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Monoclonal antibodies to human type IV collagen: Useful reagents to demonstrate the heterotrimeric nature of the molecule

(basement membranes/procollagen/immunofluorescence/immunoblotting)

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ABSTRACT **Monoclonal antibodies (mAbs) have been prepared against type IV collagen isolated from human kidney. Two mAbs, designated CIV 22 and CIV 16, were extensively characterized. CIV 22 reacted only with native type IV collagen, whereas CIV 16 also bound to fragments derived from the α1(IV) chain after reduction and alkylation of the molecule. Therefore, CIV 22 recognizes a conformational epitope on the triple helical type IV collagen, whereas CIV 16 binds to a sequential determinant in the carboxyl-terminal half of the α1(IV) chain. By immunofluorescence, typical basement membrane structures were stained with both mAbs on frozen sections of different human organs. The mAbs were used to investigate the chain composition of type IV collagen. Radiolabeled type IV collagen bound to CIV 22, proving its triple helical configuration. These native probes, containing both the α1(IV) and the α2(IV) chains, also bound to CIV 16. Since CIV 16 does not react with the isolated α2(IV) chain, both chains must be arranged in a single triple helical molecule (heterotrimer).**

Basement membranes are amorphous laminar structures in intimate contact with various cell types (epithelial cells, endothelial cells, smooth muscle cells, fat cells). They either separate two cell layers (epithelial and endothelial cells of glomerular basement membranes) or occur at the interphase of a cell layer and the underlying connective tissue (epidermal and capillary basement membranes) (1).

Basement membranes have multiple functions. They are involved in tissue morphogenesis and repair, play a role in the maintenance of tissue architecture, and form the filtration barrier of blood vessels (2). Several disorders are associated with morphological and functional changes of basement membranes (3).

Type IV collagen is a major constituent of basement membranes. It represents for instance 40% of the protein in glomerular and tubular basement membranes. A network model for its macromolecular organization has been proposed by Timpl *et al.* (4).

The structural element of type IV collagen is a triple helix with a length of 340 nm (see ref. 5 for review). Since type IV collagen is highly crosslinked by both disulfide bridges and lysine-derived bonds, numerous investigators have made use of proteases such as pepsin to solubilize its triple helical domain. Frequent interruptions of the triplet sequence Gly-X-Y (in which X and Y are unspecified amino acids) make the triple helix discontinuous and susceptible to pepsin digestion (6). Thus, several pepsin-resistant fragments, derived from at least two genetically different polypeptide chains, could be obtained. In particular, fragments with M_rs of 125,000, 95,000, and 60,000 are released from a parent α1(IV) chain, whereas fragments with M_rs of 115,000, 80,000, and 45,000 apparently originate from a distinct α2(IV) chain (7, 8). Whether the two polypeptide chains normally occur in the same triple helical molecule (heterotrimer) or are arranged in two different molecules (homotrimers) is still controversial (2, 5, 8).

Antibodies are widely used to study structural aspects and tissue distribution of the various collagens. We produced monoclonal antibodies (mAbs) against human type IV collagen and report their specificity. Using these reagents, we conclusively demonstrate the heterotrimeric nature of type IV collagen.

MATERIALS AND METHODS

Preparation of Collagens. Fragments of type IV collagen were isolated by limited digestion with pepsin (Boehringer Mannheim) from human kidneys by the method of Dixit (9) with minor modifications (8). Disulfide bonds were reduced and alkylated by dithiothreitol and iodoacetamide as described by Sage *et al.* (10). Type IV collagen was radiolabeled with ^{125}I-labeled Bolton–Hunter reagent (2000 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) (11). For the production of biosynthetically labeled type IV collagen, HT-1080 cells (ATCC CCL 121, American Type Culture Collection) were incubated for 12 hr with 5 μCi of L-[U-^{14}C]proline (250 mCi/mmol, Amersham) per ml in serum-free Dulbecco's modified Eagle's medium containing 0.05% bovine serum albumin in the presence of sodium ascorbate and β-aminopropionitrile fumarate. Subsequently proteins were precipitated from the medium with ammonium sulfate as described (12). Fibronectin was removed from the supernatant of HT-1080 cells by affinity chromatography on Sepharose-coupled gelatin (13). Other collagens were prepared as described (14).

