

Structure and Binding Properties of Collagen Type XIV Isolated from Human Placenta

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Abstract. Collagen XIV was isolated from neutral salt extracts of human placenta and purified by several chromatographic steps including affinity binding to heparin. The same procedures also led to the purification of a tissue form of fibronectin. Collagen XIV was demonstrated by partial sequence analysis of its Col1 and Col2 domains and by electron microscopy to be a disulphide-linked molecule with a characteristic cross-shape. The individual chains had a size of ~210 kD, which was reduced to ~180 kD (domain NC3) after treatment with bacterial collagenase. Specific antibodies mainly to NC3 epitopes were obtained by affinity chromatography and used in tissue and cell analyses by immunoblotting and radioimmunoassays. Two sequences from NC3 were identified on fragments obtained after trypsin cleavage. They were identical to cDNA-derived sequences of undulin, a noncollagenous extracellular matrix protein. This

suggests that collagen XIV and undulin may be different splice variants from the same gene. Heparin binding was confirmed in ligand assays with a large basement membrane heparan sulphate proteoglycan. This binding could be inhibited by heparin and heparan sulphate but not by chondroitin sulphate. In addition, collagen XIV bound to the triple helical domain of collagen VI. The interactions with heparan sulphate proteoglycan and collagen VI were not shared by the NC3 domain, or by reduced and alkylated collagen XIV. No or only low binding was observed for collagens I–V, pN-collagens I and III, and several noncollagenous matrix proteins, including laminin, recombinant nidogen, BM-40/osteonectin, plasma and tissue fibronectin, vitronectin, and von Willebrand factor. Insignificant activity was also shown in cell attachment assays with nine established cell lines.

COLLAGENS represent a large family of extracellular matrix proteins that share as a common structural element a triple helical domain composed of three polypeptide chains with repeating Gly-Xaa-Yaa sequences. So far 14 different collagens, referred to as types I–XIV, and more than 20 corresponding genes have been identified (Vuorio and de Crombrughe, 1990; van der Rest and Garone, 1991). The collagens differ in their biological functions and can be divided into major classes based on their supramolecular organization into large fibrils (types I–III, V, XI), microfibrils (type VI), networks (types IV, VIII), and anchoring fibrils (type VII). A special class of fibril-associated collagens with interrupted triple helices (FACITs)¹ has been proposed, including the types IX, XII, and XIV, which show limited sequence homology and apparently do not self assemble into large structures (Shaw and Olsen, 1991; van der Rest et al., 1991). Fibril association has so far only been demonstrated for collagen IX, which binds to the surface of collagen type II fibrils in cartilage (Vaughan et al., 1988; Eyre et al., 1987; van der Rest and Mayne, 1988).

Collagen IX consists of three different α chains, $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$, and $\alpha 3(\text{IX})$, which are connected by disulphide bridges. They fold into three relatively short triple helical domains (Col1 to Col3), separated by small nonhelical regions and a globular domain NC4 (~30 kD) present at the NH₂ terminus of the $\alpha 1(\text{IX})$ chain (Shaw and Olsen, 1991; van der Rest et al., 1991). Domains homologous to Col1 and NC4 but not to Col2 and Col3 have also been detected in collagen XII (Gordon et al., 1987, 1989; Dublet and van der Rest, 1987), which is found in several noncartilagenous matrices (Sugrue et al., 1989). Its NH₂-terminal nontriple helical domain NC3 is quite large (~200 kD) and after rotary shadowing shows a three-armed structure that is connected to a short triple helix (Dublet et al., 1989). A 3,100-residue sequence based on cDNA clones has recently been reported for the $\alpha 1(\text{XII})$ chain of chick collagen XII (Yamagata et al., 1991). This demonstrates a modular structure for NC3 with 18 fibronectin type III motifs, four von Willebrand factor domain A motifs, and a domain similar to the $\alpha 1(\text{IX})$ domain NC4. Some evidence for alternative splicing was also provided that could explain why the size of NC3 isolated so far from tissues is distinctly smaller than predicted from the entire sequence (Yamagata et al., 1991).

Two type XII-like collagens were recently separated from

1. *Abbreviations used in this paper:* EHS, Engelbreth-Holm-Swarm; FACITs, fibril-associated collagens with interrupted triple helices.

fetal bovine skin in the form of their three-armed NC3 domains (Lunstrum et al., 1991a). This, together with different immunogold localizations (Keene et al., 1991), indicates that several proteins with homologous structures may exist. Similar three-armed structures had in fact already been shown to be present in collagen VII (Lunstrum et al., 1986) and in the noncollagenous matrix protein undulin (Schuppan et al., 1990). Evidence for a novel collagen XIV was then obtained by sequencing a new Coll domain from fetal bovine tendons (Dublet and van der Rest, 1991) and from chick cDNA (Gordon et al., 1991) that was homologous to Coll of $\alpha 1(\text{IX})$ and $\alpha 1(\text{XII})$ chains. More recently, a cDNA encoding a long stretch of the chick collagen XIV NC3 domain and part of the Col2 domain has been sequenced (Trueb and Trueb, 1992). The structural data for Coll also shows that both collagen XII and XIV are likely to consist of three identical α chains. Native collagen XIV, however, has so far not been characterized, nor is its entire sequence known.

Whether collagens XII and XIV are in fact bound to the surface of collagen fibrils is still unknown, and the immunological evidence for this is rather circumstantial (Sugrue et al., 1989; Keene et al., 1991). Here, we describe the purification of collagen XIV from human placenta using neutral salt extraction as used before for the isolation of laminin isoforms (Brown et al., 1990). The identity of the extracted protein with collagen XIV was demonstrated by partial sequence analyses, electron microscopy, and proteolytic fragmentation. Collagen XIV showed distinct binding to heparin and heparan sulphate proteoglycan and to collagen VI but not to various fibril-forming collagens and several other extracellular matrix proteins. This defines new functions for collagen XIV and raises the question as to whether different FACITs do in fact have similar biological roles.

Materials and Methods

Purification of Collagen XIV and Fibronectin from Human Placenta

Fresh placentae were washed, homogenized, and extracted as previously described for the preparation of human laminin (Brown et al., 1990) except that the extraction buffer consisted of 20 mM Tris-HCl, 10 mM EDTA, 1 M NaCl, 10 mM sodium azide, 0.3 mM PMSF, 10 mM *N*-ethylmaleimide, pH 7.4, with no Triton X-100. Two 5 M NaCl precipitations were carried out as before (Brown et al., 1990). The second NaCl precipitate was redissolved in extraction buffer and dialyzed against the same buffer containing 0.1 M NaCl and without *N*-ethylmaleimide. The material was passed over a heparin-Sepharose column (80 ml), and bound material was eluted with a 0.1–0.5 M NaCl gradient (600 ml). Eluted fractions were analyzed by electrophoresis, and those containing collagen XIV were pooled, dialyzed against 50 mM Tris-HCl, 10 mM EDTA, 0.1 M NaCl, pH 8.0, then passed over an FPLC MonoQ column (HR5/5; Pharmacia Biosystems GmbH, Freiburg, Germany) equilibrated in the same buffer. Bound proteins were eluted with a 0.1–0.6 M NaCl gradient (30 ml). The peak eluting at ~ 0.4 M NaCl was concentrated and size fractionated by FPLC on Superose 6 (100 ml; Pharmacia LKB Biotechnology, Inc.) equilibrated with 0.2 M ammonium hydrogen carbonate. The first eluted peak, which contained collagen XIV, was pooled and stored at -20°C .

Reduction and alkylation of collagen XIV was carried out in 6 M guanidine. HCl, 0.1 M Tris-HCl, pH 6.9 with 0.02 M dithiothreitol (5 h, 37°C). The reaction was stopped by adding a fourfold molar excess of *N*-ethylmaleimide (2.5 h, 37°C) followed by dialysis against 0.2 M ammonium hydrogen carbonate.

