Articles

Ultrastructural Morphology and Domain Structure of a Unique Collagenous Component of Basement Membranes†

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ABSTRACT: A disulfide cross-linked collagenous fragment (7 S) has been isolated by pepsin solubilization from several tissues rich in basement membranes including bovine lung, human placenta, and the murine EHS tumor. Examination of this material by the rotary shadowing technique indicates that these fragments are similar to but not identical with the 7S collagen described recently [Risteli, J., Bächinger, H. P., Engel, J., Furthmayr, H., & Timpl, R. (1980) *Eur. J. Biochem. 108*, 239–250]. The central rodlike portion of the particles was found to be similar in length; however, the peripheral four arms of 7S particles from bovine and murine sources are 10 nm longer in comparison to those from human sources. In addition, about 5–7% of all the particles contain a fifth arm. Specific antibodies to bovine 7 S cross-react with murine 7 S but only to a rather limited extent with human 7 S. These antibodies react with antigenic sites located at the ends of the peripheral arms of the fragment as visualized directly with rotary shadowing techniques. The data are consistent with a structural difference in type IV collagens from bovine, human, and mouse which leads to pepsin cleavage at different sites in a particular noncollagenous region adjacent to 7 S. However, since bovine 7S antibodies cross-react with human and murine tissues by immunofluorescence despite the lack of complete serological cross-reactivity, it is suggested that type IV collagens from all three species have some degree of homology in this region.

Basement membranes are complex, multicomponent structures having important structural and functional roles and a wide distribution throughout the body. Collagenous components comprise a major portion of basement membranes, and at least two collagen types have been localized to morphologically identifiable basement membranes (Roll et al., 1980; Madri et al., 1980). Isolation and characterization of basement membrane collagens have been hampered by poor solubility due to cross-linking and low yields of intact native structures. In order to overcome these limitations, investigators have used a mouse tumor basement membrane to obtain large amounts of pure, native, intact acid-soluble type IV collagen (Timpl et al., 1978; Kleinman et al., 1982). Subsequent studies have indicated that this material is related to type IV collagen fragments obtained by enzyme solubilization of basement membrane rich tissues (Glanville et al., 1979; Timpl et al., 1978, 1979a,b). Biochemical, immunochemical, and tissue localization studies have greatly enhanced our understanding of the composition and structure of type IV collagen. However, it is not known whether the "domain" structure of the molecules recently described in the studies utilizing metal shadow casting methods is typical for basement membranes in organs other than placenta (Kühn et al., 1981; Timpl et al., 1981; Furthmayr et al., 1982). In the present study, we have isolated and characterized a collagenous fragment of type IV collagen from calf lung and compared it to similar material isolated from the murine EHS tumor and human placenta. Immunochemical studies indicate that this fragment is also present in native acid-soluble type IV collagen preparations, and rotary shadowing studies performed in the presence of affinity-purified antibodies confirm this notion. Because of its behavior in velocity sedimentation, this component has been named 7S collagen and is one of two defined cross-link regions noted in type IV collagen (Risteli et al., 1980; Kühn et al., 1981). Amino acid compositional data/sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoretic analyses are similar to those observed for so-called 7S collagen isolated from human placenta (Risteli et al., 1980). However, the ultrastructural morphology is distinctly different.

Materials and Methods

Collagen Preparations. Acid-soluble type IV collagen was prepared from the murine Engelbreth–Holm–Swarm tumor matrix as previously described (Roll et al., 1980). Types I, III, IV, and V collagens were prepared from human placental villi and placental membranes and bovine lung parenchyma as previously described (Madri & Furthmayr, 1980; Foellmer et al., 1982). Isolation of the 7S fragment was performed according to a modification of the methods of Risteli et al. (1980) and Furuto & Miller (1980, 1981) as outlined in Figure 1. Briefly, tissue (fresh calf lung obtained from a local slaughterhouse, human placentas obtained from the delivery suite, or murine EHS tumor matrix) is minced, washed, and digested with pepsin (Worthington) at an enzyme:substrate ratio of 1:10 (dry weight/dry weight) in diluted acetic acid (0.001 M) for 24 h at 4 °C. The resultant supernate is collected by centrifugation, and solid sodium chloride is added to 0.7 M. The resultant precipitate contains types I and III collagen. Solid sodium chloride is added to the supernate to a final concentration of 1.2 M. The precipitate formed contains types IV and V collagen. The supernate is then made 1.8 M with respect to sodium chloride, and the precipitate formed is dialyzed against 0.001 M acetic acid. After being reprecipitated by addition of solid sodium chloride to 1.8 M, the pellet is dissolved and dialyzed against dilute acetic acid

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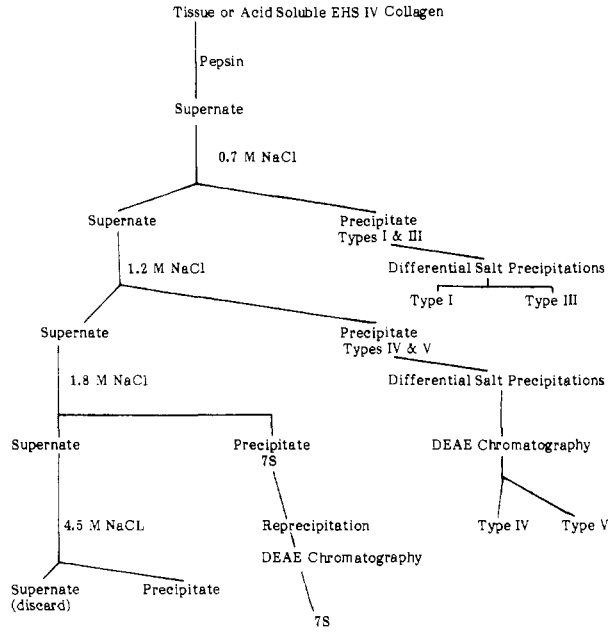

FIGURE 1: General overview of the isolation and purification scheme of the 7S fragment from basement membrane rich tissues.

and then against 0.03 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, containing 2.0 M urea and 0.2 M NaCl. The soluble material is then mixed with DEAE 52 (Whatman DE 52) equilibrated in the above buffer for 24 h at 4 °C. The nonbound fraction contains 7 S. No "intima"-type collagen as identified in this fraction was determined by immunochemical and rotary shadowing techniques (Furthmayr et al., 1983; Odermatt et al., 1983). The large collagenase-resistant fragment, Col 1 (Timpl et al., 1979a,b), 7S collagen from human placenta (Risteli et al., 1980), intima-type collagen (Odermatt et al., 1983), and affinity-purified antibodies to these materials were kindly provided by Dr. R. Timpl from the Max Planck Institute for Biochemistry in Martinsried, West Germany.