Production of Hybridomas and Testing of Supernatants with RIA. BALB/cABOM mice were immunized with purified pepsin fragments of human type IV collagen according to the schedule proposed by Stähli *et al.* (15). The hybridoma technology of Köhler and Milstein was used with some modifications (16). Hybridoma supernates were assayed for anti-type IV collagen antibodies by a solid-phase RIA. A solution (200 μl) containing collagen at 25 μg/ml in 0.1% acetic acid was dispensed in the wells of polyvinyl chloride microtiter plates (M 24, Cooke Laboratory Products, Dynatech, Alexandria, VA). After standing overnight at 4°C, the wells were filled with (*i*) 200 μl of 1% bovine serum albumin in 0.15 M NaCl/0.02 M sodium phosphate, pH 7.4 (NaCl/P_i), (*ii*) 100 μl of hybridoma supernatant (or diluted antisera or ascites), and (*iii*) 100 μl of ^{125}I-labeled rabbit antiserum against mouse immunoglobulins (Tago, Burlingame, CA; 10^5 cpm). Radiolabeling was done by the chloramine-T method (17). The

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Abbreviation: mAb(s), monoclonal antibody(ies).

wells were finally cut, and the radioactivity was assayed in a LKB 1260 Multigamma counter.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis, Autoradiography, and Immunoblotting. Proteins were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis as described by Laemmli (18). Gradient polyacrylamide gels (3–10%) were used. Gels were stained with 0.1% Coomassie blue in 10% acetic acid/25% isopropanol and destained with 10% acetic acid/10% methanol. For detection of ^{125}I-labeled proteins, gels were dried and directly exposed to Kodak XAR-5 x-ray film. For detection of ^{14}C-labeled proteins, gels were treated with EN^{3}HANCE (New England Nuclear) before exposure. For immunoblotting, proteins were electrophoretically transferred from the gels onto nitrocellulose sheets (constant current, 100 mA, 10 hr) as described by Towbin *et al.* (19). After immersion in 10% fetal calf serum/3% bovine serum albumin in 0.15 M NaCl/100 mM Tris·HCl, pH 7.4 (2 hr at 40°C), the sheets were incubated with hybridoma supernatant (overnight at 4°C) and subsequently with a peroxidase-conjugated rabbit antiserum against mouse immunoglobulins (Tago). Peroxidase was then detected with Graham–Karnovsky's solution (20).

Immunofluorescence Staining. Tissue specimens were snap frozen and cut into 5-μm sections. After fixation with ether/alcohol, 1:1 (vol/vol), sections were incubated with hybridoma supernatants (or diluted ascites) for 30 min, and tissue-bound antibody was detected with fluoresceinated anti-mouse immunoglobulin antiserum (Miles-Yeda, Rehovot, Israel). For subclass determination of the mAbs, rhodamine-conjugated subclass-specific antisera from Litton Bionetics were used as the secondary antibody. Negative controls included (*i*) incubation with a IgG$_1$/κ mAb with unknown specificity and (*ii*) incubation with secondary antibody alone.

Affinity Chromatography. mAbs CIV 22 and CIV 16 were concentrated from ascites by ammonium sulfate precipitation (40% saturation). The precipitate was dissolved in and dialyzed against 5 mM Na$_2$HPO$_4$/HCl, pH 8.0 and passed over a column of DEAE-cellulose (DE-52, Whatman). Bound proteins were eluted from the column with a linear gradient from 5 to 300 mM Na$_2$HPO$_4$/HCl, pH 8.0. The mAbs CIV 22 and CIV 16 were eluted in the first peak at 65 mM phosphate. The antibodies were pooled, dialyzed against 0.1 M Hepes (pH 7.5), and then coupled to Affi-Gel 10 (Bio-Rad).

Binding studies were performed by passing radioiodinated or biosynthetically labeled type IV collagen through columns with immobilized mAbs CIV 22 or CIV 16. Bound proteins were directly eluted with NaDodSO$_4$ sample buffer and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis.

