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Structural studies of human basement-membrane collagen with the use of a monoclonal antibody

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A monoclonal antibody monospecific for human type IV collagen was used as a structural probe to examine aspects of the macromolecular organization of basement-membrane collagen. Electron-microscopic observation of rotary-shadowed antigen–antibody complexes demonstrated a unique binding site for the antibody 55 ± 6 nm distant from the 7S cross-linking region of tetrameric type IV collagen. This observation allowed a series of studies that showed: (1) the localization of an intramolecular disulphide bridge within the helical domain of the molecule, (2) the alignment of major peptic-digest fragments of the α1(IV) chain, and (3) confirmation of the postulated antiparallel arrangement of individual molecules within type IV collagen tetramers.

Type IV (basement-membrane) collagen is an unusual and uniquely specialized member of the collagen family of proteins. In addition to its distribution being sharply delimited to basement membranes, the molecular structure and supramolecular organization of type IV collagen differ in many respects to those of the interstitial types I, II and III collagens (Kühn, 1982). The molecules of the latter are relatively rigid rods with a continuous 300 nm-long triple helix. The more flexible thread-like molecules of basement-membrane collagen are about 400 nm long and their triple helix is frequently interrupted by short non-helical regions (Schuppan *et al.*, 1980; Glanville & Rauter, 1981; Oberbäumer *et al.*, 1982). Whereas collagen I, II and III molecules assemble in a parallel staggered array of similar polarity to form fibrils, type IV collagen molecules aggregate at their ends to form tetramers. The distal end of each arm of these tetramers terminates in a globular domain that interacts with the equivalent region of adjacent tetramers. This interaction provides the sites whereby tetrameric units are covalently cross-linked into a regular flexible network (Timpl *et al.*, 1981; Duncan *et al.*, 1983).

Structural investigations of type IV collagen have typically been initiated by solubilizing tissues with pepsin (Dixit & Glanville, 1982). Although the triple-helical domain of the molecule is resistant to proteolysis, the occurrence of multiple short non-helical regions susceptible to various extents to scission results in the generation of a family of proteolytic fragments, the sizes of which depend on the extent of digestion. Electron-microscopic observations have demonstrated that pepsin initially digests the *C*-terminal globular domain. This leads to soluble tetrameric collagen containing four almost intact molecules bound together via their *N*-terminal 30 nm-long triple-helical regions, which form the 7S domains (Kühn *et al.*, 1981). Next, the triple-helical region is attacked and two major triple-helical fragments with lengths of 330 and 267 nm are set free (Glanville *et al.*, 1982). Denaturation and reduction of this material result in the release of a small proportion of α-chain fragments with M_r values of

Abbreviations used: e.l.i.s.a., enzyme-linked immunosorbent assay; α1(IV)140K and α1(IV)95K are peptic-digest fragments of the α1(IV) chain, defined by the M_r values of 140000 and 95000 respectively.

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140000 and 70000. If the former material is reduced under native conditions and treated a second time with pepsin, the 140000-M_r component is converted into a 95000-M_r fragment (Glanville & Rauter, 1981) as the major product. Such proteolytic fragments are the starting materials for amino acid sequencing studies, and therefore the alignment of these peptides within the intact molecule is of considerable interest. Also, the localization of important features, such as intramolecular disulphide bridges, in the molecule, and the orientation of individual molecules in tetrameric type IV collagen, are desirable structural goals.

A preliminary account of part of this work has already been presented (Hollister *et al.*, 1983).

Methods

Preparation of type IV collagen and CNBr-cleavage peptides

Tetrameric collagen IV was solubilized by treating human placenta, suspended in 0.5M-formic acid, with pepsin (Boehringer, Mannheim) at 4°C for 6h and purified as described previously (Glanville *et al.*, 1979). Isolation of fragments α1(IV)140K and α1(IV)95K has been published previously (Dixit & Glanville, 1982).

