Type VI Collagen Microfibrils: Evidence for a Structural Association with Hyaluronan

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Abstract. Type VI collagen, a widespread structural component of connective tissues, has been isolated in abundance from fetal bovine skin by a procedure involving bacterial collagenase digestion under nonreducing, nondenaturing conditions and gel filtration chromatography. Rotary shadowing electron microscopic analysis revealed that the collagen VI was predominantly in the form of extensive intact microfibrillar arrays. These microfibrils were seen in association with hyaluronan, which was identified by its ability to bind the G1 fragment of cartilage proteoglycan. Treatment with highly purified hyaluronidase largely disrupted the collagen VI microfibrils into component tetramers, double tetramers, and short microfibrillar sections. Subsequent incubation of disrupted collagen VI in the presence of hyaluronan facilitated a partial repolymerization of the microfibrils. In vitro binding studies have also demonstrated that type VI collagen binds hyaluronan with a relatively high affinity. These studies demonstrate that a specific structural relationship exists between type VI collagen and hyaluronan. This association is likely to be of primary importance in the growth and remodeling processes of connective tissues.

THE extracellular matrices (ECMs)[1] of connective tissues are complex and highly diverse structures comprising many different matrix components, including the fibrillar collagens, proteoglycans, and adhesive matrix glycoproteins. The spatiotemporal distributions of these various components and their complex molecular and cellular interactions define the characteristic three-dimensional arrangements of each connective tissue which are important in regulating events such as the proliferation, migration and differentiation of cells. While the role of some matrix assemblies is relatively easy to identify as exemplified by the provision of tensile strength by the major collagen types I, II, and III, the roles of other macromolecules are less clearly defined. Thus, type VI collagen and hyaluronan, both complex network-forming polymers and ubiquitous components of matrix, have been credited with important, though imperfectly understood, roles in tissue organization and remodeling (Keene et al., 1988; Bonaldo et al., 1990; Evered and Whelan, 1988).

Type VI collagen, which is expressed in virtually every tissue examined so far (Timpl and Engel, 1987; Kielty et al., 1991*a*), is synthesized by a wide range of different cell types (Hessle and Engvall, 1984; Chu et al., 1987; Peltonen et al., 1990*a*; Hatamochi et al., 1989; Colombatti and Bonaldo, 1987; Kielty et al., 1990; Fleischmajer et al., 1991). It appears to be an integral component of matrix with a pivotal influence on matrix organization and on attachment of cells within the ECM, both in normal development and tissue maintenance, and in several major disease states. Indeed, perturbations in collagen VI levels have been established in several major connective tissue disorders, including cutis laxa (Crawford et al., 1985), neurofibroma (Fleischmajer et al., 1985), atherosclerosis (Kittelberger et al., 1990), diabetes (Mohan et al., 1990), rheumatoid arthritis (Okada et al., 1990), and systemic sclerosis (Peltonen et al., 1990*b*). Collagen VI is essentially a collagenous glycoprotein which is assembled in vivo into extensive and highly flexible microfibrils which form filamentous networks interwoven among the major interstitial structures such as collagen fibrils, blood vessels, and nerves in the surrounding extracellular matrix (Bruns et al., 1986; Keene et al., 1988; Kielty et al., 1991*b*; Bray et al., 1990). The scope for collagen VI interactions with other components of matrix is large, although few associations have been specified so far. Two potential collagen VI–cell interactions have, however, been identified. Sequence analysis has revealed within the triple-helical domains of the three collagen VI chains the presence of eleven RGD putative cell-adhesion sequences, at least some of which appear to be biologically significant (Aumailley et al., 1990). An apparent protein–protein association of collagen VI with a membrane-associated chondroitin sulphate proteoglycan (NG2) has also been reported (Stallcup et al., 1990). In addition, adhesion of collagen VI to immobilized type I collagen has been demonstrated in vitro; this interaction may involve the repeating von Willebrand factor–like motifs within the globular domains of the three chains (Chu et al., 1989; Koller et al., 1989; Bonaldo et al., 1990).

1. *Abbreviation used in this paper*: ECM, extracellular matrix.

Hyaluronan is a major matrix polysaccharide with a polydisperse, high-M_r expanded coil structure (Evered and Whelan, 1988; Scott, 1989). It can occur as a free glycosaminoglycan (for example, in vitreous humor or synovial fluid), as a coat attached via receptors to cell surfaces, or as an interactive part of proteoglycans such as aggrecan. Hyaluronan is present in all connective tissue matrices, but is a major constituent of embryonic ECM where it can be detected as early as gastrulation. At this stage, it is thought to organize the ECM into a hydrated, open lattice able to support and promote cell movements. It is synthesized on the inside of the plasma membrane by the enzyme, hyaluronan synthetase, and the repeating disaccharide chains are extruded directly to the extracellular space through pores in the membrane (Prehm, 1988). There is recent rotary shadowing evidence and computer modeling information that hyaluronan is not only capable of forming defined secondary and tertiary structures, but that it is fundamentally a network-forming polymer (Scott et al., 1991). The specific mechanisms by which hyaluronan interacts with embryonic cells, tissues, and other ECM components remain to be elucidated. Hyaluronan has not been shown to be linked covalently to proteins. However, specific hyaluronan-binding proteins have now been isolated from various connective tissues (Mason et al., 1989) and from cultures of fibroblasts (Carlstedt et al., 1983) and aortic endothelial cells (Morita et al., 1990). Furthermore, cDNA sequence determinations have also shown that the hyaluronan-binding region of link protein, the G1 domain of cartilage proteoglycan, and a cell surface protein, CD44, contain structurally related tandem repeat structures (disulphide-bonded loops) which have been implicated in binding hyaluronan (Krusius et al., 1987; Dudhia et al., 1990; Aruffo et al., 1990; Culty et al., 1990). Elevated levels of hyaluronan frequently occur in tumours where it may act as a support for cell adhesion and locomotion (Knudson et al., 1988), and in diseases of connective tissues such as cirrhosis, sclerosis, and rheumatoid arthritis (Engstrom-Laurent, 1988).

The abundance, codistribution, and putative roles in cell-matrix communications of type VI collagen and hyaluronan in both normal developing tissues and certain disease conditions are suggestive of a relationship between these distinct molecular entities. We have extracted intact high-M_r assemblies containing type VI collagen microfibrils and hyaluronan from fetal calf skin, and by a combined biochemical, immunological, and electron microscopic approach we have identified a specific structural association between these molecules which has important and far-reaching implications in health and disease.

Materials and Methods

Materials

Fetal calves were obtained from the local abattoir within 1 h of maternal death. Bacterial collagenase (type 1A), PMSF, N-ethylmaleimide (NEM), benzamidine, diaminobenzidine, DTT, NP-40, DNase I (EC 3.1.21) from bovine pancreas, high-M_r hyaluronan (from bovine vitreous humor), chondroitin sulphate A and B (dermatan sulphate), heparan sulphate, hyaluronidase (EC 3.2.1.35) (type I-S from bovine testes, type IX from Streptomyces hyalurolyticus), hyaluronidase (EC 3.2.1.36) (type X from leech), chondroitinase ABC (EC 4.2.2.4), keratanase (EC 3.2.1.103), papain (EC 3.4.22.2), cetyl pyridinium phosphate, and prestained noncollagenous molecular weight markers were obtained from the Sigma Chemical Company (Poole, Dorset, UK). Tween-20 was obtained from BDH Chemicals (Poole, Dorset, UK). Sepharose CL-2B and PD10 desalting columns were supplied by Pharmacia-LKB (Milton Keynes, Bucks, UK). Peroxidase-conjugated swine IgGs to rabbit immunoglobulins were supplied by Dakopatt Ltd. (High Wycombe, Bucks, UK). Alkaline phosphatase-conjugated anti-rabbit IgG, biotinylated anti-rabbit IgG, alkaline phosphatase-conjugated extravidin, and *p*-nitrophenyl phosphate (pNPP) were obtained from the Sigma Chemical Company. Mica sheets were obtained from TAAB Laboratory Equipment Ltd. (Reading, Berks, UK). The G1 fragment of porcine cartilage proteoglycan which binds specifically to hyaluronan was a gift from Dr. Tim Hardingham and Dr. Amanda Fosang (Kennedy Institute, London, UK) (Morgelin et al., 1988; Fosang and Hardingham, 1989).