The fibronectin, which eluted with collagen XIV from the heparin-Sepharose column, was separated from it on the MonoQ column, where it eluted at 0.25–0.3 M NaCl. This material was dialyzed against 0.2 M ammonium hydrogen carbonate and stored at -20°C .

Sources of Other Proteins and of Glycosaminoglycans

A large heparan sulphate proteoglycan of low buoyant density was purified from the mouse Engelbreth-Holm-Swarm (EHS) tumor as described (Paulsson et al., 1987a). The same tumor was used to purify the laminin-nidogen complex (Paulsson et al., 1987b). Human laminin was isolated from placental extracts by procedures similar to those used previously (Brown et al., 1990). It was shown (Brown et al., unpublished) to consist of two isoforms of laminin with the chain compositions BleAmB2e and BlsAmB2e, respectively (for nomenclature, see Engel et al., 1991). Plasma fibronectin (Behringwerke AG, Marburg, Germany) was further purified on heparin-Sepharose (see above). Recombinant mouse nidogen (Fox et al., 1991) and recombinant human BM-40/osteonectin (Nischt et al., 1991) were obtained from stably transfected human cell clones. Human plasma von Willebrand factor was a kind gift of Dr. N. Heimburger (Behringwerke AG, Germany). Vitronectin was prepared from human plasma after urea activation by heparin-affinity chromatography (Yatohogo et al., 1988).

Human collagens I, III, V, and VI were solubilized by pepsin from placenta and purified following standard protocols (Miller and Rhodes, 1982; Odermatt et al., 1983). Collagen II was obtained from pepsin digested bovine hyaline cartilage (Miller and Rhodes, 1982). Truncated procollagens pNI and pNIII, possessing only the amino propeptide, and collagens I and III were obtained by neutral salt extraction from dermatoparactic sheep skin and fetal bovine skin and purified by NaCl precipitation and DEAE cellulose chromatography (Timpl et al., 1975). Collagen IV was solubilized from the EHS tumor by partial reduction (Yurchenco and Furthmayr, 1984). The NH_2 -terminal globule (N9-N2; Chu et al., 1990) of the human collagen VI $\alpha 3(\text{VI})$ chain was prepared by the recombinant methods used previously (Fox et al., 1991) and appeared after purification as a single 150-kD band (Specks et al., 1992). Reduction and alkylation of pepsin-solubilized collagen VI was performed under nonreducing conditions (Odermatt et al., 1983).

Heparan sulphate (36 kD) was obtained from the proteoglycan referred to above by alkaline degradation (Paulsson et al., 1987a). Heparin (~ 15 kD) and chondroitin sulphate were commercial products.

Proteolytic Digestions and Separation of Fragments

NC3 fragment was prepared by digesting collagen XIV with bacterial collagenase (CLSPA; Worthington, Freehold, NJ), at an enzyme-substrate ratio 1:100 for 1 h at 37°C , in 0.2 M ammonium hydrogen carbonate containing 10 mM *N*-ethylmaleimide and 0.3 mM PMSF. The digested material was mixed with an equal volume of 100 mM Tris-HCl, 20 mM EDTA, pH 8.0 then applied to a HiTrap Heparin column (5 ml; Pharmacia Biosystems GmbH) equilibrated with 50 mM Tris-HCl, 10 mM EDTA, pH 8.0. Unbound material was lyophilized and used to obtain amino acid sequences (see below). Bound material was eluted with a 0–0.5 M NaCl gradient (35 ml). The major eluted peak was concentrated, dialyzed against 0.2 M ammonium hydrogen carbonate, and stored at -20°C .

The nonbinding lyophilized collagenase fragments were dissolved in distilled water and acidified with formic acid. The material was injected onto a Nucleosil 300-10 C18 column (Macherey-Nagel GmbH & Co. KG, Düren, Germany) equilibrated with 0.1% trifluoroacetic acid and bound material was eluted with a 0–63% acetonitrile gradient (70 ml).

Collagen XIV dissolved in 0.5 M acetic acid was digested with pepsin (enzyme/substrate ratio, 1:100) for 20 h at 15°C and the reaction stopped by neutralization. After reduction with 2-mercaptoethanol, fragments were separated by electrophoresis.

To obtain tryptic fragments, collagen XIV was digested with TPCK-treated trypsin (Worthington) (enzyme/substrate ratio, 1:100) for 7 h at 37°C . The reaction was stopped by adding an equal volume of 100 mM Tris-HCl, 20 mM EDTA, pH 8, then PMSF was added to 0.3 mM. The mixture was passed over a HiTrap Heparin column (5 ml) and bound material was eluted with a 0–0.6 M NaCl gradient (50 ml). Fragments from an aliquot of the unbound fraction were applied to a FPLC MonoQ column, and bound material was eluted with a 0–0.6 M NaCl gradient (35 ml). A major peak eluting at 0.4 M NaCl was mixed with an equal volume of 8 M urea and digested with TPCK-treated trypsin (enzyme/substrate ratio, 1:100) overnight at 37°C . Digested material was lyophilized, redissolved in distilled water, and acidified with formic acid; then fragments were separated on a Nucleosil 300-10 C18 column as described above for collagenase fragments.

Analytical Methods and Rotary Shadowing

Amino acid compositions were determined after hydrolysis with 6 M HCl (16 h, 110°C) on a LC 5001 analyzer (Biotronik, Maintal, Germany). PAGE

on 3–10% gradient gels (unless otherwise indicated) was carried out in SDS. Samples were analyzed nonreduced or after reduction with 2-mercaptoethanol. Gels were calibrated with globular proteins and the laminin–nidogen complex or with CNBr peptides of collagen $\alpha 1(I)$ chain. Edman degradation was carried out in gas phase sequencer models 470A and 473 A (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Samples (0.1–1 nmol) were either chromatographically purified or were electrophoresed, electroblotted onto an Immobilon-P membrane (Millipore GmbH, Eschborn, Germany) in 10 mM $\text{Na}_2\text{B}_4\text{O}_7$, and stained with Coomassie Brilliant Blue R250, after which individual bands were excised and subjected to Edman degradation. Protein shapes were visualized by electron microscopy after rotary shadowing (Paulsson et al., 1987a, 1987b), using samples dissolved either in 0.2 M NH_4HCO_3 or in 0.1 M acetic acid.

Immunological Methods

Immunization of rabbits and radioimmunobinding and radioimmuno-inhibition assays followed standard protocols (Timpl, 1982). ELISAs were carried out as described for the binding assays. Quantitative radioimmuno-inhibition assays were established for collagen XIV ($\text{IC}_{50} \sim 25$ ng/ml), plasma fibronectin ($\text{IC}_{50} \sim 8$ ng/ml), and human placental laminin ($\text{IC}_{50} \sim 200$ ng/ml) using specific antisera. The two isoforms of laminin (see above) were of equal inhibitory capacity. For affinity purification (Timpl, 1982) of antibodies from a rabbit antiserum to collagen XIV, the serum was first passed over laminin and fibronectin immunoadsorbents and then over a collagen XIV immunoadsorbent. Antibodies displaced from the last column were then used in some of the studies. Rabbit antisera against the bovine collagen XII-like proteins TL-A and TL-B (Lunstrum et al., 1991a; Keene et al., 1991) were a kind gift of Dr. G. Lunstrum. mAbs IST-3, IST-4, and BC-1, specific for different fibronectin domains (Carnemolla et al., 1989), were kindly supplied by Dr. L. Zardi. Monoclonal antifibronectin antibody 3E3 was obtained from a commercial source (Biomol Feinchemikalien GmbH, Hamburg, Germany).

