Identification and Partial Characterization of Two Type XII-like Collagen Molecules

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Abstract. We have identified two distinct collagenous macromolecules in extracts of fetal bovine skin. Each of the molecules appears to contain three identical α-chains with short triple-helical domains of ~25 kD, and nontriple-helical domains of ~190 kD. Consistent with these observations, extracted molecules contain a relatively short triple-helical domain (75 nm) and a large globular domain comprised of three similar arms. Despite these similarities, the purified collagenase-resistant domains are distinguished by a number of criteria. The globular domains can be chromatographically separated on the basis of charge distribution. Peptide profiles generated by V8 protease digestion are dissimilar. These molecules are immunologically unique and have distinct distributions in tissue. Finally, rotary shadow analysis of purified domains identifies size and conformation differences. Structurally, the molecules are very similar to type XII collagen, but differ in tissue distribution, since both these molecules are present in cartilage, while type XII is reported to be absent from that tissue.

THERE are currently 13 identified types of structural macromolecules that contain large triple-helical domains (Mayne and Burgeson, 1987; Pihlajaniemi et al., 1987). The majority of these molecules (types I, II, III, and V) form the banded collagen fibers typically found in all connective tissues. Consistent with their ability to condense into quarter-staggered fibrils, the triple helical length of all these collagens are comparable (Burgeson, 1988). In contrast, type IX collagen is found only in cartilage, the primary corneal stroma, the nonocord sheath, and the vitreous (Irwin and Mayne, 1986; Mayne and Irwin, 1986), where it is located along the fibril surface, and in cartilage, is covalently associated with type II collagen (Wu and Eyre, 1984, 1989; Muller-Glauser et al., 1986; Eyre et al., 1987; van der Rest and Mayne, 1988). Type IX collagen has a considerably shortened triple helical domain relative to the fiber forming collagens, and its helical domains are partitioned into three short segments by nonhelical disruptions (van der Rest and Mayne, 1987). Recently, type XII collagen was identified, and its deduced sequence indicates partial homology to type IX collagen. The homology to type IX collagen suggests that these molecules are associated with the surface of banded collagen fibers in tissues other than cartilage, and the term fibril-associated collagens with interrupted triple-helices (FACIT collagens) has been proposed for type IX and XII collagens (Gordon et al., 1989*b*).

Type XII collagen was first reported in periodontal ligament (Yamauchi et al., 1986) and later identified as a unique triple helical sequence encoded by a cDNA generated from 17 d chick embryo tendon (Gordon et al., 1987). The deduced sequences showed striking similarities to chick type IX collagen, but appeared to completely lack the COL3 and NC3 domains and had a shortened COL2 domain (Dublet et al., 1987; Gordon et al., 1989*a*) more similar in size to the COL3 domain of type IX collagen. Partial sequences were obtained that indicated that type XII contained an amino-terminal NC-3 domain much larger than type IX, and this domain showed some homology with the NC-4 domain of α1(IX). Rotary shadowing of type XII identified an unusual macromolecule that contained three apparently alike nonhelical arms protruding from a central globule (Dublet et al., 1989; Gordon et al., 1990). In addition, a thinner triple helical arm also extended from the central globule. The isolated α-chain showed a molecular mass of ~220 kD. Digestion with collagenase allowed recovery of a nonhelical domain of ~190 kD. Antibodies were made to a synthetic peptide whose sequence was deduced from the cDNA (Sugrue et al., 1989). These antibodies immunoprecipitate a biosynthetic product identical to the molecule isolated from chick tissue. Immunolocalization using these antibodies indicated that type XII is present in chick tendon, perichondrium, and periosteum, but is absent from cartilage and bone (Sugrue et al., 1989).

Two molecules were isolated from calf tissue. One isolated from periodontal ligament has nearly identical sequences to chick type XII, and has a very similar rotary-shadowed image (Dublet et al., 1988). Another was isolated from bovine skin and tendon that demonstrated less sequence homology,

but again a similar rotary shadowed image (Dublet et al., 1990). From these studies the authors conclude that type XII collagen is one of a family of related macromolecules. The complete tissue distribution, ultrastructural localization, and function of these molecules is not known.

We report here the observation of two distinct molecules that have grossly similar structures to type XII collagen. In this manuscript, we refer to these as twelve-like A and B (TL-A; TL-B). The tissue distribution of these molecules is unlike that reported for type XII, since both are also found in bovine cartilage. The results agree with the prior suggestion that all these molecules represent a unique class of collagenase species, but the codistribution of these molecules with type IX collagen suggests that each may have a separate function.