A trimeric disulphide-bridged fragment was prepared by cleaving unreduced tetrameric type IV collagen with CNBr and separating the peptide mixture by chromatography on agarose 1.5m. The cysteine-containing CNBr-cleavage fragment was eluted in a peak with an M_r of approx. 90000. This fraction, when reduced, aminoethylated and rechromatographed on agarose 1.5m, resolved into two main peaks containing peptides designated 'CB1' and 'CB1-2'. These peptides were further purified on phosphocellulose equilibrated with 0.55M-NaCl/2M-urea at pH3.9 and 45°C with a superimposed gradient of 0–0.5M-NaCl in a volume of 500ml.

Construction of monoclonal antibody to human type IV collagen

Monoclonal antibody to pepsin-solubilized type IV collagen derived from human foetal membranes was constructed by using SJL/J mice, and characterized as described by Sakai *et al.* (1982). By solid-phase radioimmunoassay, this antibody binds native, but not denatured, human type IV collagen and exhibits no detectable cross-reactivity with native human collagen types I, II, III, V and native materials containing the 1α-, 2α- and 3α-chains. The antibody reacts with human type IV collagen but not type IV collagen from a variety of other mammalian species. As a control, the previously described (Hollister *et al.*, 1982) hybridoma antibody monospecific for pepsin-solubilized native human type II collagen was used. The binding of both antibodies to their respective target antigens was inhibited completely by prior specific collagenase digestion and/or prior heat denaturation, suggesting that both antibodies are directed against collagenous epitopes in or near the helical domains. In addition, isotyping indicated that both antibodies were of the $IgG_{(2b)}$ subclass. Before use and as the final purification step, both antibodies were affinity-purified on antigen–agarose columns.

Preparation of antigen–antibody complexes

To prepare antigen–antibody complexes, tetrameric type IV collagen and monoclonal antibody [either anti-(type IV) or anti-(type II)] were dissolved in 0.2M-NH_4HCO_3 and mixed to give an approximate molar ratio of antibody to antigen of 1:1 and a final concentration of tetrameric type IV collagen of 50μg/ml. This mixture was incubated at 4°C overnight.

Electron-microscopic observation of complexes

Protein samples were mixed with an equal volume of 70% (v/v) glycerol immediately before spraying on to freshly cleaved mica discs. An electron-bombarded source (Edward's) was used for shadowing the proteins with platinum at an angle of 9–10°, followed by carbon coating at 90°, and the resultant specimens were examined in the electron microscope as previously described (Kühn *et al.*, 1981). Electron micrographs at 10000–30000× magnification were prepared. Length measurements were performed by using a Numonic electronic graphic calculator on micrographs at a total magnification of 10000–15000× and using the previously determined length of 30nm for the central portion of human 7S domain (30nm) as an internal length standard (Kühn *et al.*, 1981).

Immunological techniques

The specificity of antibody binding to tetrameric type IV collagen and derived fragments was assayed by using an e.l.i.s.a. technique (Gosslau & Barrach, 1979; Rennard *et al.*, 1980). Inhibition assays were performed by preincubation of antibody with inhibitor before e.l.i.s.a. assay on type IV collagen-coated plates.

Results

Affinity-purified monoclonal anti-(human type IV collagen) antibody was found to bind with sufficient affinity to human tetrameric type IV collagen such that antigen–antibody complexes could be revealed by rotary-shadowing techniques.