Collagens and collagen fragments were analyzed for purity by $NaDodSO_4$–polyacrylamide gel electrophoresis of α chains and cyanogen bromide peptides (Laemmli, 1970; Furthmayr & Timpl, 1976) and by amino acid analysis on a Durrum D500 analyzer (Roll et al., 1980).

Antibody Preparation. Antisera to the collagens and collagen fragments were raised in female New Zealand rabbits as previously described (Madri & Furthmayr, 1980). Antisera to the 7S particle isolated from bovine lung were raised in New Zealand rabbits by utilizing intradermal injections of 1 mg of 7 S in 1 mL of 1 mM acetic acid emulsified with 1 mL of complete Freund's adjuvant every 2 weeks for 8 weeks. Two weeks after the last injection, the animals were bled from ear arteries, and after the sera were titered, the animals were exsanguinated by cardiac puncture while under anesthesia.

Antisera were affinity purified by cross-adsorption and adsorption and elution from collagen–Sepharose columns (Furthmayr, 1982). 7S antibodies were purified by cross-adsorption on types I, II, III, and V and pepsin-soluble type IV collagen–Sepharose columns and on laminin- and fibronectin–Sepharose columns followed by adsorption on and elution from a 7S–Sepharose column.

Antibody Characterization. Antibodies were characterized as type-specific by the ELISA assay and the ELISA inhibition assay and by tissue immunofluorescence and immunoperoxidase as previously described (Roll et al., 1980; Madri et al., 1980).

Rotary Shadowing Techniques. The rotary shadowing technique was adapted from Shotten et al. (1979) as previously described (Engel et al., 1981). In studies of antibody–collagen binding, a solution of a 150 mM ammonium bicarbonate (pH 8.0) was used as the buffer. Protein concentrations of 0.5 mg/mL for the collagens and variable amounts of the antibodies were used. The antibody–antigen mixtures were incubated for 1 h at 20 °C, and then aliquots of 10–30 μg in 20–60 μL were diluted into 1 mL of a 70% glycerol solution containing 15 mM ammonium bicarbonate and sprayed onto freshly cleaved mica. Controls included nonimmune immunoglobulin G (IgG) and antibodies to other collagen types as well as immune IgG incubated with other collagen types (Foellmer et al., 1983; Furthmayr et al., 1982). Grids were examined by using a Phillips 300 electron microscope at 60 kV. Images were analyzed by using a Zeiss videoplan for length and angle determination.

Tissue Immunofluorescence. Six-micrometer sections of murine, bovine, and human lung, placenta, and kidney were prepared and labeled as previously described (Madri & Furthmayr, 1980). Sections were examined with a Zeiss 14 binocular fluorescence microscope equipped with a mercury lamp and a vertical illuminator by using 450–490-nm excitation and 520-nm barrier filters. Photomicrographs were taken with Kodak Ektachrome (ASA 400) film.

Bovine aortic endothelial cells were cultured and prepared for antibody labeling as previously described (Madri et al., 1980).

Results

Isolation of 7 S from Basement Membrane Rich Tissues. We have treated calf pulmonary tissue, human placental membranes (amnion and chorion), human placental villi, and the acid-insoluble murine EHS tumor matrix with pepsin at a ratio of 1:10. After fractional salt precipitation, which removed types, I, III, IV, and V collagen, addition of 1.8 M NaCl still precipitates material. Ion-exchange chromatography on DEAE-cellulose (not shown) yields a unique fragment which has been termed 7 S with properties similar to those described by Risteli et al. (1980) and Dixit et al. (1981). The yield of this fragment from tissues from different sources and species was in the same range. Approximately 5% of the collagenous material per gram of total collagen extracted (including types I, III, IV, and V) was 7 S. The yield in general ranged from 20 to 50 mg of the fragment, starting with 10 g of washed, lyophilized tissue.

Composition of 7 S. The material purified by DEAE-cellulose chromatography migrates as a single broad band on top of a 10% polyacrylamide gel in the presence of sodium dodecyl sulfate when unreduced. This indicates a complex with an apparent molecular weight in excess of 300 000. Upon reduction and electrophoresis in sodium dodecyl sulfate–polyacrylamide gels, the material migrates as a series of bands ranging in apparent molecular weight from 300 000 to 25 000 (Figure 2a–c). These include major peptides in the bovine lung and murine tumor preparations of 25, 50, 75, and 100 kdalton. In human placental preparations, the 25 000-dalton band was faint, and the 50 000-dalton and higher chain sizes comprised the major constituents. This pattern is similar to that observed previously for 7S collagen (Risteli et al., 1980; Dixit et al., 1981). However, several minor differences are noted. In the previous report, a broad diffuse band was found at 37 000 daltons in murine tumor collagen and human tissue preparations. In contrast, this band is not detected in our preparations, and instead, we observed several discrete minor bands above and below each of the major bands of 25, 50, and

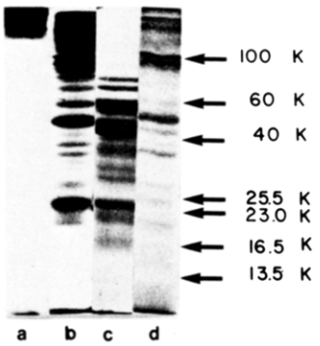

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of 7S fragments under nonreducing (a) and reducing (b–d) conditions. The samples were nonreduced samples of bovine lung 7S fragment (a) and reduced samples of bovine lung, murine EHS tumor matrix, and human placental villi 7S fragment (b, c, and d, respectively). The α chains and cyanogen bromide peptides of type I acid-soluble calf skin collagen were used as molecular weight standards and are indicated by arrows alongside the gels.