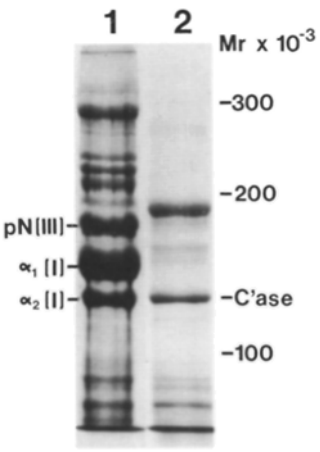

Figure 1. Electrophoretic analysis of the fetal bovine skin extract. The 2.7 M NaCl precipitate was analyzed before (lane *1*) and after (lane *2*) digestion with clostridial collagenase. Reduced samples were separated by SDS-PAGE on 5% gels and proteins visualized by Coomassie blue staining. The positions of major collagen bands and collagenase are indicated. The molecular weight scale was determined using globular protein standards.

Materials and Methods

Extraction of Fetal Bovine Skin

All steps were carried out at 4°C. Fetal calves, measuring 8–24 inches crown to rump, were obtained from Pel-Freez (Rogers, Arkansas) within 24 h of slaughter. Skins were stripped, cut into smaller pieces, weighed, and homogenized in a Waring blender with a minimal amount of extraction buffer (200 mM NaCl, 10 mM EDTA, 0.1 mM N-ethylmaleimide, 0.035 mM PMSF, 0.017 mM p-chloromercuribenzoate, and 25 mM Tris-HCl, pH 7.8). After homogenization, the extract was made 40% (wt/vol) with additional buffer and stirred for 48 h. Large pieces were removed by straining and washed with one half volume of extraction buffer. The extract and washed were combined and centrifuged (12,000 *g*, 20 min). The supernatant was isolated and DEAE-cellulose (DE52, Whatman Inc., Clifton, NJ), equilibrated in extraction buffer, was added at a ratio of 1.0 ml of packed resin per gram of original tissue weight. The DEAE-cellulose and bound material were removed by centrifugation (17,000 *g*, 20 min). The supernatant was collected and dialyzed extensively against high salt buffer (2.7 M NaCl, 1 mM EDTA, 0.1 mM N-ethylmaleimide, 0.035 mM PMSF, and 25 mM Tris-HCl, pH 7.8). The resulting salt precipitate was pelleted by centrifugation (17,000 *g*, 60 min), washed with high salt buffer, and collected after recentrifugation.

Purification of the Collagenase-resistant Domains of TL-A and TL-B Collagens

The 2.7 M NaCl precipitate was dissolved in digestion buffer (2 M urea, 200 mM NaCl, 5 mM $CaCl_2$, 0.1 mM N-ethylmaleimide, 0.035 mM PMSF, and 25 mM Tris-HCl, pH 7.8) at a ratio of 200 ml/500 g original tissue weight. The sample was treated with diisopropyl fluorophosphate (2.5 μg/ml) and dialyzed extensively against digestion buffer. Insoluble material was removed by centrifugation (125,000 *g*, 120 min). Collagenase (CLSPA, Worthington) was added to a final concentration of 50 U/ml, the sample was filter sterilized, and digestion was carried out for 5 d at room temperature. The digestion was stopped by the addition of 5 mM EDTA, insoluble material was removed by sedimentation (17,000 *g*, 20 min), and soluble proteins were precipitated by the addition of ammonium sulfate to 50% saturation. After an overnight incubation at 4°C, the precipitate was collected by centrifugation (20,000 *g*, 60 min) and dissolved in a minimal volume of sieve buffer (200 mM NaCl, 1 mM EDTA, 0.1 mM N-ethylmaleimide, 0.035 mM PMSF, and 25 mM Tris-HCl, pH 7.8). The resulting sample (see Fig. 1, lane *2*) was size fractionated by fast protein liquid chromatography (FPLC) on Superose 6 preparation grade (1.6 × 100 cm; Pharmacia, LKB Inc., Piscataway, NJ). The flow rate was 1.0 ml/min and, following a 30-min delay after sample injection, 2.0-ml fractions were collected (see Fig. 2). The heterogeneous peak in fractions 22–40 was pooled and dialyzed into ion exchange buffer (100 mM NaCl, 0.1 mM N-ethylmaleimide, 0.035 mM PMSF, and 25 mM Tris-HCl, pH 7.8) and applied to FPLC Mono-Q (HR5/5) at a flow rate of 1 ml/min. After a 10-min wash, proteins were first eluted with a 40-min linear gradient from 0.1 to 0.5 M NaCl and then a 10-min linear gradient from 0.5 to 1.0 M NaCl (see Fig. 3).

1. *Abbreviations used in this paper*: TL-A and TL-B, twelve-like A and B, respectively.