Tissue Digestion and Solubilization

Samples of skin (~2 g wet weight) were dissected and homogenized in 10 ml of 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl, 0.01 M $CaCl_2$, 2 mM PMSF, and 10 mM NEM. Bacterial collagenase (type 1A) was added to a final concentration of 0.2 mg/ml, and the digestion allowed to proceed at 4°C for 18 h with gentle stirring. The digestions were terminated by addition of EDTA to a final concentration of 15 mM. The digests were centrifuged at 10,000 *g* for 30 min and the supernatants, which contained solubilized high-M_r matrix assemblies, were retained. These extracts were used in subsequent experiments. In some experiments, high-M_r assemblies were isolated from skin after digestion with bacterial collagenase for only 4 h at 22°C. Skin samples were also digested for 18 h with 0.2 mg/ml hyaluronidase (from bovine testes) in 0.05 M sodium acetate, pH 6.0, containing 0.15 M NaCl, 2 mM EDTA, 2 mM PMSF, 5 mM benzamidine, and 10 mM NEM. The salt extracts were all digested for 4 h at 22°C with 25 μg DNase I in 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl and 0.05 M $MgCl_2$.

Isolation and Purification of Intact Collagen VI

The salt extracts were chromatographed directly without concentration under nonreducing, nondenaturing conditions on a column (1.5 × 200 cm) of Sepharose CL-2B. The column was equilibrated and eluted at room temperature with 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl. An analytical column of Sepharose 2B (1 × 20 cm) was used in some experiments. Column runs were constantly monitored at 280 nm and the column fractions comprising the void volume (V_o) and containing the excluded material, were pooled (preparation A). Intact collagen VI microfibrils represented by far the major protein of this high-M_r material. In some experiments, the excluded fraction was dialyzed extensively against distilled water, freeze-dried, and then subsequently stirred overnight at 4°C in 0.05 M Tris-HCl, pH 7.4, containing 0.15 M NaCl. Under these conditions, the intact collagen VI remained undissolved (preparation B), which facilitated the removal of soluble contaminants.

Specific Enzyme Digestions

The salt extracts, excluded high-M_r material (preparation A) and the purified collagen VI (preparation B) obtained from 18-h bacterial collagenase digests of 2 g skin were subjected to digestion either for 3 h at 22°C or overnight at 4°C with a range of highly specific glycosaminoglycan-degrading enzymes, using a ratio of 1 U enzyme activity per sample containing 40 μg uronic acid and 10 mg protein approximately. Digestions with hyaluronidase (Streptomyces hyalurolyticus or leech) were carried out in 0.05 M sodium acetate, pH 6.0, containing 0.15 M NaCl, 2 mM EDTA, 2 mM PMSF, 5 mM benzamidine and 10 mM NEM. The buffer for the chondroitinase ABC lyase digestions was 0.05 M Tris-HCl, pH 8.0, containing 0.06 M sodium acetate, 2 mM PMSF, 2 mM EDTA, 5 mM benzamidine, and 10 mM NEM, while keratanase digestions were carried out in 0.15 M Tris-HCl, pH 7.4, containing 2 mM PMSF, 5 mM benzamidine, and 10 mM NEM. Protein concentrations were determined by dry weight or by absorbance at 230 or 280 nm, using type I collagen and BSA as standards. Uronic acid determinations were carried out according to the method of Bitter and Muir (1962).

Electrophoresis and Western Blotting

The salt extracts, excluded fractions (preparation A) and purified collagen VI (preparation B) were analyzed by discontinuous SDS-PAGE on 6.5 or 8% gels (Laemmli, 1970) in the presence or absence of 10 mM DTT. Molecular weights were determined by reference to standards, which were collagenous (types I and V collagens) and noncollagenous. The prestained noncollagenous standards used were fumarase (M_r 48,500), pyruvate kinase

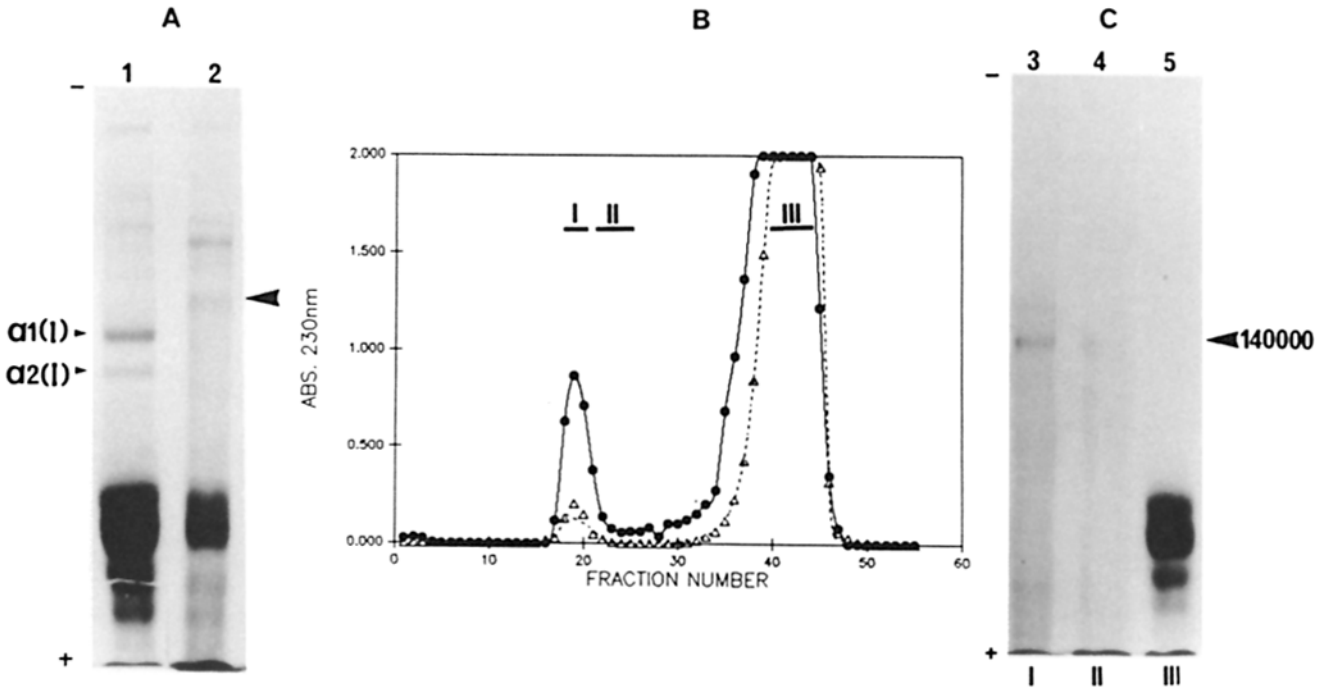

Figure 1. Isolation of high-M_r aggregates of intact type VI collagen from fetal bovine skin by digestion with hyaluronidase (bovine testes) or with bacterial collagenase under nonreducing conditions, and gel filtration chromatography. (*A*) Electrophoretic analysis on 6.5% SDS-PAGE gels under reducing conditions of the proteins solubilized by hyaluronidase digestion (track *1*) and bacterial collagenase digestion (track *2*). The position of type I collagen in the hyaluronidase digests and of the M_r 140,000 α1(VI)/α2(VI) component of type VI collagen in the bacterial collagenase digest (*large arrowhead*) are shown. (*B*) Digests were chromatographed on a column of Sepharose CL-2B under nonreducing, nondenaturing conditions. The column buffer was 0.4 M-Tris-HCl, pH 7.4. The bacterial collagenase profile is represented as a continuous line, and the hyaluronidase profile as broken lines. Fractions were pooled as indicated by the bars. (*C*) Electrophoretic analysis on 6.5% SDS-PAGE gels under reducing conditions of the proteins present in the pooled fractions obtained after chromatography of bacterial collagenase-digested skin (Tracks *3–5*). The position of the α1(VI)/α2(VI) component is indicated.

