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HSP47, a Collagen-Specific Molecular Chaperone, Delays the Secretion of Type III Procollagen Transfected in Human Embryonic Kidney Cell Line 293: A Possible Role for HSP47 in Collagen Modification[1]

Nobuko Hosokawa,*,† Christine Hohenadl,‡,2 Mamoru Satoh,*,3 Klaus Kühn,‡ and Kazuhiro Nagata*,†,4

*Department of Molecular and Cellular Biology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8397; †Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation; and ‡Max-Planck-Institut für Biochemie, 82152 Martinsried, Germany

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HSP47 is a stress protein (heat shock protein) which resides in the endoplasmic reticulum, and is postulated to function as a collagen-specific molecular chaperone. To elucidate the role of HSP47 in procollagen biosynthesis, we have established human embryonic kidney 293 cell lines, which were stably transfected with α1(III) procollagen chains with or without HSP47. 293 cells do not produce any extracellular matrix proteins including collagens, and the level of HSP47 expression is almost undetectable in this cell line. Recombinant type III procollagens in 293 cells form trypsin-resistant homotrimers, which are secreted into the medium as trimers in the presence or absence of recombinant mouse HSP47. The secretion of procollagen III was delayed in 293 cells stably transfected with proα1(III) collagen chains [293+proα1(III) cells] in comparison with human rhabdomyosarcoma cell line RD, which normally produces type III procollagens. In this study, we examined the rate of type III procollagen secretion in detail. In cells cotransfected with mouse HSP47 [293+proα1(III)+HSP47 cells], the rate of type III procollagen secretion was slower than in 293+proα1(III) cells. The binding of HSP47 with proα1(III) collagen chains was confirmed by immunoprecipitation using the chemical cross-linker, DSP. The electrophoretic mobility of proα1(III) collagen chains in 293+proα1(III) cells was slightly slower than that in RD cells, whereas the recombinant proα1(III) chains of 293+proα1(III)+HSP47 cells showed almost the same electrophoretic mobility as those of RD cells. The melting temperature (T_m) of type III procollagen in 293+proα1(III)+HSP47 cells was almost the same as that in RD cells, and the T_m in 293+proα1(III) cells was slightly higher than that in RD cells. These data suggest that the recombinant proα1(III) collagen chain is overmodified in 293+proα1(III) cells, but not in 293+proα1(III)+HSP47 cells.

Key words: HSP47, human embryonic kidney 293 cells, molecular chaperone, procollagen biosynthesis, procollagen secretion.

Collagens are major extracellular matrix (ECM) proteins synthesized in higher eukaryotes, and many steps are involved in the biosynthesis of mature collagen fibrils (*1-3*). In cells, the procollagen α-subunits are synthesized in the ER, and undergo several co- and post-translational modifications. Thus, some proline and lysine residues are hydroxylated, and several sites are glycosylated. The α-subunits of classical fiber forming collagens, such as types I, II, III, and V, are known to assemble to form homo- or heterotrimers *via* their C-termini, from which the triple helical molecules are formed in a zipper-like fashion. Only triple helical molecules, *i.e.* not individual α-subunits, are secreted into the medium. HSP47 is a heat shock protein (stress protein) that resides in the ER, and binds specifically to collagens both *in vivo* and *in vitro* (*4-6*). HSP47 is considered to function as a collagen-specific molecular chaperone (*7, 8*). HSP47 binds preferentially to unhydroxylated collagens occurring in cells after treatment with α,α′-dipyridyl (*8*). It also binds to single procollagen chains (*9, 10*) and polysome-bound nascent procollagen chains (*10*), as well as to triple helical molecules. HSP47

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Present addresses: [2] GSF-Forschungszentrum für Umwelt und Gesundheit, Institut für Molekulare Virologie, Ingolsteadter Landstrasse 1, D-85764, Oberschleissheim, Germany; [3] Institute for Developmental Research, Aichi Human Service Center, Aichi 480-0304. [4] To whom correspondence should be addressed at: Department of Molecular and Cellular Biology, Institute for Frontier Medical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8397. Tel: +81-75-751-3848, Fax: +81-75-752-9017, E-mail: nagata@chest.kyoto-u.ac.jp

Abbreviations: ECM, extracellular matrix; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; T_m, melting temperature; NEPHGE, nonequilibrium pH gradient electrophoresis; DTT, dithiothreitol; DSP, dithio*bis*(succinimidylpropionate); AGPC, acid guanidinium thiocyanate-phenol-chloroform; TCA, trichloroacetic acid; HSP, heat shock protein; GRP, glucose-regulated protein; BiP, Ig heavy chain binding protein.

dissociates from procollagens in the *cis*-Golgi compartment (*9*). These data suggest that HSP47 is involved in the biosynthesis, triple helix formation and transport from the ER to the Golgi of procollagens within the cells (*7*).

To elucidate the roles of HSP47 in the biosynthesis, modification, and secretion of procollagen, it is necessary to use cells which actively produce collagens in the presence or absence of HSP47. As the expression of HSP47 always closely correlates with that of collagens (*7, 11, 12, 26*), such cells producing collagens without HSP47 were not available, and we could not compare the secretion and modification of procollagens in the presence and absence of HSP47. Therefore, we used human embryonic kidney cell line 293, which is known to produce no ECM protein including collagens (*13*). HSP47 was expressed at an almost undetectable level in 293 cells. 293 cells were stably transfected with human proα1(III) collagen with or without the transfection of murine HSP47. This model system will be useful for examining the effects of HSP47 on collagen biosynthesis and secretion. Type III collagen is a fibril-forming homotrimeric collagen, consisting of proα1(III) chains. Both 293+proα1(III) and 293+proα1(III)+HSP47 cells synthesize and secrete recombinant type III procollagens as trypsin-resistant homotrimers, although the kinetics of triple helix formation and secretion are delayed in comparison to those for human rhabdomyosarcoma cell line RD, which was reported to produce normal proα1(III) collagens (Hohenadl, C., Kühn, K., *et al.*, manuscript in preparation).

