Purification and Characterization of Native Type XIV Collagen*

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A new molecule, type XIV collagen, with domains homologous to type IX and XII collagens has been recently discovered in pepsin extracts of fetal bovine tissues (Dublet, B., and van der Rest, M. (1991) J. Biol. Chem. 266, 6853–6858). In the present study, we describe the purification and the characterization of the intact native form of this newly discovered collagen. By using only two chromatographic steps we were able to obtain pure type XIV collagen. Furthermore, minor modifications of the protocol allowed us to perform the simultaneous large scale purification of type XII and type XIV collagens from the same tissue.

Intact type XIV collagen migrates on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as two bands of 220 and 290 kDa (reducing conditions). After collagenase treatment, a single band of 190 kDa is observed, which represents the large non-collagenous domain of the molecule (NC3). Rotary shadowing electron micrographs of intact type XIV collagen show a cross-shaped structure formed by a thin tail attached through a central globule to three identical “fingers.” These properties are similar to those previously described for intact chicken type XII collagen (Dublet, B., Oh, S., Sugrue, S. P., Gordon, M. K., Gerecke, D. R., Olsen, B. R., and van der Rest, M. (1989) J. Biol. Chem. 264, 13150–13156), but the two molecules are different gene products and have charge and glycosylation differences.

Finally, we show that the three chains of purified type XIV collagen have an apparent molecular mass of ~220 kDa and are not cross-linked to each other by bonds other than disulfide bridges. The same observation was made for type XII collagen. In both cases, the 290-kDa migrating band in SDS-PAGE is due to incomplete denaturation in electrophoresis sample buffer in the absence of urea.

Collagens represent a large family of proteins which are essential constituents of the extracellular matrix (for recent reviews, see Refs. 1–3). Based on their supramolecular structures, the collagens are divided in two main classes, fibril-forming collagens (types I, II, III, V, and XI collagens) and non-fibril-forming collagens. The latter group is heterogeneous both structurally and functionally, and a subgroup named FACIT for Fibril-Associated Collagens with Interrupted Triple helices has been defined (4), which contains at present three members, namely type IX, type XII, and the recently discovered type XIV collagen (5).

Type IX collagen, the first and best characterized FACIT, is expressed in tissues containing type II collagen. The existence of an homologue of type IX collagen in type I collagen-containing tissues was initially suggested by the isolation and sequencing of an embryonic chick tendon cDNA clone encoding a molecule, called type XII collagen, whose COL1 domain was similar to the COL1 domain of type IX collagen (6). This collagen was identified in chicken tendon extracts as a disulfide bonded polypeptide migrating in SDS-PAGE (reducing conditions) as two bands of 220 and 290 kDa. The intact native molecule was partially purified from chicken tendon and shown to produce a major 190-kDa band after collagenase treatment and reduction (7). This work and the sequencing of extended type XII collagen cDNA clones (8) showed that type XII collagen is a disulfide-bonded homotrimer with five domains. Two short triple helical collagenous domains (COL2 and COL1), containing, respectively, 152 and 103 amino acids, are spaced by a 43-amino acid-long NC2 region. The C-terminal non-collagenous domain (NC1) contains 76 residues. Very recently, the complete primary structure of the molecule has been published (9). The large N-terminal non-collagenous domain (NC3) contains 18 units of the type III motif of fibronectin and three units homologous to the von Willebrand factor A domain. On rotary shadowing electron micrographs, intact chicken type XII collagen shows a cross-shaped structure formed by a thin triple helical tail, 75 nm in length, attached through a central globule to three “finger-like” structures, each about 60 nm in length. These “fingers” and the central globule correspond to the NC3 domains and are terminated each by a small globule (7).