Protein Binding and Cell Adhesion Assays

Protein–protein interactions were analyzed with collagen XIV used as immobilized or soluble ligand following a previous protocol (Aumailley et al., 1989a) with some modifications. Wells were coated with first ligand (5 $\mu\text{g/ml}$, 50 $\mu\text{l/well}$) and then blocked with 2% milk powder dissolved in 0.05 M Tris-HCl, pH 7.2, 0.15 M NaCl (TBS). All further steps were carried out in 2% milk powder in TBS and washes between steps were with TBS, 0.04% Tween-20. Incubation with soluble ligand (0.7–50 $\mu\text{g/ml}$) followed by detection of this ligand with specific antiserum and anti-IgG conjugated to peroxidase was as previously described (Aumailley et al., 1989a). Bound peroxidase was visualized with 5-aminosalicylic acid (1 mg/ml) and 0.0045% H_2O_2 dissolved in 0.02 M Na_2HPO_4 adjusted to pH 6.8 with citric acid. Negative control values, from wells in which the soluble ligand was omitted, were subtracted from the binding data. For inhibition assays, soluble ligands were incubated with inhibitors (0.03–30 $\mu\text{g/ml}$) for 6 h at 4°C before being added to the immobilized ligand.

Cell adhesion to coated 96 well plates followed a colorimetric detection assay used previously (Aumailley et al., 1989b). Dose response profiles for collagen XIV, plasma fibronectin, and human placental laminin were determined by using the proteins at various coating concentrations (0.4–40 $\mu\text{g/ml}$, 100 $\mu\text{l/well}$). Most of the established cell lines used here have been previously described (Aumailley et al., 1989b). Serum-free culture medium was taken over a period of 24 h for radioimmunoassays.

Results

Purification and Structural Characterization of Collagen XIV and Fibronectin from Placenta

Neutral salt extracts that did not contain any detergent or other denaturing agents were prepared from washed human placenta. Extracts prepared from a single placenta contained 80–150 mg collagen XIV, 15–35 mg fibronectin, and 11 mg laminin as shown by specific radioimmunoassays at later stages of these investigations. 5 M NaCl precipitation gave rise to enrichment in these three proteins, which were partially separated from each other by affinity chromatography on a heparin column (Fig. 1). Most of the laminin did

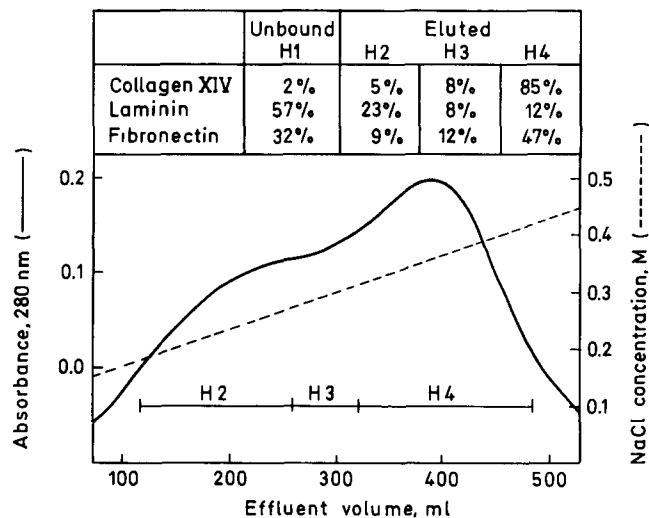


Figure 1. Separation of human placental proteins by heparin-Sepharose. The column was eluted with a linear NaCl gradient (dashed line). The distribution of collagen XIV, laminin, and fibronectin in eluted pools H2, H3, and H4 (horizontal bars) and in the unbound fraction H1 (not shown) was determined by radioimmunoassay and is shown in the table at the top of the figure.

not bind to heparin. Elution of the column with a NaCl gradient revealed a broad peak in which most of fibronectin eluted with the collagen XIV. Separation of collagen XIV from fibronectin was achieved by FPLC on MonoQ (Fig. 2), and a final step on a Superose 6 column efficiently removed contaminations of proteolytic activity that would partially cleave collagen XIV at 37°C. The final yield of collagen XIV was ~ 3 –4 mg with a low content (w/w) of laminin (3–5%) and fibronectin (1%) as determined by radioimmunoassays.

Purified collagen XIV remained near the top of the gradient electrophoresis gel when nonreduced and after reduction resolved into major bands of 340 kD and 210 kD with a fainter band at 195 kD (Fig. 3 a and b). The 340-kD band

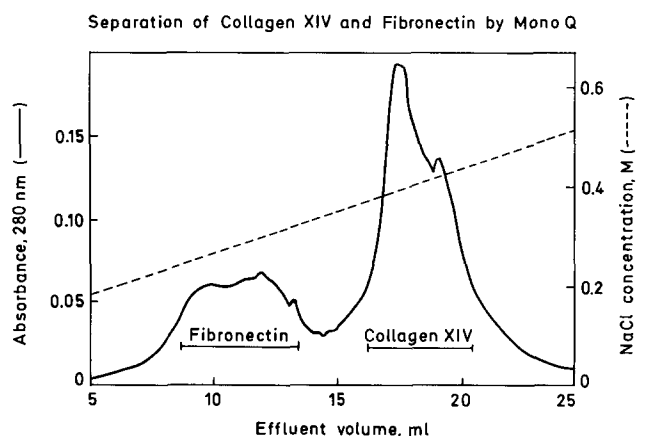


Figure 2. MonoQ separation of fibronectin and collagen XIV. The positions of fibronectin and collagen XIV (horizontal bars) were determined by electrophoresis. Low levels of laminin contamination eluted with the fibronectin. The column was equilibrated in a buffer containing 0.1 M NaCl and eluted with a linear NaCl gradient (dashed line).