In this study, we have analyzed procollagen biosynthesis, focusing on the effect of HSP47. We found that the rate of type III procollagen secretion was delayed, while overmodification of proα1(III) collagen chains was inhibited when HSP47 was coexpressed.

MATERIALS AND METHODS

Preparation of 293+proα1(III) Cells, 293+HSP47 Cells, and 293+proα1(III)+HSP47 Cells—Full length cDNA coding for the human preproα1(III) chain (*14*) was inserted into eukaryotic expression vector pCis (*15*), which contains the CMV promoter and enhancer [pCMV-proα1-(III)]. The cDNA fragment encoding the complete translated region of murine HSP47 (*16*) was inserted into vector pRC/CMV (Invitrogen, NV Leek, Netherlands), which contains the sequence coding for neomycin resistance (pRC/CMV-mHSP47). 293+proα1(III) cells were prepared by the cotransfection of 293 cells with pCMV-proα1(III) and pSV$_2$-pac (*17*), a plasmid coding for puromycin resistance. 293+HSP47 cells were obtained by transfecting 293 cells with pRC/CMV-mHSP47, and 293+proα1(III)+HSP47 cells were obtained by the transfection of 293+proα1(III) with pRC/CMV-mHSP47. In all cases, the standard calcium-phosphate precipitation method was used. Cells producing a relatively high amount of type III collagen or HSP47 were selected by Western blotting. Other stable transfectants, D1 and D44, are essentially the same as 293+proα1(III) cells except that the level of type III procollagen expression is different. M68 and M73 are other 293+proα1(III)+HSP47 cell lines, which were transfected with mHSP47 cDNA in expression vector pCXWN (*18*). All of the cells were cultured in DMEM (Dulbecco's modified Eagle's medium)/F-12, supplemented with 10% fetal bovine serum. The 293 cell derivatives were plated on poly-L-lysine coated cell culture dishes prior to harvesting. For the secretion of type III procollagens, cells were cultured in the presence of 136 μg/ml ascorbic acid phosphate (Wako Pure Chemical Industries, Osaka, Japan) for at least 16 h before sampling.

Antibodies—Polyclonal antibodies raised against human-bovine type III collagen were purchased from LSL (Japan), and monoclonal anti-HSP47 (colligin) antibodies were purchased from StressGen (Victoria, BC, Canada). Rabbit serum immunized against mouse HSP47 N-terminal peptide only recognizes mouse HSP47, *i.e.* not the human homologue (*19*). The same antibodies were used for both Western blotting and immunoprecipitation. Anti-type III collagen antibodies recognize both triple helical and incompletely folded collagen chains.

Western Blotting—Twenty micrograms of cell lysate was applied to a SDS-PAGE gel as described previously (*4*), and after blotting on to a nitrocellulose membrane, specific antibodies were added and detected with an ECL system (Amersham Life Science, Amershan, England). Densitometric analysis was performed using pdi (Protein+dna image ware system™).

Metabolic Labeling—Cells were labeled with [^{35}S]methionine (EXPRE^{35}S^{35}S, DuPont-NEN, Wilmington, DE, USA) in DMEM lacking methionine without fetal calf serum. For the pulse-chase experiments, cells were preincubated for 30 min in DMEM lacking methionine before metabolic labeling for 15 or 30 min, and then chased for various periods in medium containing excess cold methionine. After cells had been lysed in a buffer containing 1% NP-40 as described previously (*4*), the soluble fraction was analyzed by two-dimensional gel electrophoresis (NEPHGE/SDS-PAGE) (*20*) or by immunoprecipitation (*8*). Procollagens secreted into the medium were collected by TCA precipitation of the serum-free medium unless otherwise indicated.

Cross-Linking Experiments—Radiolabeled cells were cross-linked using DSP as described previously (*8*), with the minor modification that the DSP was added directly to the culture dish at a final concentration of 2 mM for 293 cell lines and of 1 mM for RD cells. After cross-linking, immunoprecipitation was performed using specific antibodies, and samples were analyzed by 8% SDS-PAGE after boiling of the immune complex in Laemmli's sample buffer containing 0.1 M DTT.

RNA Isolation and Northern Blotting—Total cellular RNA was isolated by the AGPC method (*21*). Ten micrograms of RNA was separated by denatured gel electrophoresis, and then hybridized with radiolabeled probes. The cDNA fragments used for the detection of specific RNAs were mouse HSP47 cDNA (*16*), the C-terminal region of the human proα1(III) collagen chain (3,886-4,560 bp, a kind gift from Dr. T. Tanaka, Kyoto University), and human β-actin cDNA (*22*).

Enzyme Digestion of the Procollagens—For the electrophoresis and enzyme digestion of the proα1(III) collagen secreted into the medium, metabolically labeled cells and the medium without serum were collected as described above. After measuring the TCA insoluble radioactivity of the cell lysate, the medium containing radioactivity equivalent to that in the cell lysate was precipitated with TCA, and then subjected to the following analysis. For pepsin

digestion, the TCA-precipitate was lysed in 0.5 M acetic acid containing 1% Triton X-100, and then digested with 100 μg/ml of pepsin (Sigma Chemical Co., St. Louis, MO, USA) for 16 h at 4°C (*23, 24*). For trypsin/chymotrypsin digestion, the same precipitate was dissolved in a buffer containing 0.4 M NaCl and 0.1 M Tris-HCl (pH 7.4), and then digested with 100 μg/ml of trypsin (Sigma Chemical) and 250 μg/ml of chymotrypsin (Sigma Chemical) for 2 min at 25°C (*23, 24*).

