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Extracellular Matrix 22-kDa Protein Interacts with Decorin Core Protein and Is Expressed in Cutaneous Fibrosis[1]

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A protein with an M_r of 22,000 was purified from bovine dermis. The amino acid sequence of the protein was identical to 22-kDa protein [Neame, P.J. *et al.* (1989) *J. Biol. Chem.* 264, 5474-5479] and showed highly homologous sequences to TRAMP (tyrosine rich acidic matrix protein) [Cronshaw, A.D. *et al.* (1993) *Matrix* 13, 255-266] and dermatopontin [Superti-Furga, A. *et al.* (1993) *Genomics* 17, 463-467]. The protein was proved to associate with decorin and a modified decorin with carboxymethylated cysteinyl residues, but not to assemble to hyaluronate or dermatan sulfate chains. The pyridylethylation of cysteinyl residues in the 22-kDa protein did not affect its binding activity to decorin or modified decorin. Immunohistochemical analyses revealed positive stains in endothelial cells and the periphery of collagen fibers in normal dermis but not in the fibroblasts in tissue. Collagen fibers in sclerotic regions of progressive systemic sclerosis were stained diffusely, suggesting that the 22-kDa protein increases in parallel to the accumulation of collagen in the disease. Western blotting analyses of extracts of cultured endothelial cells revealed a lower M_r protein than that from cultured fibroblasts, suggesting the presence of a molecule related to the 22-kDa protein.

Key words: decorin, dermatopontin, PSS, TRAMP, 22 kDa protein.

Extracellular matrix (ECM) plays essential roles in morphogenesis, cell differentiation, tissue repair, and cell migration. Some of the macromolecules in ECM are capable of interacting with other components during the formation of supra-molecular aggregates, and the complexes exhibit tissue-specific functions. Such aggregates are found in basement membranes (basal lamina) and in cartilage matrixes. The network polymers are covalently stabilized through binding sites to the components of basal lamina, *e.g.*, nidogen, laminin, perlecan, and type IV collagen. They are essential in maintaining tissue architecture, and function as barriers between mesenchymal tissue and epithelial compartments and as ligands for cellular receptors (*1*). Noncovalent bonds among proteochondroitin sulfate (aggrecan), hyaluronate, and link protein lead to the development in the cartilage ground substance of proteoglycan aggregates, which play important roles in maintaining the elasticity of joints on movement (*2*).

Recently, new ECM components including new types of collagens have been isolated and their biological functions demonstrated (*3-10*). In the matrix of the skin, large molecules such as collagen, elastin, or proteoglycan have been well characterized, but there remain several small molecules whose functions are poorly known. It is likely that these molecules are involved in biologically important events such as composing supra-molecular architectures or signal transduction between ECM components including cell signaling. Hence, we have tried to clarify the entity and the function of these poorly characterized components.

During the purification of low molecular ECM components, we found a decorin-associating protein that was identical to 22-kDa protein (*11*), TRAMP (*12*), and highly homologous to dermatopontin (*13*) based on the amino acid sequences. The protein is present in a considerable amount on a molar basis. In this paper, we describe a simple and improved method for isolation of the 22-kDa protein and demonstrate directly the interaction of the protein and decorin by density gradient centrifugation. We also demonstrate for the first time the pattern of localization of the 22-kDa protein in normal and diseased skin using antibodies against a synthetic carboxyl terminal peptide of the protein.

MATERIALS AND METHODS

Materials—Gel-filtration columns, TSKgel Toyopearl HW55F (50×600 mm) and TSKgel G3000 SW_{XL} (7.8×600 mm), and an anion-exchange column, TSKgel DEAE-Toyopearl (22×200 mm), were from Tosoh (Tokyo). Reverse-phase columns, COSMOSIL 5C18 AR (20×250

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Abbreviations: CB, cyanogen bromide; DMEM, Dulbecco's modified Eagle medium; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TRAMP, tyrosine rich acidic matrix protein.

mm and 4.6×250 mm), were from Nacalai Tesque (Kyoto). Pyridylethylating reagents, 4-vinyl pyridine and tri-*n*-butyl phosphine, were from Wako Pure Chemical (Osaka). TPCK-treated trypsin, lysyl endopeptidase, V8 protease, and pyroglutamyl peptidase were from Worthington Biochemical (Freehold, NJ), Wako Pure Chemical, Miles (Kankakee, IL), Toyobo (Tokyo), respectively. Lactoperoxidase (from bovine milk) and keyhole limpet hemocyanin (KLH) were supplied by Calbiochem (LaJolla, CA). Bovine dermal decorin and carboxymethylated decorin were purified and prepared as reported previously (*14*). A carboxyl terminal peptide of 22-kDa protein, RMTDY-DCEFANV, was synthesized using Fmoc derivatives. DMEM and fetal bovine serum were purchased from Gibco (Grand Island, NY) and JRH Biosciences (Lenexa, KS), respectively. FITC-conjugated Fab fragment of goat anti-rabbit IgG was from Cappel (Durham, NC), and GEL/MOUNT™ was from Biomeda (Foster City, CA). Human umbilical cord hyaluronate (M_r 3.5×10^5) and dermatan sulfate were from Seikagaku-Kogyo (Tokyo). $Na^{125}I$ was supplied by Amersham (Buckinghamshire, UK).