Previous experiments have shown that type XII collagen staining is not associated with all type I collagen-containing structures (10). This observation suggested that additional homologues of type IX and type XII collagens could exist (11). In fact, pepsin-resistant triple helical fragments of a distinct type XII-like molecule have been isolated from bovine skin and tendon and recently in chick embryonic skin (5, 11, 12). Amino acid sequence analysis showed that one fragment is derived from a reducible homotrimer, given the designation type XIV collagen, with a triple helical domain that is 64% homologous with type XII COL1 domain (5). The similarity includes size, location of cysteine residues, nature, and posi-

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1 The abbreviations used are: FACIT, fibril-associated collagens with interrupted triple helices; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Con A, concanavalin A; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyeth- ylenenitrilo)]tetraacetic acid.
tion of an imperfection in the triple helix. Other pepsin-resistant polypeptides, thought to be also derived from type XIV collagen, were purified from bovine tendon (5). Their sequence could be aligned with the COL2 domain of type XII collagen, suggesting that these two distinct FACITs could be very similar molecules present simultaneously in some tissues.

In the present study, we have identified and characterized for the first time the intact form of type XIV collagen. To investigate the functional properties of this new multidomain protein and to study in addition the role of type XII collagen which is unclear, large scale purification of the intact molecules in undenatured form is a prerequisite. We describe here a procedure for the separation and purification in substantial amounts of both type XII and type XIV colagens from bovine tissues.

MATERIALS AND METHODS

Tissue Extractions—All steps were carried out at 4°C or on ice. The leg tendons and the skin from 17-day-old chick embryos were dissected, immediately frozen in liquid nitrogen, and kept at –80°C until used. Fetal calves (5–7 months) were obtained within 1 h after slaughtering. Skins or tendons were stripped, cut into small pieces, immersed in liquid nitrogen, and milled using a grinder (Universal Multimol 20, Ika Industries) or a freezer mill (SPEX Industries, Metuchen, NJ). The resulting powders were stored at –80°C and used within 2 months. Before extraction, the powder was suspended in 10 volumes of a cold isotonic medium (0.25 M sucrose, 10 mM HEPES, pH 7, containing 2 mM EDTA, 2 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride as protease inhibitors) and left 15 min on ice. The homogenate was centrifuged 30 min at 7,500 x g and the pellet washed a second time if necessary. The residue was suspended in 10 volumes of extraction buffer (0.275 M NaCl, 50 mM Tris-HCl, pH 8, and the same protease inhibitors as above) to produce a final NaCl concentration of 0.25 M and incubated 5 h under magnetic stirring. The precipitate was separated from the insoluble material by centrifugation at 20,000 x g for 30 min. It may either be used immediately or stored at –20°C.

Batch CM-Cellulose—All steps were carried out at room temperature. The 0.25 M NaCl skin extract was diluted 4 times with distilled water, and the pH was adjusted to 7.4 with concentrated HCl. Overnight binding on CM-cellulose (CM-62, Whatman, 3.5 ml of gel/g of skin powder) was performed under gentle agitation. The gel was poured in a chromatography column and extensively rinsed (about 10 gel volumes) with 0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, 1% isopropanol. Elution was carried out with the same buffer containing 0.5 M NaCl.

For tendon extracts, the procedure was performed as described above for skin extracts with the following modifications. The extracts were diluted 5.5 times with distilled water to obtain binding of both type XII and XIV collagens on the resin. A washing of the gel with 45 mM NaCl in 10 mM Tris-HCl, pH 7.4, 1% isopropanol was included before elution with 0.1 M NaCl and 0.5 M NaCl containing buffers.

Con A-Sepharose Chromatography—The fractions enriched in type XII and type XIV collagens as judged by SDS-PAGE were pooled and chromatographed on a Con A-Sepharose (Pharmacia LKB Biotechnology Inc.) column at room temperature (0.1–0.25 ml of gel/g of initial tissue powder). The column was washed with a solution of 50 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl (10 bed volumes or more). Type XIV collagen was eluted with 10 mM methyl α-D-mananopyranoside dissolved in the same buffer. When the absorbance at 280 nm had returned to baseline, a subsequent passage of 1 M α-D-mananopyranoside was carried out, which allowed the elution of type XII collagen.