Calculation of T_m of Type III Procollagen—TCA precipitates prepared as described above were resuspended in the trypsinization buffer [0.4 M NaCl and 0.1 M Tris-HCl (pH 7.4)]. The same aliquot was heated step by step from 30°C to the indicated temperature using a thermal cycler (Techne, England) as described previously (*25*), before it was digested with trypsin/chymotrypsin for 2 min at 25°C. The radioactivity was measured quantitatively using an image analyzer (Bio-Rad Lab., Hercules, CA, USA) to calculate the T_m.

RESULTS

Two-Dimensional Gel Electrophoresis of the Cells—To determine the cellular proteins synthesized in the cell lines used in these experiments, cells were metabolically-labeled and cell lysates were analyzed by two-dimensional gel electrophoresis (NEPHGE/10% SDS-PAGE) under reducing conditions. There were some differences in the overall profiles of the labeled proteins between 293 cells and RD cells (compare Fig. 1, A and E), but the profiles were the same among the 293 cell derivatives except in the expression of transfected recombinant proteins (Fig. 1, A, B, C, and D). The expression of HSP47 and collagens was closely correlated in all cells examined to date (*7, 26*). 293 cells, which are known to produce no ECM proteins including collagens, did not express detectable levels of HSP47. The synthesis of endogenous HSP47 did not increase following

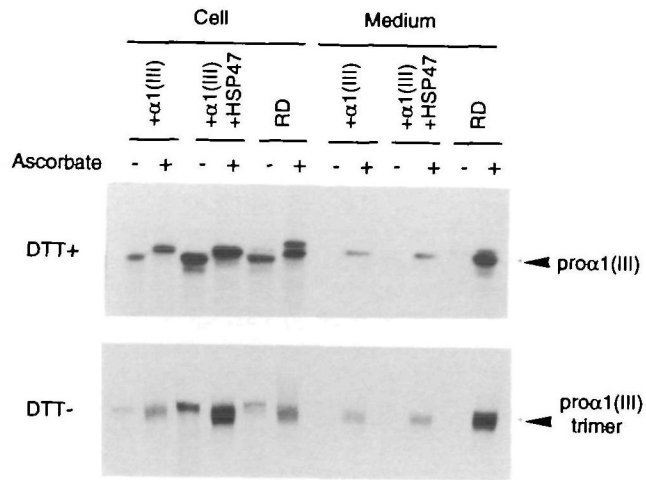

Fig. 2. **Immunoprecipitation of proα1(III) collagens synthesized in cells and secreted into the medium.** Cells were metabolically labeled as in Fig. 1, and both the cell lysate and medium were immunoprecipitated with anti-type III collagen antibodies. Samples with DTT (DTT+) and without DTT (DTT−) were separated by 8% SDS-PAGE and 5% SDS-PAGE gel, respectively.

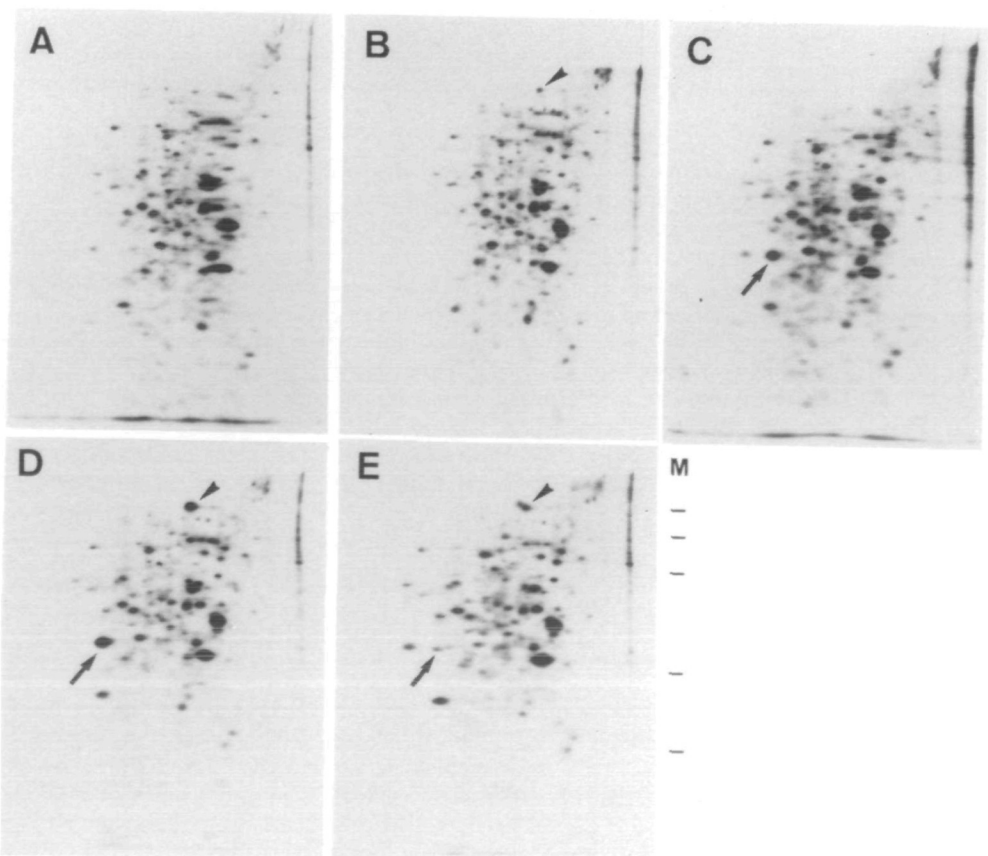

Fig. 1. **Two-dimensional gel electrophoresis.** Cells were metabolically labeled with [^{35}S]-methionine for 1 h as described under "MATERIALS AND METHODS," and newly synthesized proteins were analyzed by NEPHGE/10% SDS-PAGE under reducing conditions. Arrows indicate the position of HSP47, and arrowheads the proα1(III) collagen chain. A: 293 cells, B: 293+proα1(III) cells, C: 293+HSP47 cells, D: 293+proα1(III)+HSP47 cells, E: RD cells. The medium was supplemented with 136 μg/ml of ascorbic acid phosphate. M: prestained molecular weight standards (Bio-Rad., the sizes are 208, 144, 87, 44.1, and 32.7 kDa).

transfection of the proα1(III) collagen chain into 293 cells [293+proα1(III) cells, Fig. 1B].