Table I: Amino Acid Analysis (mol/1000 mol)

amino acid	bovine lung CL-2	human placental villi CL-2	human placental 7S*[a]
Hyp	77	92	118
Cys	25	24	18
Asp	56	51	50
Thr	21	26	26
Ser	33	32	29
Glu	120	111	91
Pro	77	79	77
Gly	300	310	326
Ala	33	34	25
Val	38	32	30
Met	10	10	12
Ile	32	27	19
Leu	52	47	49
Tyr	10	11	10
Phe	28	25	25
His	6	8	8
Hyl	40	36	46
Lys	12	13	7
Arg	30	34	34

[a] Taken from Risteli et al. (1980).

100 dalton. These most likely represent peptides generated by pepsin at minor cleavage sites caused by the enzyme to substrate ratio used.

Amino acid analysis (Table I) reveals approximately one-third glycine residues with low alanine and arginine levels and relatively high hydroxyproline:proline and hydroxylysine:lysine ratios, findings regularly observed for so-called basement membrane collagens. The amino acid compositional analysis (Risteli et al., 1980) of 7S collagen isolated from human placenta is given for comparison. As observed in Table I, there are several differences noted among the various preparations. Specifically, our preparations had higher cysteine, glutamic acid, alanine, and isoleucine values and lower hydroxyproline and glycine values than did the previous preparations. (Risteli et al., 1980; Dixit et al., 1981). When the bovine lung and human placenta preparations are compared, the human placental 7S preparations exhibited higher glycine and hydroxyproline values than the bovine lung 7S samples.

Immunochemical Analysis of Bovine Lung 7 S. Antisera to bovine lung 7S fragment raised in rabbits contain high antibody titers when tested by using an ELISA assay. Titers were found to be in the range of 1:10 000 (Figure 3a). Affinity-purified antibodies specific for acid- and pepsin-soluble collagen types I, II, III, and V, pepsin-soluble type IV collagen, and intima-type collagens did not show any activity when tested on microtiter plates coated with bovine lung 7S fragment (Figure 3a). However, affinity-purified antibodies to acid-soluble EHS tumor type IV collagen gave titers of 1:400, indicating cross-reactivity between murine acid-soluble type IV collagen and the bovine lung 7S fragment (Figure 3a). The antisera to bovine lung 7 S, affinity purified by cross-adsorption and adsorption–elution, were found not to cross-react with purified murine, bovine, and human acid- and pepsin-soluble types I, II, III, and V collagens and pepsin-soluble type IV collagens (Figure 3b).

Affinity-purified antibodies to bovine lung 7 S could be inhibited with bovine lung 7S fragment but not with the previously described human 7S collagen (Risteli et al., 1980) or the human placental 7S fragment prepared by using this procedure. Reduction of the 7S fragment had no effect on antigenic activity. Reduction and heat denaturation of the 7S fragment, however, cause a shift of the inhibition curve to the left without a change in the slope, indicating an apparent loss of some antigenic sites (Figure 3b). In addition, when bovine lung 7S antibodies were tested on microtiter plates coated with acid-soluble murine EHS tumor type IV collagen, titers of 1:160 were obtained. Acid-soluble EHS tumor type IV collagen partially inhibited the 7S antibody (Figure 3b). When purified 7S particles isolated from the EHS tumor matrix were tested, an inhibition curve was obtained with a slope identical with that observed for the bovine 7S particle, suggesting close similarity between the two preparations (Figure 3b). A subset of antibodies raised against acid-soluble EHS type IV collagen could be specifically adsorbed out on a 7S Sepharose column which gave a titer of 1:320 when tested on bovine lung 7S coated plates. These antibodies were completely inhibited by bovine lung 7S particles and the 7S particles obtained from the acid-insoluble EHS tumor matrix.

Comparison of 7 S from Bovine Lung with Human Placental Villi 7 S, the Col 1 Fragment Isolated from Human Placenta and Kidney, and "Intima-Type" Collagen Isolated from Human Placenta. Using microtiter plates coated with bovine lung 7S fragment, we noted no inhibition of antibodies to bovine lung 7 S when so-called intima-type collagen, previously described human placental 7S collagen (Risteli et al., 1980) (short and long forms), or the large collagenase-resistant fragment, Col 1, from human placenta or kidney was used as the inhibitor (Figure 3c). When human placental 7 S was used to coat microtiter plates bovine lung 7S fragment antibodies had a titer of only 1:80 (as compared to 1:10 000 when microtiter plates coated with bovine lung 7 S were used), indicating only limited cross-reactivity. In support of this finding, antibodies raised against previously described (Risteli et al., 1980) human placental 7S collagen were found to have a titer of 1:12 000 when assayed on our human placental 7S coated plates. Finally, in inhibition assays using plates coated with the previously described human placental 7S and our 7S antibodies, 7 S from human placental villi and membranes inhibited the antibodies completely, while only limited cross-reactivity was noted when bovine lung 7 S was used as the inhibitor (Figure 3d).