Collagenase Digestion—Type XIV collagen (3 µg in a 25-µl final volume) was treated with 7.5 units of purified clostripiopseptase A (bacterial collagenase, form III, Advanced Biofuels) in 0.5 M NaCl, 50 mM Tris-HCl pH 7.4, containing 10 mM N-ethylmaleimide and 6 mM CaCl2 at 30°C. After 1 h, 7.5 additional units were added and the incubation continued for 2 h. Control samples were mixed with buffer alone. Digestion was stopped by addition of 5 volumes of cold acetone. After 1 h at –20°C, the precipitates were collected by centrifugation for 10 min at 10,000 x g and redissolved in SDS-PAGE sample buffer.

Pepsin Digestion of Purified Type XIV Collagen—Type XIV collagen in Con A-Sepharose elution buffer (2 ml, 125 µg/ml) was concentrated 5 times using Centricon-100 (Amicon, M, 100,000 cut-off) at 20°C under conditions recommended by the manufacturer. The filtrate, containing low molecular weight contaminants, was discarded. After addition of 200 µl of 1 M CH3COOH in 0.25 M NaCl, the retentate was incubated with 50 µg of pepsin (from porcine stomach mucosa, Sigma, 3470 units/mg protein). After 75 min at 30°C, pepsin (15 µg) was added and the digestion was continued for 30 min. The pH was then adjusted to 6 with 2.5 M NaOH, and the solution was stored at 4°C until fractionated by HPLC (6 h in these experiments).

HPLC Separation of Pepsin-generated Fragments of Type XIV Collagen—Pepsin-digested type XIV collagen was fractionated by HPLC using an aqueous acetonitrile gradient (0–60%, 60 min) in the presence of 10 mM heptafluorobutyric acid. The column was an Aquapore RP-300 (220 x 2.1 mm; Brownlee, Applied Biosystems). The equipment was from Applied Biosystems and consisted of a model 400 solvent delivery system and a 1000 S diode array detector. Aliquots of the collected fractions were analyzed for their amino acid content, and a fraction rich in proline, hydroxyproline, and glycine was further purified on the same column using trifluoroacetic acid as ion pairing agent and an aqueous acetoniitrile gradient (0–60%, 60 min).

Antibody Production—Purified bovine type XIV collagen (10 µg emulsion with Freund complete adjuvant) was injected intramuscularly in a 5-week-old female BALB/c mouse. Booster injections were given 3 and 5 weeks later with antigen emulsified in incomplete adjuvant. Intraperitoneal boosts with antigen in Con A-Sepharose elution buffer were given 8 days and 1 day before fusion. Methods for fusion and establishment of antibody-producing hybridomas were performed using the standard procedures described by Linzenmayer and Hendrix (13). Splenocytes from the immunized mouse and spl/2-0-Ag myeloma cells were fused by polyethylene glycol 4000. Hybridomas were grown in RPMI supplemented with glutamine, glucose, penicillin-streptomycin, HEPES, pyruvate, mercaptoethanol, and 10% fetal bovine serum. Antibody secretion was screened by direct enzyme-linked immunosorbent assay against purified bovine type XIV collagen. The monoclonal antibody CY 10B8 was used in the experiments presented in this work.

Immunoblotting—Immunoblots were performed as described by Sugrue et al. (10). For the immunoblot characterization of chicken preparations, a monoclonal antibody (75d7, kindly provided by S. P. Sugrue) reacting specifically with a sequence located in the NC1 domain of chicken type XII collagen was used. Staining was done using a phosphatase-conjugated second antibody (Biosys) and the AP color development kit from Bio-Rad.

Rotary Shadowing Electron Microscopy—Proteins were dialyzed against 0.2 M ammonium bicarbonate, Samples were mixed with an equal volume of glycerol and sandwiched between freshly cleaved mica disks. Shadowing was performed as described (14). Electron micrographs were taken at 40,000 x magnification in a JEOL EX 1200 electron microscope. Replica were examined at the Centre de Microscoipe Electronique Applique à la Biologie et à la Géologie, Clermont-Ferrand University, France.