Synthesis and Secretion of Type III Procollagens—Type III procollagens synthesized in cells and secreted into the medium were analyzed by immunoprecipitation using antibodies against type III collagen (Fig. 2). The molecular weight of proα1(III) collagen increases in the presence of ascorbic acid because of the full hydroxylation and the following glycosylation of the procollagen chain (Fig. 2, DTT+). Procollagen III was not secreted into the medium in the absence of ascorbic acid phosphate, and almost all the procollagen synthesized by both RD cells and 293 cells formed disulfide-linked trimers, which was confirmed by separation of the immunoprecipitates on a non-reducing 5% gel (Fig. 2, DTT−). Western blotting was performed using specific antibodies raised against type III collagen or against mouse HSP47. The proα1(III) chain was mainly detected in the 1% NP-40 soluble fraction in all the cell lines (data not shown). Following treatment of the cells with ascorbic acid phosphate, procollagens were secreted and almost undetectable in Western blots of cell lysates of type III collagen-transfected 293 cells and RD cells (Fig. 3).

Coprecipitation of the proα1(III) Chain and HSP47—We previously reported that HSP47 binds to purified type I, II, III, IV, and V collagens *in vitro* (*6*). The expression of HSP47, however, has been reported not to correlate with type III collagen expression in developing molars in mice (*27*). We examined the binding of HSP47 and type III procollagen within cells by immunoprecipitation after cross-linking of the cellular proteins with DSP (Fig. 4). In

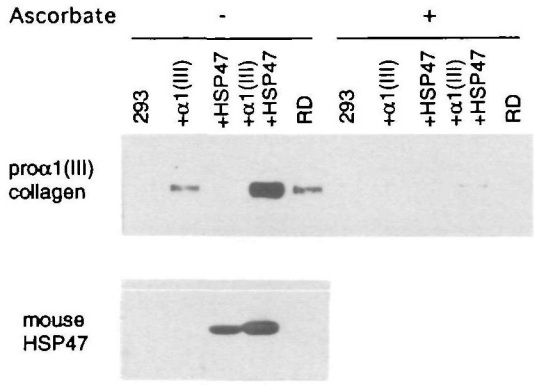

Fig. 3. **Western blot analysis.** Cells were lysed in buffer containing 1% NP-40, and the cell lysate was separated by 8% SDS-PAGE gel under reducing conditions. After blotting, type III collagens and mouse HSP47 were analyzed with specific antibodies. Anti-mouse HSP47 antibodies do not cross-react with human HSP47.