Characterization of the 7S Particle by Rotary Shadowing. When 7S fragments isolated and purified from bovine lung, murine tumor, and human placental villi and membranes were analyzed by the rotary shadowing method, some similarities as well as some striking differences were noted in comparison to previously reported images (Figure 4a,b). All share a similar overall structure, having a central core from which a number of arms radiate out. Most of the particles contain four

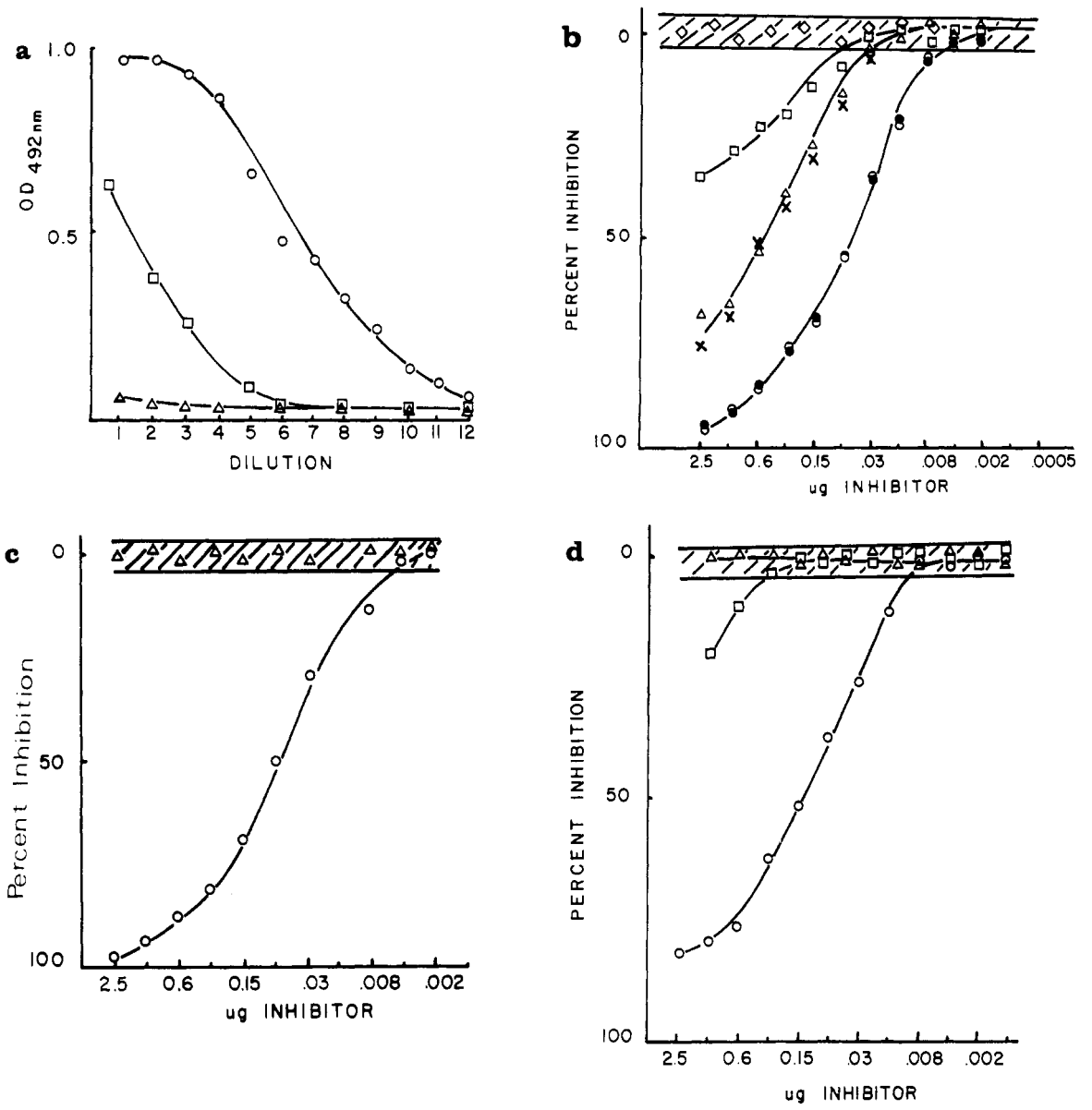

FIGURE 3: (a) ELISA titration assay of affinity-purified antibodies to various collagens and collagen fragments assayed on microtiter plates coated with bovine lung 7S fragment at a concentration of 500 ng/well. All antibodies were used at initial dilutions of 1:100, taken from stock solutions of 0.5 mg/mL. Anti-bovine lung 7 S (○); anti-acid-soluble EHS IV (□); anti-bovine calf skin acid-soluble type I, anti-bovine nasal septa pepsin-soluble type II, anti-human placental membrane and villi pepsin-soluble types III–V, and anti-bovine aorta pepsin-soluble intima type (△). (b) ELISA inhibition assay of affinity-purified antibodies to bovine lung 7S fragment assayed on microtiter plates coated with bovine lung 7S fragment at a concentration of 500 ng/well. Antibody dilution was held constant at 1:2000. Bovine lung 7S fragment (○); bovine lung 7S fragment, reduced (●); EHS tumor matrix 7S fragment (×); bovine lung 7S fragment, reduced and denatured (△); acid-soluble EHS IV collagen (□); bovine calf acid-soluble type I, bovine calf nasal septa pepsin-soluble type II, and human placental membrane and villi pepsin-soluble types I, III, IV, and V (◇). Inhibitors were serially diluted from initial concentrations of 2.5 μg/100 μL. (c) ELISA inhibition assay of affinity-purified antibodies to bovine lung 7S fragment assayed on microtiter plates coated with bovine lung 7S fragment at a concentration of 500 ng/well. Antibody dilution was held constant at 1:2000. Bovine lung 7S fragment (○); 7S collagen, long and short forms, intima-type collagen, Col 1 fragment from placenta and kidney, and human placental villi 7S fragment (△). Inhibitors were serially diluted from initial concentrations of 2.5 μg/100 mL. (d) ELISA inhibition assay of affinity-purified antibodies to human placental 7S collagen assayed on microtiter plates coated with our human placental villi 7S fragment at a concentration of 500 ng/well. Antibody dilution was held constant at 1:2000. Human placental villi CL-2 fragment (○); bovine lung CL-2 fragment (□); no inhibitor (△). Inhibitors were serially diluted from initial concentrations of 2.5 μg/100 μL.

arms; however, in all our preparations, 5–7% of the molecules contain a fifth arm (Figure 4a,b insets). The angle θ, measured as the angle of the arms as they leave the plane of the central core, was found to be 34 ± 14°. No difference was noted regardless of the tissue source.