Other Procedures—Protein concentration was determined by the method of Bradford (15) with the ready-to-use Coomassie Blue G-250-based reagent from Pierce, with bovine serum albumin as standard. SDS-PAGE were performed according to Laemmli (16). Unless otherwise indicated, the electrophoresis sample buffer contained 125 mM Tris-HCl, pH 6.8, 10% glycerol, 5% SDS, 4% 2-mercaptoethanol, and 0.007% pyronine as marker for migration. Alternatively, glycerol was omitted and 6.7 M urea was included. Apparent molecular masses on SDS-PAGE are given according to globular markers. Amino acid sequence analysis was performed by automated Edman degradation in an Applied Biosystems 473A protein sequenator using the trifluoroacetic acid conversion program provided by the manufacturer.

RESULTS

Purification of Native Type XIV Collagen from Bovine Fetal Skin—When this work was initiated, the properties of the intact type XIV collagen molecule were unknown. Because of the high sequence similarities noted between type XII and XIV collagen pepsin fragments, we assumed that the general characteristics of the two molecules would also be similar. During our purification procedures, we therefore monitored the presence of large reducible molecules with a partial sensitivity to bacterial collagenase.
Ion exchange chromatography experiments showed that two reducible collagenase-sensitive bands of 220–290 kDa were retained on CM-cellulose in our conditions. Elution of the bound proteins gave a fraction (Fig. 1, lane 2) highly enriched in these collagenase-sensitive proteins. The major contaminants (mainly type I and II collagens) were eliminated by a chromatographic step on Con A-Sepharose (Fig. 1, lane 2). After extensive wash of the gel, the collagenous material was eluted from the Con A affinity column with 10 mM methyl α-D-mannopyranoside (Fig. 1, lanes 4R and 4NR).

This protocol allows the rapid recovery of relatively large amounts of a protein fraction in which type XIV collagen (see below) represents about 90% of the Coomassie Blue-stained bands. The yields varied from 0.15 mg for 5 g of tissue to 0.7 mg for 35 g of tissue.

The Purified Molecule Is the Intact Form of Type XIV Collagen—Pepsin digestion of the purified collagen produced the accumulation of two reducible pepsin-resistant fragments of 34 and 13.5 kDa according to collagenous markers (not shown). The digest was fractionated by HPLC, and each peak was analyzed for its amino acid content. A fraction rich in proline, hydroxyproline, and glycine was further purified by HPLC. It appeared as a single polypeptide of 34 kDa on 15% SDS-gels (unreduced form, not shown). This unreduced fragment was submitted to N-terminal amino acid sequencing. The major sequence was read as XRTIQGPPGEPGRPGXP. A minor sequence (~25%) was identical to the major one but shifted by 3 residues (N+3). The first residue could not be positively identified because of the presence of some contaminating free amino acids. This sequence, from residue 3, is identical to the N-terminal sequence determined for the tryptic peptide T2 marking the transition between NC2 and COL1 in bovine α1(XIV) (5). Contrary to what was observed previously on pepsin extracted type XIV collagen fragments, the N terminus of the fragment obtained from the pepsin digestion of the purified molecule was not blocked. This could be explained by the much shorter incubation time of the sample in the presence of acetic acid.

Type XIV Collagen Has the Same General Characteristics as Type XII Collagen—Type XIV collagen has the same electrophoretic mobility in SDS gels as type XII collagen and does not enter the gels without reduction (Fig. 1, lanes 4R and NR). Furthermore, after digestion with purified bacterial collagenase, a single band at 190 kDa is observed (Fig. 1, lane 5). Rotary shadowing electron microscopy (Fig. 2) reveals that the major molecule present in the purified fraction has three fingers originating from a central globule at the end of a thinner tail (about 80 nm). The fingers (40–50 nm) are terminated by a small globule. The fingers and the tail are often kinked. These experimental results (apparent molecular masses of the intact and collagenase digested forms, reducibility and shape on rotary shadowing pictures) demonstrate that type XIV collagen is indeed very similar to type XII collagen.