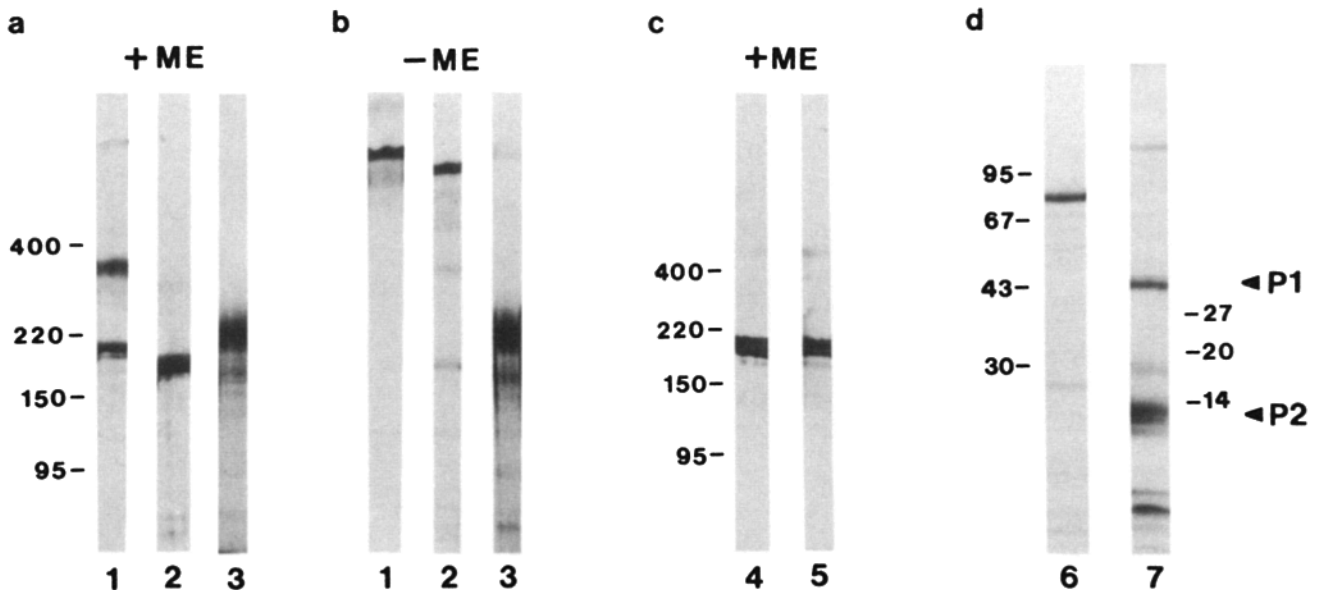


Figure 3. SDS-gel electrophoresis of purified collagen XIV, fragment NC3 and fibronectin (*a* and *b*), of further proteolytic fragments (*d*) and immunoblotting with affinity-purified antibody against collagen XIV (*c*). Lanes in *a* and *b* were loaded with purified collagen XIV (lane 1), fragment NC3 (lane 2), and placental fibronectin (lane 3). Samples were either nonreduced (-ME) or reduced with 2-mercaptoethanol (+ME). Immunoblotting (*c*) was done with an affinity-purified antibody to collagen XIV. The samples were reduced collagen XIV (lane 4) or crude placental extract (lane 5). Samples in *a-c* were analyzed on 3-10% gradient gels and the runs calibrated with reduced globular proteins (molecular masses given on the left side in kD), laminin A (400 kD) and B (220 kD) chains, and nidogen (150 kD). 8-18% gradient gels were used in *d* and loaded with a reduced trypsin digest (lane 6) and a reduced pepsin digest (lane 7) of collagen XIV. Calibration was either with globular proteins (lane 6) or with CNBr peptides of collagen $\alpha 1(I)$ chain (lane 7). The positions of fragments P1 and P2 (lane 7) are indicated by arrowheads.

was not observed in all gels even from the same collagen XIV preparation and was never seen with reduced and alkylated collagen XIV, and was therefore considered to be an artefact of incomplete reduction. Amino acid analysis demonstrated the presence of significant amounts of 4-hydroxyproline and hydroxylysine (Table I) which, however, were low enough to

Table I. Amino Acid Composition of Human Collagen XIV, its NC3 Domain, and Human Placental Fibronectin

| | Collagen XIV | NC3 | Fibronectin |
|------------------|------------------|-----|-------------|
| | (residues/1,000) | | |
| 4-Hydroxyproline | 24 | 0 | 0 |
| Proline | 68 | 52 | 72 |
| Aspartic acid | 92 | 97 | 95 |
| Threonine | 78 | 73 | 98 |
| Serine | 74 | 87 | 75 |
| Glutamic acid | 119 | 114 | 119 |
| Glycine | 114 | 117 | 182 |
| Alanine | 57 | 58 | 45 |
| Half cystine | 5 | 5 | 5 |
| Valine | 67 | 70 | 68 |
| Methionine | 7 | 24 | 3 |
| Isoleucine | 46 | 49 | 38 |
| Leucine | 72 | 79 | 52 |
| Tyrosine | 26 | 20 | 19 |
| Phenylalanine | 33 | 37 | 19 |
| Histidine | 28 | 33 | 33 |
| Hydroxylysine | 4 | 0 | 0 |
| Lysine | 41 | 43 | 28 |
| Arginine | 45 | 44 | 49 |

indicate the presence of large noncollagenous regions. Electron microscope images after rotary shadowing of the protein at neutral pH showed cross-shaped particles (Fig. 4 *A* and *B*). They consisted of thick rodlike segments (~ 50 nm) in a three-armed arrangement which were connected to a thin, 70-nm-long strand. This thin strand frequently showed a kink ~ 30 nm away from its free end. The thick rods collapsed into compact globules when collagen XIV was dissolved in dilute acetic acid (Fig. 4 *C*). The same change has been reported for the collagen XII-like component TL-B from bovine skin (Lunstrum et al., 1991a), which has recently been identified as collagen XIV by sequence analysis (Lunstrum et al., 1992). Furthermore, an antiserum against TL-B reacted much more strongly in ELISA with the placental collagen XIV than an antiserum against TL-A, indicating that TL-B and placental collagen XIV are closely related.

Brief digestion (1 h) of collagen XIV by bacterial collagenase reduced the apparent molecular mass of the constituent chains to a doublet of 180/165 kD (Fig. 3 *a*, lane 2) indicating that the triple helical domains contribute $\sim 15\%$ to the total mass. Electrophoresis of this collagenase-resistant fragment under nonreducing conditions (Fig. 3 *b*, lane 2) gave predominantly a band of slightly faster mobility than the disulphide-linked intact collagen XIV molecule (Fig. 3 *b*, lane 1), indicating that the fragment is still disulphide-linked. By analogy with the closely related molecule collagen XII, this collagenase-resistant fragment is probably the large, NH_2 -terminal NC3 domain. This NC3 domain was purified by heparin-affinity chromatography and showed an amino acid composition with negligible levels of hydroxyproline (Table I). Electron microscopy of NC3 demon-

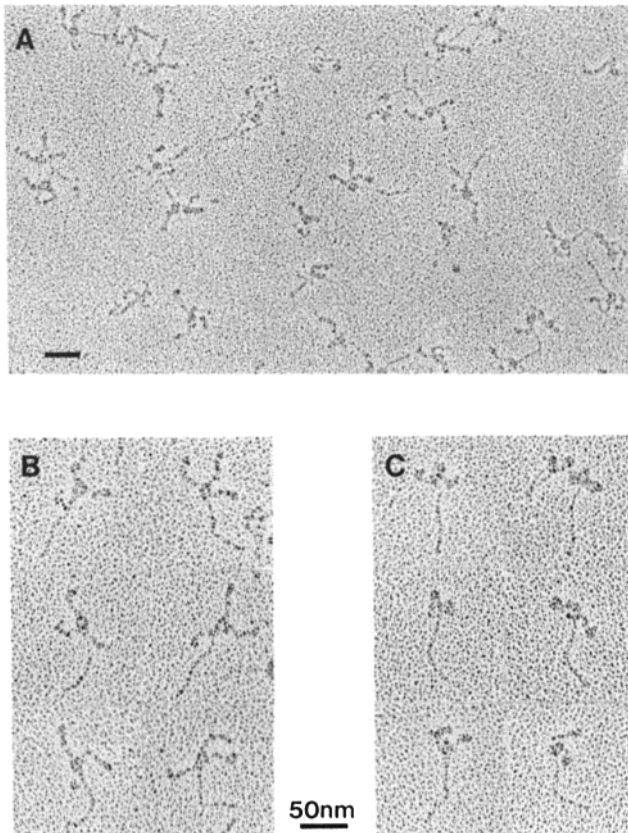


Figure 4. Rotary shadowing images of collagen XIV shown as a representative field (A) and as selected particles (B and C). Samples in A and B were dissolved in 0.2 M ammonium hydrogen carbonate pH 7.9 and in C in 0.1 M acetic acid. Bar, 50 nm.

strated the three-armed structure of the rods which lacked, however, the thin 70-nm strand (not shown).

Because tissue forms of fibronectin are not readily available (Hynes, 1990), we also purified placental fibronectin

after elution from the heparin column (Figs. 1 and 2). The purified component showed a broad band at ~ 220 kD in both reduced and nonreduced gels (Fig. 3 a and b). The amino acid composition was different to that of collagen XIV and its NC3 domain (Table I). The nature of the placental fibronectin was examined in ELISA with several specific mAbs. They detected the extra type III domain ED-A (mAb IST-3) but not ED-B (mAb IST-4), and binding was also observed for mAbs BC-1 and 3E3, which bind to epitopes common to cellular and plasma fibronectin (Carnemolla et al., 1989). Only BC-1 and 3E3 reacted with plasma fibronectin, demonstrating that the placenta-derived fibronectin contains a distinct proportion of cellular fibronectin possessing domain ED-A (Hynes, 1990).