When overall length measurements were made, significant differences were noted between the fragments obtained from the different species. Bovine lung 7S fragments were found to have an overall length of 92.8 ± 8.2 nm, which was nearly identical with that of EHS tumor matrix derived 7S fragments (92.7 ± 7.8 nm). However, human placental 7S fragments had a mean length of 77.8 ± 6.5 nm (Figure 5a,b). When measurements of the central core and peripheral arms were made, it was found that the central core region of all of these fragments was rather similar: 26.4 ± 4.1 nm for human placental 7 S vs. 25.9 ± 2.9 nm for bovine lung 7 S (Figure 5c,d) vs. 28.5 ± 4.5 nm for EHS matrix derived 7 S (data not shown). In contrast, the peripheral arms of the 7S molecules isolated from these species were significantly different (Figure 5e,f): on bovine lung 7 S, they measured 35.1 ± 4.4 nm, and on EHS tumor matrix derived 7 S, they measured 35.0 ± 2.5 nm (data not shown), vs. 24.0 ± 2.9 nm for human placental

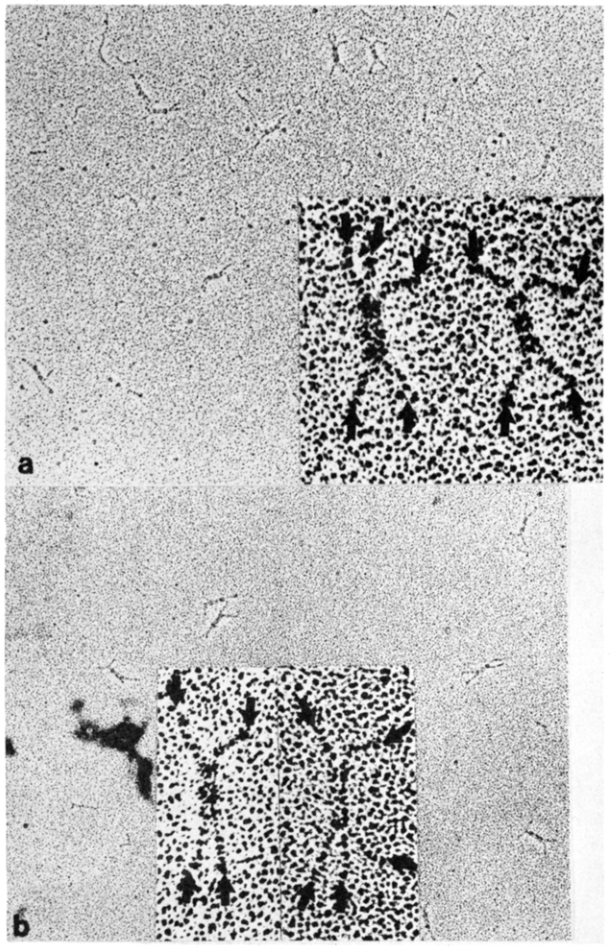

FIGURE 4: Electron micrographs of platinum–carbon replicas obtained by rotary shadowing of 7S fragments isolated from bovine lung (a) and human placental villi (b) (19500× magnification). Insets in (a) and (b) are high magnifications of 7S fragments having four and five peripheral arms (21000× magnification). Arrows indicate the positions and numbers of peripheral arms.

7 S. Examination of negatively stained samples of 7 S revealed particles similar in appearance to the rotary shadowed molecules (data not shown).

Rotary shadowing technology can be used to examine the domain structures of higher aggregates of molecules (Furthmayr et al., 1982; Furthmayr & Madri, 1982) in addition to permitting the study of the molecular morphology and domain structure of individual molecules. In combination with antibody probes, this technology can be of help to elucidate the sites or domains of interaction with specific antibodies (Furthmayr et al., 1982; Foellmer et al., 1983). As previously described, acid-soluble type IV collagen isolated from the EHS tumor is a complex molecule composed of a carboxy-terminal globular domain, a long central flexible ropelike domain, and a kinked amino-terminal domain (Timpl et al., 1981; Kühn et al., 1981; Bächinger et al., 1982; Fessler & Fessler, 1982). In addition, aggregates of molecules are observed. Aggregates revealed that collagen type IV contains two sites of association: one site which allows for the interaction of carboxy-terminal globular noncollagenous domains (NC-1) and the amino-terminal 7S domain [called CL-2 in previous reports (Furthmayr et al., 1982; Furthmayr & Madri, 1982] which allows for the interaction of up to four molecules. Pepsin treatment of acid-soluble type IV collagen yields ropelike fragments, some of which are still connected to what appear as 7S fragments, and isolated 7S fragments (Timpl et al., 1981; Kühn et al., 1981; Furthmayr et al., 1982). When polyclonal affinity-purified antibodies to EHS tumor acid-soluble type IV collagen are mixed with the antigen, binding is observed to all type IV collagen domains (Figure 6). In contrast, affinity-purified 7S antibodies or acid-soluble EHS tumor type IV collagen antibodies affinity purified on a bovine lung 7S Sepharose column are observed to bind only to the peripheral arms of 7S molecules (Figure 7a–e) and to the peripheral arms in 7S fragments seen in association with acid-soluble EHS tumor type IV collagen aggregates (Figure 8). No binding of antibodies was noted at this region when nonimmune IgG or other non-cross-reacting antibodies were used (Furthmayr et al., 1982; Foellmer et al., 1983) (data not shown).