(M_r 58,000), fructose-6-phosphate kinase (M_r 84,000), β-galactosidase (M_r 116,000) and α2-macroglobulin (M_r 180,000). Gels were stained for protein using either Coomassie brilliant blue or silver stain. Western blotting was carried out as described by Kielty et al. (1990). Proteins were electrophoretically transferred to nitrocellulose filters, and incubated at room temperature with antiserum at appropriate dilution. The identification of type VI collagen was achieved using a 1:1,000 dilution of either or both of two polyclonal antisera raised in rabbits to type VI collagen (Ayad et al., 1989; Kielty et al., 1991*b*). One of these is known to recognize specifically the α3(VI) chain and the other the α1(VI)/α2(VI) component. Positive reactions were identified after incubation with second antibody, peroxidase-labeled swine IgGs to rabbit immunoglobulins. In some experiments, the high-M_r excluded material (preparation A) and the purified intact collagen VI (preparation B) were also analyzed by SDS-PAGE on 2.5% gels as previously described (Kielty et al., 1990), using thyroglobulin (M_r 669,000) as high-M_r standard.

Analysis of Glycosaminoglycans

The glycosaminoglycan content of the extracts and of the excluded material (preparation A) was determined by electrophoresis on cellulose acetate (zip) plates after papain digestion and fractionation of glycosaminoglycans by cetyl pyridinium precipitation as previously described (Scott et al., 1981). The glycosaminoglycans were visualized after staining with Alcian blue, and identified both by reference to standards (chondroitin sulphate, dermatan sulphate, hyaluronan, and heparan sulphate) and by their susceptibility to digestion with various specific glycosaminoglycan-degrading enzymes (Streptomyces hyaluronidase, leech hyaluronidase, chondroitinase ABC lyase, keratanase, and heparanase). The relative abundance of the different glycosaminoglycans present in each sample was determined by scanning the zip plates using an LKB laser scanning densitometer at 632.8 nm.

Binding of Glycosaminoglycans to Type VI Collagen

ELISA-type solid phase assays of collagen VI-glycosaminoglycan interactions were conducted on 96-well plates (Nunclon). All incubations were carried out in PBS minus Mg^{2+} and Ca^{2+}. Wells were coated overnight at 4°C with 5 or 10 μg soluble intact bovine collagen VI (preparation A) in 100 μl PBS. To block additional protein binding sites, the wells were incubated in PBS containing 1% BSA for 2 h at 22°C. After washing, wells were incubated overnight with hyaluronan, chondroitin sulphate, dermatan sulphate, or keratan sulphate at several concentrations ranging from 0–10 μg in 100 μl PBS. The wells were washed extensively, incubated with a 1:1,000 dilution of a rabbit antibovine serum to α1(VI)/α2(VI) (Kielty et al., 1991) for 2 h at 22°C, and after a further wash, incubated with a 1:8,000 dilution of alkaline phosphatase–conjugated anti-rabbit IgG for 2 h. The wells were washed twice in PBS and then twice in alkaline phosphatase buffer (100 mM diethanolamine, pH 9.5, containing 100 mM NaCl and 5 mM $MgCl_2$), before the addition of 1 mg/ml alkaline phosphatase substrate (dNPP). The color intensity was measured at 535 nm after 10 and 30 min. In this experiment, the concentration of bound glycosaminoglycans was also determined by direct spectrophotometric microassay of washed wells after incubation with glycosaminoglycans (Farndale et al., 1982).

In another approach, the glycosaminoglycans hyaluronan, chondroitin sulphate, and dermatan sulphate (25 μg/100 μl) were dissolved in PBS and bound to wells overnight at 4°C. Additional binding sites were blocked with 1% BSA before overnight incubation with collagen VI (preparation A) (1–10 μg/μl), and immunological detection of bound collagen VI was carried out as described above.

Rotary Shadowing EM

Control and enzyme-digested aliquots of the excluded material of the salt extracts (preparation A) from 12-h digests were analyzed by rotary shadowing EM for their content of intact high-M_r extracellular matrix macromolecules using the mica sandwich technique described by Mould et al. (1985). In some experiments, the high-M_r fraction from hyaluronidase-digested skin (100 μg/ml) was preincubated with hyaluronan (0.1 or 1 mg/ml final concentration) for 30 min or 4 h at 22°C in column buffer or with the purified G1 domain of cartilage proteoglycan (1 μg G1/100 μg collagen VI)

for 3 h at 22°C according to the method of Morgelin et al. (1988) before rotary shadowing analysis. Fractions were diluted directly into 0.2 M ammonium acetate, pH 6.0, to a final concentration of ~100 μg/ml, and 5-μl droplets were sandwiched between two sheets of freshly cleaved mica. The high-M_r aggregates were allowed to absorb onto the mica surfaces for 5 min. The mica sandwiches were washed in 0.2 M ammonium acetate and then plunged into liquid nitrogen. The sandwiches were split open under nitrogen, dried in vacuo, rotary shadowed with platinum wire on a tungsten filament at an angle of 4°C, and then coated with carbon. The carbon replicas were floated off onto distilled water, and picked up on uncoated 200 mesh copper grids. Specimens were examined in a JEOL 1200 EX electron microscope at 120 kV. Length measurements of the type VI collagen and fibrillin aggregates were carried out on micrographs using a modified Microsemper software package (Synoptics, Cambridge, UK) on an Olivetti M28/Matrox PIP1024 frame store system using a line grating replica of 2,160 lines/mm.

Results

Isolation of High-M_r Aggregates from Fetal Calf Skin

The experiments presented in this paper relate to tissue obtained from a single bovine fetus from the second trimester of gestation (160 d), but identical results were obtained from a number of animals of similar ages. Digestion of fetal skin with either bacterial collagenase or hyaluronidase (bovine testes) for 18 h resulted in the release of complex mixtures of proteins and other components, the majority of which were directly soluble in the respective digestion buffers (Kielty et al., 1991*b*). However, the relative efficiencies of the two extraction procedures differed, with ~65% of the original wet weight of the bacterial collagenase-digested tissue directly soluble in the digestion buffer, compared with <40% for the hyaluronidase digests. After digestion with DNAse I and a centrifugation step, the supernatants containing solubilized molecules (designated salt extracts) were subjected to gel filtration chromatography on Sepharose CL-2B under nonreducing, nondenaturing conditions, which facilitated a clear separation of high-M_r aggregates from smaller molecules (Fig. 1 *B*). Broadly similar profiles were obtained by chromatography of the bacterial collagenase and hyaluronidase digests. The excluded peak comprised molecules with molecular masses in excess of 5×10^6. However, while the high-M_r excluded material from bacterial collagenase salt extracts represented ~15–18% of the original tissue wet weight, that from hyaluronidase-generated salt extracts represented a reduced proportion of the tissue (4–7% wet weight).

Analysis of Fractions Containing High-M_r Assemblies

Electrophoresis. Analysis of both the bacterial collagenase and hyaluronidase (bovine testes) salt extracts on 6.5 and 8% SDS-PAGE gels under reducing or nonreducing conditions, showed that both extracts contained intact high-M_r components but also a high proportion of smaller molecules with molecular masses of <70,000 (including blood proteins and digest fragments) (Fig. 1 *A*). In addition, the hyaluronidase digests contained some type I collagen. However, the major component of the excluded fraction of both extracts migrated with an apparent M_r of 140,000 under reducing conditions (Fig. 1 *C*). This component was previously identified as the α1(VI)/α2(VI) component of type VI collagen (Kielty et al., 1991). Several other high-M_r components were also present in these fractions. The type I collagen chains present in the original hyaluronidase salt extracts were not detected in the high-M_r fraction; these were eluted predominantly in the intermediate column volume. After further purification of collagen VI, the minor high-M_r components were largely removed.