RESULTS

Production of mAbs Against Type IV Collagen and Determination of Their Specificity. The major disulfide-linked triple helical domain of type IV collagen obtained from human kidney by pepsin digestion was used as immunogen. NaDodSO$_4$/polyacrylamide gel electrophoresis of this native molecule under reducing conditions revealed the characteristic fragments (Fig. 1, lane A) as described for bovine type IV collagen (8). The fragments of the α1(IV) chain exhibit a molecular mass of 125 kilodaltons (kDa), 110 kDa, 95 kDa, and 60 kDa; the fragments of the α2(IV) chain of 80 kDa and 45 kDa.

Mice hyperimmunized with native type IV collagen developed antibodies with a titer of 1:10^5 as measured by RIA. When spleen cells of these mice were fused with FO myeloma cells, hybridoma growth was observed in about 10% of the tissue culture wells. About 1% of these hybridomas had anti-type IV collagen activity in their supernatants.

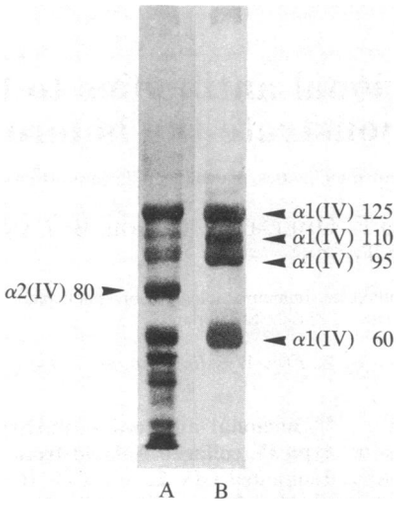

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of pepsin-extracted type IV collagen stained with Coomassie blue (lane A). Lyophilized protein (30 μg) was dissociated in NaDodSO$_4$ sample buffer and run in the presence of mercaptoethanol on 3–10% gradient gels. Major fragments are indicated by the chain they originate from and the molecular mass in kilodaltons (kDa). Immunoblotting with mAb CIV 16 (lane B). After incubation with CIV 16 hybridoma supernatant, bound antibody was detected by the immunoperoxidase method. Only fragments derived from the α1(IV) chain are stained.

The specificities of two mAbs, designated CIV 16 and CIV 22, were further assessed. Fig. 2 depicts the results of the RIA performed with these antibodies. mAb CIV 22 reacts about equally well with human and bovine type IV collagen in native conformation, whereas it does not recognize the reduced and alkylated protein in the denatured state. In contrast, mAb CIV 16 recognizes a determinant present on native type IV collagen as well as on type IV collagen after reduction and alkylation under denaturing conditions, but not on bovine type IV collagen. Therefore, the determinant recognized by CIV 16 is likely to be located on a constituent chain of human type IV collagen. To determine whether it is present on the α1(IV) or α2(IV) chain, we used the immunoblotting technique. The antibody CIV 16 stains the α1(IV) chain fragments 125 kDa, 110 kDa, 95 kDa, and 60 kDa but not the α2(IV) chain fragments 80 kDa and 45 kDa (Fig. 1, lane B). Staining of immunoblots with the antibody CIV 22 was negative. Therefore, CIV 22 recognizes a conformational epitope on type IV collagen, whereas CIV 16 binds to a sequential determinant present only on the α1 subunit.

No crossreactivity of the two mAbs with isolated human collagen types I, II, III, and V could be detected by RIA and immunoblotting.

Immunofluorescence Histochemistry. In the glomeruli of the kidney, basement membranes of capillaries, parts of the mesangial matrix and the Bowman's capsule, and tubular and vascular basement membranes were stained by mAb CIV 22 (Fig. 3*a*). Staining of kidney sections with mAb CIV 16 resulted only in faint fluorescence, which was mainly associated with some basement membrane structures of the glomeruli (Fig. 3*b*). When the sections were heated to 60°C or treated with 5 M urea prior to incubation with the mAb CIV 16 at equal concentration, brilliant staining of the same structures stainable with mAb CIV 22 was obtained. Analogous observations were made with other tissues. Therefore, the antigenic determinant recognized by mAb CIV 16 seems to be largely masked *in situ*, and dissociative pretreatment of the tissues is necessary to give mAb antibody CIV 16 access to its binding site.