Localization of the 7S Fragment in Tissues and in Cell Culture. Affinity-purified antibodies raised against bovine lung 7S fragment label the basement membranes of a variety of tissues. Sections of human, bovine, and murine kidney exhibit linear fluorescence labeling patterns of tubular and glomerular basement membranes as well as Bowman's capsule (Figure 9a,b). Bovine, human, and murine lung capillary and alveolar basement membranes (Figure 9c) as well as human placental villi capillary tufts (Figure 9d) are also labeled in linear basement membrane like distributions. Immunoferritin-labeled (Roll et al., 1980) and immunoimposil-labeled (Roll & Madri, 1982) ultrathin frozen sections of human and bovine lung confirmed the localization of the 7S fragments within the lamina densa of basement membrane (data not shown). In addition, cultured bovine aortic endothelial cells known to produce type IV collagen are labeled with anti 7 S in a lacy reticular matrix pattern (data not shown) similar to that observed with anti type IV collagen (Madri et al., 1981).

Discussion

Currently, it is held that type IV collagen is composed of at least three different domains including a large carboxy-terminal globular domain called noncollagenous domain 1 (NC-1) which is involved in aggregation of two type IV molecules (Timpl et al., 1981; Furthmayr & Madri, 1982). The central domain is a collagenous flexible ropelike domain of approximately 330-nm length containing several noncollagenous regions in which the typical collagen triplet sequence Gly-X-Y is interrupted by noncollagen sequences. The third domain is the amino-terminal collagenous and noncollagenous domain (called the 7S domain) which is involved in the association of type IV molecules via parallel–antiparallel association of up to four 7S domains. The noncollagenous region close to the 7S collagen domain has been labeled the noncollagenous domain 2 (NC-2) and is felt to be the site at which pepsin acts to cleave and subsequently solubilize 7S collagens.

The 7S fragment, a major cross-linking domain of type IV collagen in basement membranes, was isolated from several tissues rich in basement membranes. Amino acid analyses of 7S fragments are similar to but not identical with those reported previously for 7S collagen (Risteli et al., 1980). In contrast to previous preparations of 7S collagen, solubilization and isolation were achieved after only one pepsin digestion. This is most likely due to a more extensive digestion at the higher pepsin concentrations used and may account for the minor differences in amino acid composition. Electrophoretic analysis of the 7S fragments also gave results similar to those published previously. The 7S fragment is known to contain significant amounts of noncollagenous structures at least some of which are cleaved from the 7S fragment by collagenase treatment (Risteli et al., 1980). The loss of these structures by the use of multiple protease steps in the previous preparations is suggested by the amino acid composition, in par-

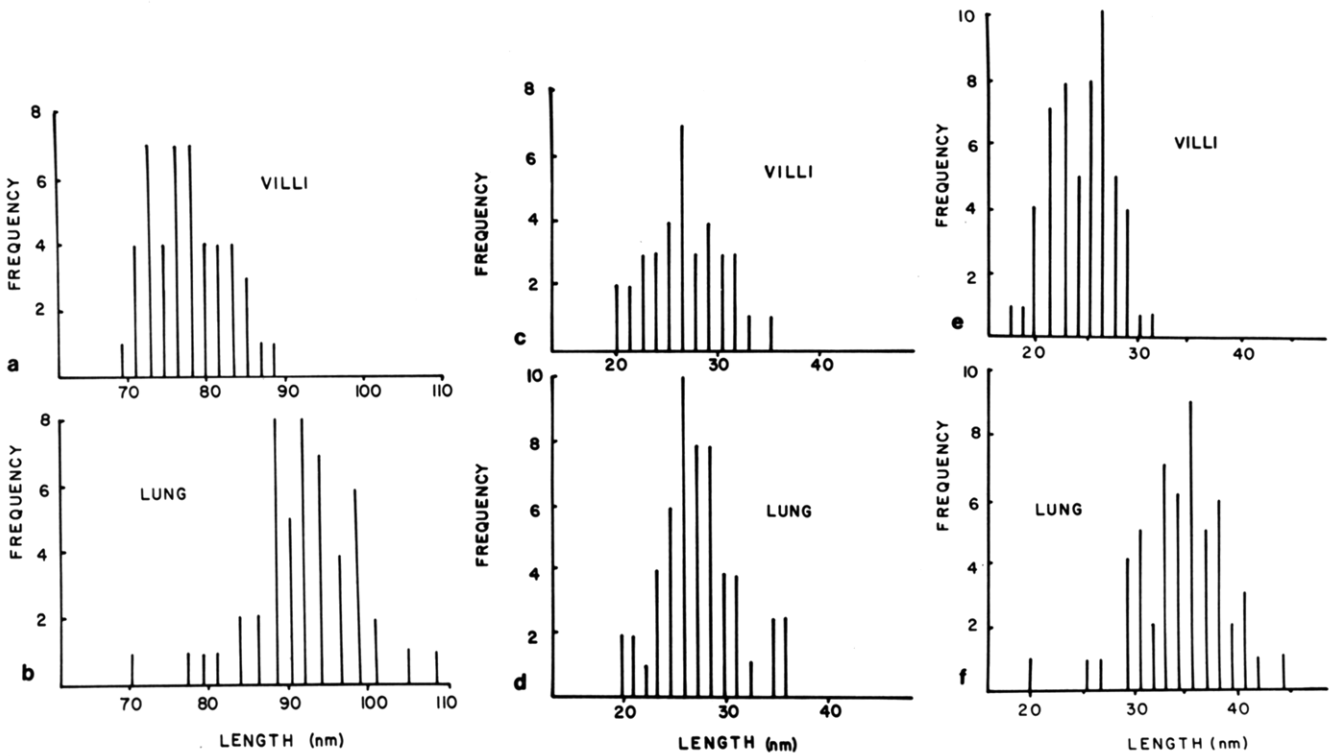

FIGURE 5: Frequency distributions of overall length and domain lengths of 7S fragments isolated from bovine lung and human placental villi. (a) Overall length distribution of human placental villi 7S fragments. (b) Overall length distribution of bovine lung 7S fragments. (c) Central core length distribution of human placental villi 7S fragments. (d) Central core length distribution of bovine lung villi fragments. (e) Peripheral arm length distribution of human placental villi 7S fragments. (f) Peripheral arm length distribution of bovine lung villi fragments.

