins, certain stress proteins such as glucose-regulated protein 78 (GRP78<sup>1</sup> or Ig heavy chain-binding protein [BiP]) and GRP94 are thought to facilitate translocation into the ER and then to assist in folding, oligomeric assembly and sorting in the ER (Kozutsumi et al., 1988; Melnick et al., 1992). The folding enzymes, which in-

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1. *Abbreviations used in this paper:* ARF, ADP ribosylation factor; BiP, Ig heavy chain binding protein; CEF, chick embryo fibroblast; DSP, dithiobis (succinimidylpropionate); endo H, endoglycosidase H; GRP, glucose-regulated protein; HSP, heat shock protein; KDEL, Lys-Asp-Glu-Leu sequence; PDI, protein disulfide isomerase; RDEL, Arg-Asp-Glu-Leu sequence.

clude prolyl *cis-trans* isomerase and protein disulfide isomerase (PDI), are involved in the posttranslational modification of proteins such as procollagen (Chessler and Byers, 1993; Chessler et al., 1993). The sequential formation of disulfide bonds in the ER is required for normal folding and for subsequent transport to the Golgi apparatus and beyond (Koivu and Myllyla, 1987; Hurlley and Helenius, 1989). These soluble ER-resident proteins have the sequence Lys-Asp-Glu-Leu (KDEL) at their carboxy terminus, which acts as an ER-retention signal (Munro and Pelham, 1987). Similarly, some ER-resident type I transmembrane proteins contain an ER-targeting motif consisting of two lysine residues at the carboxy terminus (Nilsson et al., 1989; Jackson et al., 1993).

Heat shock protein 47 (HSP47) is a 47-kD heat-inducible stress protein found in collagen-producing cells, where it functions as a collagen-binding glycoprotein (Nagata et al., 1986, 1988; Saga et al., 1987; Nakai et al., 1992). HSP47 has an Arg-Asp-Glu-Leu sequence (RDEL) at the COOH terminus, and it has been observed to localize to the ER (Saga et al., 1987; Nakai et al., 1990; Hirayoshi et al., 1991; Takechi et al., 1992). We previously demonstrated that HSP47 was coprecipitated with newly synthesized procollagen chain(s) using a chemical cross-linker, and that HSP47 was stably associated with those procollagen chain(s) when the cells were treated with  $\alpha$ ,  $\alpha'$ -dipyridyl, an iron-chelating agent that blocks prolyl and lysyl hydroxylation, thus preventing the stable triple-helix formation of procollagen (Nakai et al., 1992). HSP47 was therefore postulated to be a molecular chaperone-like protein resident in the ER.

In addition to this functional relationship between HSP47 and procollagen, the expression of HSP47 is always closely correlated with the expression of various types of collagen. The synthesis of both HSP47 and type I collagen are decreased after the malignant transformation of fibroblasts (Nagata et al., 1988; Nakai et al., 1990). Conversely, the synthesis of both HSP47 and type IV collagen are markedly increased during the differentiation of the mouse teratocarcinoma cell line F9 (Kurkinen et al., 1984; Takechi et al., 1992). We recently reported that both HSP47 and type I and III collagens are clearly induced during the progression of rat liver fibrosis caused by the administration of carbon tetrachloride (Masuda et al., 1994). These observations suggest that HSP47 might be important in the synthesis, processing, and secretion of collagen in various types of cells. Recent studies have shown that the receptors for the KDEL motif are localized to the Golgi membrane, and they mediate intracellular transport from the Golgi apparatus to the ER (Semenza et al., 1990; Lewis and Pelham, 1990, 1992; Tang et al., 1993). Although there are many reports dealing with the function of ER-resident chaperone proteins, and the sequential interaction of these chaperone proteins with unfolded substrate proteins in the ER was recently reported (Hammond and Helenius, 1994a; Melnick et al., 1994), to our knowledge, there are few reports that clearly show where these chaperones associate with and dissociate from their substrates. In this report, we have analyzed the specific location where HSP47 associates with and dissociates from the newly synthesized procollagen chains, by coprecipitation and pulse-label/chase experiments using a cross-linker, as well as by confocal microscopic observation.

## Materials and Methods

### Chemicals and Reagents

The chemical cross-linker dithiobis (succinimidylpropionate) (DSP) was purchased from Pierce Chemical Co. (Rockford, IL). Chromatographically purified bacterial collagenase (form III) and recombinant endoglycosidase H (endo H) were obtained from Advance Biofactures Co. (Lynbrook, NY) and Boehringer Mannheim GmbH (Mannheim, Germany), respectively. Monensin sodium salt, brefeldin A, L-ascorbic acid phosphate magnesium salt, and bafilomycin A1 were purchased from Wako Junyaku (Osaka, Japan).  $\alpha$ ,  $\alpha'$ -dipyridyl was obtained from Nacalai Tesque (Kyoto, Japan). Rabbit antiserum raised against rat type I collagen was obtained from LSL Co. Ltd. (Tokyo, Japan). Rabbit polyclonal and rat monoclonal (11D10) IgGs against chick HSP47 had been made previously (Saga et al., 1987; Nakai et al., 1992), and were used after affinity purification on chick HSP47-coupled Sepharose 4B column. Rabbit serum raised against the 15 amino acid residues at the NH<sub>2</sub> terminus of mouse HSP47 was also made, which specifically recognized mouse but not chick HSP47. EXPRE<sup>35S</sup> and L-[2,3-<sup>3</sup>H]proline were purchased from New England Nuclear (Boston, MA). The anti-Golgi 58K protein mAb (Bloom and Brachear, 1989; Donaldson et al., 1990; Ktistakis et al., 1991) for staining the Golgi apparatus and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal IgG against PDI and mouse monoclonal IgG against  $\alpha$ -mannosidase II were kind gifts from Dr. T. Yoshimori (Kansai Medical University, Osaka, Japan) and Dr. G. Warren (Imperial Cancer Research Fund, London, U.K.), respectively. All other reagents were purchased from Wako Junyaku and Nacalai Tesque.

### Cell Culture and Metabolic Labeling

Chick embryo fibroblasts (CEFs) were maintained in Vogt's GM medium, and cell populations were passed with a 0.25% trypsin-1 mM EDTA solution. The experiments were conducted between passages three and eight. Briefly  $6 \times 10^5$  cells were plated onto a 35-mm plastic dish, and were incubated at 37°C for 16–24 h in 5% CO<sub>2</sub>. The subconfluent cultures were then rinsed with methionine-free medium or proline-free medium, preincubated in the same medium for 30 min, then labeled with 0.2 mCi/ml of [<sup>35</sup>S]methionine or 0.1 mCi/ml of L-[2, 3-<sup>3</sup>H]proline in the presence of 2–2.5% dialyzed FCS for the indicated periods.

### Cross-linking and Immunoprecipitation

Detailed protocols have been previously described (Nakai et al., 1992). Briefly, cultured CEFs were rinsed with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS and incubated with 200  $\mu$ l of trypsin (0.025%)–EDTA (1 mM) at 37°C for 3 min. Then 800  $\mu$ l of ice-cold medium containing 10% FCS to block trypsin activity was added to the trypsin-EDTA. After rinsing, the cell suspension was incubated with 2 mM DSP (stored at a concentration of 0.1 M in DMSO) on ice for 30 min, rinsed with 2 mM glycine in PBS to block DSP activity, rinsed with PBS again, and then lysed with a 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  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml pepstatin) containing 0.5 mg/ml gelatin on ice for 15 min. The excess gelatin served to prevent the binding of procollagen to HSP47 in the cell extract after cell lysis by competing with the endogenous procollagen. After centrifugation at 12,000 g for 15 min, the supernatant was divided into two equal aliquots. One aliquot was immunoprecipitated with the rabbit polyclonal IgG against HSP47, and the other half was immunoprecipitated with the rabbit antiserum raised against rat type I collagen, as previously described. The samples were resuspended in Laemmli's sample buffer containing DTT, boiled for 5 min, and analyzed by SDS-PAGE as previously described (Nakai et al., 1990, 1992). The gels were dried and exposed on Fuji HR-H film (Fuji Photo Film Co., Ltd., Tokyo, Japan) after treatment with 1 M sodium salicylate.

### Confocal Scanning Laser Microscopy

CEFs were cultured on coverslips for ~12 h in the presence or absence of 5  $\mu$ g/ml ascorbate. The cells were then treated with or without several inhibitors for the secretory pathway, and then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. After permeabilization with either 0.2% Triton X-100 in PBS or with 0.1% Triton X-100 plus 0.05% SDS in PBS for 4 min, the coverslips were rinsed and then blocked with 10% nonimmune goat serum and 2.5% skim milk (Yukijilushi Milk Co.,

Sapporo, Japan) in PBS overnight at 4°C. The cells were then incubated with a mixture of the primary antibodies: (30 µg/ml rat anti-HSP47 monoclonal IgG (11D10) and 1:200 diluted rabbit antiserum raised against rat type I collagen (LSL Co. Ltd.)). The primary incubation was carried out in PBS containing 10% nonimmune goat serum and 2.5% skim milk. Goat anti-rat IgG coupled to FITC and rhodamine-conjugated goat anti-rabbit IgG were used as the secondary antibodies at a dilution of 1:200. The coverslips were mounted in a drop of Slow Fade™ (Molecular Probes, Inc., Eugene, OR) to reduce photobleaching. All the specimens were observed using a laser scanning confocal apparatus (MRC-600; Bio-Rad Microscience Division, Herts, U.K.), and photographed with Fujichrome 100D film and Kodak Ektachrome 100 Plus film (Eastman Kodak Co., Rochester, NY).

### **Trypsin and Collagenase Treatments**

The immunocomplexes precipitated with protein A-agarose (20 µl bed vol) were incubated with 20 µg/ml trypsin at 37°C for 4 min or with 33 µl of collagenase buffer (20 U/ml collagenase, 50 mM Tris-HCl, pH 7.5, 2 mM PMSF, 10 mM CaCl<sub>2</sub>) at 37°C for 30 min. These samples were then boiled for 5 min in Laemmli's sample buffer and DTT, and then applied to an SDS-polyacrylamide gel for electrophoresis.

### **Construction of an Expression Vector and Transfection**

The truncated HSP47 lacking the RDEL sequence at the COOH terminus was constructed using a mouse HSP47 cDNA (MH47) by PCR (Saiki et al., 1988) using oligonucleotides A (5'-CCGGATCCATGGTGGACAA-CCGTGGCTTC-3') and B (5'-CCGGATCCCTACATCTGTCTCCCT-TGGG-3') as primers. Primer B contains a complementary sequence for the mouse HSP47 cDNA (Takechi et al., 1992) between positions 1478 and 1495, and a stop codon, followed by a BamHI site at the 5' end. The product (0.6 kb) from the PCR was cut with BamHI and subcloned into a pUC19 vector predigested with the same enzymes. This product was confirmed by sequencing, and then subcloned as a XhoI-EcoRI fragment into a pMH47a construct (Takechi et al., 1992) predigested with XhoI and EcoRI. The RDEL-deleted MH47 was digested with EcoRI and HindIII, added to a BglII linker, and ligated into the BglII site of the expression vector pcDEBA (Nakabeppu et al., 1993). This expression vector was designated as MH47ΔRDEL and was transiently transfected into CEFs by the calcium phosphate method. The secretion of this mutant HSP47 was examined in an *in vitro* assay.