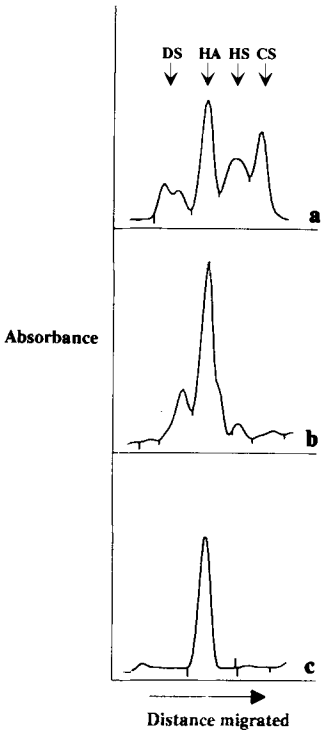

Figure 2. Analysis of glycosaminoglycans present in a salt extract and excluded fraction (preparation *A*) of 18-h bacterial collagenase-digested fetal bovine skin, by cellulose acetate electrophoresis after papain digestion and cetyl pyridinium chloride fractionation. Plates were scanned at 632.8 nm using a laser densitometer. (*A*) Control, showing the electrophoretic positions of dermatan sulphate (*DS*), hyaluronan (*HA*), heparan sulphate (*HS*), and chondroitin sulphate (*CS*). (*b*) Salt extract, containing hyaluronan and low levels of dermatan sulphate and heparan sulphate. (*C*) Excluded fraction, containing hyaluronan.

Uronic Acid Content. The abundance of uronic acid-containing molecules varied significantly between the bacterial collagenase and hyaluronidase (bovine testes) salt extracts, and between the excluded fractions of these digests. This result largely reflects the fact that this hyaluronidase digestion will have removed hyaluronan as well as chondroitin 4 and 6 sulphate. The bacterial collagenase salt extracts from 2 g skin had a substantial total uronic acid content (15.6 mg), but there was a reduced amount in the hyaluronidase salt extracts (5.1 mg). Similarly, the total uronic acid content of the collagenase-digested high-M_r material was 3.7 mg, while the corresponding fraction from the hyaluronidase digests contained barely detectable levels. A subsequent analysis of the glycosaminoglycans content of the digests and excluded fractions by cellulose acetate (zip plate) electrophoresis resulted in the identification of several glycosaminoglycans. The major component in the collagenase-digested material was hyaluronan, with low levels of dermatan sulphate and heparan sulphate also detected in the original extracts, but not in the excluded fraction (Fig. 2). Hyaluronan was not detected in the hyaluronidase extracts.

Rotary Shadowing EM. The high-M_r assemblies isolated and partially purified from the collagenase and hyaluronidase extracts by gel filtration chromatography (preparation A) were visualized by rotary shadowing electron microscopy. By far the major matrix macromolecule isolated from both digests was type VI collagen. There were, however, differences in the sizes of these collagen VI assemblies from the two sources. The collagenase-extracted collagen VI extracted was identified as very extensive twisting microfibrils, in some cases in excess of 6 μm in length (Kielty et al.,

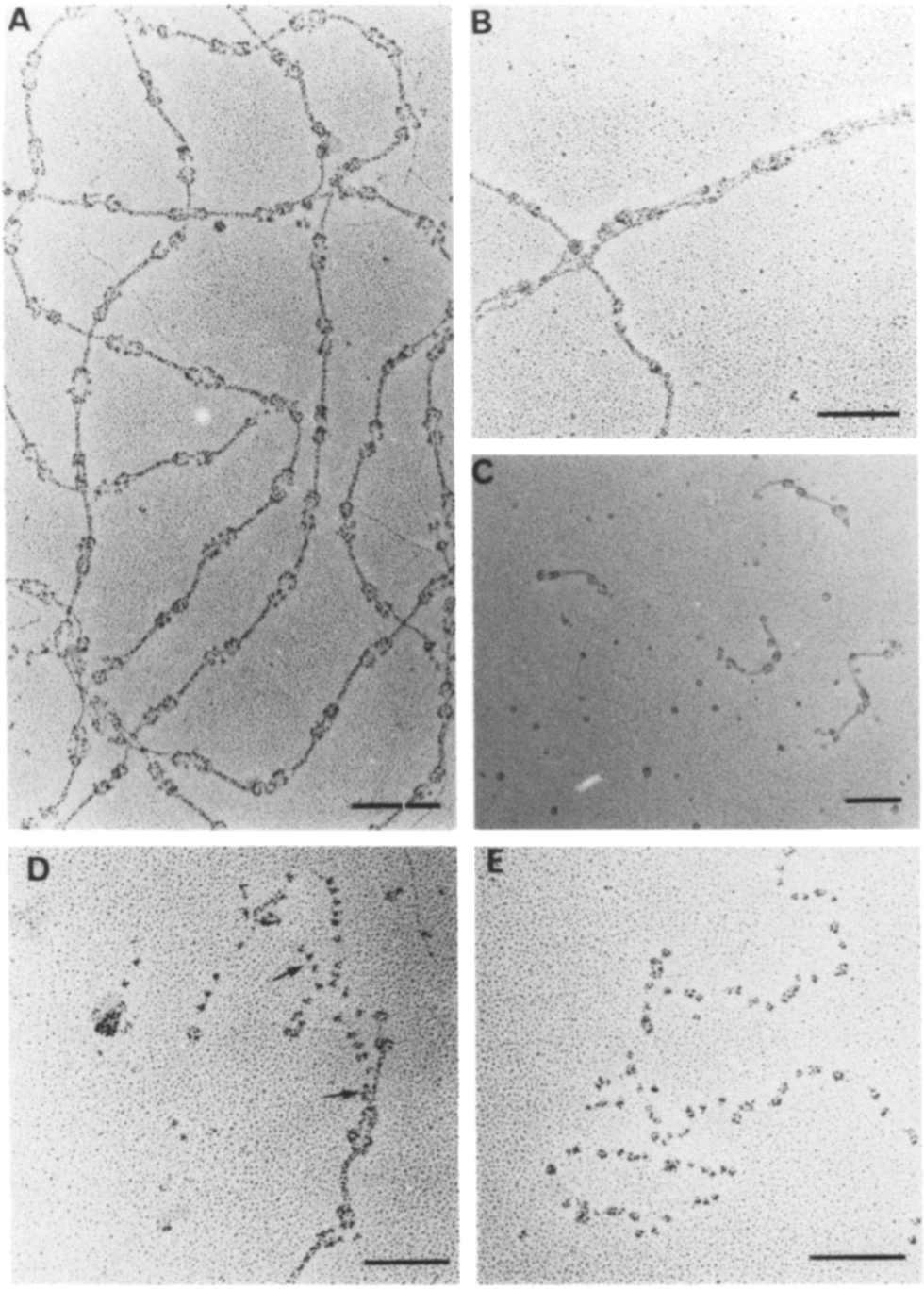

Figure 3. Electron micrographs after rotary shadowing of type VI collagen isolated from fetal bovine skin after digestion by bacterial collagenase or hyaluronidase (bovine testes) in the presence of protease inhibitors, and fractionated by gel filtration on Sepharose CL-2B. (*A*) Large intact microfibrils of collagen VI isolated from 18-h bacterial collagenase-digested skin (preparation A). (*B*) Large intact microfibrils and aggregated microfibrils of collagen VI isolated from 4-h bacterial collagenase-digested skin. (*C*) Individual tetramers and double tetramers of collagen VI isolated from 18-h hyaluronidase-digested skin. (*D*) Association of hyaluronan with collagen VI microfibrils isolated from 18-h bacterial collagenase-digested skin. The hyaluronan (indicated by *arrows*) has been visualized by decoration with the G1 fragment of porcine cartilage proteoglycan. (*E*) A single strand of bovine vitreous hyaluronan decorated with the G1 fragment. Bars, 100 nm.