The epithelial basement membrane of the skin and the

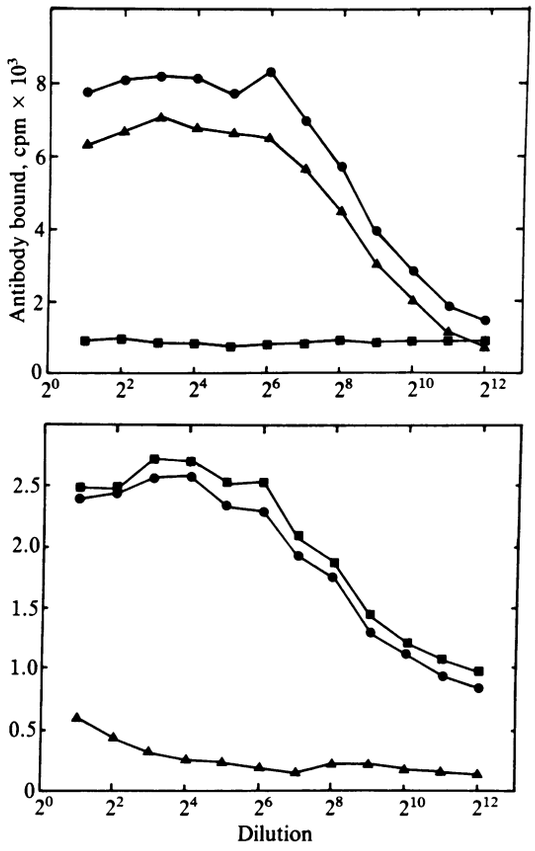

FIG. 2. Solid-phase RIA of mAb CIV 22 (*Upper*) and mAb CIV 16 (*Lower*). Titration curves were obtained by serial 1:1 dilutions of hybridoma supernatants on plates coated with native human type IV collagen (●), reduced and alkylated human type IV collagen (■), and native bovine type IV collagen (▲). cpm are not corrected for background activity.

basement membranes of blood vessels and accessory organs also reacted with CIV 22 (Fig. 3*c*). The staining of striated (Fig. 3*d*) and smooth muscle was associated with the basement membranes surrounding each muscle fiber and the basement membranes of capillaries. Organs like spleen and lymph node (Fig. 3*e*) showed the expected fragmented staining pattern of the discontinuous basement membranes of the sinusoids. In contrast, the other blood vessels exhibited a linear, continuous staining. A variety of other organs tested (including lung, placenta, and tendon) showed staining of typical basement membrane structures. The only exception observed was the basement membrane of the corneal epithelium and the Descemet's membrane, which could not be stained with either antibody. The anterior lens capsule stained brightly (Fig. 3*f*). Structures other than basement membranes were consistently negative.

The heavy chain subclass and the light chain type of the mAbs was determined on kidney sections with rhodamine-conjugated secondary antibodies. CIV 22 is an IgG_1/κ antibody, and CIV 16, an IgG_1/λ antibody.

Affinity Chromatography of Radioiodinated Type IV Collagen. Pepsin-extracted native type IV collagen was radioiodinated. $NaDodSO_4$/polyacrylamide gel electrophoresis of this labeled probe under reducing conditions revealed a banding pattern similar to the material prior to labeling (Fig. 1, lane A). Prominent bands belonging to the α1(IV) chain fragments of 125 kDa, 95 kDa, and 60 kDa as well as the α2(IV) 80-kDa fragment can be seen (Fig. 4, lane A). This labeled probe was used for affinity chromatography. Radiolabeled, native type IV collagen was retained on mAb CIV 22 immobilized on agarose. $NaDodSO_4$/polyacrylamide gel electrophoresis of the eluted protein revealed essentially all chain fragments derived from the major triple helix of type IV collagen (Fig. 4, lane C). A similar banding pattern was obtained by affinity chromatography of labeled type IV collagen on agarose-bound mAb CIV 16 (Fig. 4, lane D). No binding of the labeled probe to agarose-bound MOPC 21 mouse myeloma protein (IgG_1/κ) occurred (Fig. 4, lane B). Similar results were obtained by immunoprecipitation (data not shown).