## **Results**

### **Inhibition of Procollagen Secretion in the ER**

CEFs cultured in the presence or absence of 5 µg/ml ascorbate phosphate ester for 12 h were labeled with [<sup>35</sup>S]methionine for 10 min and then cross-linked with DSP for 30 min. The detergent-soluble proteins were immunoprecipitated with anti-HSP47 and anticollagen antibodies, as described in Materials and Methods. The secretion of procollagen into the culture medium was delayed without the addition of a bovine embryo extract containing ascorbate, a cofactor for prolyl-4-hydroxylase, prolyl-3-hydroxylase, and lysyl hydroxylase (Fig. 1 A, lanes 9–12). The addition of ascorbate phosphate ester, a longer-lived derivative of ascorbate, induced the active secretion of procollagen, which was demonstrated by the disappearance of the procollagen band after 20–40 min of the chase period (Fig. 1 A, lanes 13–16). The molecular sizes of procollagen bands without ascorbate were slightly different from those with ascorbate because of the hydroxylation of proline and lysine residues and the subsequent O-glycosylation (Fig. 1 A, lane 9 vs lane 13). In the presence of ascorbate, the procollagen that was coprecipitated with HSP47 using the anti-HSP47 antibody disappeared more rapidly than that in the absence of ascorbate (Fig. 1 A, lanes 5–8 vs lanes 1–4).

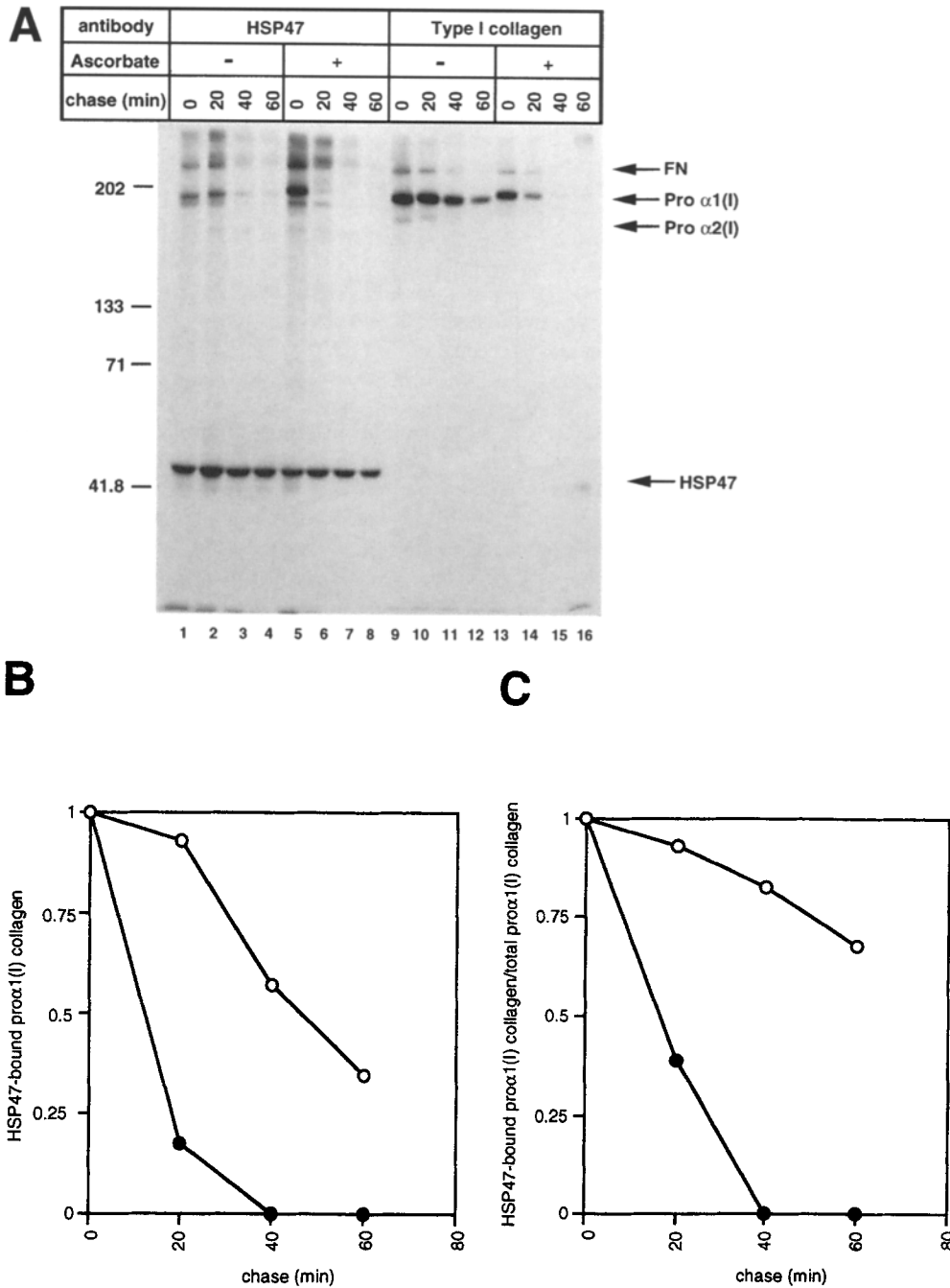
Quantitative examination more clearly showed the prolonged binding of HSP47 to underhydroxylated procollagen during the chase periods (Fig. 1, B and C). Procollagen α1(I) chain bound to HSP47 decreased much slower in the absence of ascorbate than in the presence of ascorbate (Fig. 1 B). The HSP47-bound α1(I) chain/total α1(I) chain of procollagen decreased rapidly in the presence of ascorbate, whereas it decreased much slower in the absence of ascorbate (Fig. 1 C). Fig. 1 C suggests that the prolonged binding of procollagen to HSP47 in the absence of ascorbate is not merely caused by the large accumulation of procollagen in the ER. These results indicate that the underhydroxylated procollagen remained bound to HSP47 for a much longer period than normal procollagen.

When the cell lysates were immunoprecipitated with the anticollagen antibody, HSP47 was not detected in the immunocomplex (Fig. 1, lanes 9–16). This was partly caused by the poor labeling efficiency of HSP47 after pulse labeling for a short period because the half life of HSP47 is very long (>48 h; Nakai et al., 1992). Previously, we clearly showed that the immunocomplexes precipitated with the anticollagen antibody contained detectable levels of HSP47 by labeling the cells with [<sup>35</sup>S]methionine for longer periods or by Western blot analysis using anti-HSP47 antibody (Nakai et al., 1992). Nevertheless, it should be noted that only a small fraction of newly labeled HSP47 bound to procollagen in the ER (Fig. 1, lanes 9–16), as reported for BiP (Hurtley et al., 1989). Colocalization of procollagen with HSP47 was confirmed by confocal microscopic observation after the CEFs were double stained with anti-HSP47 and anticollagen antibodies, and visualized with FITC-conjugated and rhodamine-conjugated secondary antibodies, respectively. Since almost all of the procollagen was secreted out of the cells in the presence of ascorbate, only HSP47 was immunostained within the cells (Fig. 2, D–F). In the absence of ascorbate, however, a strong yellow staining was observed on the confocal micrograph, indicating the colocalization of HSP47 and procollagen. This yellow staining, consisting of HSP47 plus procollagen, coincided with the HSP47 staining pattern, which indicated that the HSP47 and procollagen complexes had accumulated in the ER.

In the absence of ascorbate, some portion of the procollagen was localized to a site distinct from the ER, possibly in the Golgi (Fig. 2, A–C, also see Fig. 7). In a previous paper, we showed that procollagen was inhibited from being secreted and remained bound to HSP47 for a much longer period when the CEFs were treated with α, α'-dipyridyl, an iron-chelating agent that blocks prolyl and lysyl hydroxylation (Nakai et al., 1992). This inhibition was also shown to occur in the ER by confocal microscopic observation (Fig. 2, G–I). These results indicated that procollagen was not dissociated from HSP47 when the secretion of procollagen was inhibited in the ER, either by ascorbate deprivation or by treatment with α, α'-dipyridyl. Based on these results, we performed the following experiments in the presence of ascorbate phosphate ester to examine the secretion of procollagen under more normal conditions.