Purification of 22-kDa Protein—Newborn calf dermis (2 kg) obtained from a local slaughterhouse was homogenized and extracted with 50 mM Tris-HCl buffer, pH 7.5, containing 4 M guanidine HCl at 4°C. The homogenates were centrifuged at 100,000×*g* and the supernatants were desalted by dialysis against deionized water, lyophilized, and finally dissolved in 100 mM Tris-acetic acid buffer, pH 8.1, containing 6 M urea and 0.1 M NaCl. The sample was filtered on a column of TSK gel Toyopearl HW 55F (50×600 mm) equilibrated with the same buffer. Fractions of 10.5 ml were collected at flow rate of 5 ml/min. The absorbance at 280 nm was recorded. Fractions with K_{av} = 0.5-0.7 were diluted with three volumes of 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and applied to an anion-exchange column of TSK gel DEAE-Toyopearl (22×200 mm), which was eluted with a linear gradient from 0.1 to 1.0 M NaCl. Fractions eluted at approximately 0.7 M NaCl were applied to reverse-phase HPLC with a COSMOSIL 5C18 AR column (20×250 mm) equilibrated with 10 mM Tris-HCl buffer, pH 7.5 (neutral reverse-phase HPLC). Proteins were eluted with a linear gradient from 25 to 50% acetonitrile. Fractions eluted at around 35% acetonitrile were rechromatographed using the same column equilibrated with 0.1% TFA (acidic reverse-phase HPLC). And the column was eluted with a linear gradient from 20 to 50% acetonitrile over 80 min at a flow rate of 9.9 ml/min. Fractions of 9.9 ml were collected and the absorbance at 210 nm was monitored. Finally the fractions eluted at 35-38% acetonitrile were collected. The gel-filtration and the anion-exchange chromatographies were performed at room temperature, and the reverse-phase HPLC was carried out at 40°C. Proteins were visualized with Coomassie Brilliant Blue R250 after Tricine SDS-polyacrylamide gel (16.5% T, 3% C) electrophoresis (PAGE) (*15*).

Western Blotting—Proteins separated on SDS-PAGE gel were electroblotted onto an Immobilon™ PVDF membrane (Millipore, Bedford, MA) according to the method of Towbin *et al.* (*16*).

Enzymatic and Chemical Digestion—The purified protein was pyridylethylated (*17*). The pyridylethylated protein (1 mg) was digested with 15 μg of TPCK-trypsin in 50 mM HEPES buffer, pH 8.0, for 4 h, or with 20 μg of V8 protease in 0.1 M ammonium bicarbonate buffer, pH 7.8, for 16 h at 37°C. A 100-μg sample of the protein was digested with 6 μg of lysyl endopeptidase in 50 mM Tris-HCl buffer, pH 9.0, for 16 h at 37°C. A 500-μg sample of the protein dissolved in 0.1 M phosphate buffer, pH 8.0, containing 5 mM dithiothreitol and 10 mM EDTA was incubated with 12 mU of pyroglutamyl peptidase at 37°C for 4 h. To obtain CB peptides, 500 μg of the protein was cleaved by 0.1% CB in 70% formic acid for 20 h at 4°C in the dark.

Separation of Fragments—Peptides generated by enzymatic digestion or CB peptides were separated on a COSMOSIL 5C18 AR column (4.6×250 mm) with a linear gradient from 0 to 60% acetonitrile over 100 min at 40°C at a flow rate of 1 ml/min.

Amino Acid Sequencing—The amino acid sequences of the protein and its fragments were determined with a pulse-liquid phase sequenator (ABI 477A/120A, Applied Biosystems, Foster City, CA).

Antibody Production against a Carboxyl Terminal Peptide—A synthetic carboxyl terminal peptide (RMTDY-DCEFANV) (10 mg) was conjugated with KLH (2.5 mg) in 50 mM phosphate buffer, pH 7.0, containing 10 mM glutaraldehyde for 5 h at 37°C. The mixture was dialyzed against 0.1 M phosphate buffer, pH 7.2. The KLH-conjugated peptide was injected to New Zealand White rabbits subcutaneously five times at intervals of 2 weeks. The titer of the antibodies was measured by dot immunoblotting onto the transfer membrane.

Cell Culture—Human dermal fibroblasts from normal individuals and endothelial cells from porcine aorta and bovine pulmonary artery were cultured on cover glasses in DMEM supplemented with 10% fetal bovine serum, 100 μg/ml ascorbate, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37°C under humidified 5% CO_2-95% air.

Extraction of Cultured Fibroblasts and Endothelial Cells—Cultured human dermal fibroblasts and bovine pulmonary arterial endothelial cells were extracted with 50 mM Tris-HCl buffer, pH 7.5, containing 6 M urea and 0.1 M NaCl. The extracts were chromatographed on a gel-filtration column of TSKgel G3000 SW_{XL} (7.8×600 mm) equilibrated with the same buffer. Fractions with K_{av} = 0.5-0.7 were separated by Tricine SDS-PAGE and Western blotted as described, then immunostained with anti-synthetic peptide antibodies.

Cytochemistry—Cells cultured on cover glasses were fixed with absolute methanol for 10 min and rinsed in PBS. The antibodies against the carboxyl terminal peptide of 22-kDa protein were incubated for 30 min at room temperature. The nonspecific reaction was blocked by treatment with PBS containing 1% skim milk for 30 min before incubation. After washing with PBS, FITC-conjugated goat anti-rabbit IgG in PBS containing 1% skim milk was incubated at room temperature for 30 min. The specimen was finally mounted in GEL/MOUNT™ for microscopic observation.

Immunohistochemistry—Skin biopsy specimens were taken from normal individuals and the sclerotic lesions of patients with progressive systemic sclerosis (PSS), which is characterized by dermal sclerosis due to an increase of collagen and abnormally thickened fibers. Formalin-fixed and paraffin-embedded sections were sliced in 3 μm sections and deparaffinized. After blocking internal peroxidase

activity with 0.3% hydrogen peroxide in methanol, the sections were rinsed in PBS for 30 min. To prevent non-specific binding of rabbit immunoglobulins, PBS containing 1% skim milk was used. The sections were incubated with the anti-peptide serum diluted 100-fold with PBS containing 1% skim milk. After washing with PBS, the sections were colored with an alkaline phosphatase kit in StrAviGen® MULTILINK IMMUNODETECTION SYSTEM according to a manufacturer's protocol.

Radioiodination of 22-kDa Protein—A 20-μg sample of the protein was labeled with 0.5 mCi of $Na^{125}I$ by the lactoperoxidase method (*18*). Free iodine was removed by passage through a TSK-Octadecyl 4PW column (4.6×10 mm), and the labeled protein was purified on a TSK-Octadecyl 4PW column (4×30 mm). The labeled protein was checked for purity by Tricine SDS-PAGE, then subjected to analysis with a BAS-2000 Bio·Imaging Analyzer (FUJIX, Tokyo).