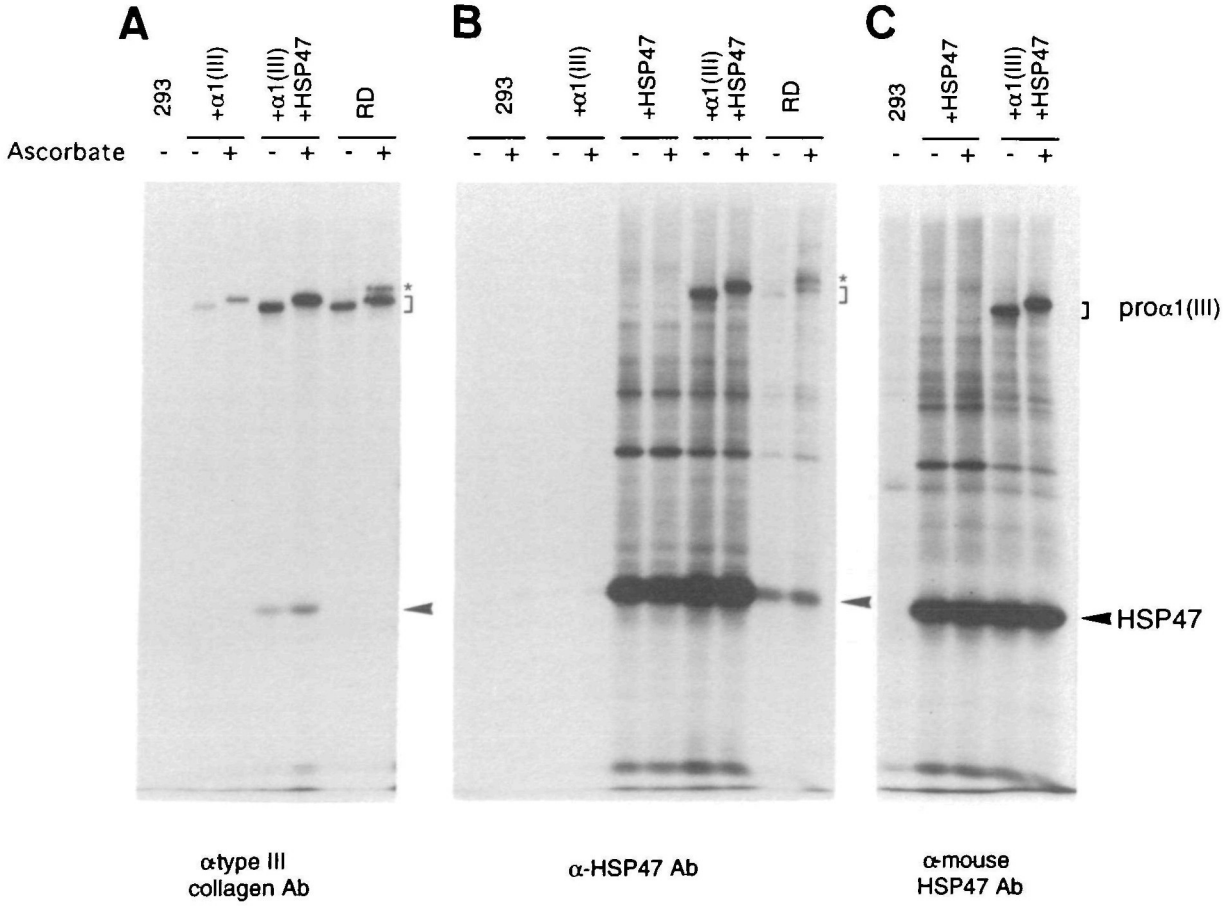

Fig. 4. **Coprecipitation of type III procollagens with HSP47 in cells.** Cells were metabolically labeled as described under "MATERIALS AND METHODS," and after cross-linking using DSP, cell lysates were immunoprecipitated with specific antibodies and then analyzed by 8% SDS-PAGE under reducing conditions. A: anti-type III collagen antibodies, B: anti-HSP47 antibodies, which recognize both human and mouse HSP47, C: anti-mouse HSP47 antibodies, which do not cross react with human HSP47. * shows the slower migrating band of RD cells coprecipitated with both anti-type III collagen antibodies and anti-HSP47 antibodies.

both RD cells, which naturally produce type III procollagen, and 293+proα1(III)+HSP47 cells, which harbor recombinant type III collagen and HSP47, the binding of HSP47 and the proα1(III) chain was confirmed using either anti-type III collagen or anti-HSP47 antibodies. Although the band of HSP47 coprecipitated with anti-type III collagen antibodies for RD cells is very faint in Fig. 4A, it was clearly detected in samples labeled for a longer period or detected on Western blotting (data not shown). This was due to the much lower labeling efficiency of HSP47 compared with that of procollagen, as we reported previously (*8*). Several other chaperone proteins in the ER have been reported to be coprecipitated with type I procollagens and HSP47 (*8, 28*). A comparison of 293+HSP47 cells with 293+proα1(III)+HSP47 cells revealed that most of the chaperone proteins, including GRP78 (BiP) and GRP94, were directly coprecipitated with HSP47 in the absence of collagen chains (Fig. 4; Hosokawa, N., *et al.*, unpublished observation). In RD cells, an additional band exhibiting slower electrophoretic mobility than that of the proα1(III) chain (Fig. 4, A and B, indicated by *) was coprecipitated with anti-type III collagen antibodies and with anti-HSP47 antibodies. We are now examining if this band represents another type of collagen chain or some other protein tightly associated with type III collagen.

Establishment of Other 293 Cell Lines Expressing Different Levels of Both the proα1(III) Collagen Chain and HSP47—Although the level of type III procollagen secreted into the medium during the 1 h labeling period was almost the same for 293+proα1(III) and 293+proα1(III)+HSP47 cells, the level of synthesis of the proα1(III) chain was higher in 293+proα1(III)+HSP47 cells than in 293+proα1(III) cells (Fig. 2). This observation suggests that the secretion of type III procollagen is delayed in 293+proα1(III)+HSP47 cells. The different levels of procollagen synthesis in these cell lines were confirmed by Northern blot analysis (Fig. 5). A pulse-label and chase experiment confirmed that the secretion of the proα1(III) collagen chain was slower in 293+proα1(III)+HSP47 cells than in 293+proα1(III) cells (data not shown).

To exclude the possibility that the delayed procollagen secretion in 293+proα1(III)+HSP47 cells was due to the higher level of proα1(III) chain expression, we selected other 293 cell lines expressing different levels of proα1(III) and HSP47 by Western blotting of cell lysates in the absence of ascorbic acid phosphate. D1 and D44 are cell lines expressing low and high levels of proα1(III) chains, respectively, without the transfection of recombinant HSP47. The M68 and M73 cell lines exhibit high levels of HSP47 expression with low and high levels of type III procollagen synthesis, respectively. Figure 6 shows the results of Western blot analysis of these newly selected 293 cell lines, in comparison to previous cell lines, and these lines were used for the following analyses. The levels of type III collagen in D1 and D44 cells were comparable to those in M68 and M73 cells, respectively. The addition of ascorbate caused the depletion of intracellular collagen in all of these cell lines. Densitometric analysis of Fig. 6 revealed that the relative amounts of type III procollagen in the cell lysates without ascorbate were 0.42, 1.30, 0.88, and 1.62 for D1, D44, M68, and M73 cells, respectively, when that for RD cells was taken as 1.00.

Secretion of Type III Collagen Was Delayed on Co-Expression of HSP47—The secretion of type III collagen was examined in these cell lines by means of pulse label and chase experiments combined with immunoprecipitation. In Fig. 7A, the kinetics of type III procollagen secretion by RD cells are shown in the presence or absence of ascorbic acid phosphate. Cells were pulse labeled with [^{35}S]methionine for 15 min and then chased for the indicated periods. When ascorbic acid phosphate was added to the medium, the proα1(III) chain was secreted into the medium for between 15 to 30 min of the chase period, which is consistent with a previous report (*29*). In the absence of ascorbic acid, the molecular size of the proα1(III) chain was observed to increase gradually on 8% SDS-PAGE , and only a trace