### **Treatment of CEFs with Brefeldin A**

Brefeldin A is known to release coat proteins and ADP ri-

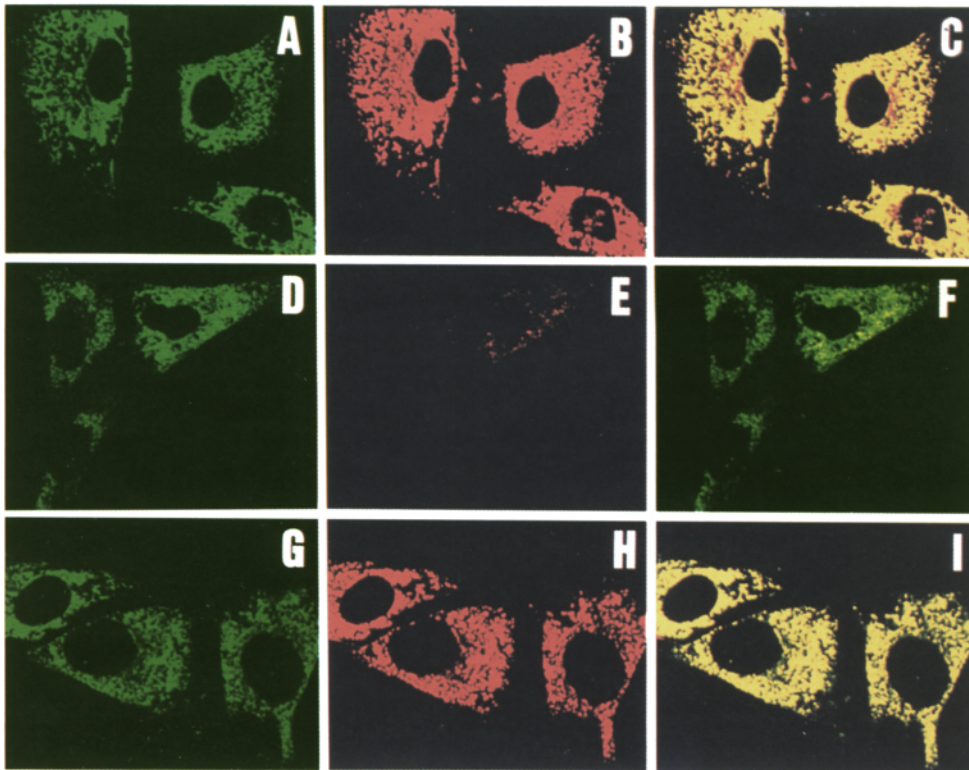


**Figure 1.** Effects of ascorbate on the secretion of procollagen and the interaction of procollagen with HSP47. CEFs were preincubated in the presence or absence of 5  $\mu\text{g/ml}$  ascorbate phosphate for 12 h, pulse labeled with 0.2 mCi/ml [ $^{35}\text{S}$ ]methionine for 10 min, and then chased in medium containing excess cold methionine for the indicated time periods. The cells were trypsinized, incubated with the cross-linking reagent, and treated with the lysis buffer. Immunoprecipitation was performed as described in Materials and Methods. (A) The immunocomplexes precipitated with anti-HSP47 antibody (lanes 1–8) or anti-type I collagen antiserum (lanes 9–16) were applied to an SDS–8% polyacrylamide gel, electrophoresed, and exposed on Fuji HR-H film. The number on top of each lane indicates the duration of the chase period in minutes. (B) Kinetics of procollagen coprecipitated with HSP47 in the presence or absence of ascorbate phosphate. (C) Kinetics of the HSP47-bound procollagen/total intracellular procollagen ratio. After the gels were exposed on Fuji HR-H film, the relative density of the bands corresponding to  $\alpha 1(\text{I})$  procollagen was estimated using a one-dimensional image analyzer (The Discovery Series, Quantity One; PDI, Huntington, NY). The value of the relative density before chase was designated as arbitrary one unit. *Open circles*,  $\alpha 1(\text{I})$  procollagen in the absence of ascorbate phosphate; *closed circles*,  $\alpha 1(\text{I})$  procollagen in the presence of ascorbate phosphate.

bosylation factor (ARF) from the Golgi stack, and thus inhibits the transport of secretory proteins from the ER to the Golgi (Lippincott-Schwartz et al., 1989, 1990; Klausner et al., 1992; Duden et al., 1994). CEFs were pretreated with or without 2  $\mu\text{g/ml}$  brefeldin A for 30 min, pulse-labeled with [ $^{35}\text{S}$ ]methionine for 10 min, and then chased for the indicated periods in the presence of excess cold methionine. The cells were then treated with a cross-linker, and the cell extract was immunoprecipitated with anti-HSP47 and anticollagen antibodies as described in Materials and Methods. Although almost all of the pulse-

labeled procollagen had been secreted from the cells after 40 min in the absence of brefeldin A (Fig. 3 A, lanes 9–12), the secretion of procollagen was completely inhibited by the presence of brefeldin A (lanes 13–16). The amount of procollagen and collagen chains secreted into the culture medium was examined directly by SDS-PAGE, and these results also showed the complete inhibition of all protein secretion into the medium (Fig. 3 B).

Immunoprecipitation analysis using the anti-HSP47 antibody after cross-linking revealed that the procollagen, which could not be secreted in the presence of brefeldin A,



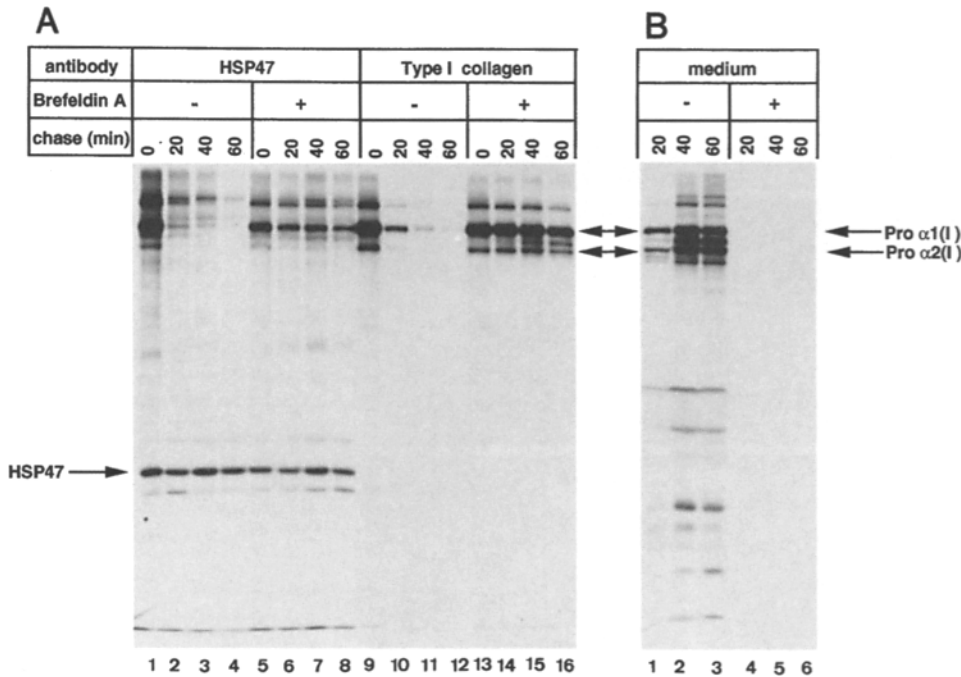
**Figure 2.** Localization of HSP47 and procollagen in cells treated with ascorbate and  $\alpha, \alpha'$ -dipyridyl. CEFs were preincubated in the presence (D–I) or absence (A–C) of 5  $\mu\text{g/ml}$  ascorbate phosphate for 12 h, and then treated with 0.3 mM  $\alpha, \alpha'$ -dipyridyl for 2 h (G–I). The cells were fixed, permeabilized, and double stained with the rat anti-HSP47 mAb (11D10) and the rabbit anti-type I collagen antiserum. Visualization was performed with FITC-conjugated goat anti-rat IgG for HSP47, and with rhodamine-conjugated goat anti-rabbit IgG for procollagen. Images were obtained with a scanning laser confocal microscope separately for HSP47 (A, D, and G) or type I procollagen (B, E, and H), and by overlapping them (C, F, and I). In the double staining with both FITC and rhodamine images, the yellow color indicates the overlap of the two fluorescent antibodies (C, F, and I).

remained bound to HSP47 for up to 60 min, whereas most of the procollagen had dissociated from HSP47 after 20 min under normal conditions (Fig. 3 A, lanes 1–4 and 5–9). Cells were double-stained with anti-HSP47 and anticollagen antibodies and observed by confocal microscopy. Anticollagen antibody stained many small vesicles after treatment with brefeldin A (Fig. 4 B). When green (HSP47) and red (collagen) staining were superimposed, it was clearly shown that HSP47 and procollagen colocalized in these small vesicles (Fig. 4 C). Anti-HSP47 antibody also strongly stained the ER structure (Fig. 4 A), indicating that most of the HSP47 remained in the ER, even after treatment with brefeldin A. This is consistent with the observation that only a small fraction of HSP47 bound to procollagen in the ER, as mentioned above (Fig. 1). Staining with anti-PDI antibody indicates that the appearance of small vesicles by treatment with brefeldin A did not result from the destruction of the ER structure because this treatment did not affect the reticular meshwork structure of the ER (Fig. 5 B). Besides the ER staining pattern, staining with anti-PDI antibody also showed the colocalization of PDI with HSP47 in the small vesicles (yellow spots). To examine whether these small vesicles are derived from the dispersion of the Golgi stack, we stained the cells with anti-p58 antibody to stain the Golgi structure. This anti-p58 antibody recognizes the Golgi protein known as “58K protein,” which binds directly to microtubules and is postulated to be involved in anchoring the Golgi apparatus to microtubules *in vivo* (Bloom and Brashers, 1989; Ktistakis et al., 1991; Saraste and Svensson, 1991). It should be noted this p58 protein in the Golgi is

not the same one as an intermediate compartment marker protein, hence the name “p58.” Anti-p58 antibody clearly stained the Golgi structure in CEFs in the presence of ascorbate (Fig. 5 C). However, no staining was observed with this antibody in the cells treated with brefeldin A (Fig. 5 D). Although it is noteworthy that the loss of p58 staining in brefeldin A-treated cells possibly resulted from the membrane uncoating, these results seem to be consistent with the previous report that brefeldin A treatment causes a disappearance of the Golgi structure (Lippincott-Schwartz et al., 1989; Ktistakis et al., 1991). These observations suggest that these small vesicles observed after treatment with brefeldin A are distinct from the ER and the Golgi.

When brefeldin A was removed from the medium, these small vesicles disappeared after 60 min, and the anti-HSP47 antibody again revealed an ER staining pattern, whereas the staining with the anticollagen antibody was located in the Golgi and then disappeared (data not shown). When the cells were treated with  $\alpha, \alpha'$ -dipyridyl and then with brefeldin A, the staining patterns of the cells stained with anti-HSP47 and anticollagen antibodies were indistinguishable from cells treated with  $\alpha, \alpha'$ -dipyridyl alone, thus showing the ER pattern (Fig. 5 A). This result indicated that these small vesicles did not appear when the secretion of procollagen from the ER was inhibited by  $\alpha, \alpha'$ -dipyridyl treatment, and that the appearance of these vesicles was not due to an artifact caused by brefeldin A treatment. These results also imply that the small vesicles might represent an intermediate compartment between the ER and Golgi.





**Figure 3.** Effects of brefeldin A on the interaction of procollagen with HSP47. The cells were cultured in medium containing 5  $\mu\text{g/ml}$  ascorbate phosphate for 12 h, and were then preincubated in the presence or absence of 2  $\mu\text{g/ml}$  brefeldin A for 30 min. The cells were pulse-labeled with 0.2 mCi/ml [ $^{35}\text{S}$ ]methionine for 10 min, and were chased in medium containing excess cold methionine. Cross-linking and immunoprecipitation were performed as described in Materials and Methods. (A) Immunocomplexes precipitated with the anti-HSP47 antibody (lanes 1–8) or the anti-type I collagen antibody (lanes 9–16) were applied to a SDS–8% polyacrylamide gel, electrophoresed, and exposed on Fuji HR-H film. (B) Culture medium was collected and then analyzed by SDS-PAGE after various time periods of chase. The number on top of each lane indicates the duration of the chase period in minutes.