Other Methods

Indirect immunofluorescence and mAb production have been described previously (Sakai et al., 1986). Preparation of polyclonal antisera and cross-reactivity testing are detailed in an accompanying paper (Keene et al., 1991). Techniques including V8 protease digestion, Western blotting, rotary shadowing, and length measurements are described elsewhere (Lunstrum et al., 1986, 1987; Morris et al., 1986; Bachinger et al., 1990).

Results

Bovine skin was obtained from 8–24-in (crown to rump lengths) fetal calves. Skins were extracted in 0.2 M NaCl buffer in the cold, and fractionated by differential NaCl precipitation. The electrophoretic profile of the disulfide bond reduction products of the 2.7 M NaCl precipitate is shown in Fig. 1, lane *1*. Comparison of the Coomassie blue–visualized pattern with known collagen markers allowed the identification of pN α1(III), α1(I), and α2(I) chains as has been previously reported in fetal calf skin extracts (Fujii and Kuhn, 1975), as well as numerous unidentified species. An aliquot of the extract shown in lane *1* was extensively digested with Clostridial collagenase, and the electrophoretic profile of the products is shown in lane *2*. Collagenase digestion considerably simplified the pattern, and generated a major protease resistant band with an apparent M_r of 190,000 in addition to other species and the added collagenase.

Further fractionation of the extract under nondenaturing conditions has proven difficult. Therefore, isolation of the collagenase-resistant portion of the molecule was effected. The fractionated extract shown in Fig. 1, lane *1* was subjected to exhaustive digestion with Clostridial collagenase, and the product equivalent to that shown in Fig. 1, lane *2* was fractionated by FPLC using a Superose 6 molecular sieve column under nondenaturing conditions. The absorbance at 220 nm of the effluent is shown in Fig. 2. The absorbance pattern indicates that the peak between fractions 22 and 40 is heterogeneous. Alternate peak fractions from 22–40 were analyzed by gel electrophoresis after disulfide bond reduction (Fig. 2, *inset*). A single major electrophoretic species was visualized by Coomassie blue staining. Fractions 22–40 were pooled and applied to FPLC Mono-Q ion exchange resin in neutral buffer under nondenaturing conditions, and bound materials were eluted with a linear gradient of NaCl

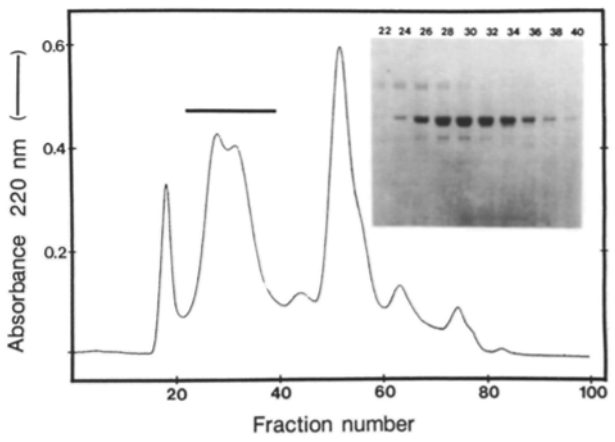

Figure 2. Molecular sieve FPLC of collagenase-digested extracts. As described under Materials and Methods, initial fractionation of collagenase-resistant products was performed on Superose 6. (*inset*) Alternate fractions indicated by the bar were analyzed by gel electrophoresis. Reduced samples were separated by SDS-PAGE on 5% gels before staining with Coomassie blue. The major species present migrates with an apparent M_r of 190,000.

concentration. The absorbance profile at 220 nm is indicated in Fig. 3 *A*.

Two distinct absorbance peaks are seen. Gel electrophoretic analysis of the disulfide bond reduction products of fractions 29–33 (peak 1), and 42–45 (peak 2) is shown in Fig. 3 *B*. The fractionated proteins are better than 95% pure as judged by the electrophoretic stain pattern. Materials in identical electrophoretic positions fractionate into two classes of charge density. To further investigate the identities of Mono-Q fractionated materials, each was separately evaluated by rotary shadowing, Western blotting, and distribution within various tissues by indirect immunofluorescence. mAbs were individually raised to the materials in the two pooled Mono-Q fractions. Polyclonal antibodies were also made in rabbits to the Mono-Q peak 1.