Partial Sequence Analysis of Collagen XIV

To confirm the identity of the placental collagen XIV, limited NH_2 -terminal sequencing was carried out on selected peptides. We started by a selective approach for the triple helical domains Col1 and Col2. Pepsin digestion of collagen XIV, which should release Col1 and Col2 in intact form, showed an electrophoresis pattern with several bands with fast mobilities of $< \sim 30$ kD when compared to collagen I peptides (Fig. 3 d). The same NH_2 -terminal sequence was found for fragments P1 (28 kD) and P2 (13 kD) and came from a junction region between a noncollagenous and a collagenous domain. When compared to the known chick collagen XIV sequences (Gordon et al., 1991; Trueb and Trueb, 1992), the peptide sequence showed slightly higher identity (8/14 residues) with the junction between domains NC3 and Col2 than with the sequence at the junction between NC2 and Col1 (7/14 residues). In support of a localization of this sequence to the NC3/Col2 junction was the interpretation of the data to mean that P2 corresponds to domain Col2 and P1 to an uncleaved combination of domains Col1-Col2. The peptide sequence showed a similar level of identity to chick collagen XII in this region (Table II). The remaining bands gave het-

Table II. Partial Amino Acid Sequences of Human Collagen XIV Fragments and Their Correlation to Chick Collagen XII and XIV Sequences

| | Human collagen XIV sequence | Match to | | |
|--------|---|--------------------|--------------------|--------------|
| | | Chick collagen XII | Chick collagen XIV | |
| | | Positions | Identity (%) | Identity (%) |
| P1, P2 | VALGPAGPPGGPGL | 2748–2761 | 57 | 57 |
| C1 | GPPGGPGLRGPXGQ | – | – | – |
| C2 | GPQGIPGGVGSPPGRDGSF | 2837–2854 | 50 | –† |
| C3 | GPPGHLGVP | 3028–3036 | 56 | 67 |
| C4 | GTPGFPGNAGVPGTPGER GLTGIXGEXGNPVGVTQ | 2967–2998* | 67 | 76 |
| C5 | GPSGLSIQGMP | 2799–2809 | 64 | 91 |
| C6 | GPIGIPGTPGVPGITSM | 2861–2878 | 56 | 87‡ |
| T1 | ASAXAITGPPELITSE | 1379–1395 | 29 | –† |
| T1' | LTWDPTSR | 1585–1592 | 38 | 75 |

Fragments were generated by digestion with pepsin (P), bacterial collagenase (C), or trypsin (T). A dot over P indicates 4-hydroxyproline. Identity of peptide sequences with corresponding published sequences of chick collagen XII (Yamagata et al., 1991) and chick collagen XIV (Gordon et al., 1991; Trueb and Trueb, 1992) is shown. The numbering of the chick collagen XII sequence is from Yamagata et al. (1991). X, nonidentified residues.

* A three-residue gap was introduced into the collagen XII sequence, as described in Gordon et al. (1991), to allow alignment.

† This part of the chick collagen XIV sequence has not yet been published.

‡ Match for residues 4–18 of peptide C6. The chick collagen XIV sequence corresponding to residues 1–3 is not yet known.

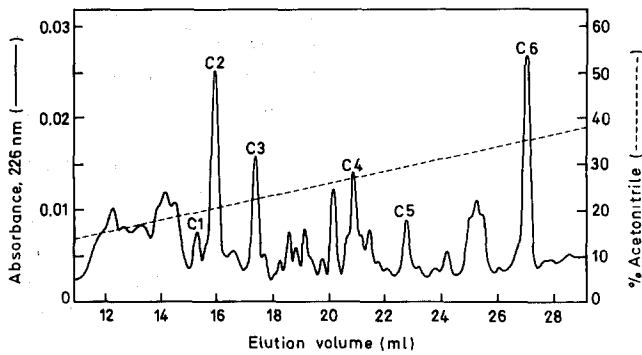


Figure 5. Elution profile of small collagenase-derived peptides of collagen XIV from HPLC C18 reverse phase column. NH₂-terminal sequences were determined for peptides in labeled peaks C1–C6 (Table II). The column was in 0.1% trifluoroacetic acid and was eluted with a linear acetonitrile gradient (dashed line).

erogeneous sequences, indicating fuzzy ends generated by pepsin cleavage.

Further sequence data were generated from small collagenase-derived collagen XIV peptides that did not bind to a heparin column. Reverse-phase chromatography showed a profile of limited complexity (Fig. 5), and six major peaks, C1 to C6, gave clearly identifiable sequences (Table II). Peptides C5 and C6 matched the Col2 region of chick collagen XIV and peptides C3 and C4 matched its Col1 region. These peptides, and in addition peptide C2, also matched the corresponding chick collagen XII sequences, although with lower levels of identity (Table II). Peptide C1 showed no strong correspondence to any collagen XII or collagen XIV sequence

and may originate from a less conserved region of collagen XIV. An overall identity of 59% (59/100 residues) was seen between peptides P1/P2 and C2–C6 and the corresponding chick collagen XII sequences. Only incomplete sequence data are available from the Col1 and Col2 domains of bovine (Dublet and van der Rest, 1991) and chick (Gordon et al., 1991; Trueb and Trueb, 1992) collagen XIV, but of the sequences that could be matched, 90% identity (43/48 residues) was seen with bovine collagen XIV and 76% identity (44/57 residues) with chick collagen XIV. Thus, the data clearly show that our placental collagen possesses the domains Col1 and Col2 of collagen XIV.

Sequences of the NH₂-terminal domain NC3 were obtained from large tryptic fragments. A trypsin digest of collagen XIV produced a major 80-kD band (fragment T1; Fig. 3 d) together with several minor bands in the range of 20–140 kD. Passage over a heparin affinity column produced only little binding of a few smaller fragments that did not give clear NH₂-terminal sequences. The nonbound fragment T1 was further purified on a MonoQ column. An internal fragment T1' was then generated by a second cleavage with trypsin in 4 M urea and was purified by chromatography on a C18 reverse phase column. The NH₂-terminal sequences of collagen XIV fragments T1 and T1' were identical to cDNA-predicted sequences of human undulin (Just et al., 1991) corresponding to positions 160–176 and 366–373 in clone Un1. T1' could also be matched to a sequence in the NC3 domain of chick collagen XIV (Trueb and Trueb, 1992), and the two sequences showed some identity with two different fibronectin type III motif sequences within the NC3 domain of chick collagen XII (Table II).