Interaction of 22-kDa Protein with Decorin, Hyaluronate, or Dermatan Sulfate Chain—The radiolabeled protein (~1×10^6 cpm) was incubated for 30 min at room temperature with decorin, hyaluronate, or dermatan sulfate chain under associative conditions in 0.15 M potassium acetate buffer, pH 6.3, or dissociative conditions in the presence of 4 M guanidine-HCl in the same buffer. Total volumes and densities were adjusted to 5.4 ml and 1.46 g/ml by addition of CsCl, respectively, giving final concentrations of decorin, hyaluronate, and dermatan sulfate chain of 92.6, 370, and 18.5 μg/ml, respectively. To analyze the binding ability of the modified proteins, cysteinyl residues of decorin and the radiolabeled 22-kDa protein were carboxymethylated and pyridylethylated, respectively. After ultracentrifugation at 150,000×g for 48 h at 15°C, aliquots of the samples were collected from the bottom of the tube and separated into nine fractions designated A1 to A9 (in the case of association) or D1 to D9 (in the case of dissociation).

RESULTS

Purification of 22-kDa Protein—SDS-PAGE after gel-filtration of crude extracts from bovine dermis revealed that the protein with an M_r of 22,000 was rich in the relatively low molecular weight proteins (K_{av}=0.5–0.7). The fractions containing the 22-kDa protein were further separated by anion-exchange and reverse-phase chromatographies as shown in Figs. 1 and 2. The final yield was about 50 mg from 2 kg wet weight of calf dermis. The purification of 22-kDa protein is summarized in Table I. The final purity of the material was checked by Tricine SDS-PAGE. Even when the gel was overloaded, no contaminating protein bands appeared; only the single band at M_r 22,000 and its dimer at M_r 44,000 were seen (Fig. 2B). The purified protein thus obtained was used for subsequent investigations.

Amino Acid Sequencing—The pyridylethylated protein was digested with three proteases and CB. The amino acid sequences of the fragments were determined as described in "MATERIALS AND METHODS." Twelve peptide peaks were obtained in peptide mapping of TPCK-trypsin fragments and the sequences are shown in Table II. Homology search revealed that these sequences are identical with those of a 22-kDa protein from bovine dermis (*11*) and with a partial sequence of TRAMP from porcine skin (*12*), and homologous to the deduced amino acid sequence from cDNA of dermatopontin from human fibrosarcoma (*13*). No amino acid sequence was given by direct sequencing of the undigested protein, but a definite sequence was obtained

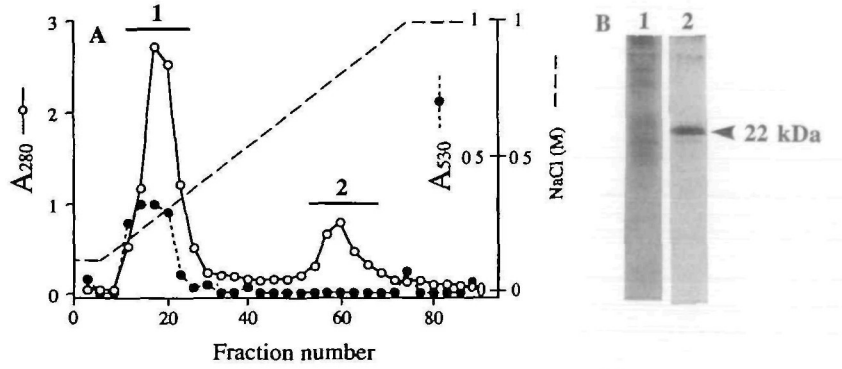

Fig 1 **TSKgel DEAE-Toyopearl anion-exchange chromatography of the pool from the gel-filtration chromatography.** A. A column of TSKgel DEAE-Toyopearl was eluted with a linear gradient from 0.1 to 1.0 M NaCl over 160 min at a flow rate of 5 ml/min Fractions of 10 ml were collected. The absorbance at 280 nm was recorded and uronate was monitored at 530 nm B: Lanes 1 and 2 the fractions indicated by bars in A were pooled and electrophoresed on 16 5% T, 3% C Tricine SDS-PAGE.

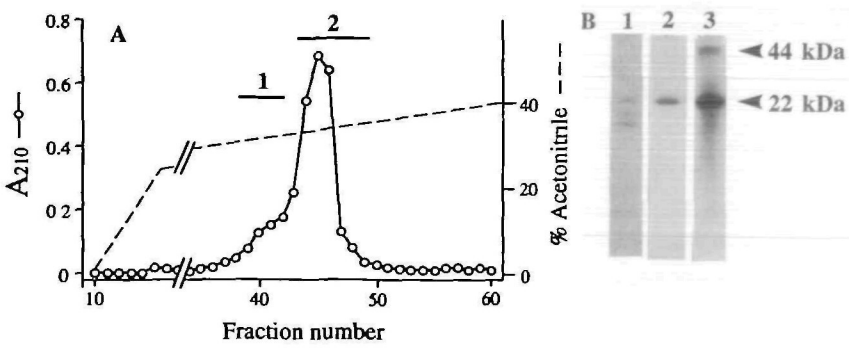

Fig. 2 **Purification of the M_r 22,000 protein with a neutral reverse-phase HPLC column** A· The pooled fraction indicated by bar 2 in Fig. 1A was chromatographed on a reverse-phase COSMOSIL 5C18 AR column. The column was eluted with a linear gradient of 25–50% acetonitrile over 80 min at a flow rate of 5 ml/min. Fractions of 5 ml were collected and the absorbance at 210 nm was recorded. B: Lanes 1 and 2: the fractions indicated by bars in A were pooled and electrophoresed as in Fig. 1; lane 3: 25 μg of the pooled fraction indicated by bar 2 in A was electrophoresed on the same gel to check purity.

after digestion of the protein with pyroglutamyl peptidase. These results indicated that the amino terminus of the purified protein was pyroglutamic acid. This and the amino acid sequences obtained by lysyl endopeptidase, V8 protease, and CB, confirmed that the purified protein was identical to the 22-kDa protein. The alignment of the peptides generated by digestion with these reagents is shown in Fig. 3, in comparison with the two related proteins described above.