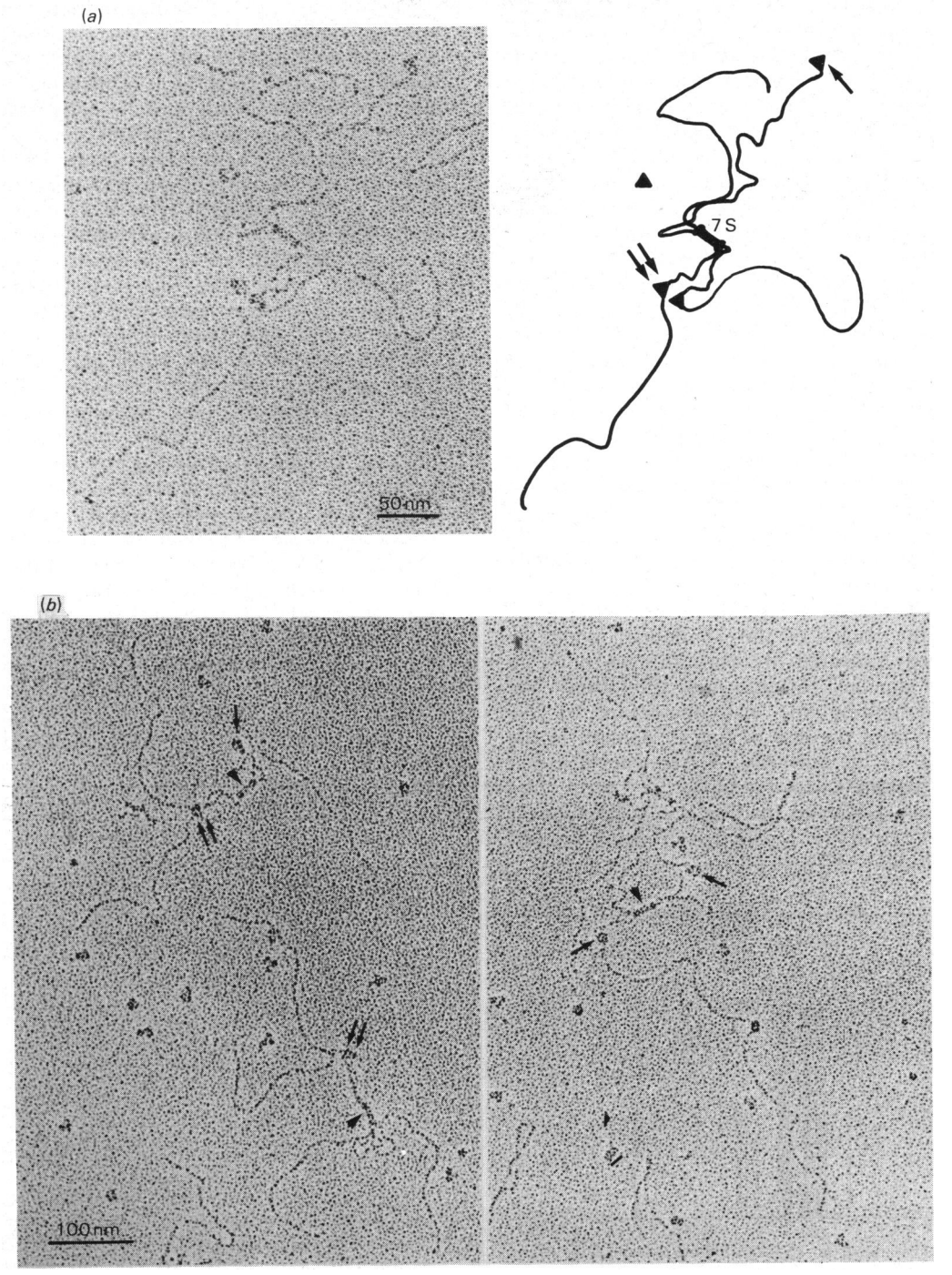

Fig. 1. *Electron micrographs of type IV collagen–antibody complexes*

(*a*) A tetrameric type IV collagen 'spider' complexed with monoclonal antibody is depicted. Note the symmetric and equidistant binding of antibody to adjacent arms of the spider. The globular profile indicated by a single arrow may represent an antibody molecule, or, more probably, represents a terminal globular NC1 domain of the molecule that has survived proteolytic removal. (*b*) 'Spiders' of tetrameric type IV collagen complexed with antibody. The arrowheads denote the 7S cross-linking regions. Note the apparent 'cross-linking' of two adjacent arms of the spiders by bivalent antibody (double arrow) and the symmetrical distribution of antibody (single arrow) with respect to the 7S domain.