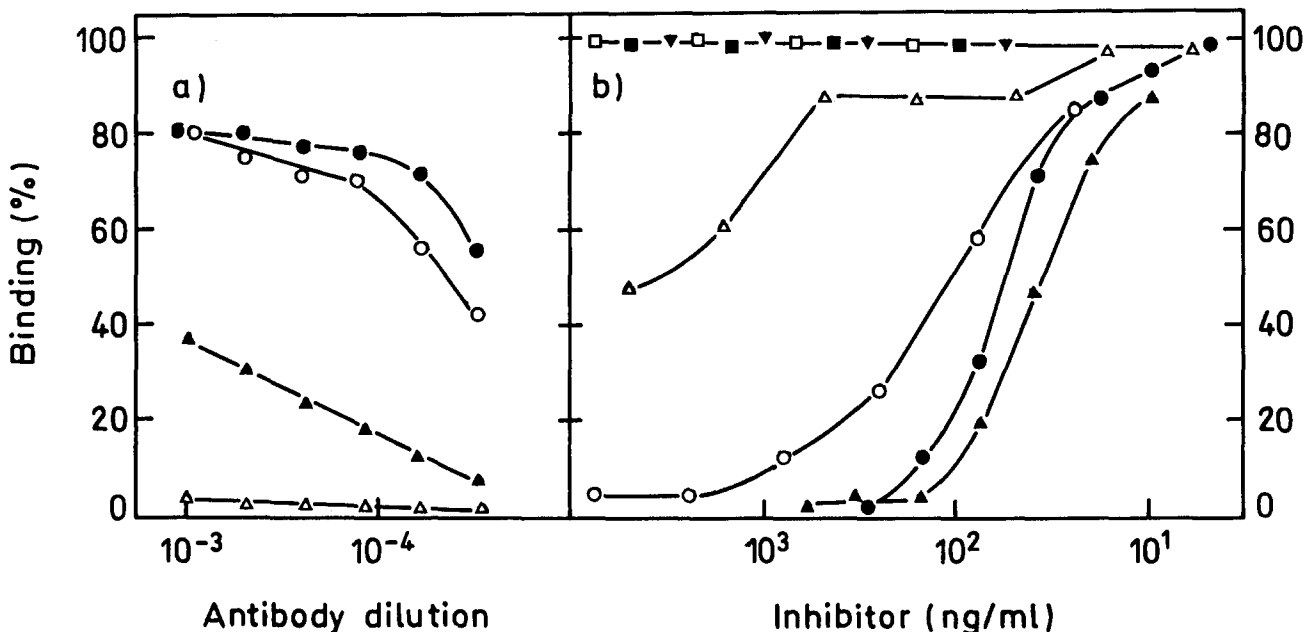


Figure 6. Characterization of antibodies against collagen XIV by radioimmunobinding (a) and inhibition (b) assays. Binding (a) was determined with a rabbit antiserum before (closed symbols) and after (open symbols) affinity-purification on a collagen XIV immunoabsorbent. Binding curves are shown for ¹²⁵I-labeled collagen XIV (○, ●) and plasma fibronectin (△, ▲). In the inhibition assay (b), a fixed dilution of antiserum to collagen XIV was incubated with nonlabeled inhibitors before the addition of ¹²⁵I-labeled collagen XIV. Inhibitors used were collagen XIV (●), reduced and alkylated collagen XIV (○), fragment NC3 of collagen XIV (▲), pepsin digest of collagen XIV (△), plasma fibronectin (■), von Willebrand factor (□), and vitronectin (▼). The globular domain N2–N9 of collagen VI α3 chain was also not active as an inhibitor (not shown).

Immunochemical Specificity and Cell and Tissue Studies

A rabbit antiserum against human collagen XIV showed a strong binding in radioimmunoassays (Fig. 6 a) and ELISA to collagen XIV, a moderate to strong binding to fibronectin, and weaker reactions with placental laminin and collagen IV. After affinity purification, the antibodies displaced from a collagen XIV column (see Materials and Methods) retained a strong reaction with collagen XIV (Fig. 6 a) but showed negligible or no binding for the other proteins. Specificity was also demonstrated by radioimmuno-inhibition assays (Fig. 6 b). Collagen XIV was strongly inhibitory with $IC_{50} \sim 20$ ng/ml, whereas >100-fold higher concentrations of placental laminin, fibronectin, von Willebrand factor, vitronectin, and NH_2 -terminal globular domain of collagen $\alpha 3(VI)$ chain had no significant effect. Studies with the purified NC3 domain showed an inhibition profile similar to that of collagen XIV, indicating that NC3 contributes most of the immunogenic epitopes. Reduced and alkylated collagen XIV also produced almost complete inhibition, indicating that the epitopes are not dependent on disulphide bonds. Pepsin digestion, however, caused a considerable loss in inhibitory capacity (Fig. 6 b).

Immunoblotting of reduced collagen XIV with the affinity-purified antibody showed a distinct reaction with the 210/195-kD doublet band (Fig. 3 c) and in some experiments with the intermittently observed 340-kD band. The same blotting patterns were observed with the crude placental extract, demonstrating the lack of substantial amounts of a larger variant of collagen XIV (Fig. 3 c). The radioimmuno-inhibition assay was used to study various biological fluids. Human normal sera from eight adult donors lacked any significant amounts of cross-reacting antigen (<20 ng/ml). The same negative result (<50 ng/ml) was found for serum-free culture medium from seven established human tumor cell lines (HT1080, HBL-100, A375, SAOS-2, A431, SCII, and SV40Wi26). An exception were rhabdomyosarcoma RD cells that secreted during a 24-h period ~ 240 ng/ml collagen XIV.

Cell Adhesion Activity of Collagen XIV

Both collagen XII (Yamagata et al., 1991) and collagen XIV (Gordon et al., 1991) possess a potentially cell-adhesive RGD sequence located at the COOH-terminal end of the Col2 domain. To examine cell attachment activity, we compared the dose-response profile of collagen XIV with those of human placental laminin and fibronectin in cell attachment assays (Fig. 6). The established cell lines used were HT 1080 (human fibrosarcoma), HBL-100 (human mammary epithelium), A431 (human epidermoid), A375 (human melanoma), SCII (human epidermal carcinoma), Rugli (rat glioma), RN22 (rat schwannoma), F9 (mouse teratocarcinoma), and B16F10 (mouse melanoma). They all showed strong adherence to laminin and fibronectin with plateau values in the range of 1.1–1.8 (OD units) and coating concentrations required for half maximal color yields of 0.4–0.8 $\mu\text{g/ml}$ for laminin and of 2–5 $\mu\text{g/ml}$ for fibronectin. Six of the cell lines did not attach at all to collagen XIV. Only HT 1080 (Fig. 7) and Rugli cells showed some weak adhesion to collagen XIV but at high coating concentrations of 40 $\mu\text{g/ml}$. Thus, the activity might be entirely attributable to the

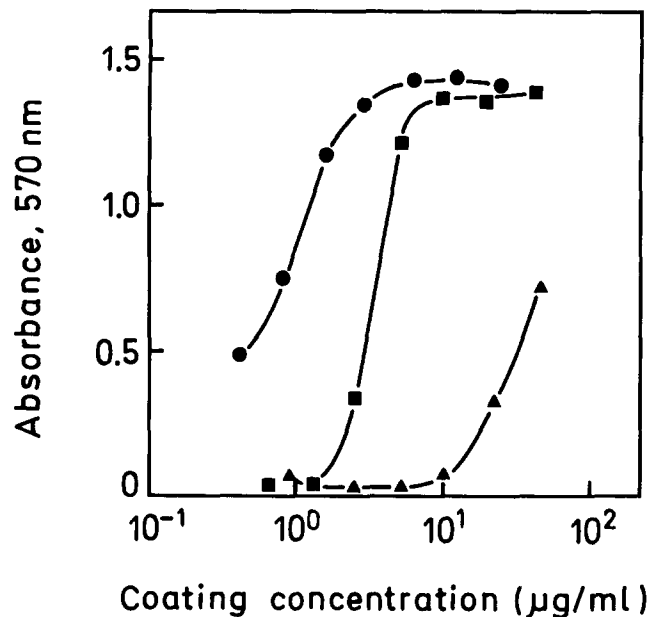


Figure 7. Dose-response profiles of HT 1080 cell attachment to various substrates. Wells were coated with placental laminin (●), fibronectin (■), or collagen XIV (▲). Relative numbers of attached cells were determined by a colorimetric assay.

laminin contamination ($\sim 3\%$) in collagen XIV. Some low adhesion activity (0.5 OD units), but at 3 $\mu\text{g/ml}$, was found for B16F10 cells.