1991*b*) (Fig. 3 *A*). Thin filaments apparently emerged from a high proportion of the beaded "junction" domains and microfibril ends. When the tissue digestion by bacterial collagenase was stopped after only 4 h, the collagen VI subsequently isolated was present predominantly as enormous linear microfibrillar aggregates (Fig. 3 *B*). In contrast, the collagen VI present in the solubilized high-M_r fraction obtained by hyaluronidase digestion was predominantly in the form of single and double tetramers, and short microfibrillar sections (Fig. 3 *C*). In a separate experiment in which hyaluronan was visualized by decoration with G1 fragment of cartilage proteoglycan, we identified a frequent association of hyaluronan with the junctional domains and end regions of collagen VI microfibrils (Fig. 3, *D* and *E*).

Digestion of Glycosaminoglycans in Collagenase-solubilized High-M_r Fractions

The effects on the integrity of the high-M_r collagen VI assemblies (preparations A and B) of digestion with highly purified hyaluronidase (Streptomyces and leech), keratanase, and chondroitinase ABC, respectively, were investigated by assessing alterations in chromatographic behavior on an analytical column of Sepharose 2B, by comparing electrophoretic profiles under reducing and nonreducing conditions, and by rotary shadowing electron microscopic analysis.

(*A*) The chromatographic profiles of extracts digested with highly purified hyaluronidase showed significant differences from that characteristic of the undigested control (Fig. 4).

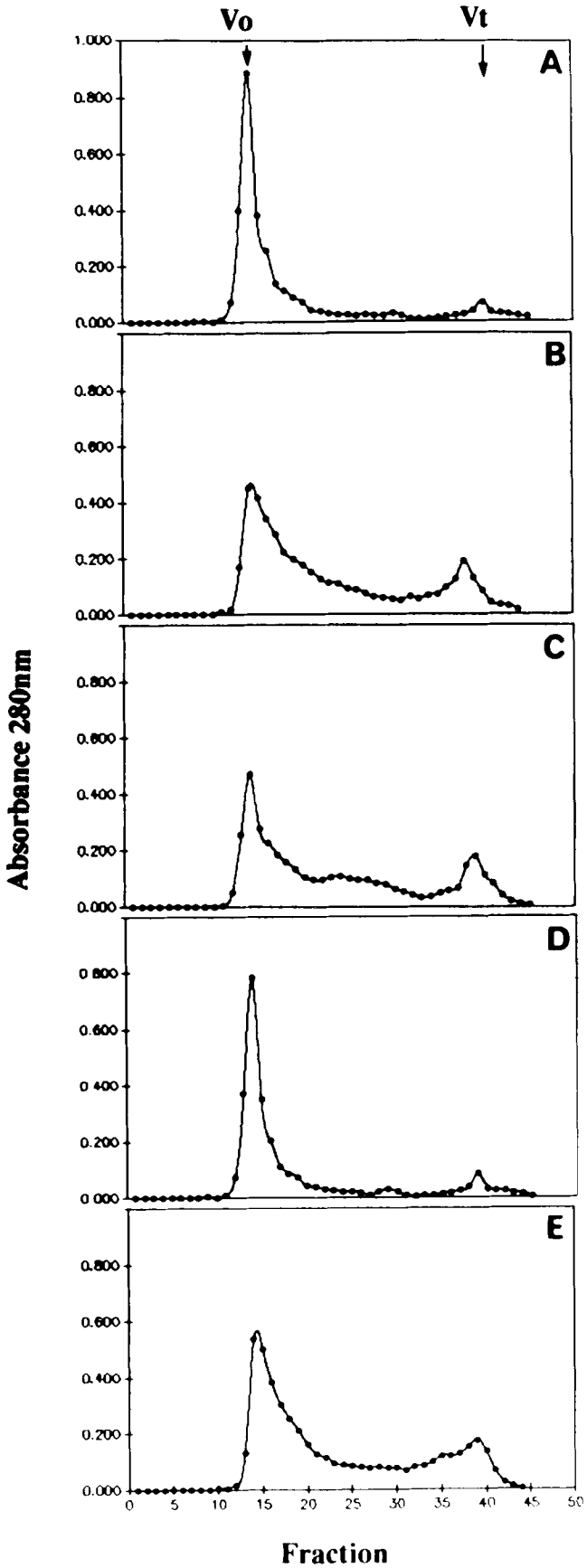

Figure 4. The effects of treatment with specific glycosaminoglycan-degrading enzymes for 3 h at 22°C on the chromatographic behavior of the excluded fraction of 18-h bacterial collagenase-digested skin (preparation *A*). Samples were chromatographed at 22°C on an analytical column of Sepharose CL-2B under nonreducing, nondenaturing conditions. The column buffer was 0.05 M Tris-HCl, pH 7.5, containing 0.4 M NaCl. Columns were continuously monitored at OD_{280}. The positions of the void volume (V_o and total volume (V_t) are indicated. (*A*) Untreated control. (*B*) Streptomyces hyaluronidase. (*C*) Leech hyaluronidase. (*D*) Chondroitinase ABC lyase. (*E*) Keratanase.

There was both a reduction in the proportion of material eluting at the position of V_o relative to the control and concomitant broadening of this peak, with some material of reduced molecular mass now included in the column volume. Digestion with chondroitinase ABC, which would degrade any dermatan sulphate or residual chondroitin sulphate, had no effect on the chromatographic profile of any of the extracts, but after keratanase treatment, there was a broadening of the main peak which had previously eluted exclusively in the V_o position.

(*B*) The electrophoretic profiles of control and enzyme-treated extracts, high-M_r fractions (preparation A) and further purified collagen VI (preparation B) were analyzed on 6.5, 8, and 2.5% SDS-PAGE gels. There were no apparent differences in the mass of the control and enzyme-treated α1(VI/α2(VI) component resolved under reducing conditions on 6.5 or 8% gels and detected by protein staining or by Western blotting (Fig. 5, *A* and *B*). On 2.5% SDS-PAGE gels under nonreducing conditions, the purified collagen VI preparation resolved into a diffuse high-M_r component with an apparent M_r of 1,000,000 and a second band of apparent M_r of 500,000 (determined by reference to thyroglobulin) together with some material that failed to penetrate the gel (Fig. 5 *C*). Similar profiles were observed for the keratanase- and chondroitinase ABC lyase-treated samples, but those fractions digested with purified hyaluronidase contained only the M_r 500,000 component. After reduction, all protein ran at the dye front.

(*C*) Rotary shadowing EM of the hyaluronidase-digested samples revealed that the enzyme treatment had a profound effect on the morphology of the collagen VI microfibrils (Fig. 6). After 30 min incubation, disruption to microfibril integrity was already apparent (Fig. 6, *A–C*), with dramatically reduced microfibrillar lengths and single and double tetramers appearing after 2 h digestion (Fig. 6, *D–F*) and predominating after overnight digestion (Fig. 6, *G–I*). There was, however, no discernible difference in the gross morphology of the collagen VI microfibrils of the untreated control, the chondroitin ABC lyase–digested, or keratanase-digested samples. Neither did the treatment have any disaggregating effect on the loose aggregates of adjacent microfibrils observed predominantly in the 4-h digests (see Fig. 3 *B*).