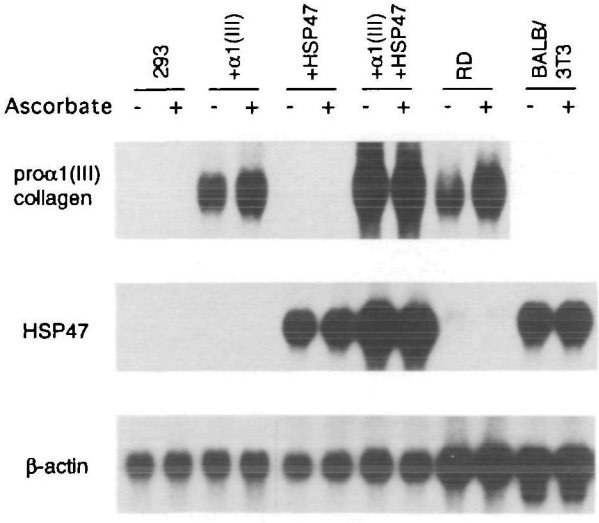

Fig. 5. **Northern blot analysis.** Cellular RNA was isolated by the AGPC method. Ten micrograms of total RNA was electrophoresed, and then hybridized with cDNA probes to detect proα1(III) collagen, HSP47, and β-actin.

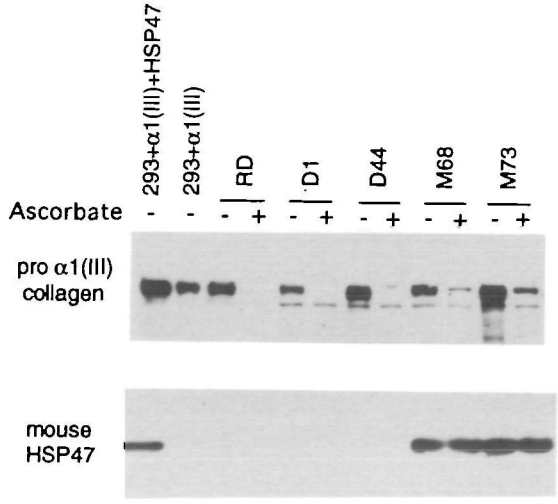

Fig. 6. **Western blot analysis of 293 cell lines expressing different levels of proα1(III) collagen chains.** Cell lysates were prepared and analyzed by 8% SDS-PAGE as in Fig. 3. 293 cell lines expressing different levels of both proα1(III) chains and HSP47 were selected. D1: low level of proα1(III) collagen expression, D44: high level of proα1(III), M68: low level of proα1(III) and high level of HSP47, M73: high levels of both proα1(III) and HSP47.