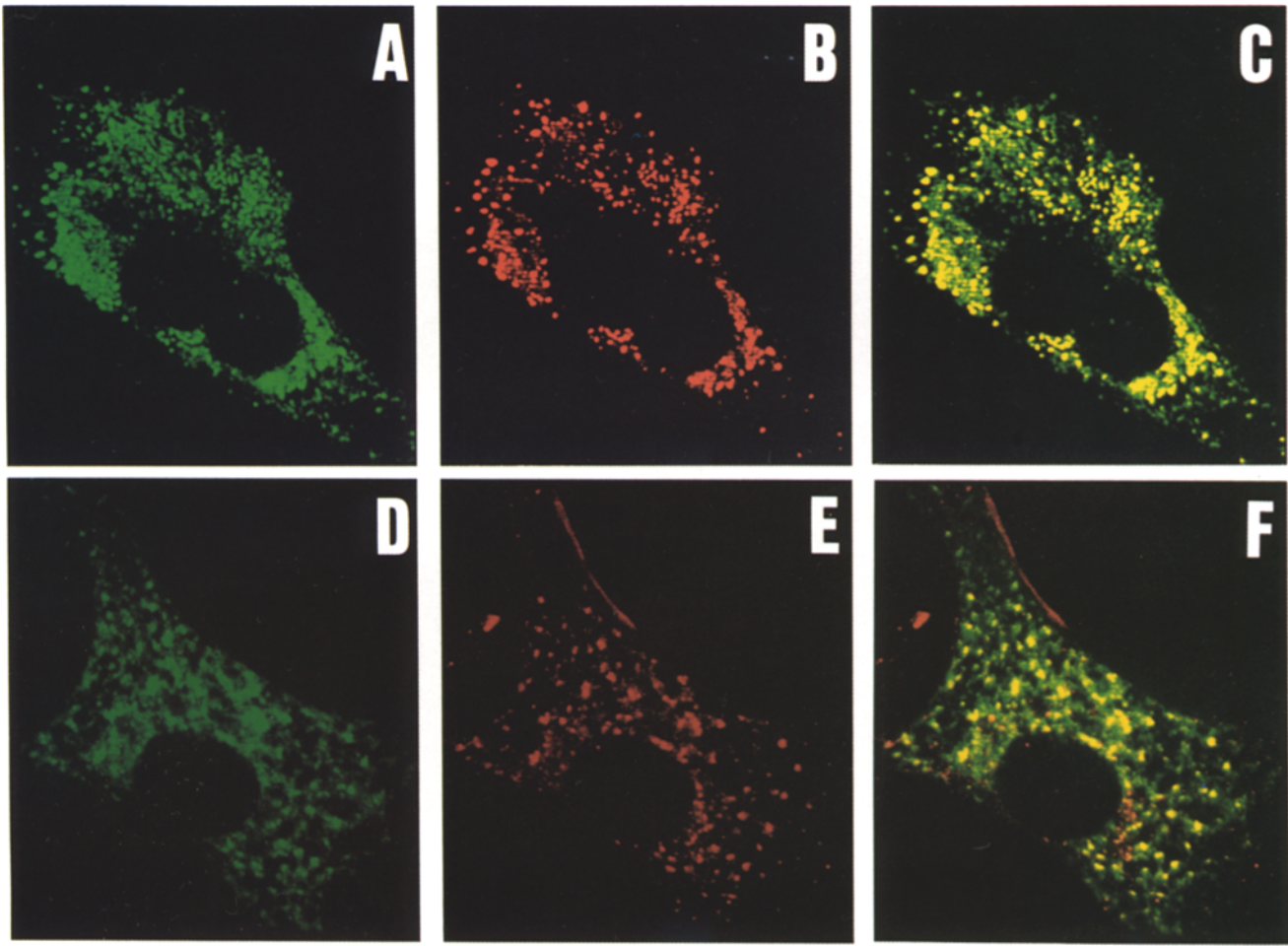
When cells were treated at 15°C for 30 min, the secretion of procollagen was inhibited, and the procollagen was shown to be bound to HSP47, as previously reported (Nakai et al., 1992). The treatment of the cells at this temperature is known to cause the inhibition of transport between the ER and Golgi, presumably at the intermediate compartment (Tartakoff, 1983; Schweizer et al., 1988, 1990; Saraste and Svensson, 1991). We next examined whether these small vesicles containing both procollagen and HSP47 could be observed when the cells were treated at low temperatures. Since protein synthesis is inhibited at this low temperature and the localization of procollagen was not visualized in the presence of ascorbate because of active secretion, we first incubated the CEFs in the absence of ascorbate for 12 h to allow procollagen accumulation in the ER, and then incubated the cells at 15°C in the presence of ascorbate. As shown in Fig. 4 F, inhibition of procollagen secretion was observed to occur in small vesicles that were similar to those observed after treatment of the cells with brefeldin A (Fig. 4 C). These vesicles that had accumulated at 15°C contained both HSP47 and procollagen (yellow staining). Staining with anti-HSP47 antibody again revealed that a considerable amount of HSP47 remained in the ER (Fig. 4 D). This result again indicated that the procollagen was not dissociated from HSP47 in the intermediate compartment.

#### Treatment of CEFs with Monensin and Bafilomycin A1

Monensin is an ionophore for  $\text{Na}^+$ ,  $\text{K}^+$ , and protons, and it is known to inhibit intracellular transport within the Golgi complex, preferentially between the *cis*- or *medial*-Golgi

and *trans*-Golgi compartment (Tartakoff, 1983; Rosa et al., 1992). CEFs were incubated with or without 5  $\mu\text{M}$  monensin for 60 min, pulse-labeled with [ $^{35}\text{S}$ ]methionine, and then chased for various periods. In the presence of monensin, the secretion of procollagen was inhibited for up to 60 min, which was similar to the treatment with  $\alpha$ ,  $\alpha'$ -dipyridyl or brefeldin A (Fig. 6 A, lanes 13–16). However, these accumulated procollagens were not coprecipitated with HSP47 after the chase period, when the cell extract was immunoprecipitated with the anti-HSP47 antibody (Fig. 6 A, lanes 5–8). Procollagen bound to HSP47 decreased rapidly in monensin-treated cells during the chase period, as was seen in the nontreated cells (Fig. 6 A, lanes 1–4). Therefore, while the secretion of procollagen was inhibited by treatment with monensin, the procollagen dissociated normally from HSP47, even in the presence of monensin.

Similar results were obtained when the secretion was inhibited by treatment with bafilomycin A1, which is a strong inhibitor of the vesicular-type  $\text{H}^+$ -ATPase and is known to inhibit secretion at the *trans*-Golgi network (Umata et al., 1990; Henomatsu et al., 1993). After treatment of the CEFs with 1  $\mu\text{M}$  bafilomycin A1 for 60 min, the cells were chased for various time periods, and the dissociation of procollagen from HSP47 was similarly observed in the presence of monensin (data not shown). There is a possibility, however, that the [ $^{35}\text{S}$ ]methionine-labeled procollagen was simply displaced from HSP47 by unlabeled, newly synthesized procollagen, which could result in the apparent dissociation of procollagen from HSP47 during the chase period. This possibility was excluded by treating the cells with cycloheximide after pulse labeling to



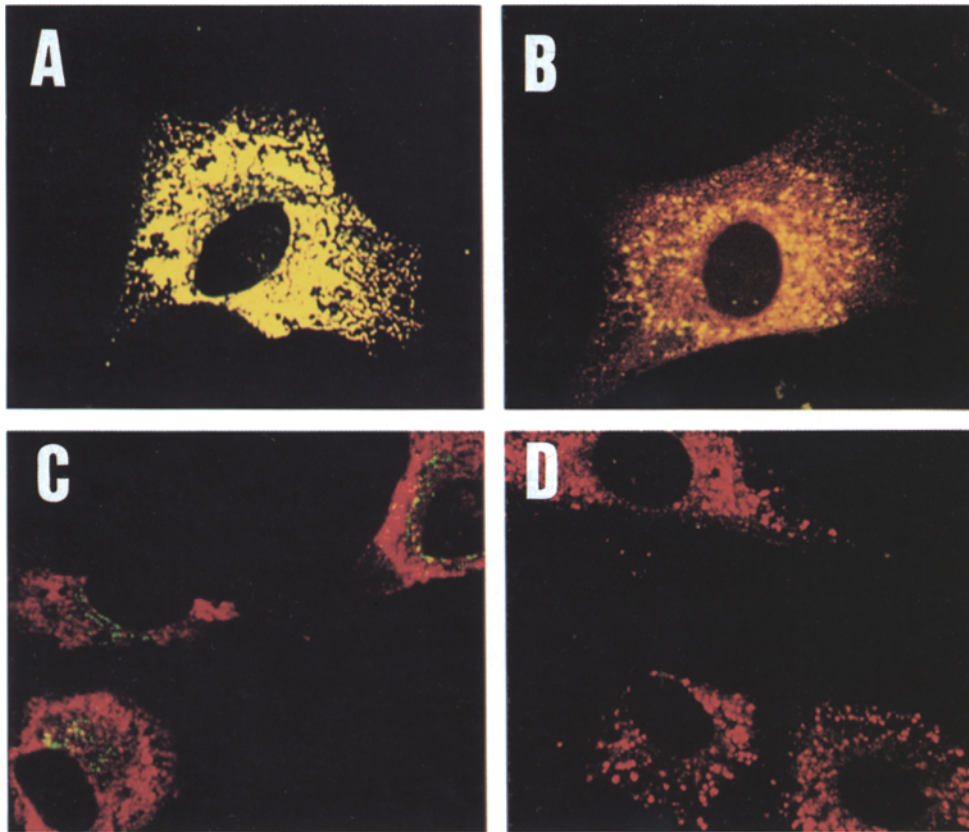
**Figure 4.** Effects of brefeldin A and low temperature on the localization of HSP47 and procollagen. CEFs were cultured in medium with (A–C) or without (D–F) 5  $\mu\text{g/ml}$  ascorbate phosphate for 12 h. The cells were then treated with 2  $\mu\text{g/ml}$  brefeldin A for 30 min (A–C), or they were incubated for 2 h at 15°C (C). The cells were fixed, permeabilized, double stained with the combination of the rat anti-HSP47 mAb (11D10) and the rabbit anti-type I collagen serum, followed by incubation with the FITC-conjugated goat anti-rat IgG and the rhodamine-conjugated goat anti-rabbit IgG, respectively, as the secondary antibodies. Images were obtained with a scanning laser confocal microscope separately for HSP47 (A and D) or type I procollagen (B and E), and by overlapping them (C and F). In the double staining with both FITC and rhodamine images, the yellow color indicates the overlap of the two fluorescent antibodies (C and F).

prevent procollagen synthesis. Even in the presence of cycloheximide, the dissociation of procollagen from HSP47 was similarly observed after the treatment of the CEFs with monensin or bafilomycin A1 (data not shown).

This dissociation of procollagen from HSP47 in the presence of monensin or bafilomycin A1 was demonstrated more directly by confocal microscopic observation. After 60 min of monensin treatment, the CEFs were immunostained with anti-HSP47 and anticollagen antibodies. As shown in Fig. 7 A, procollagen accumulated within these cells. The site of procollagen accumulation, however, was clearly distinct from the localization of HSP47, resulting in the red staining characteristic of collagen molecules. Some yellow staining in the ER demonstrated that the newly synthesized procollagen was bound to HSP47 because protein synthesis was not blocked in this experiment. The site where the procollagen had accumulated should be the Golgi compartment, because of the reported inhibitory mechanism by monensin (Tartakoff, 1983; Rosa et al., 1992). To identify this site more clearly, we tried to

stain the CEFs with anti-mannosidase II antibody. Unfortunately, however, this antibody did not cross-react with the chick protein. The site of procollagen accumulation in the monensin-treated cells was next examined by staining the cells with anti-p58 antibody (Golgi marker). Immunostaining demonstrated the colocalization of the procollagen and p58 protein in these cells (Fig. 7 C). Although the secretion of procollagen was similarly inhibited by treatment of the cells with bafilomycin A1, it was clear that the procollagen no longer colocalized with HSP47 (Fig. 7 B).