Interaction of 22-kDa Protein and Proteoglycans—To confirm the binding of the 22-kDa protein and decorin, we used the method of CsCl density gradient centrifugation under associative and dissociative conditions. Before the experiment, we analyzed the purity of the radiolabeled 22-kDa protein by Tricine SDS-PAGE followed by the image analysis with BAS 2000. The labeled protein showed a single but broadened band at a position slightly larger than 22 kDa (Fig. 4).

The radioactivity of the 22-kDa protein was mostly recovered in the low buoyancy fraction (A9) in the absence of decorin under associative conditions (Fig. 5A), while in the presence of decorin, the main radioactive peak shifted to the middle of the density gradient ($\rho = 1.4$-1.5 g/ml), which corresponds to the location of decorin alone. The displacement of the radioactivity indicated the interaction of the protein and decorin. When centrifuged under dissociative conditions, the main peaks derived from the radiolabeled protein and decorin separated into D9 and D5, respectively (data not shown). On the other hand, hyaluronate and dermatan sulfate chain clearly separated from the radiolabeled 22-kDa protein and did not show the association with the protein even under associative conditions (Fig. 5, B and C).

TABLE I. **Purification of 22-kDa protein.**

Steps	Fraction	Content of 22-kDa protein in the fraction (%)
Gel-filtration	$K_{av} = 0.5$-0.7	20
Anion-exchange	~0.7 M NaCl	80
Neutral reverse-phase HPLC	~35% acetonitrile	~100
Acidic reverse-phase HPLC	35-40% acetonitrile	~100

TABLE II **Amino acid sequences of peptides generated by digestion of pyridylethylated 22-kDa protein with TPCK-trypsin.**

Peptide peaks generated by digestion with trypsin	Sequence[a]
T1	QWK
T2	DRQWK
T3	RCPYSCW
T4	SIFNK
T5	GATTTFSAVER
T6	FIMCR
T7	QGFSYQCPHGQVVVAVR
T8	MTDYDCEFANV
T9	AGMEWYQTCSNNGLVAGFQSR
T10	YFESVLDREWQFYCCR
T11	CPYSCWLTTEYPGHYGEEMDMISY-NYDYYMR
T12	QWNYACMPTPQSLGEPTECWWEEI-NR

[a]The amino acid residues are given by the one-letter code.

To clarify the structural prerequisites for the interaction of the 22-kDa protein and decorin, disulfide bonds in these molecules were cleaved by pyridylethylation and carboxymethylation, respectively, and their interactions were investigated. The radioactivity of the 22-kDa protein in the presence of the carboxymethylated decorin was recovered in fractions A4-A7, while in the absence of the modified decorin, the radioactive peak of the 22-kDa protein accumulated in the low buoyancy fraction (A9) (Fig. 6A). On the other hand, in the presence of the intact or the carboxymethylated decorin, the radioactive peaks of the pyridylethylated 22-kDa protein moved to fractions A5-A7 and A4-A6, respectively, in comparison with those of modified 22-kDa protein alone (Fig. 6, B and C). These results indicated that decorin and 22-kDa protein, whether intact or with modified cysteinyl residues, interact with each other.

Cytochemistry and Immunohistochemistry—The distribution of the 22-kDa protein was immunohistochemically investigated using antibodies against the synthetic carboxyl terminal peptide. In the normal human skin specimen,

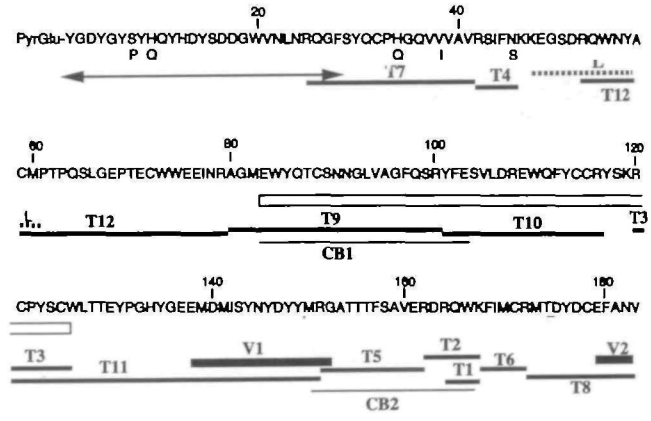

Fig. 3. **Amino acid sequence of the M_r 22,000 protein, and peptide alignment of the protein in comparison with TRAMP and dermatopontin.** Top: 22-kDa protein reported by Neame *et al.* (*11*). Middle: dermatopontin (*13*). Amino acid sequences identical to those of the 22-kDa protein are omitted. Empty bar indicates the sequence of TRAMP (*12*). Bottom: peptide alignment of the 22-kDa protein. V, L, T, and CB indicate digestion products with V8 protease, lysyl endopeptidase, TPCK-trypsin, and CB peptides, respectively. Sequences given by pyroglutamyl peptidase digestion are shown by a bar with arrowheads.

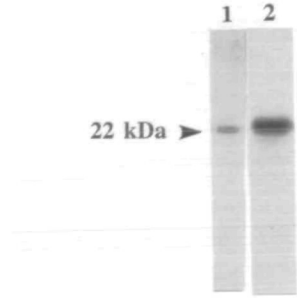

Fig. 4 **Purity check of the ^{125}I-labeled 22-kDa protein.** Lane 1: Tricine SDS-PAGE pattern of unlabeled 22-kDa protein stained with Coomassie Brilliant Blue R250. Lane 2: Pattern of the labeled 22-kDa protein in the same gel analyzed as described in the text.