Polymerization of Collagen VI Tetramers with Hyaluronan

An excluded fraction from collagenase-digested skin (preparation A) was pretreated with leech hyaluronidase overnight at 4°C and then incubated for 30 min or 4 h at 22°C in the presence or absence of added bovine vitreous hyaluronan (0.1 mg/ml or 1 mg/ml final concentration) before visualiza-

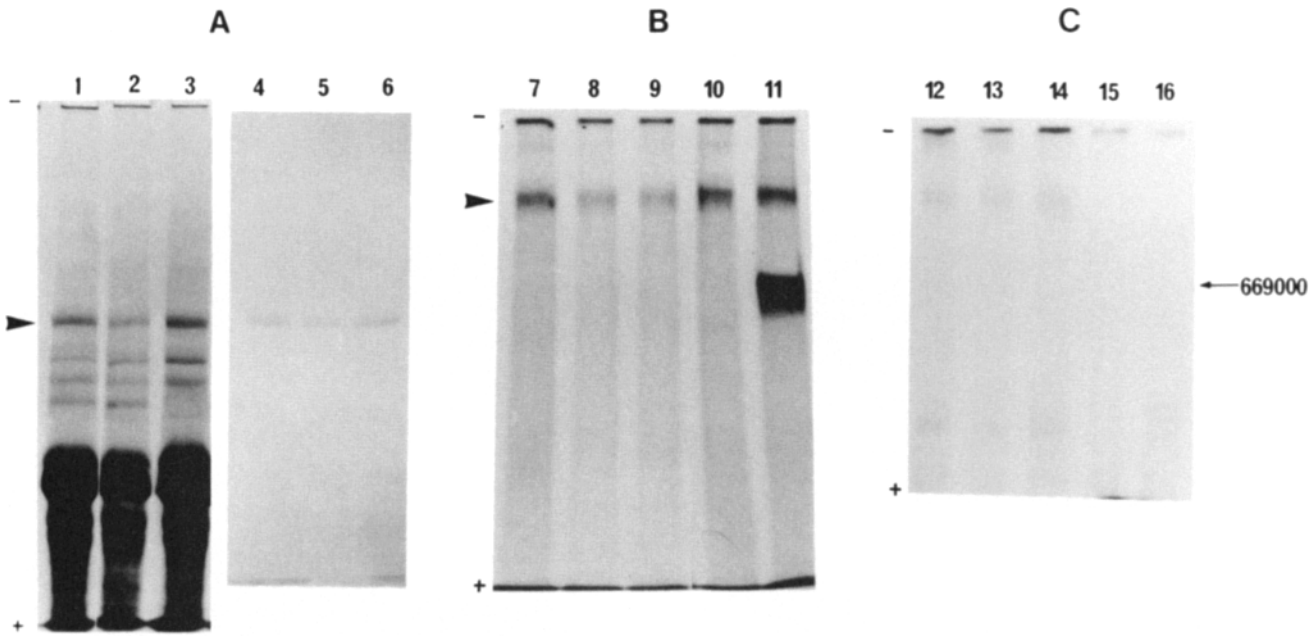

Figure 5. Electrophoretic analysis of type VI collagen before and after treatment with specific glycosaminoglycan-degrading enzymes. There was no degradation of the individual chains of collagen VI (indicated by *large arrowheads*) under any of these conditions (*A* and *B*). However, digestion with Streptomyces hyaluronidase or leech hyaluronidase did effect a reduction in the macromolecular mass of collagen VI from high-M_r aggregates (*C*). Resolved proteins were detected by staining with Coomassie brilliant blue (tracks *1–3* and *7–16*) and the α1(VI)/α2(VI) component was identified by Western blotting using specific polyclonal antiserum (Kielty et al., 1991*b*) (tracks *4–6*). (*A*) Salt extracts from 18-h bacterial collagenase-digested skin analyzed on 6.5% SDS-PAGE gels under reducing conditions. (Tracks *1* and *4*) Untreated controls; (tracks *2* and *5*) samples treated with leech hyaluronidase; (tracks *3* and *6*) samples treated with keratanase. (*B*) Purified collagen VI (preparation *B*) analyzed on 8% SDS-PAGE gels under reducing conditions. (Track *7*) Untreated control; (tracks *8–11*) samples treated with Streptomyces hyaluronidase, leech hyaluronidase, chondroitinase ABC lyase, and keratanase, respectively. Note: the chondroitinase ABC lyase enzyme preparation contains carrier protein. (*C*) Purified collagen VI (preparation *B*) analyzed on 2.5% SDS-PAGE gels under nonreducing conditions. (Track *12*) Untreated control; (tracks *13–16*) samples treated with keratanase, chondroitinase ABC lyase, Streptomyces hyaluronidase, and leech hyaluronidase, respectively.

tion by rotary shadowing electron microscopy (Fig. 7). While the control (zero time) samples contained mostly single tetramers (Fig. 7 *E*), those incubated for 30 min in the presence of hyaluronan contained both tetramers and short microfibrillar sections (Fig. 7, *B* and *F*), and the 4-h samples contained substantially longer collagen VI microfibrils of up to 15 tetramer units in length (Fig. 7, *C, G, H*). This experiment therefore provided clear evidence that the added hyaluronan had facilitated an in vitro polymerization of collagen VI tetramers.

Binding of Intact Type VI Collagen to Hyaluronan

The binding of hyaluronan, keratan sulphate, chondroitin sulphate, and dermatan sulphate to intact type VI collagen was investigated in ELISA-type binding studies in one of two ways. Soluble intact collagen VI (preparation A) was immobilized in microtitre wells and incubated with different concentrations of glycosaminoglycans, or glycoaminoglycans were adsorbed onto the wells and incubated with collagen VI. In the first case, the binding of different glycosaminoglycans to immobilized collagen VI clearly showed that incubation with hyaluronan was most effective in blocking the interaction of collagen VI with antibody (Fig. 8 *A*). Of the other glycosaminoglycans tested, only keratan sulphate showed any inhibition, and this was at a much higher concentration. When glycosaminoglycans were bound to the wells and subsequently reacted with collagen type VI, the presence of hyaluronan led to the retention and detection of most collagen VI, with keratan sulphate also apparently binding weakly to collagen VI (Fig. 8 B). Despite the poor attachment of glycosaminoglycans to the wells, the similar findings between the two approaches indicate a specific interaction between collagen VI and hyaluronan.

Discussion

We have presented biochemical, immunochemical, and ultrastructural evidence for an interaction between type VI collagen and the long, unbranched polysaccharide, hyaluronan. This analysis was facilitated by our recent development of an efficient method for extracting intact type VI collagen microfibrils from fetal tissues after removal of the fibrillar collagen framework (Kielty et al., 1991*b*). This protocol releases abundant and extensive collagen VI microfibrillar arrays, which confirm the primary location of this collagen in vivo among the interstitial collagen fibrils. In the present work, we have demonstrated that the collagen VI extracted after hyaluronidase-induced tissue disruption differed in several important respects from the bacterial collagenase-released material. The yield of hyaluronidase-extracted collagen VI was not only markedly lower than that released by bacterial collagenase digestion, but it was also predominantly