Under identical conditions, affinity-purified monoclonal anti-(human type II collagen) antibody (of the same $IgG_{(2b)}$ isotype) did not bind to tetrameric type IV collagen. Fig. 1 demonstrates representative profiles obtained. In addition to the previously described linear replicas of tetrameric type IV collagen 'spiders', discrete globular (and occasionally somewhat triangular) profiles approx. 15nm in diameter are observed. These globular profiles, in numerous instances, appear in close association with and/or overlay the arms of the 'spider'; in other instances, such profiles are distant and distinct from the collagenous profile.

Control experiments with tetrameric type IV collagen alone or antibody alone demonstrated that the profiles observed in the mixture of tetrameric type IV and antibody represent specific antibody–type IV collagen complexes.

From examination of multiple micrographs, it was apparent that the binding of antibody to type IV collagen occurs with high frequency at a restricted location with respect to the 7S cross-linking region. In order to examine this specificity in detail, a number of electron micrographs of antigen–antibody complexes were selected in which the length of the 7S domain could be accurately measured. By using this length as an internal standard for each molecule (30nm), the antibody-binding site was determined as the distance from the end of the 7S domain to the midpoint of the antibody. A frequency-distribution histogram is depicted in Fig. 2. Approx. 75% of the total observed binding occurs between 45 and 63nm from the 7S region in a roughly bimodal distribution.

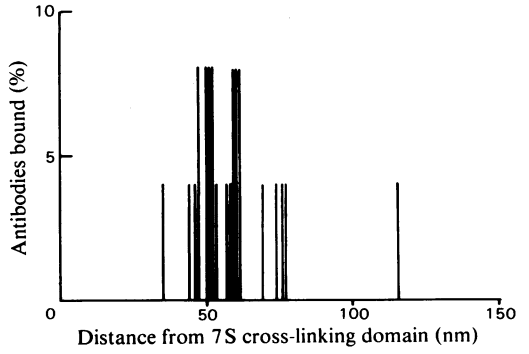

Fig. 2. *Frequency-distribution histogram relating the observed frequency of binding of monoclonal antibody to the distance (in nm) from the 7S cross-linking domain of type IV collagen*

Note that 76% of the observed binding occurs between 45 and 63nm from this region; no binding was observed to the distal half of the arms of tetrameric type IV collagen.

Multiple instances of binding of more than one antibody molecule to a single type IV 'spider' are observed, and representative examples are illustrated in Fig. 1. Two patterns of multiple binding are observed. Frequently, two antibody molecules bind to adjacent arms of a 'spider', or in a *cis* or *trans* configuration with respect to the 7S region, or both. The second pattern was the apparent 'cross-linking' of adjacent arms of a 'spider' by a single antibody molecule, an expected result of the binding of bivalent antibody to adjacent epitopes.

Fig. 3(*a*) shows the disc-gel-electrophoresis pattern of the cysteine-containing CNBr-cleavage fragment before and after reduction. Before reduction the fragment has an M_r of approx. 90000. After reduction, two major peptides, CB1 and CB1-2, are visible that have M_r values of approx. 35000 and 50000 respectively. Additional peptides, not yet characterized but presumably originating from the α2(IV) chain, can also be seen. Sequence analysis of peptides CB1 and CB1-2 revealed the same *N*-terminal amino acid sequence (Dieringer, 1982). Thus the larger peptide is a double peptide produced by the incomplete cleavage of methionine residues in the unreduced starting material. The intact 90000-M_r fragment had a c.d. spectrum characteristic of collagen and was visible, after rotary-shadowing in the electron microscope, as about 50nm-long rods, indicating an intact triple-helical conformation (Dieringer, 1982).