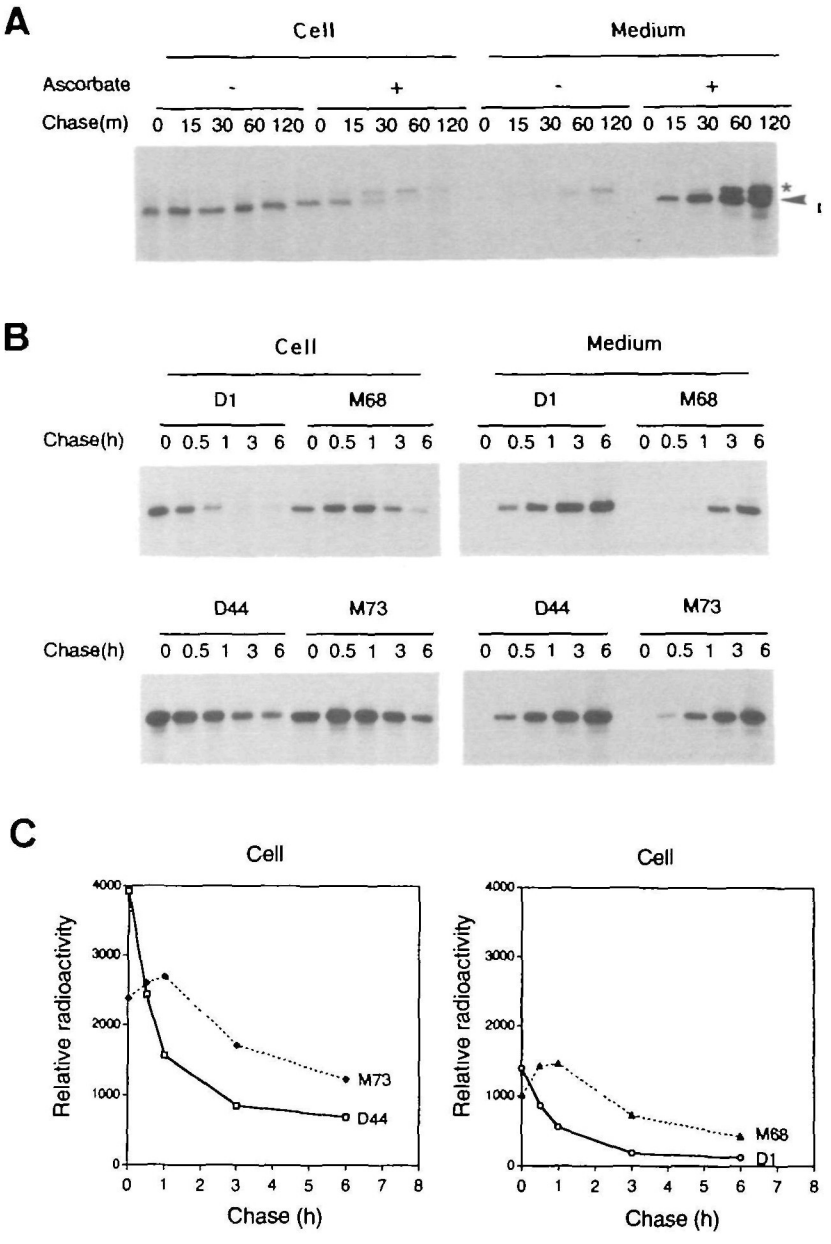

Fig. 7. **Kinetics of type III procollagen secretion.** Cells were pulse-labeled for 15 min for RD cells or 30 min for 293 cell lines, and then chased for the times indicated. Proα1(III) collagen chains in the cell lysates were detected by immunoprecipitation using specific antibodies. Procollagens secreted into the medium were analyzed by either immunoprecipitation in RD cells or TCA precipitation in 293 cell derivatives. Samples were subjected to 8% SDS-PAGE under reducing conditions. All the pulse-chase experiments were repeated two to four times. A: RD cells, B: 293+proα1(III) cells (D1 and D44), and 293+proα1(III)+HSP47 cells (M68 and M73), C: quantitative analysis of type III procollagen in cell lysates (Fig. 5B), using an image analyzer. * denotes the slower migrating band of RD cells, as in Fig. 4.

amount of procollagen was detected in the medium. For the 293 cell lines, cells were cultured in the presence of ascorbic acid, and were labeled for 30 min to determine the type III procollagens expressed at low levels in D1 and M68 cells. These cells were chased for a longer period than for RD cells. An autoradiogram of the pulse-chase experiment on D1, D44, M68, and M73 cells is shown in Fig. 7B. The relative radioactivity measured quantitatively with an image analyzer is shown in Fig. 7C. In both D1 and D44 cells, pulse-labeled procollagen III disappeared exponentially from the cells with an approximate $T_{1/2}$ of 1 h, and was rapidly secreted into the medium irrespective of the level of proα1(III) synthesis. On the contrary, in M68 and M73 cells, in which recombinant HSP47 is overexpressed, procollagen secretion was much more delayed and the $T_{1/2}$ of secretion was about 6 h, including a lag period in the initial phase of the pulse/chase period.

Enzyme Digestion of Radiolabeled Procollagens—To determine if there are differences in the type III procollagens synthesized by RD cells and those synthesized by the 293 cell lines, procollagens secreted into the medium were digested with trypsin/chymotrypsin or pepsin. Differences in the electrophoretic mobility were seen on 8% SDS-PAGE after digestion with either trypsin/chymotrypsin or pepsin (Fig. 8, C, D, and E). The α1(III) collagen chain secreted by 293+proα1(III) cells (D1 and D44 cells) migrated slightly slower than normal type III collagen from RD cells, while the electrophoretic mobility of the α1(III) chain in 293+proα1(III)+HSP47 cells (M68 and M73 cells) was almost the same as that in RD cells. Differences in electrophoretic mobility of α1(III) procollagen chains were similarly observed for intracellular collagen (Fig. 8A, 8% SDS-PAGE reducing gel) and for secreted procollagen (Fig. 8B, 5% reducing gel), in the absence of protease digestion. These data suggest that overmodification of type III collagen occurred in 293+proα1(III) cells, whereas the modification

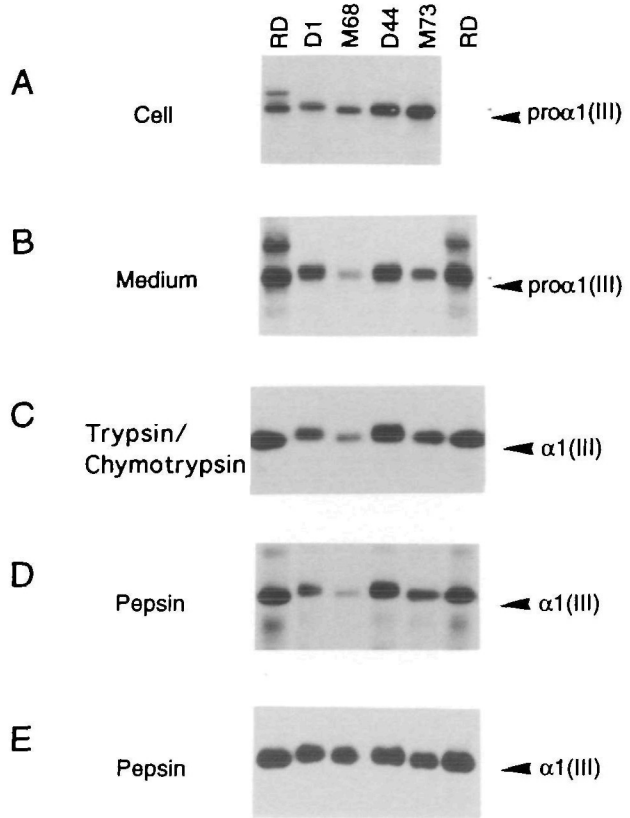

Fig. 8. **Different electrophoretic mobilities of type III procollagens.** Cells were metabolically labeled for 3 h and then lysed in a buffer containing 1% NP-40. Samples containing the same TCA-insoluble radioactivity were used (see "MATERIALS AND METHODS"). A: Cellular proα1(III) chains immunoprecipitated with specific antibodies, B: type III procollagen in the medium precipitated with TCA, C: trypsin/chymotrypsin-resistant collagen chains in the medium, D: pepsin digestion of the procollagens secreted into the medium, E: pepsin digestion of secreted procollagens, same as in panel D except that the samples were loaded so that radioactivity almost equal to that of pepsin-resistant type III collagen band was obtained. Samples were subjected to 8% SDS-PAGE reducing gel, and were run for longer than usual; the samples in panel B were analyzed with a 5% reducing gel.