These results indicated that the procollagen had already dissociated from HSP47 in the *cis*- or *medial*-Golgi compartment. When the cells were treated with monensin and then the cell lysates were treated with endo H, fibronectin bands which were identified with an antifibronectin antibody became endo H sensitive (data not shown). This suggests that monensin inhibited the secretion at the *cis*-Golgi compartment because fibronectin should be endo H resistant if it enters the *medial*-Golgi compartment (Kornfeld



**Figure 5.** Localization of HSP47, procollagen, PDI, and p58 after treatment with brefeldin A combined with or without  $\alpha, \alpha'$ -dipyridyl. CEFs were cultured in the presence of 5  $\mu\text{g/ml}$  ascorbate phosphate for 12 h. (A) The cells were then preincubated with 0.3 mM  $\alpha, \alpha'$ -dipyridyl for 1 h followed by treatment with 0.3 mM  $\alpha, \alpha'$ -dipyridyl plus 2  $\mu\text{g/ml}$  brefeldin A for 30 min. The cells were treated with (B and D) or without (C) 2  $\mu\text{g/ml}$  brefeldin A for 30 min. Cells were fixed, double stained and visualized by scanning confocal microscopy as described in Fig. 4. Antibodies used for double staining were as follows (fluoresceins used to visualize the first antibody are also shown in parentheses): (A) rat anti-HSP47 mAb (FITC) and rabbit anti-type I collagen serum (rhodamine), (B) rat anti-HSP47 mAb (FITC) and rabbit anti-PDI antibody (rhodamine), (C) rabbit anti-HSP47 antiserum (Texas red) and mouse anti-p58 mAb (FITC), and (D) rabbit anti-type I collagen serum (Texas red) and mouse anti-p58 mAb (FITC). Overlapped images obtained by scanning confocal microscopy were shown for each set of the double staining.

(FITC), and (D) rabbit anti-type I collagen serum (Texas red) and mouse anti-p58 mAb (FITC). Overlapped images obtained by scanning confocal microscopy were shown for each set of the double staining.

and Kornfeld, 1985). Thus, from all the available data described above, the dissociation of procollagen from HSP47 was concluded to occur in the *cis*-Golgi network.

#### **Endo H Sensitivity of HSP47 before and after Deletion of the RDEL Sequence at the COOH Terminus**

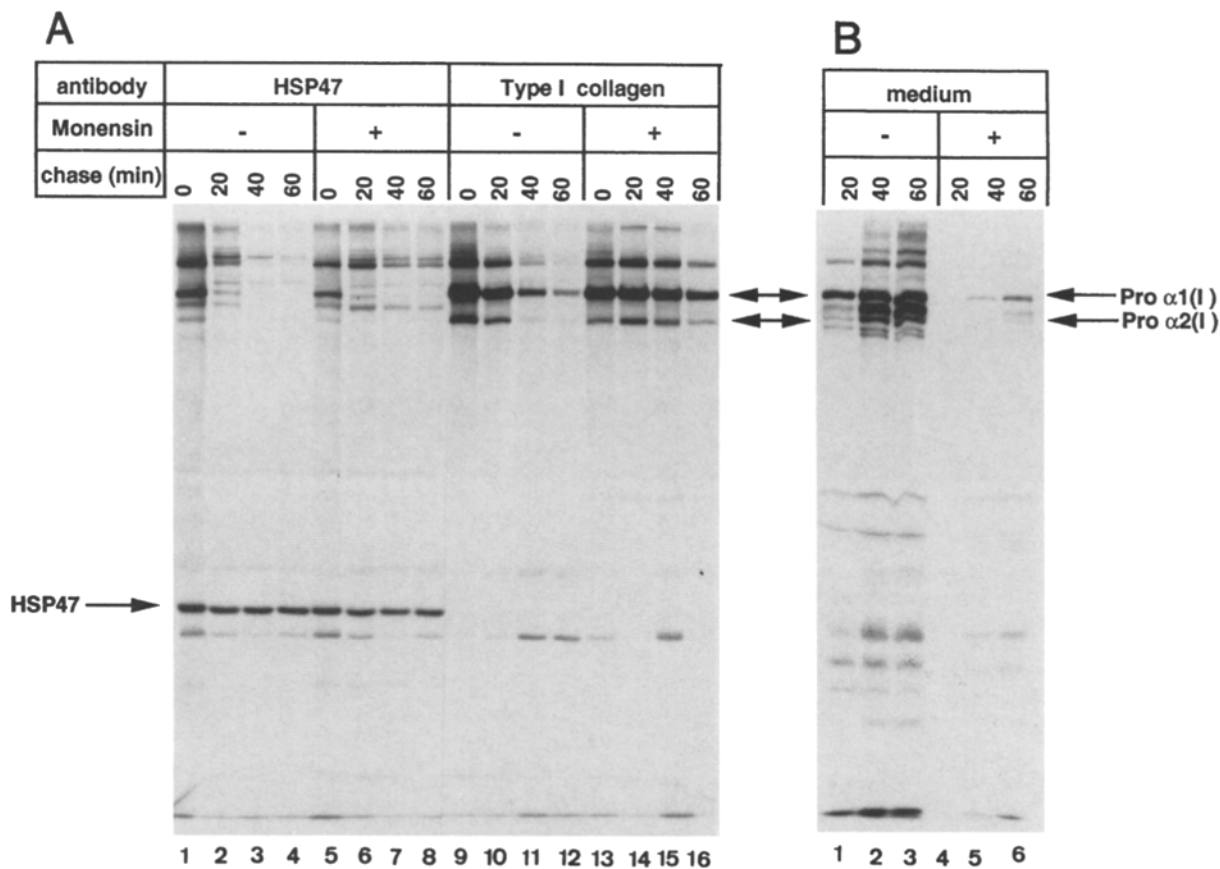
In the above experiments, we noticed that HSP47 was still endo H sensitive even after treatment of the cells with brefeldin A (data not shown). It has been reported that ER-resident proteins such as GRP94 acquire resistance to treatment with endo H after the cells have been treated with brefeldin A for 3–6 h (Lippincott-Schwartz et al., 1989, 1990). This results from a redistribution of Golgi proteins, including glycosidation enzymes into the ER. To confirm whether HSP47 still remained endo H sensitive even after it entered the *medial*- or *trans*-Golgi cisternae, as well as to confirm that the RDEL sequence at the COOH terminus actually acts as an ER-retention signal, we prepared an RDEL sequence-deleted cDNA construct from mouse HSP47 cDNA (Takechi et al., 1992). We then transfected it into the CEFs and examined the endo H sensitivity of HSP47 that was secreted into the medium. We used the mouse HSP47 for the deletion of the RDEL sequence because we can discriminate the transfected mouse HSP47 from the endogenous chick HSP47 by specific antibodies. The RDEL-deleted mouse HSP47 (MH47 $\Delta$ RDEL) was transfected into the CEFs by the calcium phosphate method, and was then immunoprecipitated with anti-chick and anti-mouse HSP47 antibodies 3 d later. As shown in

Fig. 8 A, the amount of transfected RDEL-deleted HSP47 decreased during the chase period, but did not decrease in the presence of monensin. This antibody did not recognize the endogenous chick HSP47 (Fig. 8 A, lanes 1 and 2), and the amount of endogenous HSP47 did not change during the chase period (data not shown). RDEL-deleted HSP47 was secreted into the medium in the absence of monensin, whereas the secretion was greatly reduced in the presence of monensin (Fig. 8 B). The secreted HSP47 was examined for endo H sensitivity after being precipitated with the anti-mouse HSP47 antibody, and Fig. 8 C clearly shows that the secreted HSP47 again failed to acquire resistance to endo H. The RDEL-deleted HSP47, which accumulated in the cell after monensin treatment, was also shown to be endo H sensitive (data not shown). This inability to acquire endo H resistance suggests that the glycosylation enzymes in the Golgi complex could not gain access to HSP47. The above results also revealed that the RDEL sequence at the COOH terminus of HSP47 acts as an ER-retention signal, as the KDEL sequence does (Munro and Pelham, 1987).

#### **HSP47 Can Bind to Both the $\alpha 1(I)$ Single Chain and Mature Procollagen**

Since molecular chaperones like HSP70 and the GroE complex are known to bind only to the immature form of the polypeptide and become dissociated from their substrates once the proteins adopt their mature form, it was of interest to examine whether HSP47 can bind to the imma-





**Figure 6.** Effects of monensin on interaction of procollagen with HSP47. CEFs were cultured in medium containing 5  $\mu\text{g/ml}$  ascorbate phosphate for 12 h, and were preincubated in the presence or absence of 5  $\mu\text{M}$  monensin. The cells were pulse-labeled with 0.2 mCi/ml [ $^{35}\text{S}$ ]methionine for 10 min, and were then chased in medium containing excess cold methionine. The cross-linking and immunoprecipitation experiments were performed as described in Materials and Methods. (A) Immunocomplexes precipitated with the anti-HSP47 antibody (lanes 1–8) or the anti-type I collagen antibody (lanes 9–16) were applied to an SDS–8% polyacrylamide gel, electrophoresed, and exposed on Fuji HR-H film. (B) Medium was collected and analyzed by SDS-PAGE after various time periods of chase. The number on top of each lane indicates the duration of the chase period in minutes.

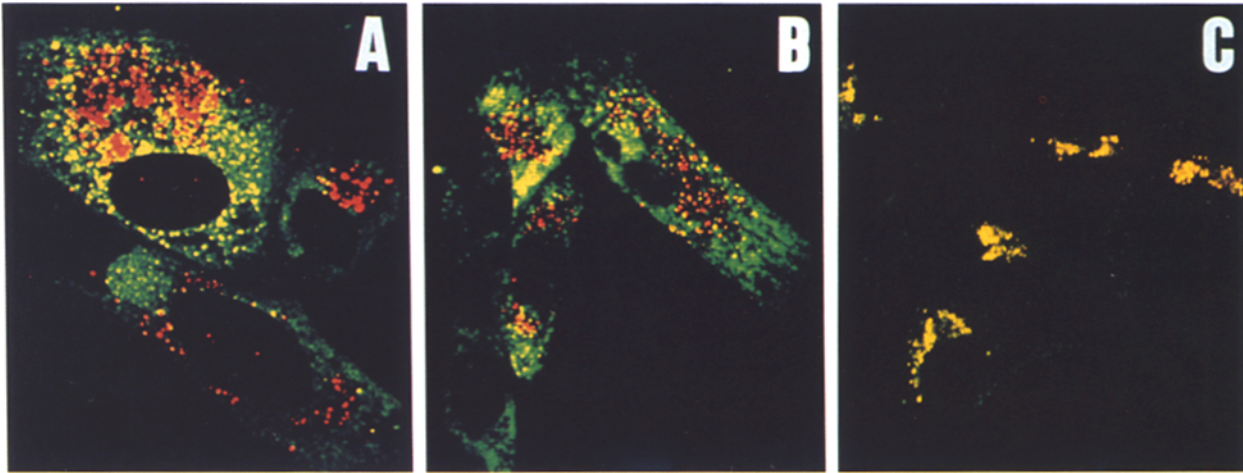
ture form and/or to the mature form of procollagen molecules, or even to the  $\alpha 1(I)$  polypeptide chain.