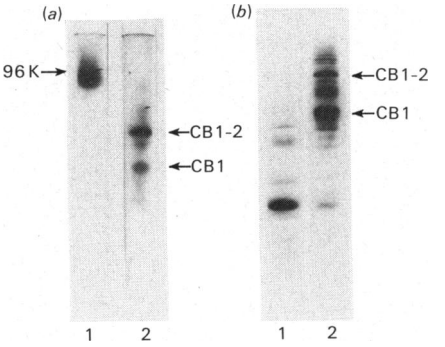

Fig. 3. *Electrophoretograms of type IV collagen CNBr-cleavage peptides*

(*a*) Sodium dodecyl sulphate/7%-(w/v)-polyacrylamide-gel electrophoretogram of the trimeric CNBr-cleavage peptide isolated from CNBr digestion of unreduced polymeric type IV collagen before (lane 1) and after (lane 2) reduction with 2-mercaptoethanol. The arrow labelled '96K' depicts the electrophoretic position of the α1(I) chain. (*b*) Sodium dodecyl sulphate/20%-(w/v)-polyacrylamide-gel electrophoretogram of the products of CNBr digestion of fragments α1(IV)95K (lane 1) and of α1(IV)140K (lane 2). The electrophoretic positions of peptides CB1 and CB1-2 are indicated.

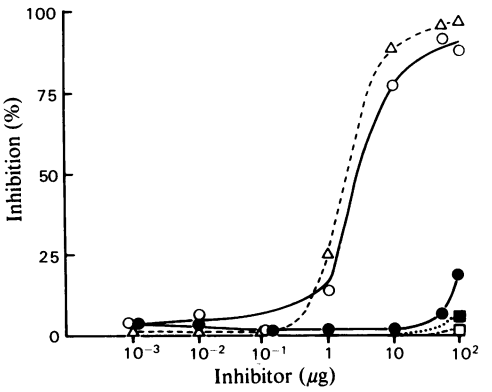

Fig. 4. *Inhibition of the binding of monoclonal antibody to native polymeric type IV collagen*

Inhibitors were mixed in various amounts with a fixed dilution of monoclonal antibody and incubated overnight before assay on microtitre plates coated with native polymeric type IV collagen. Inhibitors: ○, native, and ●, denatured polymeric type IV collagen; △, the trimeric CNBr-cleavage peptide; □ and ■, isolated constituent peptides CB1 (□) and CB1-2 (■).

The CNBr digests of the α1(IV)140K and α1(IV)95K peptic-digest fragments (Fig. 3*b*) demonstrate the presence of peptides CB1 and CB1-2 in the α1(IV)140K digest, but not in that of α1(IV)95K. The remaining peptide pattern is similar, indicating that the α1(IV)95K is a fragment of α1(IV)140K, in agreement with previous reports (Dixit & Kang, 1979).

E.l.i.s.a. assays demonstrate that the small cysteine-containing CNBr-cleavage peptide binds the monoclonal antibody (results not shown). Inhibition assays indicate that the binding of antibody to polymeric type IV collagen could be blocked by prior incubation of antibody with the trimeric CNBr-cleavage fragment (Fig. 4). The observed inhibition is as complete as that of tetrameric type IV collagen, indicating complete immunological identity. On a molar basis the magnitude of inhibitory activity of the peptide was not as high as that of the tetrameric type IV collagen, which may relate to slight modifications of the antigenic site(s) during preparation, isolation and purification procedures.

Discussion

We have demonstrated the specific binding of a monoclonal antibody to native tetramers of human type IV collagen with sufficient affinity such that antigen–antibody complexes are observable by rotary-shadowing techniques. The observed interaction of monoclonal antibody with tetrameric type IV collagen is such that 75% of total binding was observed between 45 and 63nm from the 7S region. Notably, no binding was observed to the distal half of the tetramer arms. Under the assumption of a single epitope, these data indicate a location of 55±6nm (S.D.) from the 7S region.