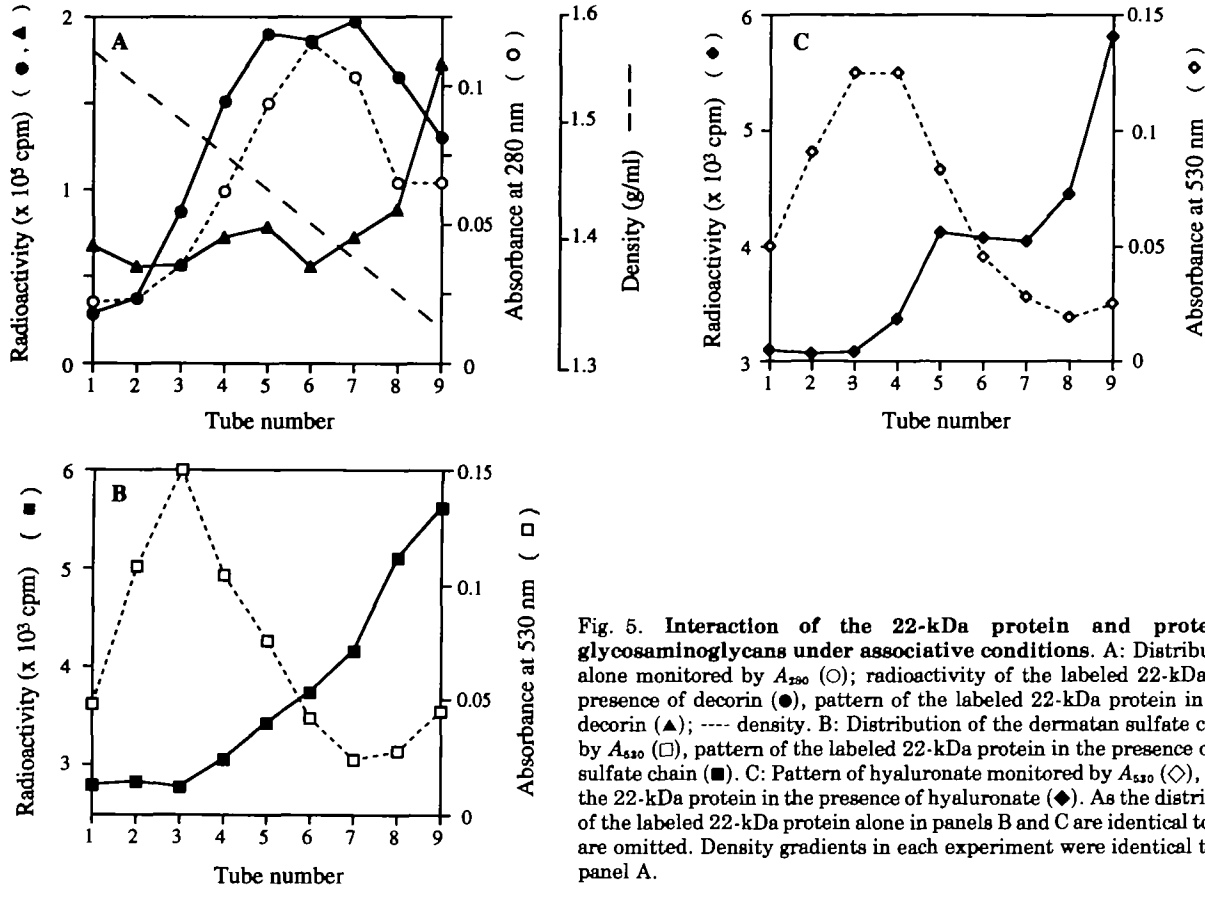

Fig. 5. **Interaction of the 22-kDa protein and proteoglycans or glycosaminoglycans under associative conditions.** A: Distribution of decorin alone monitored by A_{280} (○); radioactivity of the labeled 22-kDa protein in the presence of decorin (●), pattern of the labeled 22-kDa protein in the absence of decorin (▲); ---- density. B: Distribution of the dermatan sulfate chain monitored by A_{530} (□), pattern of the labeled 22-kDa protein in the presence of the dermatan sulfate chain (■). C: Pattern of hyaluronate monitored by A_{530} (◇), radioactivity of the 22-kDa protein in the presence of hyaluronate (◆). As the distribution patterns of the labeled 22-kDa protein alone in panels B and C are identical to that in A, they are omitted. Density gradients in each experiment were identical to that shown in panel A.