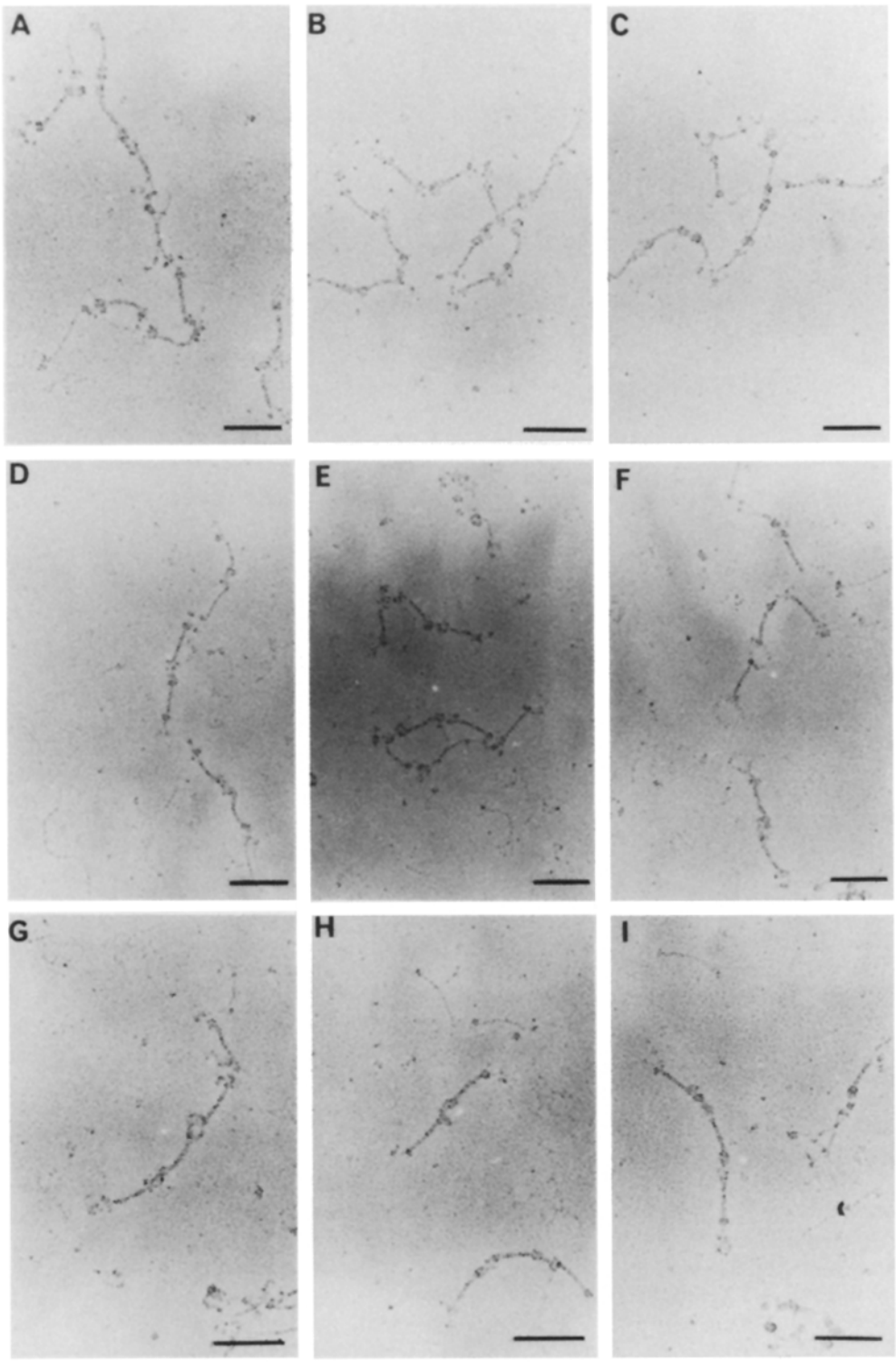

Figure 6. Electron micrographs after rotary shadowing of collagen VI (preparation *A*) after treatment with leech hyaluronidase at 22°C for 30 min (*A–C*), 2 h (*D–F*) and overnight (*G–I*). A progressive depolymerization of collagen VI microfibrils is apparent under these conditions.

in the form of single and double tetramers and short microfibrillar sections. Another notable difference between the collagen VI extracted by the two enzyme treatments was the lack of ultrastructural evidence for any association with thin filamentous strands at tetrameric junctions or extruding from microfibrillar ends such as are frequently observed in the bacterial collagenase–released collagen VI. These initial observations prompted us to investigate further the molecular basis of these gross structural differences, and the possibility of a physiological relationship between collagen VI and hyaluronan.

We have demonstrated the presence of abundant hyaluronan in the fetal skin extracts and in the high-M_r aggregates isolated by gel filtration chromatography. Indeed, hyaluronan makes the principle contribution to the uronic acid content of this tissue, although low levels of dermatan sulphate and heparan sulphate were also detected in this tissue. The effects on the intact collagenase-released type VI collagen

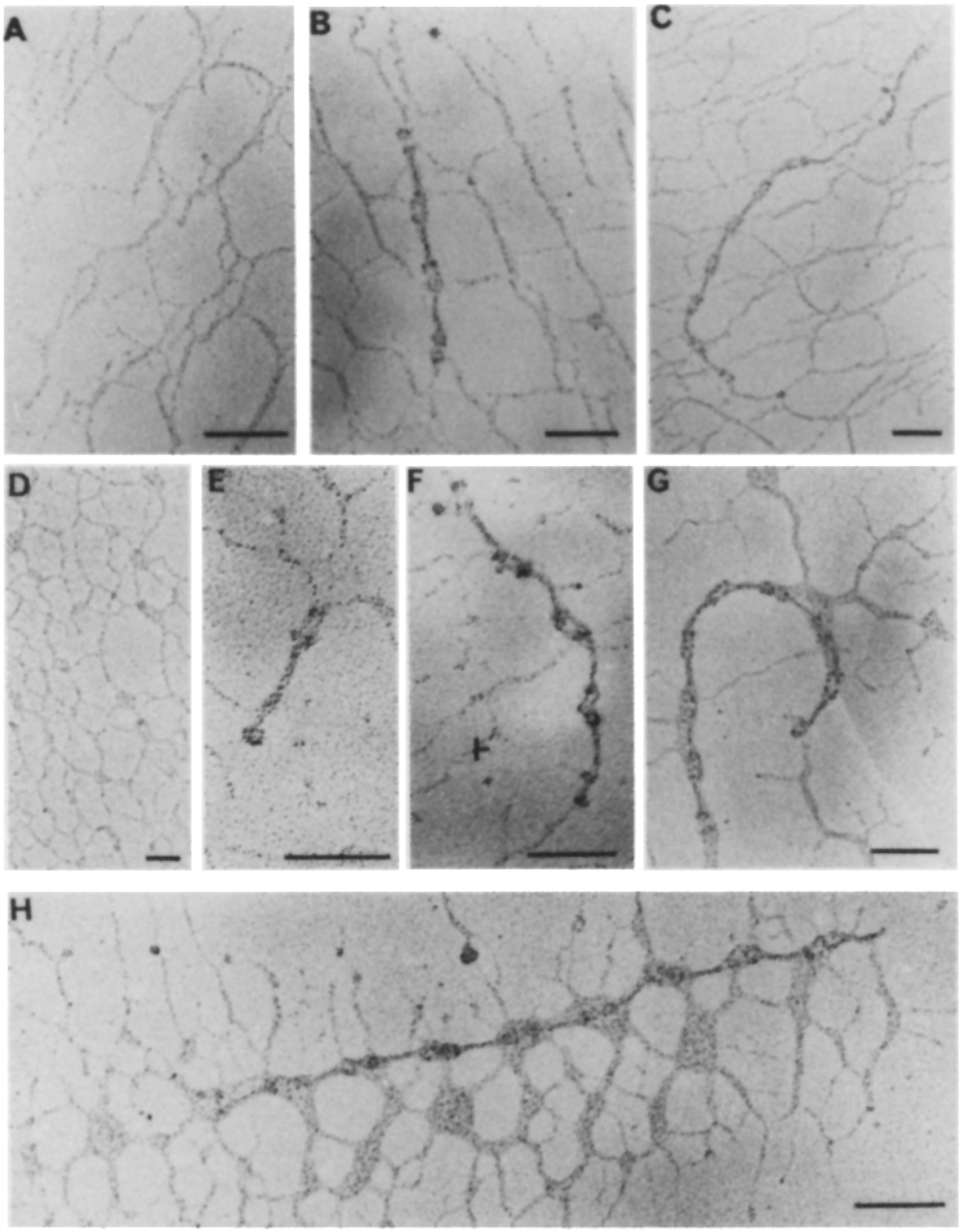

Figure 7. Electron micrographs after rotary shadowing of collagen VI (preparation *A*) after treatment with leech hyaluronidase and subsequent incubation for 30 min or 4 h at 22°C in the presence of 0.1 mg/ml (*A–C*) or 1 mg/ml bovine vitreous hyaluronan (*D–H*). The association of hyaluronan with the globular junctional and end domains of collagen VI tetramers and microfibrillar sections can be clearly seen. *A* and *D* contain hyaluronan only. Control samples (zero incubation time) contained mostly collagen VI tetramers and double tetramers (*E*). After 30 min of incubation, short collagen VI microfibrillar arrays could be detected (*B* and *F*). After 4 h of incubation, longer microfibrils of up to 15 tetramer units were frequently observed (*C, G, H*). Bars, 100 nm.

microfibrils of treatment (in the presence of protease inhibitors) with highly purified hyaluronidase, chondroitinase ABC, or keratanase, were investigated using a combination of gel filtration chromatography, electrophoresis, and rotary shadowing EM. This approach demonstrated that while the individual chains, triple-helical monomers, dimers, and tetramers were undegraded by any of these treatments, there was a shift in the size distribution of the collagen VI microfibrils after hyaluronidase digestion, with short microfibrillar sections, tetramers, dimers, and also individual chains generated by this treatment. A small amount of collagen VI depolymerization was also observed on treatment with keratanase, although the physiological significance of this observation is unclear. The chondroitinase ABC lyase treatment failed to influence the size distribution or morphology of the collagen VI microfibrils. We have also been able to demonstrate in a converse approach, that incubation of hyaluronidase-digested collagen VI with an excess of purified high-M_r hyaluronan facilitates a partial repolymerization of the microfibrils. A separate ultrastructural investigation of a high-M_r excluded fraction revealed that hyaluronan, clearly visualized by decoration with the G1 fragment of cartilage proteoglycan, was frequently associated with the globular junctional and end domains of intact collagen VI microfibrils. Finally, we have shown in an in vitro assay that collagen VI is indeed able to bind strongly to hyaluronan. It also appears to interact with keratan sulphate, but with a much lower affinity. Taken together, these data provide compelling evidence for a physiological association between collagen VI and hyaluronan which has important implications for their biological roles and mechanisms of action in tissue organization and remodeling.