Available data supports the conclusion that type IV collagen molecules are heteropolymers consisting of two α1(IV) and one α2(IV) chains (Trüeb *et al.*, 1982; Qian & Glanville, 1984), and previous studies employing the monoclonal antibody used for the present study have demonstrated immunoprecipitation of newly synthesized human type IV collagen containing α1(IV) and α2(IV) in a 2:1 ratio (Sakai *et al.*, 1982). If type IV collagen tetramers are the result of the interaction of identical heteropolymeric molecules, it then follows that localization of specific antibody-binding sites with respect to the 7S region will yield useful structural information concerning the parallel or antiparallel organization of the four collagen molecules within that domain. The observed symmetric binding of antibody to equidistant sites on the arms of type IV tetramers and the observed 'cross-linking' of adjacent arms of the tetramer are consistent only with the latter possibility. This is in agreement with data on the binding of monoclonal antibody to both human (Foellmer *et al.*, 1983) and chicken (Mayne *et al.*, 1984) type IV collagen tetramers. The antibody-binding data, together with biosynthetic (Fessler & Fessler, 1982; Oberbäumer *et al.*, 1982) and structural studies (Weber *et al.*, 1984; Babel & Glanville, 1984; Qian & Glanville, 1984), confirms the antiparallel alignment of the type IV collagen molecules in the tetramer.

Since the CNBr-cleavage-derived triple-helical peptide described here contains the relevant antigenic binding site(s) for the monoclonal antibody, this peptide must be localized to the antibody-binding region of the type IV molecule about 55nm from the 7S domain. Therefore this region must also contain intramolecular disulphide bridges. This particular area of the collagen molecule appears to be highly immunogenic, since two other monoclonal antibodies directed against peptic-digest-derived human and chick type IV collagen respectively were found to bind there (Foellmer *et al.*, 1983; Mayne *et al.*, 1984). The reason for this high antigenicity may be the presence of two special structural features in this region. Firstly, as demonstrated in the present paper, this region contains intramolecular disulphide bridges that cross-link the three α-chains of the molecule. Steric considerations suggest that such bridges must occur in discontinuities of the helix, but the absence of alterations in the thread-like profiles observed by rotary shadowing suggests that such discontinuities are small. Secondly, it

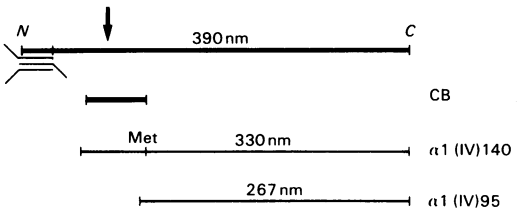

Fig. 5. *Schematic representation of type IV collagen molecule and the alignment of constituent peptide fragments*
The 7S domain at the *N*-terminus (*N*) is indicated and the arrow depicts the antibody-binding site. In addition, the alignment of the trimeric CNBr-cleavage peptide (CB) and of the peptic-digest single-chain fragments α1(IV)140K and α1(IV)95K are shown. *C*, *C*-terminus.

exhibits a particularly high flexibility, which is presumably caused by an accumulation of non-triple-helical regions (Hofmann *et al.*, 1984). The disulphide bridges appear to protect this region from proteolysis by pepsin. The large peptic-digest fragment, α1(IV)140K, contains the CNBr-cleavage peptide CB1. Reduction of this large fragment followed by further pepsin treatment yields a fragment, α1(IV)95K, that no longer contains peptide CB1. This also indicated that peptide CB1 is located at the *N*- or the *C*-terminus of α1(IV)95. As the α1(IV)140K fragment contains both α1(IV)95K and peptide CB1, and peptide CB1 is located 55nm from the 7S domain, then peptide CB1 must be located at the *N*-terminus of the α1(IV)140K fragment.

The localization of the helical disulphide-bridged 90000-M_r CNBr-cleavage fragment, which contains peptide CB1, therefore permitted the alignment of both the 140000-M_r and the 95000-M_r α1(IV) peptic-digest fragments (Fig. 5). A comparison of the flexibility profiles of the 330nm-long and 267nm-long triple-helical fragments with that of the intact molecule has also suggested that the two fragments terminate at or near the *C*-terminus of the triple helix of the molecule (Hofmann *et al.*, 1984). In confirmation, sequence studies have shown that the *C*-terminal end of the α1(IV)95K fragment carries a short non-collagenous amino acid sequence that is part of the *C*-terminal globular domain of the α1(IV) chain (Weber *et al.*, 1984; Babel & Glanville, 1984).

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