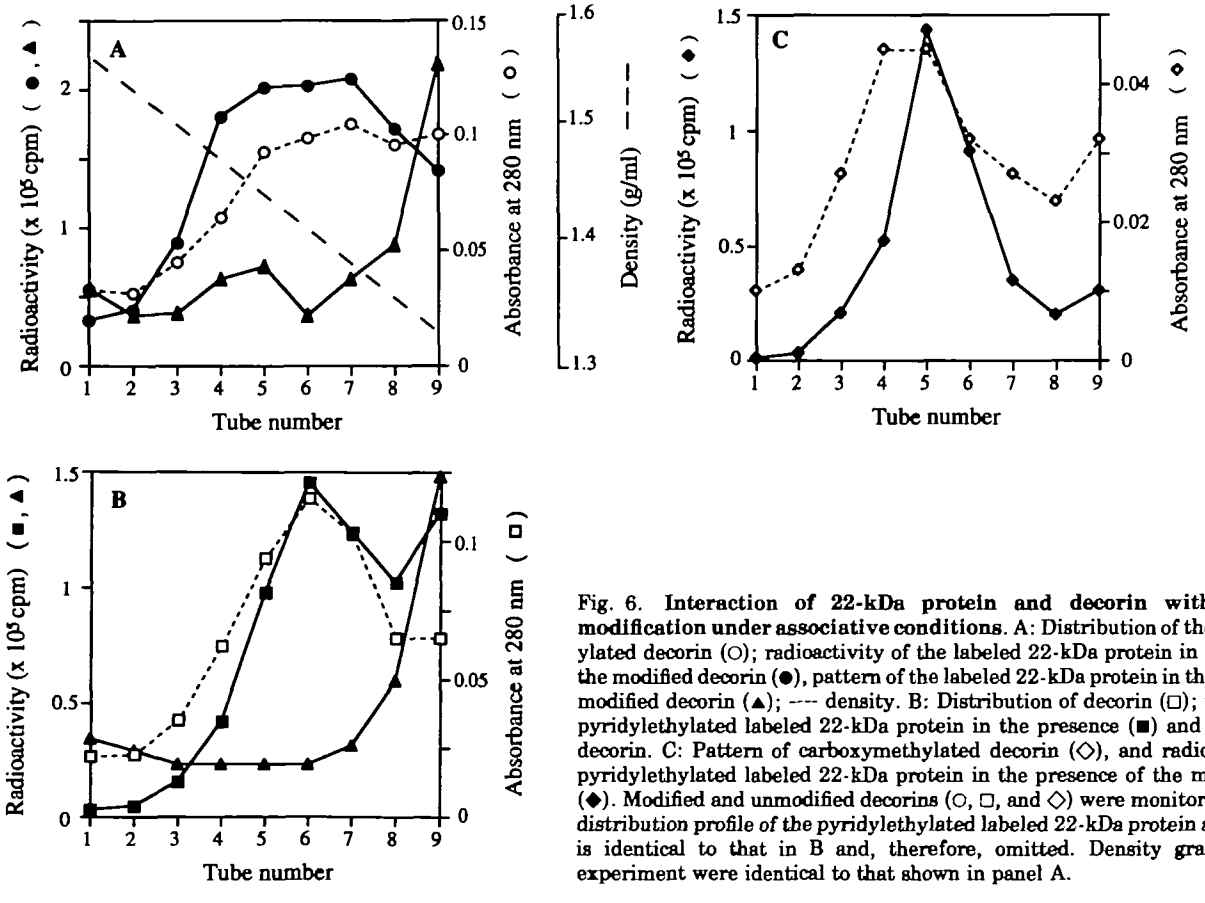

Fig. 6. **Interaction of 22-kDa protein and decorin with or without modification under associative conditions.** A: Distribution of the carboxymethylated decorin (○); radioactivity of the labeled 22-kDa protein in the presence of the modified decorin (●), pattern of the labeled 22-kDa protein in the absence of the modified decorin (▲); ---- density. B: Distribution of decorin (□); radioactivity of pyridylethylated labeled 22-kDa protein in the presence (■) and absence (▲) of decorin. C: Pattern of carboxymethylated decorin (◇), and radioactivity of the pyridylethylated labeled 22-kDa protein in the presence of the modified decorin (◆). Modified and unmodified decorins (○, □, and ◇) were monitored by A_{280}. The distribution profile of the pyridylethylated labeled 22-kDa protein alone in panel C is identical to that in B and, therefore, omitted. Density gradients in each experiment were identical to that shown in panel A.

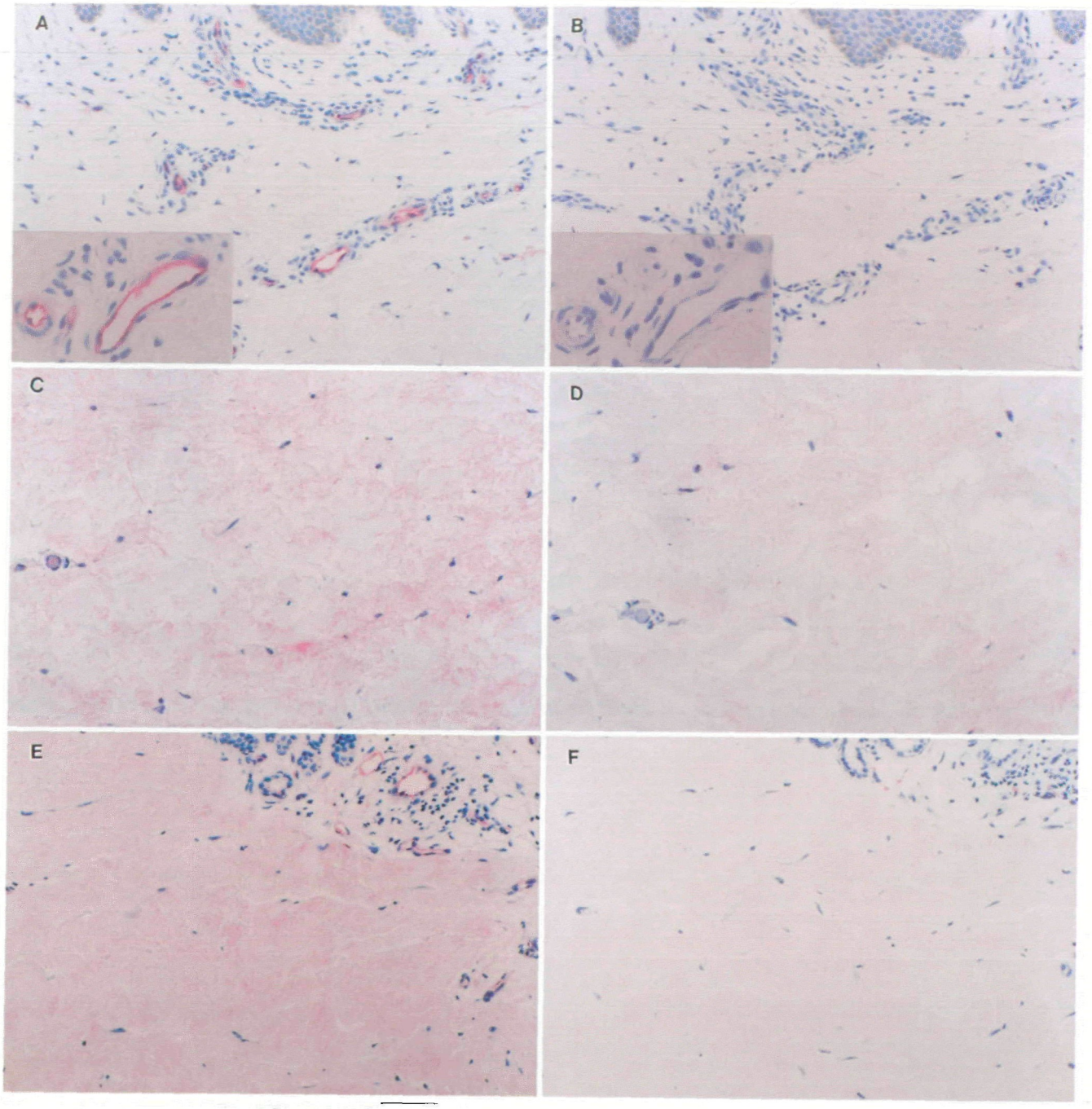

Fig. 7. **The distribution of 22-kDa protein in the skin.** Paraffin-embedded sections were incubated with antibodies against the carboxyl terminal peptide of the 22-kDa protein (A, C, and E) and with preimmune serum (B, D, and F) as negative controls. Tissues were taken from normal individual (A-D) and from sclerotic lesion of a PSS patient (E, F). Pinkish tone represents the positive stain. Magnifications are ×200 and ×400 (inlets).