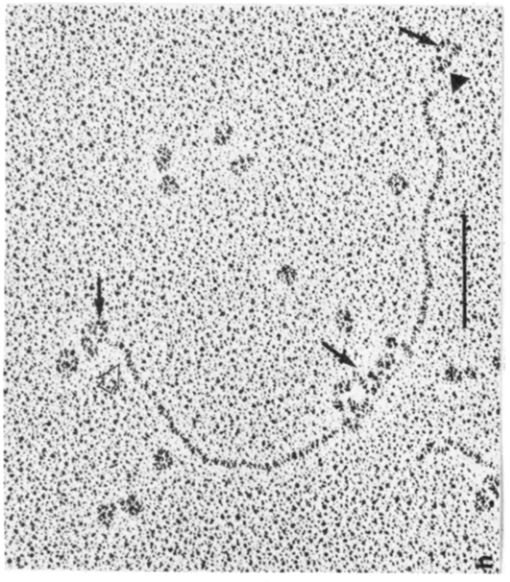

FIGURE 6: Electron micrograph of a platinum–carbon replica of acid-soluble EHS tumor type IV collagen incubated with polyclonal antibodies to the collagen. Note antibody (IgG) molecules bound to all the domains of the collagen molecule including the CL-1 and 7S domains as well as to the ropelike helical domain. The bar represents 1000 Å.

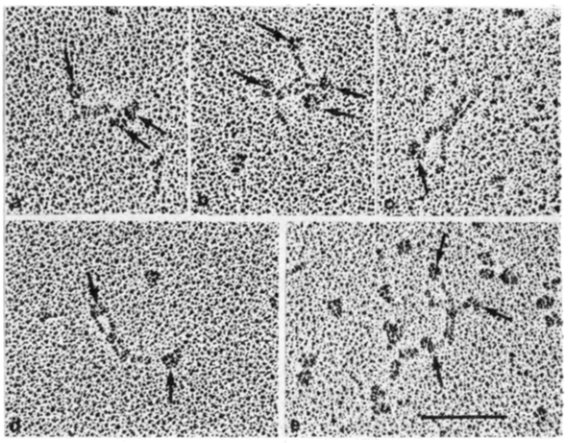

FIGURE 7: Electron micrographs of platinum–carbon replicas of bovine lung 7S fragments incubated with affinity-purified antibodies to bovine lung 7S fragment (a–c) or affinity-purified antibodies (on a 7S column) raised against acid-soluble EHS tumor type IV collagen (d and e). Note the binding of the antibodies to the peripheral arms of the 7S fragments. The bar represents 1000 Å. Arrows denote antibody binding.

ticular, by the higher glycine and hydroxyproline values. It is likely that in our preparations the 7S fragments have retained more of their noncollagenous structures. The overall structure of the 7S fragment isolated from various tissues and species is similar to that previously reported for 7S collagen, being composed of an array of four triple-helical segments aligned in such a way as to form an "X-shaped" particle with a central core and four peripheral arms. However, various measurements of 7S fragments isolated from bovine lung, the EHS tumor matrix, and human placenta (by using our procedures) revealed some differences among these preparations.

The difference in the average length of the particles from bovine lung and murine tumor as compared to those from human placenta can be explained by a difference in length of the peripheral arms. This finding may underscore structural differences of type IV collagen of various species not observed by using other protease procedures (Dixit et al., 1981; Mayne et al., 1982). When pepsin and collagenase were used sequentially in the preparation of 7S collagen, all samples regardless of tissue or species source showed complete serological cross-reactivity and a peripheral arm length of about 27 ● 2 nm when measured (Dixit et al., 1981; Kühn et al., 1981). In other studies using limited pepsin digestion of various chicken tissues, although an 7S domain was identified by using rotary

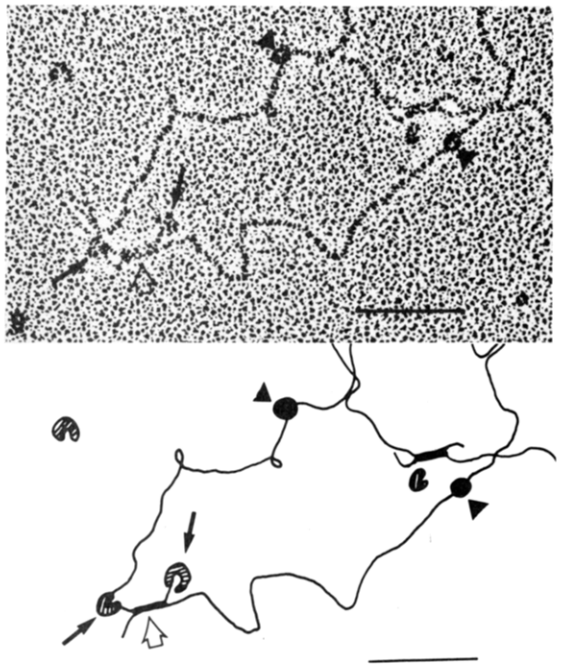

FIGURE 8: Electron micrograph of a platinum–carbon replica of an aggregate of acid-soluble EHS tumor type IV collagen incubated with antibodies to bovine lung 7S fragment. Note the binding of antibody molecules to the peripheral arms of the 7S domains in the aggregates. The schematic diagram illustrates antibody binding to the 7S domain. The bar represents 1000 Å. Arrowheads = NC-1 domain (the globular domain of type IV collagen); open arrows = 7S domain; solid arrows = antibody binding (● = NC-1, hatched horseshoe = antibody). Antibodies raised against acid-soluble EHS tumor type IV collagen and purified on a 7S column gave similar results (data not shown). The carboxy-terminal globular domain (NC-1) of type IV collagen is similar in mass to the IgG molecule (M_r 150 000). The two can be distinguished from each other by examining their positions relative to the 7S domain. The above two NC-1 domains are 387 and 391 nm away from the 7S domains in type IV aggregates. In contrast, the antibodies are noted approximately 30 nm from the central core of the 7S domain.