These results indicate that (*i*) the radiolabeled type IV collagen retained its native triple helical conformation, since it is bound by mAb CIV 22, and (*ii*) both the α1(IV) and α2(IV) chains must be present in the major triple helix, since both are retained by antibody CIV 16, which by itself does not react with the α2(IV) subunits. In order to rule out the possibility that the α1(IV) and α2(IV) chains cosegregate because two hypothetical homotrimeric molecules, $[\alpha 1(IV)]_3$ and $[\alpha 2(IV)]_3$, are crosslinked to each other by lysine-derived

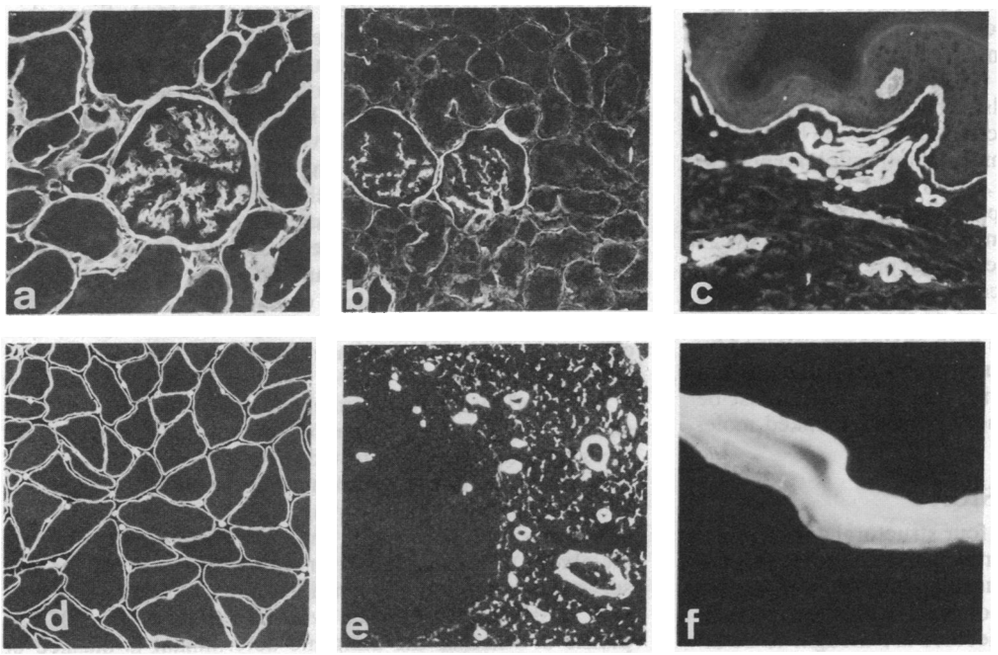

FIG. 3. Immunofluorescence on frozen sections of human organs with mAb CIV 22 (except *b*, stained with mAb CIV 16). Typical basement membrane structures are stained. (*a*) Kidney. (*b*) Kidney stained with mAb CIV 16. Only weak staining confined mainly to some glomerular basement membranes can be seen. (*c*) Skin. (*d*) Skeletal muscle. (*e*) Lymph node. (*f*) Anterior lens capsule.

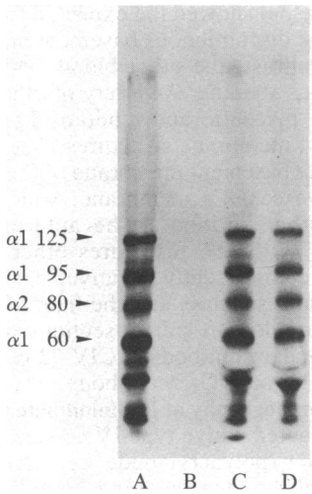

FIG. 4. Affinity chromatography of radiolabeled type IV collagen. $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions (bands visualized by autoradiography) of radiolabeled type IV collagen (lane A) and of the same radioiodinated probe eluted from agarose-bound mAb CIV 22 (lane C) and agarose-bound mAb CIV 16 (lane D). No protein is retained on agarose-bound MOPC 21 mouse myeloma protein (control, lane B). Typical fragments of pepsin-treated type IV collagen are indicated by the chain from which they originate and the molecular mass in kDa.