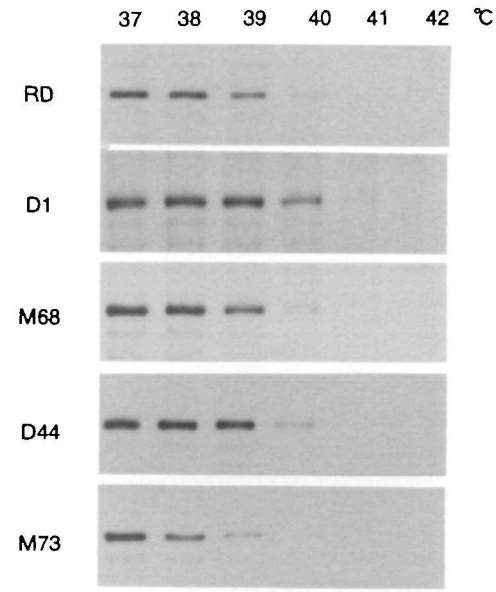

Fig. 9. **Thermal stability of type III procollagens.** T_m was analyzed as described under "MATERIALS AND METHODS." After trypsin/chymotrypsin digestion, samples were analyzed by 8% SDS-PAGE under reducing conditions, and an autoradiogram of the α1(III) collagen chain is shown.

in 293+proα1(III)+HSP47 cells was similar to that observed in RD cells. There was no significant difference in the percentage of trypsin- or pepsin-resistant collagen as to the total type III procollagen secreted into the medium between 293 transfectants and RD cells (data not shown).

Thermal Stability of the Type III Procollagen—The differences in procollagen modification described above may affect the triple helix conformation of procollagen. Thus, the thermal stability of each procollagen was examined using a thermal cycler as described previously (*24*). The melting temperature (T_m) of type III procollagen secreted into the medium of RD cells was 39.5°C, in agreement with a previous report (*30*). Although the procollagen III secreted from 293+proα1(III)+HSP47 cells (M63 and M73) showed almost the same T_m value as that from RD cells, the procollagen from 293+proα1(III) cells (D1 and D44) exhibited a melting temperature of approximately 1°C higher (Fig. 9). These data also support the hypothesis that posttranslational overmodification of procollagens occurs in 293+proα1(III) cells and that the modification in 293+proα1(III)+HSP47 cells is normal, as seen in RD cells.

DISCUSSION

293 cells are characterized by a complete absence of ECM proteins, including collagens (*11*). This cell line was confirmed to produce an almost undetectable level of HSP47 (Fig. 1A). We used this cell line as a model to examine the procollagen biosynthesis and to elucidate the function of HSP47 in this process. We found that the trimer formation of proα1(III) collagen chains in 293 cells is delayed in comparison to that in RD cells, which synthesize type III procollagens normally (Hohenadl, C., Kühn, K., *et al.*, manuscript in preparation). This delay in trimer formation is essentially the same in the presence and absence of recombinant HSP47, a postulated molecular chaperone for procollagen biosynthesis in the ER. We also found that 293 cells synthesize and secrete type III procollagen in a trimer form, which is trypsin/chymotrypsin-resistant (Figs. 2 and 8), and that 293 cells exhibit prolyl hydroxylase activity (Hohenadl, C., Kühn, K., *et al.*, manuscript in preparation). In this study, we established several 293 cell lines expressing different levels of proα1(III) and HSP47, to examine the effect of HSP47 on procollagen biosynthesis in detail. The extracellular secretion of type III procollagen by these cells was found to be delayed if recombinant HSP47 was coexpressed (Fig. 7, B and C). The secretion of proα1(III) collagen was more delayed in 293+proα1(III) cells than in RD cells, and overexpression of HSP47 further inhibited this secretion. As we reported previously for type I procollagens, HSP47 also binds with type III procollagens within the ER. The delay in secretion in the presence of HSP47 may be due to the transient binding of HSP47 with

newly synthesized proα1(III) collagen chains in the ER (Fig. 4).

To determine the physiological significance of the coexpression of HSP47 in procollagen biosynthesis, we analyzed the proα1(III) collagens synthesized by these cell lines. The type III procollagen of 293+proα1(III) cells showed slightly slower electrophoretic mobility than that of normal procollagen III of RD cells. This is consistent with the observation that procollagens retained in the ER for longer periods than usual are overmodified, which is in many cases due to overhydroxylation (*31*). Although we have not analyzed the amino acid composition for the detection of hydroxylation, it is conceivable that the type III procollagen in 293+proα1(III) cells is overhydroxylated. The slightly higher T_m of type III procollagen in 293+proα1(III) cells than in RD cells also supports this suggested overmodification. When HSP47 is coexpressed in 293 cells, the electrophoretic mobility and the T_m of type III procollagen are almost the same as those seen in RD cells.

Collectively, these results suggest that type III procollagens are overmodified in 293+proα1(III) cells but not in 293+proα1(III)+HSP47 cells. Overmodification of transfected type III procollagens is inhibited when HSP47 is coexpressed. One possible role of HSP47 deduced from these experiments is its involvement in the proper modification of procollagen chains. The level of HSP47 expressed in 293 cells was very low, and we did not detect the upregulation of HSP47 in the proα1(III)-transfected 293 cell lines. The expression of recombinant HSP47 in 293 cells was much higher in comparison to intrinsic HSP47 expression in RD cells, but expression is likely within the physiological range because the level of transfected HSP47 expression was similar to that in mouse BALB/3T3 cells (Fig. 5). The ratio of HSP47 to procollagens may be important, and more HSP47 would be necessary in 293 cell lines than RD cells because larger amounts of procollagens are retained in the ER due to delayed secretion. Thus, HSP47 may regulate the modification of procollagens in the ER through regulation of the accessibility of collagen-modification enzymes to newly synthesized procollagen chains. Some factor(s) other than HSP47 may be required in 293 cell lines to assist in the rapid synthesis and secretion of correctly aligned type III procollagen triple helices, as observed in RD cells, which normally secrete type III collagen. These 293 cell lines would be useful as a model for further analysis of the involvement of these factors in the process of type III procollagen biosynthesis.

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