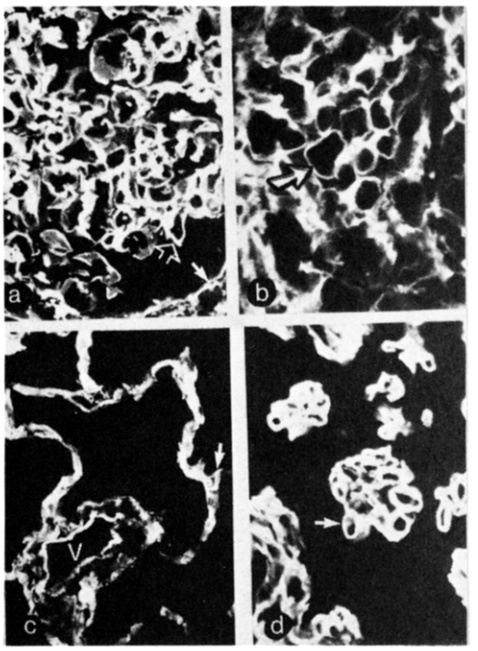

FIGURE 9: Photomicrographs of immunofluorescence labeling of basement membrane rich tissues with affinity-purified antibodies to bovine lung 7S fragment. (a) Human glomerulus labeled with antibody revealed uniform linear labeling of glomerular capillary basement membranes (open arrow) and Bowman's capsule basement membrane (solid arrow) (150× magnification). (b) High-power field of human glomerulus capillary tuft showing uniform linear labeling of glomerular capillary basement membranes (arrow) (300× magnification). (c) Human lung tissue labeled with antibody revealed linear labeling of endothelial basement membranes underlying arterioles (V) and capillary (arrow) endothelium (150× magnification). (d) Human placenta villi capillary tufts labeled with antibody revealed uniform linear labeling of capillary basement membranes (arrow) (150× magnification).

shadowing techniques, the peripheral arm lengths were variable, some being in the range of 215 nm (Mayne et al., 1982). Since hydrolysis at this site is felt to generate 7S fragments and the lengths of the peripheral arms of the 7S molecule generated from bovine lung, EHS tumor matrix, and human placenta vary by 10 nm [cf. Kühn et al. (1981)], it is likely that there are differences in the size or primary structure of the NC-2 domain in various species (Figure 10).

The difference in length of ther peripheral arms of the various 7S fragments accounts for the lack of immunological identity observed in the ELISA assays for 7 S from bovine, murine, and human sources. We have shown that antibodies to bovine lung 7S particles interact with antigenic sites at or close to the ends of the peripheral arms. Since human placental 7S particles have shorter peripheral arms in comparison to 7S particles isolated from murine or bovine tissues, it is likely that the major antigenic site(s) are located in a region which is removed with pepsin treatment of human tissues and is not recovered on 7S fragments in this case. This explanation is supported not only by data obtained with the rotary shadowing technique and serological methods but also by the reactivity of bovine 7S antibodies on tissue sections of human origin. The latter finding suggests homology between collagen type IV of human and bovine origin in the region which is retained on the 7S fragments isolated from calf tissue after pepsin digestion (Figure 10). The partial cross-reactivity observed between human and bovine 7S antibodies and the EHS type IV collagen 7S domain is compatible with the notion that these antibodies

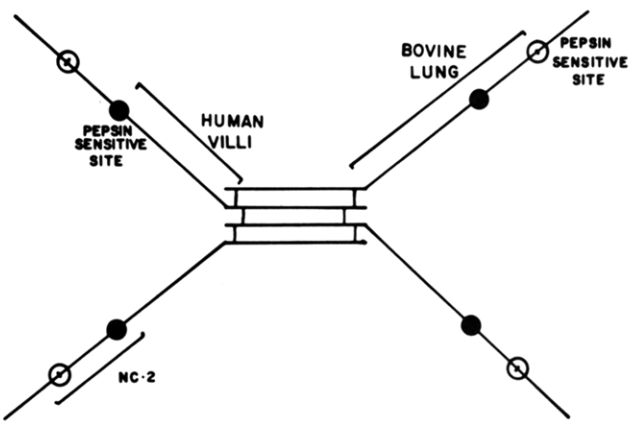

FIGURE 10: Schematic diagram of the 7S fragment isolated by pepsin solubilization. The differences in length of the peripheral arms noted in bovine lung, murine tumor, and human placenta preparations are thought to be due to differences in the relative susceptibilities of the noncollagenous domains located on the peripheral arms of the spiderlike molecules. NC-2 = noncollagenous domain 2; (⊙) pepsin-sensitive site for bovine lung and murine tumor matrix type IV collagen; (●) pepsin-sensitive site for human placental type IV collagen.

recognize additional determinants which are common to murine, bovine, and human 7S particles.

The presence of a fifth peripheral arm in 5–7% of the molecules in the preparations of the 7S fragment (Figure 7a,b) raises interesting possibilities about which we can only speculate at this time. It could be representative of yet another (collagen) molecule which would bridge linear arrays of type IV collagen aggregates (Furthmayr & Madri, 1982). To date, we have been unable to separate this small subset of fragments

from the majority of the 7S fragments. Perhaps the development of monoclonal antibody probes which recognize these structures will allow for the isolation of sufficient material for chemical analyses.

The rotary shadowing technique was used to directly determine antigenic site(s) on the collagen molecules. This approach may prove to be very useful in correlating tissue labeling on an ultrastructural level with the domain structure and molecular organization of the basement membrane constituents. Preliminary reports using monoclonal antibodies to the flexible ropelike helical domain of type IV collagen have shown a homogeneous pattern of labeling throughout selected renal basement membranes (Foellmer et al., 1982, 1983). As (monoclonal) antibody probes are developed for the various specialized domains of the type IV molecule [such as the NC-1 (CL-1) and 7S (Cl-2) cross-linking domains], specific organization patterns or arrays may become apparent in immunoelectron microscopic studies. This approach could yield significant new information regarding the molecular organization of the collagens as well as other basement membrane components within basement membranes.

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