Type XII collagen Is Absent from the Type XIV Collagen Preparation—Dublet and van der Rest (5) have shown that pepsin-generated fragments from both type XII and XIV collagens can be isolated from bovine fetal skin. In consequence, it was important to look for a possible contamination of our preparation by α1(XII). As no antibody reacting with bovine type XII collagen was available, we used the protocol described above to partially purify chicken skin type XIV collagen and tested its immunoreactivity toward a monoclonal antibody to chicken α1(XII). As shown in Fig. 3, we could purify from chicken embryonic skin a molecule which has the same electrophoretic mobility as bovine type XIV collagen (compare lanes 1 and 2, Fig. 3A). No type XII collagen can be detected by immunoblotting in this preparation (Fig. 3B, lane 2).

Type XII and XIV Collagens Can Be Separated on the Basis of Their Different Binding on CM-Cellulose and Con A-Sepharose—Having shown that type XIV collagen can be obtained in a pure form, we have investigated the conditions that would allow the separation of type XII and type XIV collagens.

A 0.25 M NaCl extract from chicken tendon (Fig. 4, lanes 1) was diluted to 45 mM NaCl final concentration allowing both type XII and type XIV collagens to bind to CM-cellulose. Raising the NaCl concentration to 0.1 M resulted in the elution of material with 220–290 kDa apparent molecular mass immunoreactive toward the anti-type XII collagen monoclonal antibody (Fig. 4, lanes 3). The 0.1 M NaCl CM-cellulose eluate was applied to a Con A-Sepharose column.

Fig. 1. SDS-polyacrylamide gel electrophoresis of selected fractions obtained in the course of bovine skin type XIV collagen purification. The fractions were run on 6% acrylamide gels with (R) or without (NR) reduction. Lane 1, 20 μg of 0.25 M NaCl bovine skin extract; lane 2, 20 μg of 0.5 M NaCl CM-cellulose eluate; lane 3, fraction passing through Con A-Sepharose; lane 4, 3 μg of 10 mM α-D-methylmannopyranoside eluate from Con A-Sepharose; lane 5, the same as in lane 4 treated with bacterial collagenase.

Fig. 2. Rotary shadowing electron micrographs of type XIV collagen. Bovine skin type XIV collagen purified as described under "Materials and Methods" was dialyzed against 0.2 M ammonium bicarbonate before rotary shadowing. Magnification 200,000 ×; bar, 50 nm.
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FIG. 3. Electrophoretic characterization of type XIV collagen partially purified from chicken fetal skin. Bovine (lanes 1) and chicken (lanes 2) fetal skin type XIV collagens were purified according to the method described in the first paragraph of "Results" and run on 6% SDS-gels (reducing conditions). Chicken tendon type XII collagen was included as control (lanes 3). A, Coomassie Blue-stained gel; B, immunoblot with a monoclonal antibody to chicken type XII collagen.

After washing of the gel, 10 mM methyl α-D-mannopyranoside (conditions efficient for the release of type XIV collagen) did not result in the elution of detectable amounts of type XII collagen (Fig. 4, lanes 4), while 1 M methyl α-D-glucopyranoside permitted the desorption of type XII collagen (Fig. 4, lanes 5). These results show that type XII and XIV collagens have different binding properties to CM-cellulose and Con A-Sepharose. This latter difference between two FACITs isolated from the same species and tissue probably reflects differences in glycosylation. In view of the importance of sugars in recognition phenomena, this result is an indication that these two very similar molecules may have different interactive properties.

Elution of type XIV collagen from CM-cellulose was obtained with 0.5 M NaCl. A significant amount of type XII collagen was still present in this fraction (Fig. 4, lanes 6). This contamination could be eliminated by Con A-Sepharose chromatography using the conditions described in the preceding paragraph (Fig. 4, lanes 7 and 8). This protocol does not permit however the complete purification of chicken type XII and type XIV collagens, contrary to what we obtained in preparation from bovine tissue (see below).