The chemical nature of the association between collagen VI and hyaluronan is not clear from the present work. How-

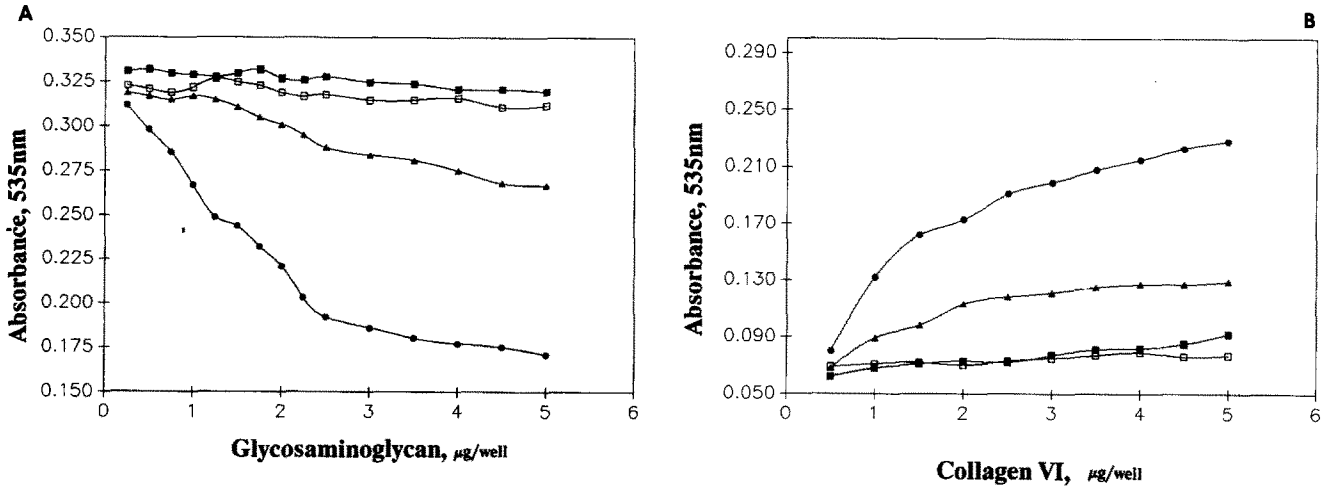

Figure 8. Binding of glycosaminoglycans to collagen VI. The binding of different glycosaminoglycans to collagen VI was determined by two modified ELISA procedures using a specific polyclonal antibody to collagen VI. (*A*) Flat bottom microtitre plates were coated with 10 μg intact collagen VI (preparation *A*) overnight at 4°C. The collagen-coated wells were washed, blocked with 1% BSA, and incubated overnight at 4°C with 0–10 μg of hyaluronan (●), chondroitin sulphate (■), dermatan sulphate (□), and keratan sulphate (▲). The proportion of collagen VI that remained unmasked by bound glycosaminoglycan was determined by ELISA. (*B*) Plates were coated overnight at 4°C with 25 μg hyaluronan (●), chondroitin sulphate (□), dermatan sulphate (■), or keratan sulphate (▲), washed, blocked with 1% BSA, then incubated overnight at 4°C with 0–10 μg intact collagen VI (preparation *A*). Collagen VI bound to immobilized glycosaminoglycan was determined by ELISA.

ever, the available ultrastructural information points to the primary involvement of the (largely nonhelical) terminal regions of collagen VI, and not the central rod-like helical domain, in this interaction. Possibilities include a relatively nonspecific hydrophobic association between hyaluronan and the globular domains of collagen VI such as that envisaged as a basis of potential hyaluronan interactions with proteins and lipid membranes by Scott (1989), or possibly a highly specific (hydrophobic) interaction between hyaluronan and a hyaluronan-binding site. One family of proteins with a hyaluronan-binding motif has been identified and this includes cartilage proteoglycan, link protein, and the cell surface hyaluronan receptor, CD-44 (Aruffo et al., 1990; Culty et al., 1990). We have been unable to identify any such obvious motif in the chains of collagen VI; however, they may combine to form a new class of hyaluronan-binding domain.

The hyaluronan network is an integral structure, which appears not to require other components to hold it together (Scott et al., 1991). Indeed, the formation of extensive and continuous secondary and tertiary structures appear to be driven by interactions between the large repeating hydrophobic patches on alternate sides of this molecule. Furthermore, the overall strength of the meshwork is likely to be dependent on the size of the component hyaluronan molecules, with shorter molecules with fewer potential cross-links generating weaker meshworks, for example, in rheumatoid arthritis where a pathological decrease in the size of hyaluronan molecules apparently affects the lubricating qualities of synovial fluid. Hyaluronan also interacts with proteoglycans, cell membranes and receptors such as CD-44 with very high specificity (Evered and Whelan, 1988; Gallagher, 1989; Aruffo et al., 1990; Culty et al., 1990). Thus, this relatively simple long unbranched polysaccharide is capable of enormous specificity and versatility. Type VI collagen is, in contrast, a highly complex macromolecule. The extent of its complexity has recently become apparent with the elucidation of the complete cDNA sequence (Bonaldo et al., 1989; Bonaldo and Colombatti, 1990; Chu et al., 1988, 1989, 1990; Trueb et al., 1989) with evidence for discrete (potentially functional) sequence motifs in all three chains and tissue-specific alternative splicing of the α2(VI) and α3(VI) chains. Furthermore, although the sequence of intracellular events leading to the formation of monomers, dimers, and tetramers has been well established in biosynthetic studies (Engvall et al., 1986; Colombatti and Bonaldo, 1987), the chemical basis of the polymerization of type VI collagen tetramers into microfibrils in the extracellular space is not well established. The involvement of aldehyde-derived cross-links has been virtually excluded, and disulphide bonds also appear not to play a primary role in this interaction (Wu et al., 1987; Kielty et al., 1991*b*), which might be hydrophobic or ionic in nature. It is therefore particularly tempting to speculate on a possible role for hyaluronan in directing the polymerization of collagen VI microfibrils in the extracellular space proximal to the cellular basement membrane from which it is extruded. However, it is far from clear whether the association of hyaluronan with collagen VI is of such fundamental structural importance, whether it may play a role in lateral aggregation of collagen VI microfibrils, or whether it simply acts as a primary stabilizing influence on the collagen VI microfibrillar network. What is clear is that removal of hyaluronan renders these microfibrillar structures far less stable entities with increased susceptibility to depolymerization. At a functional level, hyaluronan might act to modulate collagen VI-matrix or -cell interactions, for example, by masking cell- or collagen-binding sequences, or by acting in a synergistic manner with collagen VI to strengthen specific interactions. Some clues as to which, if any, of these options may apply could come from analysis of certain disease conditions where perturbations in the structure and abundance of both hyaluronan and type VI collagen have been recorded. Further work is currently in progress to elucidate not only the

molecular basis of the specific collagen VI-hyaluronan interaction, but also the physiological significance of the collagen VI interactions in vivo with hyaluronan, and possibly other glycosaminoglycans or proteoglycans.

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