Hybridomas were selected by their ability to specifically Western blot the separated Mono-Q pools used as immunogens, and by their ability to direct fluorescent staining of fetal bovine skin. Hybridoma lines selected for further study were cloned by serial dilution, and expanded using standard techniques. Analysis of two mAbs by Western blot of mono-Q–separated materials (Coomassie blue stained; Fig. 3 *B*) is shown in Fig. 4. One cell line, C1J, produced antibodies that reacted specifically with the peptides present in peak 1. This antibody recognized the 190-kD peptide and the faster electrophoretic species (a presumed degradation product) as seen in Fig. 4 *A*. The C1J antibody did not react with the peptides in peak 2. Antibodies produced by another cell line, 1011G, reacted only with peptides in peak 2. Both the major 190-kD peptides and the slightly faster peptide (believed to be a degradation product of the larger peptide) were blotted, as shown in Fig. 4 *B*. Polyclonal antibodies raised to materials in pool 1 did not cross react with materials in pool 2 by ELISA assay or Western blots, and produced a blot pattern identical to that shown in Fig. 4 *A* (data not shown). The materials in peak 1 are referred to as the globular domain of a type XII-like molecule, TL-A, and those in peak 2 as the globular domain of TL-B.

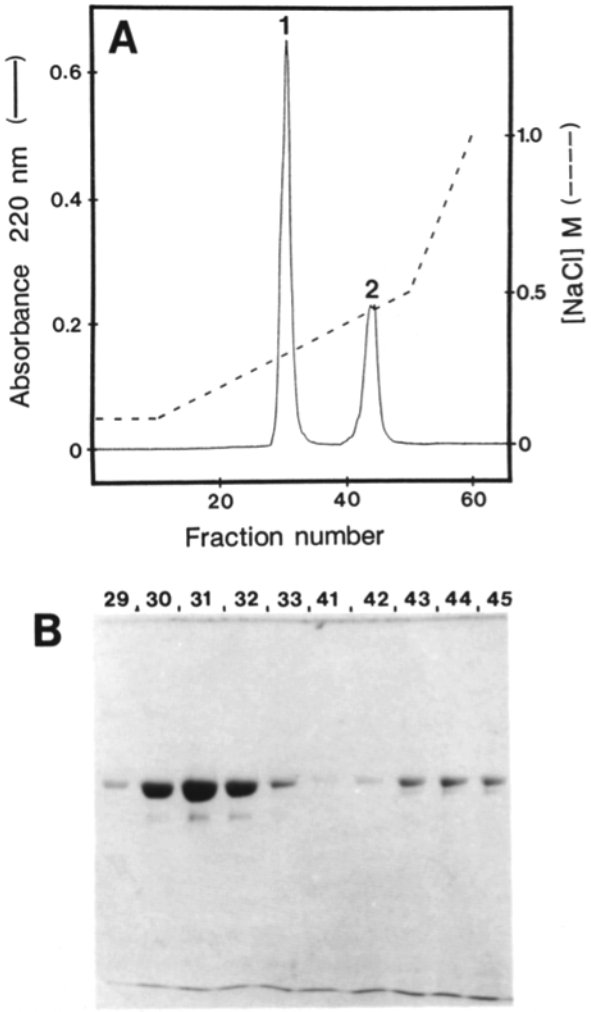

Figure 3. Separation of two collagenase-resistant species by ion exchange FPLC on Mono Q. (*A*) Absorbance profile for Mono Q elution showing the separation of two peaks, 1 and 2, present in pooled molecular sieve fractions. (*B*) Selected fractions were analyzed by SDS-PAGE on a 5% gel under reducing conditions. Proteins were visualized by Coomassie blue staining.

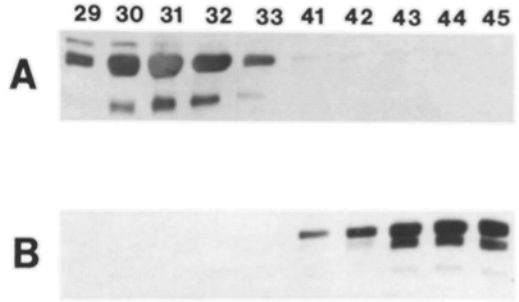

Figure 4. Immunoblot characterization of specific mAbs Mono Q fractions (see Fig. 3 *B*) were separated by electrophoresis and transferred to nitrocellulose for Western blot analysis. (*A*) mAb C1J specifically reacting with peak 1. (*B*) mAb 1011G specifically reacting with peak 2.