Binding Properties of Collagen XIV for Other Extracellular Matrix Proteins

Because collagen XIV shows strong affinity for heparin (Fig. 1), we decided to study its interaction with a large heparan sulphate proteoglycan known to be produced by EHS tumor cells (Paulsson et al., 1987a) as well as by fibroblasts (Here-mans et al., 1989). Immobilized proteoglycan showed a distinct reaction with soluble collagen XIV, with half maximal binding at ~ 10 $\mu\text{g/ml}$ (Fig. 8 a). A similar binding profile was also observed for soluble proteoglycan and immobilized collagen XIV. The binding could be inhibited by heparin ($IC_{50} = 0.1$ $\mu\text{g/ml}$) and heparan sulphate ($IC_{50} = 8$ $\mu\text{g/ml}$) but not by chondroitin sulphate (Fig. 8 b) up to a concentration of 500 $\mu\text{g/ml}$. This indicates a specific interaction which is likely to be mediated by the heparan sulphate side chains of the proteoglycan. The NC3 fragment of collagen XIV and reduced and alkylated collagen XIV did not bind to the proteoglycan.

A large variety of other extracellular matrix or plasma proteins were inactive ligands for soluble collagen XIV (up to a concentration of 50 $\mu\text{g/ml}$) with an example illustrated in Fig. 8 a. They included pepsin-solubilized human collagens I, III, and V and bovine collagen II, EHS tumor collagen IV, neutral salt-soluble collagens I and III, and the truncated procollagens pNI and pNIII. Further inactive ligands were placental laminin, EHS tumor laminin-nidogen complex, nidogen, BM-40/osteonectin, plasma and placenta fibronectin, vitronectin, von Willebrand factor, and the NH_2 -terminal globular domain of collagen $\alpha 3(VI)$ chain. These potential interactions also remained negative when examined

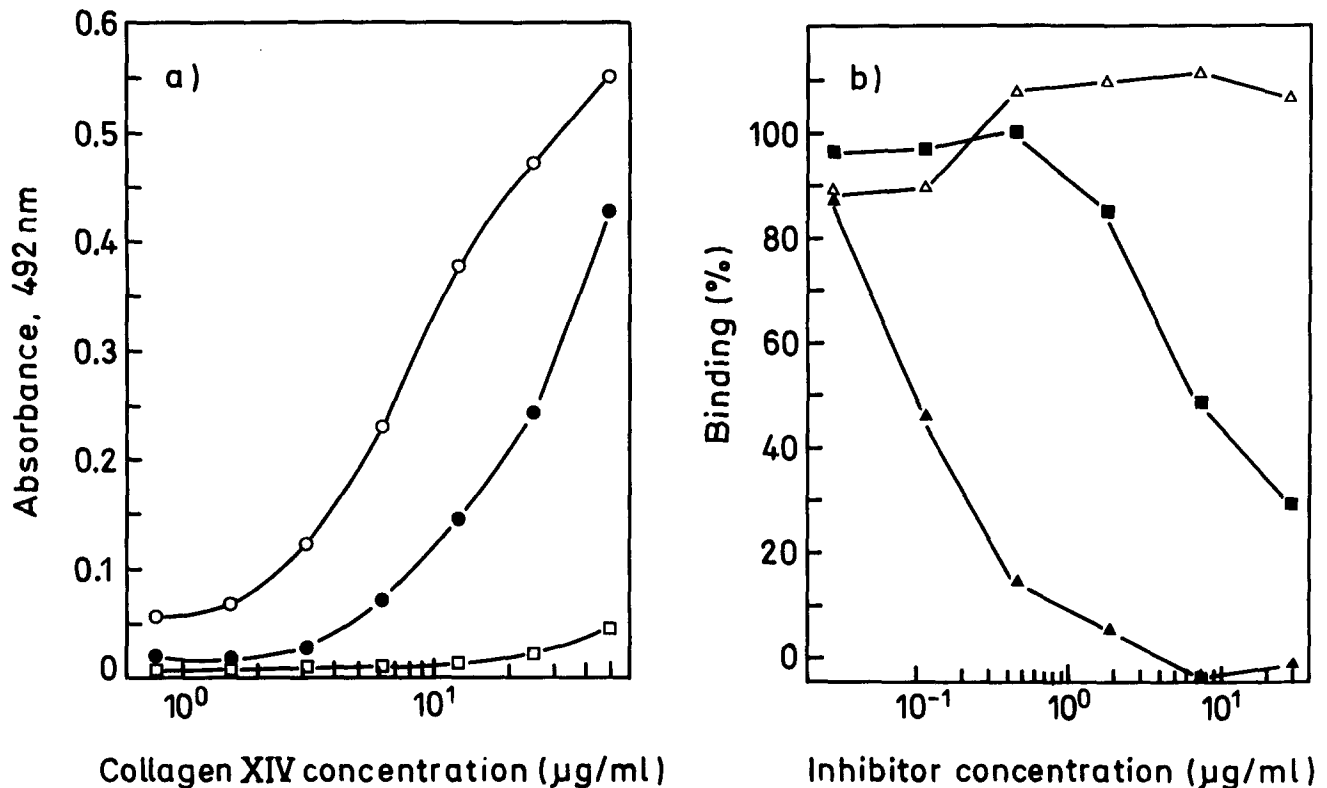


Figure 8. Interaction of human collagen XIV with extracellular matrix components. (a) Binding of soluble collagen XIV to immobilized heparan sulphate proteoglycan (○), collagen VI (●), and vitronectin (□) was determined by an ELISA procedure. (b) Inhibition of binding of collagen XIV to heparan sulphate proteoglycan. Soluble collagen XIV was incubated with heparin (▲), heparan sulphate (■), or chondroitin sulphate (△) before being added to immobilized proteoglycan. Results are expressed as percent of binding seen in the absence of inhibitors.

with immobilized collagen XIV, although this could not be studied with soluble collagens I, II, III, and V due to the lack of sufficiently strong or specific antisera for these ligands. Collagen XIV did, however, bind to pepsin-solubilized human collagen VI, both when collagen XIV and when collagen VI was used as the immobilized ligand (Fig. 8 a). This interaction was not shared by the NC3 fragment of collagen XIV. Partial reduction of collagen VI or heat denaturation (20 min, 50°C), which unfolds the triple helix (Odermatt et al., 1983), substantially reduced binding activity.

Discussion

We have purified a large disulphide-linked protein with the characteristic properties of collagen XIV from human placental neutral salt extracts. Electron microscopy showed a four-armed structure with three thick rods corresponding to a large globular domain NC3 originally identified in collagen XII (Dublet et al., 1989). This domain is connected to a 70-nm-long thin thread that is sensitive to bacterial collagenase, a treatment that also removes all 4-hydroxyproline and hydrolysine residues. This indicates that the 70-nm thread corresponds to the collagenous region of the molecule. The homology of this region with domains Coll and Col2 of bovine and chick collagen XIV (Dublet and van der Rest, 1991; Gordon et al., 1991; Trueb and Trueb, 1992) was shown directly by determining 116 of its sequence positions. Because the sequences were closer to the collagen XIV than

the collagen XII sequence of chick, there is no doubt about the identification of the human placental protein. Furthermore, several lines of evidence rule out significant contamination of the preparation with human collagen XII. First, virtually all of the human collagen XIV molecules in the preparation exhibited a change in shape at acidic pH similar to that described for the collagen XII-like TL-B from fetal bovine skin (Lunstrum et al., 1991a). TL-B was recently identified as collagen XIV by sequence analysis (Lunstrum et al., 1992). Such a change in shape is not seen with TL-A (Lunstrum et al., 1991a), which represents bovine collagen XII (Lunstrum et al., 1992). Second, the NC3 domains of bovine collagens XII and XIV could be clearly separated from one another on an FPLC MonoQ column, with no cross-contamination detectable by mAbs specific for the two molecules (Lunstrum et al., 1991a). Because the human collagen XIV was purified on MonoQ with similar buffer conditions and eluted at a similar NaCl concentration (~0.4 M) to bovine collagen XIV, it is likely that the human collagens behave similarly to their bovine counterparts and would be separated by this purification step if collagen XII were present. Third, none of the human collagen XIV peptide sequences showed higher homology with collagen XII than with collagen XIV.