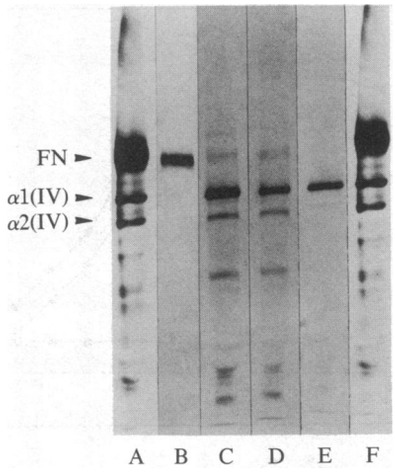

FIG. 5. Affinity chromatography of biosynthetically labeled type IV procollagen. $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions (bands visualized by fluorography) of biosynthetically labeled type IV procollagen and other proteins secreted by HT-1080 cells (lanes A and F), fibronectin removed from the HT-1080 supernatant (lane B), type IV procollagen eluted from CIV 22-agarose (lane C), type IV procollagen eluted from CIV 16-agarose (lane D), and the α1(IV) procollagen chain retained on CIV 16-agarose after passage of an aliquot of HT-1080 supernatant that had been denatured and reduced prior to chromatography (lane E). FN, fibronectin; α1(IV), α1(IV) procollagen chain; α2(IV), α2(IV) procollagen chain.

bonds, type IV collagen synthesized in the presence of an inhibitor of crosslinking was used for the following experiments.

Affinity Chromatography of Biosynthetically Labeled Type IV Procollagen. Type IV procollagen was synthesized *in vitro* by HT-1080 cells in the presence of [^{14}C]proline. Besides type IV procollagen, these cells produce mainly fibronectin (12). Thus, $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions revealed three major bands: fibronectin (subunit 230 kDa), the pro-α1(IV) chain (180 kDa), and the pro-α2(IV) chain (165 kDa) (Fig. 5, lanes A and F). Initial affinity chromatography experiments showed that fibronectin was retained, together with the procollagen molecule, on agarose-bound mAbs CIV 22 and CIV 16. In order to exclude linkage of the procollagen chains mediated by fibronectin, this molecule was removed from the HT-1080 supernatant by absorption on gelatin-sepharose (Fig. 5, lane B). After this step, type IV procollagen molecules were retained by agarose-immobilized mAb CIV 22 as well as CIV 16, and both the pro-α1(IV) and the pro-α2(IV) chains could be eluted from either immunoadsorbent (Fig. 5, lanes C and D). After denaturation and reduction of the probe by heating to 50°C in a buffer containing 5 mM dithiothreitol, no protein bound to the CIV 22-agarose, whereas CIV 16-agarose bound exclusively the α1(IV) procollagen subunit (Fig. 5, lane E). These results again demonstrate that mAb CIV 22 recognizes a conformational determinant present only on the native type IV procollagen molecule, whereas CIV 16 binds to a sequential determinant present on the pro-α1(IV) chain but lacking on pro-α2(IV). Since both chains are retained by the CIV 16 immunosorbent column when the sample is not reduced prior to chromatography, the two chains must be arranged in a single, heterotrimeric, disulfide linked molecule.

DISCUSSION

Type IV procollagen consists of four structural domains that are arranged in the following order: (*i*) 7S domain (aminoterminal end), (*ii*) noncollagenous domain 2, (*iii*) major triple helix, and (*iv*) noncollagenous domain 1 at the carboxyl-terminal end. The procollagen molecules are thought to be arranged in a three-dimensional network, with the 7S domain and the noncollagenous domain 1 as crosslinking sites (21). Antigenic determinants occur on each domain of the collagen molecule. Studies with polyclonal antisera showed that the antigenic regions in collagen consist both of sequential and conformational (helical) determinants (22).