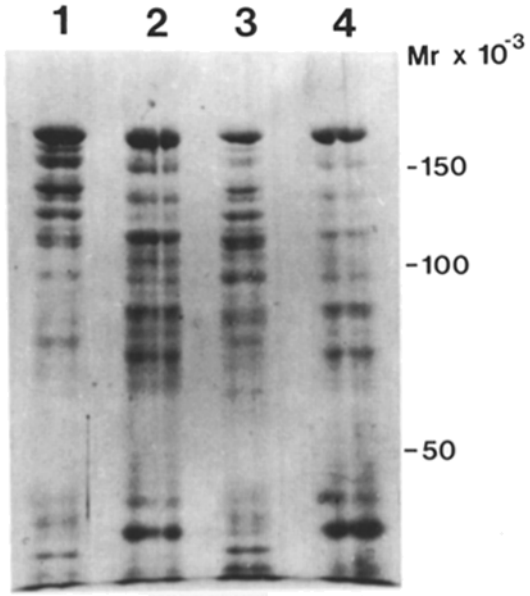

Figure 5. Comparison of the globular domains of TL-A and TL-B by V_8 protease peptide mapping. The peak 1 (lanes *1* and *3*) and peak 2 (lanes *2* and *4*) 190-kD bands were excised and digested with V_8 protease at 0.5 μg/ml (lanes *1* and *2*) and 1.0 μg/ml (lanes *3* and *4*). The resulting peptides were then separated by SDS-PAGE on a 10% gel and stained with Coomassie blue. A scale for globular protein molecular weight standards is indicated.

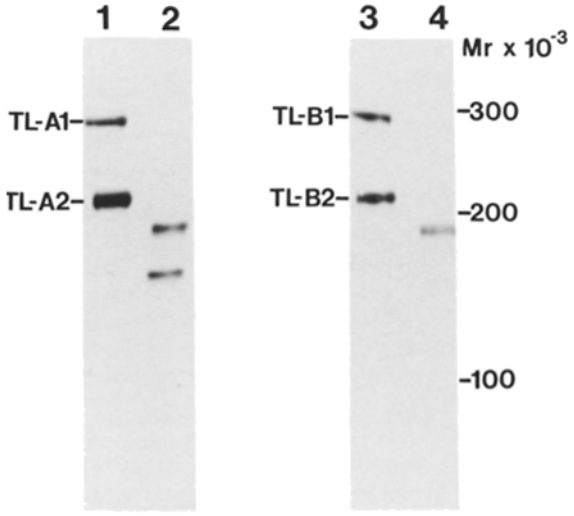

Figure 6. Identification and comparison of the collagenous forms of TL-A and TL-B present in tissue extracts. Salt-precipitated samples before (lanes *1* and *3*) and after (lanes *2* and *4*) collagenase digestion (see Fig. 1) were separated by SDS-PAGE on a 5% gel and analyzed by Western blotting with the C1J (lanes *1* and *2*) and 1011G (lanes *3* and *4*) mAbs.

The differential immunological reactivity of the globular domain of TL-A versus TL-B to both monoclonal and polyclonal antibodies, the separation of the globular domain of TL-A from that of TL-B by ion exchange under nonreducing conditions, and the observation of what appears to be distinguishable degradation products from the separated species, together suggest that the domains of TL-A and TL-B derive from separate molecules. To test this hypothesis, peptide maps from the two mono-Q–separated molecules were compared after V8 protease digestion (Fig. 5). The different sizes of the proteolytic products confirm differential susceptibility of the amino acid sequences to V8 protease. Similar results were obtained after tryptic digestion (not shown).

Despite the observed apparent amino acid sequence differences between the globular domains of TL-A and TL-B, both derive from molecules with similar gross structures. Western blots of extracted bovine skin proteins before and after collagenase digestion (equivalent to the material shown in Fig. 1) with the anti-TL-A and anti-TL-B mAbs are shown in Fig. 6. The blot pattern seen with the anti-TL-A antibodies (Fig. 6, lanes *1* and *2*) is essentially identical to the pattern observed using the anti-TL-B mAbs (Fig. 6, lanes *3* and *4*). Each shows two electrophoretic species: TL-A1 and TL-B1 (M_r = 300,000); and TL-A2 and TL-B2 (M_r = 220,000). The reason for the electrophoretic separation of TL-A1 from TL-A2, or TL-B1 from TL-B2 is not clear, but these differences are lost upon collagenase digestion. Both TL-A and TL-B digest to single species with collagenase. The faster migrating band in lane 2 is a degradation product of the globular domain and is seen in these preparations to varying degrees.

Rotary-shadowed images of purified, nondenatured globular domains of TL-A and TL-B were compared after spraying each from either bicarbonate buffer or dilute acetic acid solution (Fig. 7). The images are similar, but distinguishable. When sprayed from either neutral or acidic pH buffers, the images of TL-A are comparable (Fig. 7, *a* and *b*). Each molecular fragment appears as a trident, with three apparently identical arms extending from a central globule. Each arm is 55 nm in length, and often the arms display a characteristic kink about half-way along their length. The TL-B fragments also appear as three arms projecting from a central globule when sprayed from neutral pH buffer, but in contrast to TL-A, each arm is shorter (45 nm; Fig. 7 *c*) and the arms of both molecules appear to terminate in a small globule. When sprayed from acid, the arms of TL-B appear to fold into compact globules (Fig. 7 *d*).