Two more sequences were generated from the NC3 domain of human collagen XIV. One of these showed a high level of identity with a chick collagen XIV NC3 sequence (Trueb and Trueb, 1992), and both showed partial identity to segments within fibronectin type III repeats of chick colla-

gen XII (Yamagata et al., 1991). In addition, all 24 residues were identical to cDNA-derived sequences of human undulin (Just et al., 1991). Undulin was recently described as a non-collagenous protein lacking any 4-hydroxyproline and with a three-armed shape (Schuppan et al., 1990). Because complete sequence identity of 24 residues is higher than expected by chance for two related proteins, this indicates that a substantial portion of the sequences of human undulin and the NC3 domain of collagen XIV are identical. Indeed, Trueb and Trueb (1992) have recently shown that the amino acid sequence from the NC3 domain of chicken collagen XIV shows 71% identity with the sequence of human undulin and concluded that collagen XIV is a variant of undulin. The sequences reported here confirm this conclusion and provide further evidence that undulin and collagen XIV are probably products of the same gene, the sequence differences observed by Trueb and Trueb being due to species variation. We also isolated a tissue form of fibronectin from placenta, characterized by the presence of the extra type III domain ED-A. Because the molecular mass (220 kD) did not change after reduction, this fibronectin has apparently lost a small COOH-terminal disulphide-bridging region. This may occur for example by cleavage with plasmin (Jilek and Hörmann, 1977). Whether this fibronectin fragment is deposited in tissues in this form or is an artefact of extraction and purification is unknown.

Specific antibodies against collagen XIV were generated by affinity purification. These failed to demonstrate larger forms of collagen XIV than described here (~210-kD polypeptides) from the placenta extracts. A large form (~340 kD) has been predicted for collagen XII from cDNA sequencing (Yamagata et al., 1991) and shown in preliminary studies to exist in cell culture and cartilage (Lunstrum et al., 1992; Watt et al., 1992). The 340-kD band that was sometimes observed in our studies is unlikely to represent a larger intact form of collagen XIV because it was unstable in the presence of 2-mercaptoethanol. Radioimmunoassays demonstrated negligible secretion of collagen XIV for several human tumor cell lines except for a rhabdomyosarcoma. Considering the widespread occurrence of collagen XIV (TL-B) in tissues as revealed by immunostaining (Keene et al., 1991), this could indicate that only a few cultured cells maintain collagen XIV production or that cell transformation decreases expression. The serum levels of collagen XIV did not exceed those of other extracellular matrix proteins (Risteli and Risteli, 1987), indicating a firm anchorage of this protein to the extracellular matrix.

Such anchorage was demonstrated by the necessity to increase the ionic strength above physiological levels to ~1 M NaCl for the extraction of substantial amounts of collagen XIV from placenta (Brown et al., unpublished observations). This then led to the finding that collagen XIV has a distinct affinity for heparin, requiring ~0.4 M NaCl for dissociation. It was also shown that the NC3 domain possesses a binding site(s) for heparin. Binding sites for heparin have been assigned to type III domains in fibronectin (Hynes, 1990) and to A domains in von Willebrand factor (Mohri et al., 1989; Sixma et al., 1991). Homologs of these domains are also present in collagen XII (Yamagata et al., 1991) and are likely to exist in collagen XIV. The precise localization of such binding domains has now become feasible.

Collagen XIV also showed distinct binding to a basement

membrane-derived heparan sulphate proteoglycan. Complex formation could be inhibited by heparin and with lower affinity by heparan sulphate, demonstrating that the glycosaminoglycan chains and not the core protein of the proteoglycan are responsible for binding. A similar or identical proteoglycan is also produced by fibroblasts (Heremans et al., 1989). Together with the immunolocalization of collagen XIV to matrices produced by fibroblasts (Keene et al., 1991), this indicates that binding to proteoglycan may be of relevance *in situ*. A surprising observation was that the heparin-binding domain NC3 did not bind to the heparan sulphate proteoglycan. This could indicate that several heparin-binding sites exist on collagen XIV, including within the triple helix, and/or that the release of NC3 produces subtle changes in conformation and affinity which may allow binding to the sulphate-rich heparin but not to the undersulphated heparan sulphate (Pejler et al., 1987) in agreement with the different affinities shown by inhibition assays (Fig. 8 *b*). Preliminary studies with chymotryptic digests have in fact indicated that collagen XIV may possess more than one heparin-binding site (Brown et al., unpublished).

The only other significant binding activity of collagen XIV so far observed was with pepsin-solubilized collagen VI from human placenta. This truncated collagen VI consists mainly of the 100-nm-long triple helical domain and some small residual structures of the adjacent globular domains N1 and C1 (Odermatt et al., 1983; Chu et al., 1987, 1988). Immunoelectron microscopy has shown that collagens XIV (Keene et al., 1991) and VI (von der Mark et al., 1984; Bruns et al., 1986; Keene et al., 1988) are found in close vicinity to the major cross-striated collagen fibrils. Although collagen XII shows only limited colocalization with collagen VI (Keene et al., 1991), similar colocalization studies with collagen XIV have yet to be carried out. Because the NC3 domain does not bind, it may be that the triple helix of collagen XIV associates with that of collagen VI. Both triple helical domains, that of collagen XIV as shown here and that available on the surface of collagen VI microfibrils (Furthmayr et al., 1983; Bruns, 1984; Bruns et al., 1986), have about the same length (70 nm), which would allow a perfect match over their entire length.

Quite a few other extracellular matrix proteins, however, were not active in binding assays with collagen XIV. They included in particular the fibril-forming collagens I–III and V and the pN-collagens I and III. The latter were included in this study because of the immunoelectron microscopical evidence (Fleischmajer et al., 1981, 1990) for their localization on the surface of major collagen fibrils. Our data are different to those reported for undulin, which has been reported to bind to collagens I and III, as well as collagen VI (Just et al., 1991), and do not of course exclude the possibility that intact fibrils rather than monomeric collagens provide the appropriate binding structures for collagen XIV. Several basement membrane proteins and other noncollagenous extracellular matrix proteins also failed to bind collagen XIV. However, when studied by the same assay, distinct interactions have been found between laminin, collagen IV, nidogen, BM-40, proteoglycan, and fibronectin (Aumailley et al., 1989a, 1993; Mayer et al., 1991; Fox et al., 1991; Nischt et al., 1991), demonstrating the utility of the procedure. We also failed to demonstrate significant cell adhesiveness of collagen XIV with a panel of tumor cells that together pos-

sess a large variety of integrins involved in cell matrix interactions, i.e., $\alpha 1\beta 1$ to $\alpha 6\beta 1$ and $\alpha v\beta 3$ (e.g., Sonnenberg et al., 1990). Some weak activity was found, however, for B16F10 melanoma cells. This could correlate with the observations of Yamagata et al. (1991), who showed distinct adhesion of B16F10 cells to a synthetic RGD-peptide designed according to the chick collagen XII sequence. Nevertheless, collagen XIV does not seem at all comparable to strong and widely recognized adhesion substrates such as laminin, fibronectin, and collagens IV and VI.

The group of collagens referred to as FACITs (Shaw and Olsen, 1991; van der Rest et al., 1991) have been proposed to be associated with the major fibrillar collagens in tissues. While we observed no binding of collagen XIV to isolated fibril-forming collagens, the association could take place directly with a higher order structure of the intact fibril or indirectly via a bridging molecule. Although an immunoelectron microscopic study indicated that most of the collagen XIV was found in close vicinity to the major collagen fibrils, it was not associated with all fibrils in the preparation and in some instances it was localized between fibrils (Keene et al., 1991), indicating that collagen XIV may bind to multiple components of the extracellular matrix. This concept is supported by our data, which suggest a possible association of some FACITs with microfibrils and proteoglycans.

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