In order to investigate the functional properties of these FACITs, purification of substantial amounts of type XII and XIV collagens is needed. We have thus carried out the large scale purification of both type XII and XIV collagens from bovine tendon extracts using the protocol described above. The purified fractions were analyzed after SDS-PAGE by Coomassie Blue staining of the gel and immunoblotting using a monoclonal antibody raised against bovine type XIV collagen (Fig. 5). As shown in Fig. 5A, the purified fractions had similar electrophoretic behavior (apparent molecular mass 220–290 kDa). These proteins gave rise to unique bands of 190 kDa after bacterial collagenase digestion (not shown). As revealed by the immunoblot, the bovine type XII collagen fraction was not contaminated by type XIV collagen (Fig. 5B, lane 1). The same blot proved in addition that in tendon (as in skin) the protein eluting with 10 mM methyl α-D-mannopyranoside was actually type XIV collagen (lanes 2). In consequence, the purification protocol described here allows the rapid recovery of relatively high amounts of both type XII

FIG. 4. Electrophoretic analysis of the main fractions obtained during partial purification of type XII and XIV collagens from chicken tendon. The different steps of the purification protocol and the correspondence between lane numbers and the nature of each fraction are described under "Results" except for lanes 2, which represent the CM-cellulose unbound fraction. SDS-PAGE was performed using 6% acrylamide separating gels. A, Coomassie Blue-stained gel; B, immunoblot with a monoclonal antibody to chicken type XII collagen.

FIG. 5. Electrophoretic pattern of collagens purified from fetal bovine tendon. Collagens XII and XIV were purified as summarized in Fig. 4 and analyzed after electrophoresis in 6% acrylamide gels by Coomassie Blue staining (A) or immunostaining of a transfer with a monoclonal antibody to bovine type XIV collagen (B). 1, type XII collagen preparation; 2, type XIV collagen preparation.
and XIV collagens (respectively, 40 and 70 μg/g initial powder) from a single bovine tendon extract.

The 290-kDa Band Is Due to an Incomplete Denaturation in Laemmli Sample Buffer—Type XII and XIV collagens are homotrimeric molecules (5,7). It is therefore intriguing to observe two major bands in SDS-PAGE, one at 220 kDa and the second at 290 kDa (apparent molecular masses). Several experiments have been reported which unsuccessfully attempted to elucidate this problem in intact type XII collagen preparations from chicken tendon (7). We now show that inclusion of 6.7 M urea in the electrophoresis sample buffer results in the appearance of the 220-kDa band only.

In 3.5% acrylamide gels, all the polypeptides present in the bovine type XIV collagen preparation enter the gel under reducing conditions regardless of the presence of urea (Fig. 6, lane 1). They are sensitive to bacterial collagenase and a single band of 190 kDa, corresponding to the NC3 domain of α1(XIV), is observed after collagenase treatment (Fig. 6, lane 2). If 6.7 M urea is included in the sample buffer, the most striking effect is the disappearance of the 290-kDa band with a concomitant increase in intensity of the 220-kDa band (Fig. 6, lane 3).

In experiments not described here, we have checked using 6 and 15% acrylamide gels that no polypeptide, distinct from the 220-kDa band, appear after type XIV collagen urea treatment. In consequence, the 290-kDa form is not an α1(XIV) chain associated with some other polypeptide. In addition, if the 290-kDa band is cut out and boiled in the presence of urea-containing sample buffer in reducing conditions, it migrates as a 220-kDa band on SDS-PAGE. This result confirms that the increase in the 220-kDa band intensity actually represents a conversion of the band from 290 kDa to 220 kDa. The same results were obtained with chicken type XII collagen. In consequence, the 290-kDa bands observed in intact α1(XII) and α1(XIV) collagens are due to an incomplete denaturation in SDS-reducing sample buffer in the absence of urea. Additional experiments indicate that urea treatment by itself is unable to promote the conversion of the 290-kDa band into the 220-kDa band. Simultaneous treatment with urea and a reducing agent is required to cleave the masked disulfide bond responsible for the presence of the 290-kDa band on SDS-PAGE (not shown).

**DISCUSSION**

In this report, we have presented the characterization of the native form of a new collagen molecule, type XIV collagen, which has been recently discovered as a 10-kDa pepsin-resistant fragment in extracts of fetal bovine tissues. Our results show that the triple helical pepsin-resistant domains represent less than 15% of the intact molecule.