positive staining was seen in the cytoplasm of endothelial cells and at the peripheries of some collagen fibers in the deep dermis (Fig. 7, A and C), whereas the cytoplasm of fibroblasts failed to react. In the specimens taken from the lesional skin of PSS patients, positive staining was observed diffusely in and around almost all the collagen fibers in the upper dermis as well as in the deep dermis, where sclerotic and abnormally thickened collagen fibers accumulated (Fig. 7E). Staining in the endothelial cells of the PSS patients was faint.

To examine whether fibroblasts and endothelial cells produce the 22-kDa protein, a cytochemical method was used. Clear stains were observed diffusely in the cytoplasm of these cells (Fig. 8, A and C). In addition, Western blotting of extracts of cultured fibroblasts and endothelial cells followed by immunostaining showed a clear positive band at 22 kDa and about 20 kDa, respectively (Fig. 9).

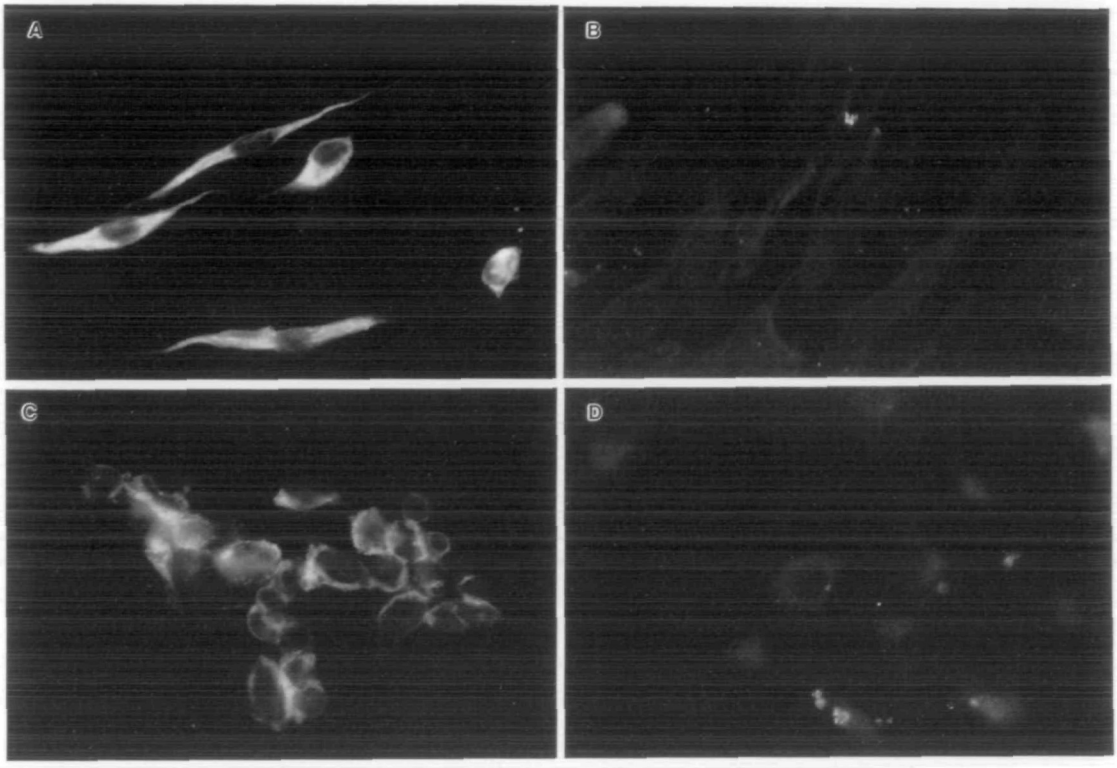

Fig. 8 **Fluorescence micrographs of 22-kDa protein in the cultured cells.** A and B, human dermal fibroblasts; C and D, porcine aortic endothelial cells. B and D are negative controls for A and C, respectively, using the preimmune rabbit serum. Magnifications are ×400.

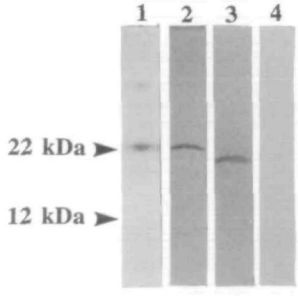

Fig. 9. **Western blotting of the extracts from the cultured fibroblasts and endothelial cells followed by the immunostaining with the antibodies against the 22-kDa protein.** Each extract was prepared for Tricine SDS-PAGE and Western blotted as described in the text. Lane 1, purified 22-kDa protein; lane 2, extract from cultured fibroblasts; lane 3, extract from endothelial cells. Lane 4 represents the negative control with the preimmune rabbit serum.

DISCUSSION

We purified a protein of M_r 22,000 which is identical to 22-kDa protein from bovine dermis described by Neame *et al.* (*11*) based on the amino acid sequence. Closely homologous proteins have been reported as TRAMP in porcine skin (*12*) and dermatopontin from human fibrosarcoma (*13*). The amino acid sequence of our protein corresponded to that from Glu 83 to Trp 127 of TRAMP, whose full sequence has not been determined. The deduced amino acid sequence of cDNA from dermatopontin has been shown to have 97% homology to bovine 22-kDa protein (*13*). These data suggest that these are genetically the same protein, and the differences in their amino acid sequences reflect differences among species.

The protein was recovered with a yield of 25 μg/g wet weight of bovine skin by our improved method, while TRAMP was recovered at the concentration of 4 μg/g wet weight of porcine skin (*12*). The difference in yield may reflect the inclusion in our extraction buffer of 4 M guanidine-HCl instead of 6 M urea. In addition, our use of the reverse-phase column seems important because it can achieve separation and desalting of the protein simultaneously, thus obviating the need for dialysis.