First, the binding of HSP47 to the immature single polypeptide form of type I procollagen was examined *in vivo* by immunoprecipitation after a short pulse labeling. Immediately after the CEFs were labeled with [ $^{35}\text{S}$ ]methionine for 10 min, the cell lysates were immunoprecipitated with anti-HSP47 and anticollagen antibodies, either with or without cross-linking with DSP. These immunoprecipitates were then electrophoresed in the presence or absence of dithiothreitol (DTT).

Fig. 9 A shows that the procollagen precipitated with the anticollagen antibody consisted of mainly two bands, upper and lower bands that were both collagenase sensitive (Fig. 9 A, lanes 3 and 4). Type I collagen is known to form a triple helix at the COOH terminus by forming intermolecular disulfide bonds between two  $\alpha 1(I)$  and one  $\alpha 2(I)$  polypeptide chains (Rosenbloom et al., 1976; Koivu and Myllyla, 1987; Chessler et al., 1993a). The upper band, under nonreduced conditions, is therefore thought to be a trimer form of procollagen because a trimer form of C-propeptide, (C-propeptide) $_3$ , appeared after treatment with collagenase in the absence of DTT (lane 4), and the lower

band is thought to be a monomer form of procollagen before it forms triple helices. This upper band disappeared in the presence of DTT, and two bands, pro- $\alpha 1(I)$  and pro- $\alpha 2(I)$  chains of procollagen, appeared instead (Fig. 9 B, lane 3), which again suggests that the high molecular weight band is the trimer form of procollagen. Anti-HSP47 antibody precipitated both the trimer and monomer forms of procollagen in addition to HSP47 itself under nonreduced conditions (Fig. 9 A, lane 1). Thus, it was clearly shown that HSP47 could bind to the monomer forms of procollagen as well as to the trimer form.

To examine whether HSP47 could bind to the mature triple-helix form of procollagen within the cells, we exploited the resistance of the triple-helical region of procollagen against proteinase digestion. Procollagen consists of two nonhelical regions at the  $\text{NH}_2$  and COOH termini, plus a helical domain that is resistant to trypsin digestion. Since only a small number of methionine residues are found in the helical region of type I collagen, the CEFs were labeled with [ $^3\text{H}$ ]proline instead of [ $^{35}\text{S}$ ]methionine in this experiment. After preincubation in proline-free medium in the presence or absence of brefeldin A or  $\alpha$ ,  $\alpha'$ -dipyridyl, the cells were pulse-labeled with [ $^3\text{H}$ ]proline



**Figure 7.** Effects of monensin and bafilomycin A1 on the localization of HSP47 and procollagen. CEFs were cultured in medium containing 5  $\mu\text{g/ml}$  ascorbate phosphate for 12 h, and then treated with either 5  $\mu\text{M}$  monensin for 60 min (A and C) or with 1  $\mu\text{M}$  bafilomycin A1 for 60 min (B). (A and B) The cells were fixed, permeabilized, double stained with the combination of the rat anti-HSP47 mAb (11D10) and the rabbit anti-type I collagen antiserum, followed by incubation with the FITC-conjugated goat anti-rat IgG and the rhodamine-conjugated goat anti-rabbit IgG, respectively, as the secondary antibodies. (C) Cells were also stained with the combination of the rabbit anti-type I collagen serum and the mouse anti-p58 mAb (C), followed by incubation with the Texas red-conjugated goat anti-rabbit IgG and the FITC-conjugated goat anti-rat IgG, respectively, as the secondary antibodies. Images obtained by scanning laser confocal microscopy were visualized as described in the legend of Fig. 4.

for 30 min and then chased for various time periods in the presence of excess proline. Because HSP47 contains such a small number of proline residues, HSP47 could not be detected, even after immunoprecipitation with anti-HSP47. Procollagen was precipitated with the anticollagen antibody (Fig. 10 B, lanes 7–12), and the procollagen that was bound to HSP47 was coprecipitated with the anti-HSP47 antibody (Fig. 10 A, lanes 7–12). When the immunocomplex precipitated with the anticollagen antibody was treated with trypsin, the procollagen bands in the cells treated with  $\alpha, \alpha'$ -dipyridyl were completely digested (Fig. 10 B, lanes 1–3), whereas the procollagen in brefeldin A-treated cells was resistant to trypsin digestion (Fig. 10 B, lanes 4–6).

HSP47-bound procollagens were also treated with trypsin. HSP47-bound procollagen that had been treated with  $\alpha, \alpha'$ -dipyridyl was completely digested, whereas the HSP47-bound procollagen in cells pretreated with brefeldin A was trypsin resistant (Fig. 10 A, lanes 1–6). These results indicated that HSP47 can bind to the triple-helix form of procollagen (Fig. 10), as well as to the single  $\alpha 1(I)$  polypeptide chain form of procollagen (Fig. 9).

## Discussion

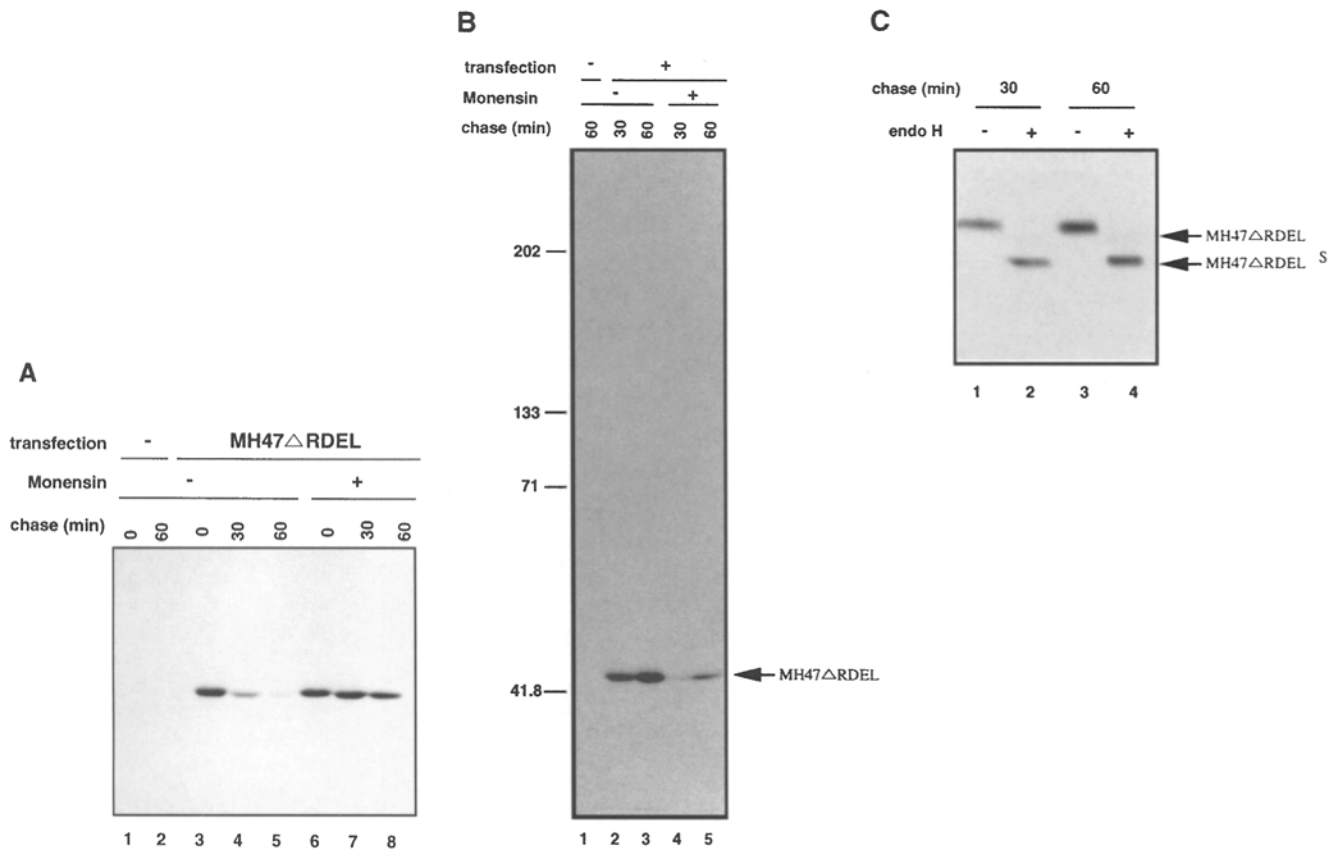
### HSP47 Binding and Dissociation of Procollagen

HSP47, a heat shock-inducible glycoprotein localized in the ER, was found to bind to both collagen and gelatin (Nagata et al., 1986; Saga et al., 1987). HSP47 was previously reported to bind transiently to procollagen in the ER using *in vivo* cross-linking and immunoprecipitation techniques with anti-HSP47 and anticollagen antibodies, combined with pulse-label and chase methods (Nakai et al., 1992). In normal cells, the dissociation of HSP47 from procollagen precedes the secretion of procollagen out of the cells. However, HSP47 remains bound to procollagen for

much longer periods of time when the cells are heat shocked or treated with  $\alpha, \alpha'$ -dipyridyl. Thus, HSP47 can be presumed to be a molecular chaperone-like protein specific for collagen. Similarly, the underhydroxylated procollagen was retained to be bound to HSP47 for a much longer time in the ER compared to normally hydroxylated procollagen, as shown in Fig. 1.

Thus, it was of interest to determine exactly when this chaperone-like protein binds to the procollagen polypeptide chain and where it dissociates from its substrate, procollagen. In this report, we have examined whether HSP47 can bind to nascent procollagen polypeptide chains, and we have determined the sites within the cells where HSP47 dissociates from procollagen using several inhibitors against procollagen secretion. The binding and colocalization of HSP47 with procollagen were examined by immunoprecipitation analysis combined with *in vivo* cross-linking, and by confocal microscopic observation after double labeling with anti-HSP47 and anticollagen antibodies, respectively. Short-term labeling of the cells with [ $^{35}\text{S}$ ]methionine followed by immunoprecipitation with anti-HSP47 and anticollagen antibodies revealed that HSP47 was bound to single nascent peptides that are sensitive to collagenase. This suggests that HSP47 was bound to procollagen polypeptide chains immediately after the procollagen had cotranslationally entered the ER as single  $\alpha 1(I)$  or  $\alpha 2(I)$  polypeptides. This conclusion is also supported by the coprecipitation of HSP47 and nascent collagenous polypeptides when the polysome-containing fraction of the cell lysates is precipitated with anti-HSP47 and anticollagen antibodies (Sauk et al., 1994).