In the present study, we describe two mAbs against pepsin-extracted type IV collagen. One of them, mAb CIV 22, is directed against a helical determinant on the native type IV collagen. In contrast, monoclonal antibody CIV 16 binds to a sequential determinant on the α1(IV) chain. The antibody recognizes its binding site on the α1(IV) chain regardless of whether this chain exists in the free form or associated to a triple helical molecule. In immunoblotting, the α1(IV) 60-kDa fragment is the smallest fragment stained. Since this fragment has been demonstrated to be located at the carboxyl terminus of the α1(IV) chain (6), mAb CIV 16 must recognize a determinant in the carboxyl-terminal half of the α1(IV) chain. mAbs against the triple helical domain of type IV collagen from human sources have been prepared by Sakai *et al.* (23), Foellmer *et al.* (24), and SundarRaj and Wilson (25). The antigenic determinant of the latter is not yet fully characterized. Scheinmann and Tsai described a mAb recognizing a determinant related to the 7S domain of human type IV collagen (26). Fitch *et al.* (27) and Mayne *et al.* (28) produced mAbs against chicken type IV collagen, which were specific for epitopes on the 7S, F3, and $(F1)_2F2$ domains. Chain-specific mAbs, like CIV 16, have not been described by these authors.

Immunofluorescence histochemistry with mAb CIV 22 showed characteristic staining of basement membrane structures in different organs tested, demonstrating that type IV collagen is a common component of basement membranes. With our antibodies, no staining of the basement membranes of the corneal epithelium and endothelium (Descemet's membrane) and the posterior lens capsule could be observed. These structures remained also unstained in chicken with mAbs against chicken type IV collagen (27, 29). The puzzle of whether the antigenic determinant is masked or

absent in these basement membranes or whether a genetically different type IV molecule is present remains open.

In all tissues tested, staining with mAb CIV 16 could only be achieved after dissociative pretreatment of the sections. Obviously, the antigenic determinant is masked *in situ* probably by juxtaposed noncollagenous molecules. Von der Mark and Oecalan (30) and Linsenmayer *et al.* (31) described mAbs against chicken type V collagen that, for recognition of the epitope *in situ*, required pretreatment of tissue sections with acetic acid or pepsin. After dissociative treatment, the tissue distribution of the antigenic determinant bound by mAb CIV 16 was the same as that of the epitope recognized by mAb CIV 22.

Antisera have been widely used for structural studies of type IV collagens (32–35). For such studies, well-characterized mAbs are even a more powerful tool. Mayne *et al.* used mAbs against epitopes of different domains of chicken type IV collagen to assess the order of the pepsin-resistant fragments (36). Whether the α1(IV) and α2(IV) chains of type IV collagen are arranged in two different homotrimers or are present in a single heterotrimer has been controversial (2, 5, 8). The conflicting data point to the possibility of tissue as well as species differences in the chain composition of collagens in different basement membranes. To study the chain composition of biosynthetically labeled human type IV collagen, Sakai *et al.* (23) used a mAb against a helical determinant of type IV collagen for immunoprecipitation. Both pro-α1(IV) and pro-α2(IV) chains were recovered from the immune complex, indicating the heterotrimeric nature of the molecule. However, the possibility that hypothetical homotrimers $[\alpha1(IV)]_3$ and $[\alpha2(IV)]_3$ share the same antigenic determinant recognized by their mAb could not be ruled out.

Using a conformation-specific mAb in combination with a chain-specific mAb, we prove immunologically the heterotrimeric nature of human type IV collagen. Affinity chromatography experiments were done with the mAbs CIV 22 and CIV 16 and two radiolabeled probes: (*i*) radiolabeled pepsin-extracted type IV collagen, which might contain some cross-links between fragments of the α1(IV) and α2(IV) chains, and (*ii*) biosynthetically labeled type IV procollagen that was synthesized in the presence of β-aminopropionitrile and, therefore, lacks lysine-derived bonds. Both collagen probes bound to mAb CIV 22, proving their triple helical configuration. The native probes also bound to mAb CIV 16, and the bound material contained both the α1(IV) and the α2(IV) chains. Since it was demonstrated above that mAb CIV 16 recognizes a sequential determinant present only on the α1(IV) chain, the α2(IV) chain must be arranged in a single triple helical molecule together with the α1(IV) chain, thus proving the heterotrimeric nature of human type IV collagen.

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