The exact structure of intact type XIV collagen was unknown, although preliminary microscopy observations suggested that type XII and type XIV collagens could be very similar in size and in shape (11). The protocol described here allows the purification, in two chromatographic steps, of a reducible molecule presenting properties closely resembling those of type XII collagen. It migrates in standard SDS-PAGE as two bands of 220–290 kDa after reduction. After bacterial collagenase treatment, a major band of 190 kDa is observed. Rotary shadowing visualization of the purified molecule reveals a cross-shaped structure with a central globule, three fingers, and a collagenous tail.

Our data demonstrate that this protein is distinct from type XII collagen and actually represents type XIV collagen. First, pepsin digestion of the intact purified protein produces a major fragment which contains the COL1 domain of α1(XIV) as shown by N-terminal amino acid sequencing. Second, a monoclonal antibody we raised against bovine type XIV collagen does not recognize a 220–290-kDa collagenous molecule purified from bovine tendon using conditions by which type XII collagen from chicken tendon is obtained. Third, the chicken protein isolated with the same protocol used to purify bovine type XIV collagen is not immunoreactive toward a monoclonal antibody to chicken type XII collagen. This latter result shows in addition that type XIV collagen purified from chicken skin by our protocol is not contaminated by type XII collagen. This fact will be important in additional studies aiming at the elucidation of the specific roles of each of these collagens.

The isolation of the collagenase-resistant portions of two “twelve-like” collagens (TL-A and TL-B) has been described, and antibodies specific for each form have been obtained (17). This group very recently reported in abstract form the identification of these molecules as type XII and type XIV collagens respectively (18). On immunoblots of fractionated extracts, two bands were observed after reduction (17): TL-A1 (300 kDa) and TL-A2 (220 kDa), observation which confirms our previous results with type XII collagen (7), and TL-B1 (300 kDa) and TL-B2 (220 kDa), in agreement with the results presented here for type XIV collagen.

In the present report, we demonstrate that the 290-kDa bands do not represent α1(XII) or α1(XIV) chains associated with some other polypeptides. These bands result from an uncleaved disulfide bond. In consequence, our data strongly suggest that two disulfide bonded α1(XII) or α1(XIV) chains migrate in standard SDS-PAGE with anomalous apparent molecular masses of 290 kDa.

The exact role of type XII and type XIV collagens is still unknown. Type XII collagen was localized by immunofluorescence studies in type I containing dense connective tissues such as tendon, ligaments, periosteum, and perichondrium (10). The demonstrated covalent bonds between type IX and type II collagens (19,20), together with the homology of type IX, type XII, and type XIV collagens in their COL1 domains (5,6), suggest that these molecules are closely associated with or even part of the fibril assembly (4,21). The results recently

**Fig. 6. Urea treatment of type XIV collagen results in the disappearance of the 290-kDa band.** Bovine type XIV collagen was incubated first in the absence (lanes 1 and 3) or in the presence (lanes 2 and 4) of bacterial collagenase as described under “Materials and Methods.” Samples were boiled 3 min in electrophoresis sample buffer (125 mM Tris-HCl, 10% glycerol, 5% SDS, 4% 2-mercaptoethanol, and pyronine as tracer; lanes 1 and 2) or in the same buffer where glycerol was omitted and 6.7 M urea was included (lanes 3 and 4). SDS-PAGE was performed using a 3.5% acrylamide separating gel.
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published on TL-A and TL-B immunoelectron microscopy localization in various tissues show that these two twelve-like molecules are present on the surface of banded collagen fibers, with their noncollagenous domain extending from the fibers (22). These results are in favor of an interaction of type XII and XIV collagens with type I collagen-containing fibrils. This interaction might involve the COL1 domains of the FACITs, as previously proposed (23). The purification of these molecules in intact form will allow the characterization of their individual binding properties to fibrils, to other matrix components and to cells, as well as their possible role in collagen fibril formation.

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