On the other hand, it is clear from the *in vitro* experiments that HSP47 binds to collagens that have already formed a triple helix (Nagata et al., 1986; Natsume et al., 1994). In this report, we have also shown the binding of HSP47 with mature triple-helix forms of procollagen (Fig.



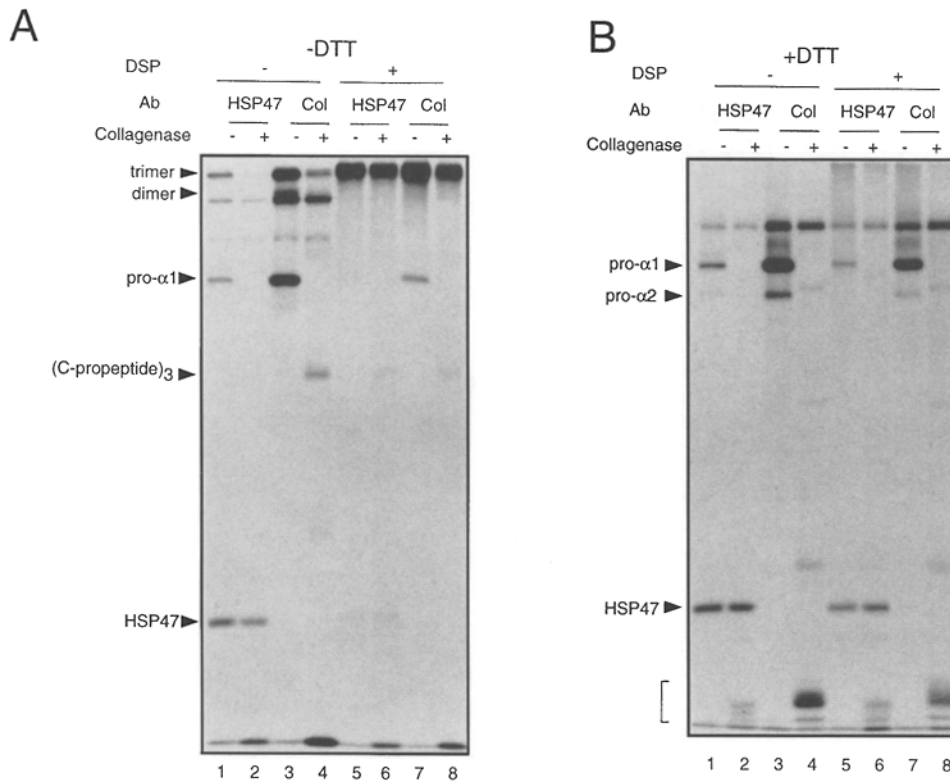
**Figure 8.** The RDEL sequence at the COOH terminus of HSP47 acts as an ER-retention signal. The construct containing the RDEL-truncated mutant mouse HSP47 from its COOH terminus (MH47 $\Delta$ RDEL) was transfected by the calcium phosphate method into the CEFs. (A) The cells transfected with MH47 $\Delta$ RDEL were preincubated in the presence (lanes 6–8) or absence (lanes 1–5) of 5  $\mu$ M monensin, pulse labeled with 0.2 mCi/ml [<sup>35</sup>S]methionine for 30 min, and then chased in medium containing excess cold methionine for the indicated periods. After treatment with the lysis buffer, aliquots of the cell extract were immunoprecipitated with the rabbit anti-mouse HSP47 antibody. The immunocomplexes were subsequently analyzed by SDS-PAGE and autoradiography. (B) Time course of the secretion of MH47 $\Delta$ RDEL transfected and expressed in CEFs. Culture medium from A was collected after 30 or 60 min of chase, immunoprecipitated with the anti-mouse HSP47 antibody, and then analyzed by SDS-PAGE. (C) MH47 $\Delta$ RDEL secreted into the culture medium was sensitive to endo H treatment. The immunocomplexes in B were treated with or without 55 U/ml of endo H at 37°C for 12 h, and were then applied to an SDS–8% polyacrylamide gel. MH47 $\Delta$ RDEL<sup>S</sup> is the form digested by endo H.

10). In the future, we will examine whether HSP47 binds to nascent single-polypeptide chains as an early event, with interactions continuing during the folding and triple-helix formation of procollagen. The possibility must also be considered that HSP47 binds independently to monomeric polypeptide chains and to mature triple-helical forms of procollagen.

In other experiments, we examined where the HSP47 dissociates from procollagen within the cells. The inhibition of procollagen secretion in the ER induced by  $\alpha$ ,  $\alpha'$ -dipyridyl or by the depletion of ascorbate did not cause any dissociation of HSP47 from procollagen. Brefeldin A has been reported to inhibit protein secretion and dramatically disintegrate the Golgi apparatus, resulting in the redistribution of Golgi membranes in the ER. Uncoated tubules emanating from the Golgi complex are observed rapidly after the addition of brefeldin A, suggesting that they are the structural intermediates that carry the Golgi membranes to the ER. The intermediate compartment, however, is shown to remain intact in brefeldin A-treated cells (Lippincott-Schwartz et al. 1989, 1990; Klausner et

al., 1992). The immediate effect of brefeldin A is to prevent nucleotide exchange, thereby preventing ARF binding to the Golgi membrane and subsequent ARF-dependent assembly of coated vesicles (Donaldson et al., 1992; Tanigawa et al., 1993). Thus, brefeldin A would block anterograde traffic from ER and Golgi without affecting retrograde transport from Golgi complex to ER (Klausner et al., 1992).

Treatment of the cells with brefeldin A or at a low temperature (15°C) did not cause the dissociation of HSP47 from procollagen, which was examined biochemically by coprecipitation analysis using either antibody. These treatment paradigms caused the accumulation of small vesicles, where both HSP47 and procollagen were colocalized, although most of the HSP47 that did not associate with procollagen remained in the ER. Similar small vesicles that were identified as the intermediate compartment were reported to be observed after treatment of the cells with brefeldin A or at 15°C (Lippincott-Schwartz et al., 1989, 1990). These small vesicles, which were distinct from the ER, disappeared after removing brefeldin A from the me-



**Figure 9.** HSP47 binds to nascent single polypeptide chains of procollagen. CEFs were cultured in medium containing 5  $\mu\text{g/ml}$  ascorbate phosphate for 12 h. Immediately after pulse labeling with 0.2 mCi/ml [ $^{35}\text{S}$ ]methionine for 10 min, the cells were collected, incubated without (lanes 1–4) or with (lanes 5–8) the cross-linking reagent for 30 min at 0°C, and were then extracted by the addition of lysis buffer without (lanes 1–4) or with (lanes 5–8) 0.5 mg/ml gelatin. Aliquots of the cell extract were mixed with the rabbit anti-HSP47 antibody (lanes 1, 2, 5, and 6) or with the rabbit anti-type I collagen antiserum (lanes 3, 4, 7, and 8). The immunocomplexes were incubated with or without 20 U/ml collagenase for 30 min at 37°C, and were then eluted with the SDS sample buffer. The immunocomplexes were analyzed by 8% SDS-PAGE under nonreducing (A) and reducing (B) conditions. (C-propeptide)<sub>3</sub>, the C-propeptide of procollagen that was cross-linked by intermolecular disulfide bonds between the three procollagen chains. *Bracket*, procollagen bands digested with collagenase.

dium or upon returning the temperature to 37°C. Since the anti-p53 antibody (Schweizer et al., 1988) did not react with CEFs, we could not identify these vesicles as intermediate compartments. However, the above mentioned experimental results strongly suggest that these vesicles were intermediate compartments. Thus, it is probable that procollagen does not dissociate from HSP47 neither in the ER nor in the intermediate compartment. Recently, Hammond and Helenius (1994b) showed that the misfolded G protein of the temperature-sensitive vesicular stomatitis virus tsO45 is associated with both calnexin and BiP/GRP78, but moves to the intermediate compartment only with BiP/GRP78. In this report, it was demonstrated that when the secretory pathway was blocked with brefeldin A, the complex of normal folded procollagen with HSP47 (and perhaps with PDI as well) accumulated in the intermediate compartment. We will try to identify whether there are other chaperone proteins contained in these small vesicles.

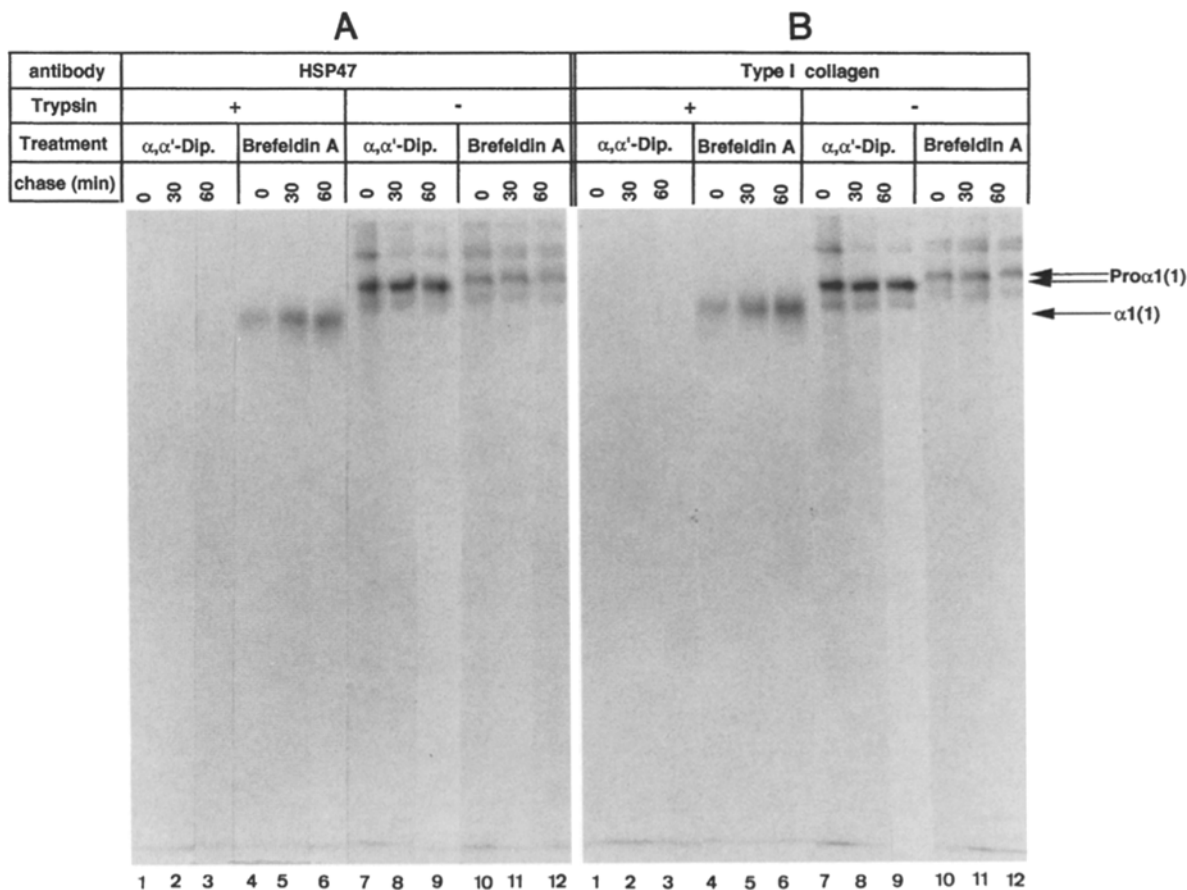
When the cells were treated with monensin or bafilomycin A1, the dissociation of HSP47 from procollagen was clearly observed, whereas the secretion of procollagen was inhibited. Monensin is a well-characterized ionophore that binds Na<sup>+</sup>, K<sup>+</sup>, and protons (Tartakoff, 1983). Bafilomycin A1 is a specific inhibitor for the vacuolar-type H<sup>+</sup> ATPase, and it is known to inhibit the secretion of the proteins from the *trans*-Golgi network (Umata et al., 1990; Yoshimori et al., 1991; Henomatsu et al., 1993). Treatment of