The distinctive shapes of the globular domains of TL-A and TL-B, and the collagenase sensitivity of the parent molecules encouraged us to evaluate the undigested extracts for molecules containing these fragments. Partially fractionated extracts of fetal bovine skin (equivalent to the materials in Fig. 1, lane *1*) were sprayed from neutral solution and evaluated by rotary shadowing. In addition to images consistent with their identification as type I collagen and pN-type III collagen, we observe abundant molecules that appear to contain globular domains similar to those predicted for TL-A and TL-B. A field of several representative molecules is shown in Fig. 8. In addition to the three-armed structures described above, these molecules contain an additional, but thinner arm, ~75 nm in length. Assuming that this thin arm represents the triple-helical, collagenase sensitive portion of the molecule, the length of this arm indicates that it should contain three peptides, each M_r 25,000. Given the anomalously slow electrophoretic mobilities of triple-helix–derived

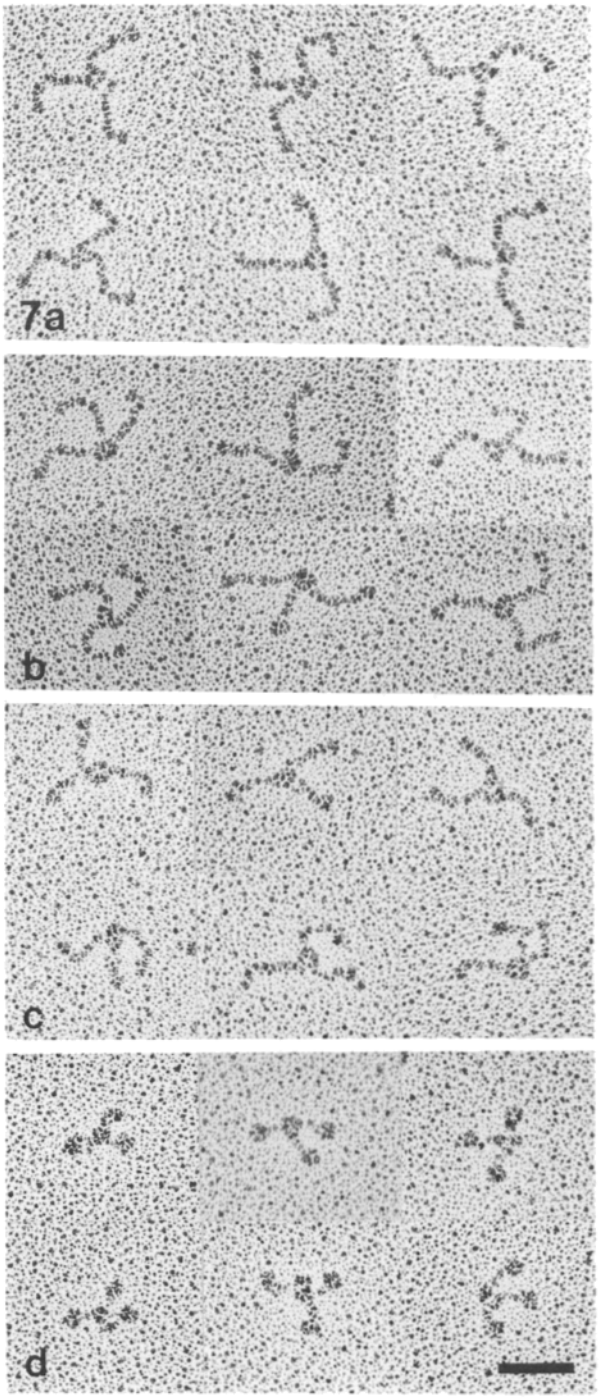

Figure 7. Rotary shadow analysis of the globular domains of TL-A and TL-B. Mono-Q–generated peak 1 (*a* and *b*) and peak 2 (*c* and *d*) materials (as shown in Fig. 3) were sprayed from either 0.2 M ammonium bicarbonate (*a* and *c*) or 0.2 M acetic acid (*b* and *d*), magnification 200,000×; bar, 50 nm.

peptides, this estimate is consistent with the differences in electrophoretic mobility observed between TL-A2 and the TL-A globular domain, and between TL-B2 and TL-B globular domain shown in Fig. 6.

The availability of mAbs specific to TL-A and TL-B allowed us to determine the tissue distribution of these collagens. Fetal bovine skin, tendon, and cartilage were evaluated. The distributions of the molecules were distinctive and often complementary, but sometimes overlapping. Both molecular species are present in nearly all connective tissues examined. In skin, TL-A is present throughout the dermis, but the most intense fluorescence is concentrated within the papillary dermis (Fig. 9 *A*). In contrast, TL-B–directed fluorescence is uniformly distributed throughout the reticular dermis, but is considerably diminished and localized only around hair follicles within the papillary layer (Fig. 9 *B*). In hyaline cartilage, TL-A is limited to the articular surface and around blood vessel canals (not shown), while TL-B exhibits a punctate localization throughout the cartilage matrix (Fig. 9, *E* and *F*). In tendon, the antibodies direct similar staining of the sheath and fibrillar collagen bundles (Fig. 9, *C* and *D*).