To examine the association of the protein and decorin, we performed CsCl density gradient ultracentrifugation in the presence or the absence of decorin. The 22-kDa protein comigrated with decorin under associative conditions, though it was separated from decorin under dissociative conditions. On the other hand, no such a comigration was seen when the 22-kDa protein was centrifuged in the presence of hyaluronate or dermatan sulfate chain. It was also found that the binding activity of the 22-kDa protein to decorin was not influenced by cleavage of the intramolecular disulfide bonds of the 22-kDa protein or decorin.

The association of the 22-kDa protein and decorin was reported by Lewandowska *et al.* (*19*) using antibodies against the protein. They showed that the antibodies failed to detect the 22-kDa protein precoated on a well by postadsorption of the mixture of decorin and biglycan. They also showed that the 22-kDa protein sustained BALB/c 3T3 cell attachment and that this activity was inhibited by decorin or biglycan and/or its glycosaminoglycan sidechains, but not by their core proteins. They concluded that

the 22-kDa protein associates with decorin or biglycan through its glycosaminoglycan side-chains. We showed that the 22-kDa protein interacted with decorin but not with dermatan sulfate chain, indicating that the 22-kDa protein binds to the core protein of the decorin molecule. CsCl density gradient centrifugation has the advantage of allowing the behavior of molecules in the density gradient to be observed directly, while Lewandowska *et al.* used indirect methods for analyzing the association of the 22-kDa protein and dermatan sulfate chain. The discrepancy between their result and ours may reflect this difference in analysis.

The 22-kDa protein associated with decorin in the absence of 4 M guanidine-HCl but not in its presence. In addition, during purification, the 22-kDa protein was separated from residual decorin by elution with the linear gradient of NaCl on ion-exchange chromatography. These observations indicate that the interaction between the 22-kDa protein and decorin is probably ionic.

The specific mRNA for the 22-kDa protein was found to be expressed in skeletal muscle, heart, pancreas, and cultured fibroblasts using cDNA of dermatopontin (*13*), and the protein was found to be present in the extracts of several tissues including skin by immunoblotting (*20*). Although the 22-kDa protein is distributed in such a wide variety of tissues, its pattern of distribution has not been elucidated. We thought it important to know the actual pattern of distribution of the 22-kDa protein in healthy as well as diseased tissues to get more information about the protein. Therefore, we tried to reveal the localization of the protein in the skin by an immunohistochemical method. Our antibodies against synthetic peptide recognized only a single band at 22-kDa in the crude extract of dermis by Western blotting, and the synthetic peptide showed no homology with other proteins. Hence our antibodies are specific for the 22-kDa protein. In normal human skin, the stain showed shaggy pattern mainly around some of the collagen fibers in the deep dermis and, unexpectedly, within the dermal endothelial cells.

The specificity of the positive staining in the endothelial cells was confirmed by two methods. In the cytochemical study, a clear cytoplasmic stain was obtained and, unexpectedly, a band of about 20 kDa in the extract reacted with our antibodies in the Western blotting. The clear stain in the cytoplasm of endothelial cells can be explained by the presence of this protein that is cross-reactive with our antibodies. It is possible that this 20-kDa protein is a cell-specific splicing form of the 22-kDa protein. Alternatively, it may reflect a difference in the modification of the molecule. Because TRAMP, the porcine homologue of the 22-kDa protein, is reported to be sulfated at the tyrosyl residues (*20*) and there are no acceptor sequences for oligosaccharide chains in bovine 22-kDa protein, the difference in the M_r may reflect the low degree of sulfation in the molecule from the endothelial cells. The features of this protein are now under investigation.

In contrast to the endothelial cells in tissue, no immunoreactive products were seen in the cytoplasm of tissue fibroblasts. In cultured fibroblasts, however, a clear cytoplasmic stain was obtained in the cytochemical study, and a band with an M_r of 22,000 from their extracts reacted with our antibodies. As no band was seen other than that of M_r 22,000 in our experiment, the positive stain in the cells is likely to reflect the residual end-product. Thus the 22-kDa protein is the product of the fibroblasts. This raises the possibility that some matrix components transmit signals to the fibroblasts in tissue to facilitate secretion of the 22-kDa protein into the ECM.

In contrast to normal skin, in the specimens taken from the PSS patients, diffuse positive stains increased in almost all the abnormally accumulated thick collagen fibers in the superficial as well as deep dermis, although only a faint stain was recognized in the endothelial cells. These data suggest that the accumulation of collagen and the 22-kDa protein increased in parallel in PSS.

Concerning collagen fibrillogenesis, MacBeath *et al.* reported that TRAMP accelerates collagen fibril formation and decreases fibril diameters to about two-thirds (70–100 nm) *in vitro* but does not modify D-periodic banding patterns (*21*). Decorin also affects collagen fibrillogenesis by delaying fibril formation and decreasing the diameters of fibrils (*22, 23*). In relation to the smaller diameters of collagen fibrils, Hayes and Rodman (*24*) and Perlish *et al.* (*25*) reported that the population of collagen microfibrils with smaller diameters (peak value, 30–50 nm) increased in the dermis of PSS compared with normal (70–140 nm). Fleischmajer and Perlish reported a significant increase of dermatan sulfate in the dermal extracts from PSS patients (*26*). This result suggests the increase of decorin in the dermis of PSS, because dermal dermatan sulfate derives from a side-chain of decorin, which is a major proteoglycan in the skin (*27*). Together with the influence of decorin on collagen fibrillogenesis *in vitro*, the increase of decorin in PSS may explain the accumulation of collagen composed of microfibrils with smaller diameters in this disease. Our histochemical observations also support the notion that overexpression of the 22-kDa protein is involved in the pathophysiology of PSS, and that the 22-kDa protein affects collagen fibril formation *in vivo* as it does *in vitro*.

Our results suggest that the 22-kDa protein interacts with decorin and collagen to form a supra-molecular aggregate that functions as architecture in maintaining structures and as a scaffold for cells by controlling extracellular matrix assembly.

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