the cells with monensin inhibits the intracellular transport of newly synthesized plasma membrane proteins and secretory proteins, including procollagens and proteoglycans, within the Golgi complex (Uchida et al., 1979; Tartakoff, 1983). Although monensin may have multiple sites of action depending on the cell type, our results suggest that monensin inhibited translocation between the *cis*- and *medial*-Golgi compartments because the accumulated procollagen was colocalized with p58 (a Golgi marker) and monensin-inhibited fibronectin was endo H sensitive (data not shown). Thus, HSP47 must have been dissociated from the procollagen between intermediate compartment (*cis*-Golgi network) and the *cis*-Golgi compartment. This is the first report identifying where a molecular chaperone protein dissociates from its substrate in the central secretory pathway.

#### Dissociation of HSP47 from Procollagen

Molecular chaperones such as HSP70 and GroEL are known to have ATP binding and weak ATPase activities (Chandrasekhar et al., 1986; Liberek et al., 1991). HSP70 is now thought to dissociate from its substrates by changing its affinity for them after it binds ATP (Palleros et al., 1994). However, HSP47 has neither ATP-binding nor ATPase activities (Nagata, K., and M. Satoh, unpublished observation). It is therefore of interest to elucidate how HSP47 dissociates from procollagen. Previously, we re-





**Figure 10.** HSP47 binds to the trypsin-resistant triple helix form of procollagen. CEFs were cultured in the medium containing 5  $\mu\text{g/ml}$  ascorbate phosphate for 12 h, and were preincubated in either 2  $\mu\text{g/ml}$  brefeldin A or in 0.3 mM  $\alpha, \alpha'$ -dipyridyl for 30 min. The cells were pulse labeled with 0.167 mCi/ml [ $^3\text{H}$ ]proline for 30 min, and were then chased for 30 or 60 min in medium containing excess cold proline. Cross-linking and immunoprecipitation were performed as described in Materials and Methods. The immunocomplexes precipitated with the anti-HSP47 antibody (A) or the anti-type I collagen antibody (B) were treated with (lanes 1–6) or without (lanes 7–12) 20  $\mu\text{g/ml}$  trypsin for 4 min at 37°C, and were then applied to an SDS–8% polyacrylamide gel.

ported that HSP47 dissociates from collagen when the buffer pH is lowered to pH 6.3 in an *in vitro* binding assay using collagen-coupled Sepharose 4B affinity beads (Saga et al., 1987), and that the coprecipitation of procollagen with HSP47 decreases in pH 6.0 buffer (Nakai et al., 1990, 1992). When the cells are treated with the proton ionophore carbonyl *m*-chlorophenylhydrazine (CCCP) in a low pH (pH 6.3) medium, the coprecipitation of procollagen with HSP47 is decreased (Nakai et al., 1992). These results show that the dissociation of HSP47 from collagen can be regulated by pH. We have reported very recently, however, that the majority of HSP47 bound to collagen dissociates in a pH-independent but concentration-dependent manner in an *in vitro* binding analysis using surface plasmon resonance, the *BIAcore* system (Natsume et al., 1994). According to the results obtained by surface plasmon resonance, the dissociation constants of HSP47 for types I–IV collagens ranges from  $10^{-7}$  to  $10^{-6}$  M, and HSP47 dissociates very rapidly from collagen molecules once the free HSP47 concentration in the flow buffer is lowered (the dissociation rate constant of HSP47 for type I collagen is  $2.36 \times 10^{-2} \text{ s}^{-1}$ ).

Considering these results obtained from both *in vitro* and *in vivo* experiments, the intracellular interaction of

HSP47 with newly synthesized collagen molecules could be outlined as follows. Immediately after single chains of procollagen cotranslationally enter the ER, HSP47 binds to these single polypeptide chains. Since the type I procollagen forms a triple helix at the COOH terminus, the newly synthesized polypeptide chains need to be protected from being folded or aggregated until the completion of translation. The binding of HSP47 to the newly synthesized single chains might be beneficial in preventing the folding or the aggregation of these nascent polypeptides. As we previously reported, other ER-resident proteins such as GRP78/BiP and PDI might be involved in the formation of these complexes because these three ER proteins (BiP, PDI, and HSP47) form a complex in the ER (Nakai et al., 1992). HSP47 remains bound to these triple helices of procollagen in the ER, as well as in the transport vesicles. Recently, we determined stoichiometry of the binding of recombinant mouse HSP47 to type I collagen using surface plasmon resonance (Natsume, T., and K. Nagata, unpublished observations); more than seven molecules of HSP47 bind to a single triple helix of type I collagen. Although the physiologic role of HSP47 binding to procollagen in the above processes is obscure, there is the possibility that HSP47 is involved in the processing of pro-

collagen, such as prolyl and lysyl hydroxylation or the transport of the procollagen from the ER to the Golgi compartments. Alternatively, HSP47 (colligin) has been reported recently to have an inhibitory action against the degradation of procollagens in the ER (Jain et al., 1994). This is more conceivable considering the similarity in the primary structures of HSP47 and the serpin (serine protease inhibitor) super family (Hirayoshi et al., 1991; Takechi et al., 1992).

When the HSP47/procollagen complex enters the *cis*-Golgi compartment, the concentration of free HSP47 decreases rapidly since HSP47 might be retrogradely transported back from the Golgi to ER via a retrieval flow with KDEL receptors. KDEL receptors are found to be concentrated mainly in the Golgi membranes (Semenza et al., 1990; Lewis and Pelham, 1990; Tang et al., 1993), and we have clearly confirmed in the present work that the RDEL sequence at the COOH terminus of HSP47 actually acts as an ER-retention signal by deleting this sequence from the HSP47 cDNA. Although we have not confirmed whether the RDEL sequence is actually recognized by KDEL receptors, the possible scavenging of HSP47 from the Golgi compartment may cause the absence of free HSP47 there, which was confirmed by immunoelectron microscopy (Saga et al., 1987; Kambe et al., 1994). This rapid decrease in the free HSP47 concentration could cause the rapid dissociation of HSP47 from procollagen molecules in the *cis*-Golgi compartment because of their high dissociation rate constant ( $K_d = 10^{-2} \text{ s}^{-1}$ ; Natsume et al., 1994). Although we clearly observed the pH-dependent dissociation of collagen from HSP47 in *in vitro* experiments (Saga et al., 1987; Nakai et al., 1992), we cannot confirm at the present time whether this pH-dependent dissociation actually occurs *in vivo*. Since the pH in the *trans*-Golgi compartment is known to fall as low as pH 5.7 (Anderson and Orci, 1988; Wilson et al., 1993), it should be interesting to determine whether a small portion of the HSP47 is transported into the *trans*-Golgi cisternae with procollagen and whether it dissociates there from procollagen in a pH-dependent manner.

### Correlational Expression of HSP47 with Collagen Genes

As a possible molecular chaperone, HSP47 should be closely related to collagen processing and/or secretion, as well as to the inhibition of procollagen degradation in the ER. In addition to these functions, the expression of HSP47 is always closely correlated with the expression of the various types of collagens. In fibroblasts, the synthesis of both HSP47 and type I collagen decreases after malignant transformation by Rous sarcoma virus (Nagata et al., 1986; Clarke et al., 1993), simian virus 40 (Nakai et al., 1990), and the activated *c-Ha-ras* oncogene (Nagata et al., 1991). In contrast, both HSP47 and type IV collagen synthesis increase markedly during the differentiation of the mouse teratocarcinoma cell line F9 after treatment with retinoic acid alone or with retinoic acid plus dibutyryl cAMP (Kurkinen et al., 1984; Takechi et al., 1992). Interestingly, HSP47 synthesis is not observed in cells where collagen synthesis is not detected, such as mouse myeloid leukemic M1 cells and mouse pheochromocytoma PC12 cells (Nagata et al.,

1991). Such a correlation between the expression of HSP47 and several types of collagen has also been reported in rat cells (Clark et al., 1993). We also observed a strong correlation in the regulation of HSP47 with collagen under pathological conditions *in vivo*: the synthesis of HSP47, as well as type I and III collagens, are dramatically increased during the progression of rat liver fibrosis induced by carbon tetrachloride (Masuda et al., 1994). *In situ* hybridization analysis shows that only cells adjacent to the collagen fibrils in the carbon tetrachloride-treated rat liver expressed large amounts of HSP47 mRNA, whereas cells far from the collagen fibrils did not. Thus, HSP47 is the stress protein that has substrate specificity, and the regulation of which is also closely correlated with this substrate, collagen.

Such a correlation in the expression between HSP47 and various collagens is also observed during the development of the mouse embryo. For example, HSP47 is strongly expressed in cartilaginous regions, especially in chondrocytes in the proliferation and maturation zones of epiphyseal growth plates, where the expression of type II collagen was also prominent. In contrast, both type I collagen and HSP47 were localized to the extracellular matrix and osteoblasts in the vascularization zone during ossification (Masuda, H., and K. Nagata, manuscript submitted for publication). Kambe et al. (1994) also reported that HSP47 was expressed in the epiphyseal cartilage and in cultured chondrocytes from chick embryos. These correlations in the expression of HSP47 with various types of collagen suggest an important role for HSP47 in the processing and/or secretion of collagens in various types of cells. The mechanism of HSP47 induction with respect to collagen formation, as well as the function of HSP47 as a molecular chaperone specific for collagen, still remain to be elucidated in detail in the near future.

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