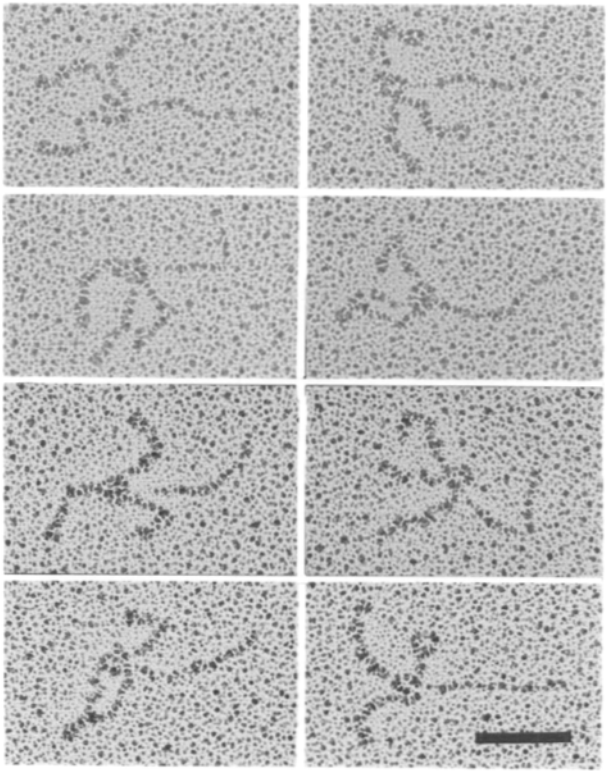

Figure 8. Identification of probable TL-A and TL-B collagens. Samples of solubilized 2.7 M NaCl precipitates were equilibrated in 0.2 M ammonium bicarbonate and analyzed by rotary shadowing. The selected molecules shown have a triple-armed globular domain and a collagen helix ~75 nm in length. Bar, 50 nm.

Discussion

The data presented above are consistent with the identification of two collagen molecules whose gross structures are similar to those recently reported for type XII collagen (Dublet and van der Rest, 1990), but are distinct from each other. The observations that the globular domains of TL-A and of TL-B can be separated without denaturation by ion exchange chromatography, that monoclonal or polyclonal antibodies made to either species demonstrate no cross reactivity, and that they show distinguishable tissue distributions

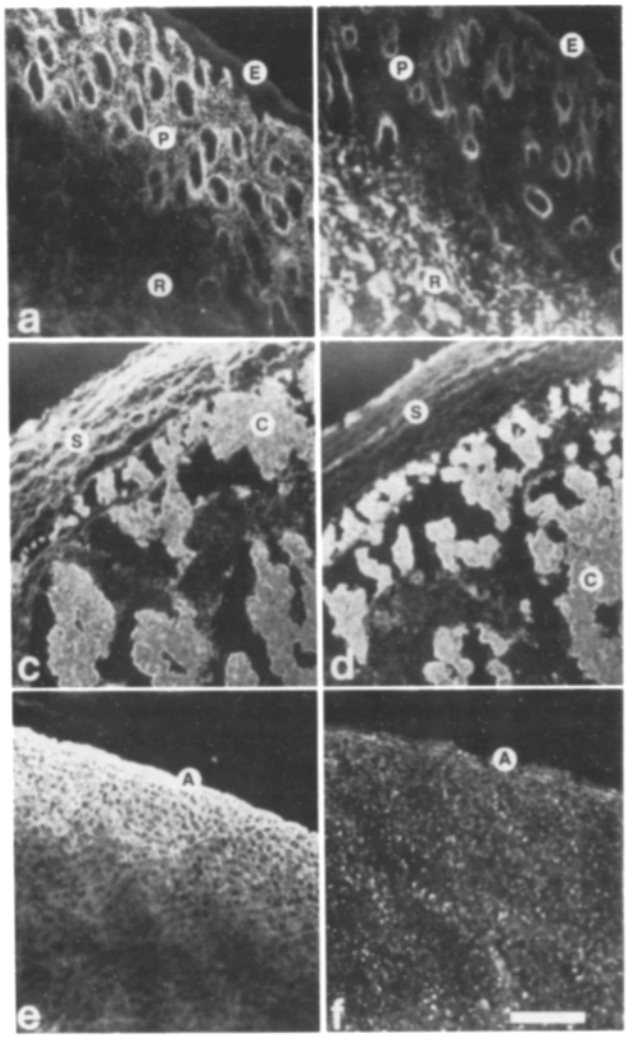

Figure 9. Distribution of TL-A and TL-B collagens in fetal bovine tissue. Immunofluorescent staining directed by mAbs C1J (*a*, *c*, and *e*) and 1011G (*b*, *d*, and *f*) was compared in skin (*a* and *b*), tendon (*c* and *d*), and articular cartilage (*e* and *f*). *E*, epithelium; *P*, papillary dermis; *R*, reticular dermis; *S*, tendon sheath; *C*, collagen bundles; *A*, articular surface of femoral head. Bar, 2 mm.

support this conclusion. To date, we have been unable to demonstrate any electrophoretic, immunological, or chromatographic heterogeneity within the TL-A or TL-B peptides, suggesting that both TL-A and TL-B are homotrimeric molecules.

The relationship of TL-A and TL-B to type XII collagen remains an open question. The sizes of TL-A and TL-B are both comparable to those reported for α1(XII), and the rotary shadowing patterns are strikingly similar (Dublet et al., 1989; Gordon et al., 1990). However, the tissue distribution of these molecules contradicts their identification as type XII, as published data indicate that type XII is not present in chick cartilage, although type XII messenger RNA was detected in cartilage by RNAse protection assays (Sugrue et al., 1989). It is possible that mammalian type XII will demonstrate a more widespread tissue distribution. A bovine homologue of chick type XII isolated from periodontal ligament is 93% homologous within COL-1. A second type XII-like molecule present in skin and tendon is 62% homologous within this domain (Dublet and van der Rest, 1990). We assume that either TL-A or TL-B is identical to this molecule, but at present our evidence cannot be directly compared to this data. It is interesting to note that only one pepsin-resistant collagen sequence (COL1) has been identified in skin, while the present data indicate that at least two unique NC-3 domains are present in this tissue.

Amino acid sequences of type XII collagen (Gordon et al., 1989*a*) indicate that it lacks a domain comparable to type IX NC-3 that is responsible for the attachments of chondroitin sulfate and for crosslinking to type II collagen. The facile extractability of TL-A and TL-B from all tissues suggests that the majority of these collagens are not covalently stabilized to banded collagen fibers, at least not in fetal tissues. Strong homologies between the COL1 domains of type XII and type IX collagens have been reported (Gordon et al., 1987, 1989*b*), and it has been suggested that such homology predicts an association of the type XII-like molecules with the surface of banded collagen fibers (Sugrue et al., 1989). Ultrastructural immunolocalization of TL-A and TL-B is consistent with this hypothesis (Keene et al., 1990).

The present data are consistent with the published concept that type IX, type XII, TL-A, and TL-B are members of a structurally related family of collagen molecules. However, the observation that type IX, TL-A, and TL-B are all present in cartilage, and that TL-B and type IX are uniformly distributed throughout the hyaline cartilage matrix suggest that the individual members of this family may serve different functions. Unique functions for TL-A and TL-B are suggested by the complementary tissue distributions seen for those molecules in skin, cartilage, and blood vessels (not shown). The present data do not allow insights into what these functions might be.

The relationship of TL-A1 to TL-A2, and TL-B1 to TL-B2 is unclear. The simplest interpretation of the size relationship is that the slower electrophoretic form is a dimer of the faster and has an anomalous migration. Since both convert to a single three-armed fragment upon collagenase treatment, the purported nonreducible crosslink resides within the triple-helical fragment, as has been proposed for type XII collagen (Dublet et al., 1989). It is quite possible that TL-A1 or TL-B1 become covalently associated with other macromolecules. In addition to independent molecules like those shown by rotary shadowing in Fig. 6, we frequently see similar molecules closely associated with rod-like molecules ~300 nm in length (not shown). Presumably, these are either type I or type III collagens. It is not known if the close associations seen by rotary shadowing are coincidental, or if they result from covalent bonding between TL-A or TL-B molecules. It is also possible that the slow electrophoretic mobilities of TL-A1 and TL-B1 result from increases in their Stoke's radii due to some factor other than increased peptide length, or result from posttranslational carbohydrate additions. However, the sharpness of the electrophoretic bands argues against the latter. Also, the type XII equivalent of TL-A1 and TL-B1 does not stain with Alcian blue, and its electrophoretic mobility is not altered by chondroitinase ABC digestion (Dublet et al., 1989).

We have observed two anti-TL-A–immunoreactive electrophoretic species in cell culture with slower electrophoretic mobilities than TL-A1 and TL-A2, that upon collagenase

digestion yield a single resistant fragment larger than the globular domain of TL-A (Keene et al., 1991; and our unpublished data). This observation suggests that TL-A is synthesized in a precursor form, distinguishing TL-A from the reported characteristics of type XII collagen (Dublet et al., 1989).

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