ANTIBODIES TO COLLAGENASE-RESISTANT TERMINAL REGIONS OF PRO-TYPE IV COLLAGEN RECOGNIZE WHOLE BASEMENT MEMBRANE AND 7 S COLLAGEN

S. GARBISA[o,*], L. A. LIOTTA[o], K. TRYGGVASON[+] and G. P. SIEGAL[o]

[o]*Laboratory of Pathophysiology, NCI-NIH, Bethesda, MD 20205, USA; *Institute of Histology and Gen. Embryology. University of Padova, Italy* and [+]*Department of Clinical Chemistry and Medical Biochemistry, University of Oulu, Finland*

Received 20 March 1981

1. Introduction

Biosynthetic studies on basement membrane type IV collagen indicate that it is first synthesized as two different chains, proα1(IV) and proα2(IV). The prefix 'pro' in these names denote the presence of non-helical globular end-regions in the molecule. Current data from pulse-chase studies [1,2] show little or no conversion of the pro-IV chains and it has been proposed that at least a portion of the non-helical end-region of the pro-IV chains are retained and participate in the 3-dimensional structure of basement membrane matrix [3,4]. Here we describe the preparation of antibodies directed against at least 1 collagenase-resistant pepsin and chymotrypsin-sensitive disulfide bonded end-region in each of the proα1(IV) and proα2(IV) chains. The tissue localization of the antigenic determinants is studied by immunofluorescence. Competition radioimmunoassay with these antibodies shows recognition of reduced and alkylated 7 S collagen, supporting the possibility that this recently identified collagen is a cross-linked domain of type IV collagen [5].

2. Materials and methods

2.1. *Purification and characterization of antigens*

[^{14}C]Proline-labeled pro-type IV collagen was extracted from organ cultures of the murine EHS sarcoma with 0.5 M acetic acid and purified by NaCl precipitation as in [2]. This collagen shows in SDS-PAGE after reduction the typical double band mobility corresponding to the apparent M_r-value of 170 000 and 185 000 and can be purified to homogeneity. The same procedure was used to obtain non-radiolabeled type IV collagen, except that the tumor tissue culture was carried out using a non-labeled proline-containing medium (RPMI 1640).

[^{3}H]Mannose-labeled pro-type IV collagen was generously supplied by Dr C. Clark (Philadelphia, PA).

Pepsinized lens capsule collagen containing Cl, C and D chains was generously supplied by Dr E. Miller (Birmingham, AL).

Type I collagen was extracted from guinea pig skin as in [6].

αAαB collagen was purified from human placentae obtained from normal deliveries as in [7].

Laminin was a generous gift of Dr V. Terranova (National Institute of Dental Research, NIH).

Fibronectin was purified by gelatin column chromatography from the culture media of human fibroblasts [8,9].

2.2. *Preparation and purification of antibodies*

New Zealand white adult rabbits were injected subcutaneously at 2 different sites on the back with 0.1 mg of native pro-type IV collagen in 0.5 M acetic acid emulsified with an equal volume of Freund's complete adjuvant. Booster injections were repeated as above after intervals of 3 and 10 weeks. Blood was collected from ear veins and the serum was stored in aliquots at −70°C. Possible contaminant antibodies reacting against laminin were removed by passing the antiserum through a 5 mg laminin-Sepharose affinity column prepared using 5 ml of CNBr-activated Sepharose-4B (Pharmacia). PBS (pH 7.4) was used as equilibrating and running buffer. In order to remove

Abbrevations: BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediamidetetraacetic acid; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TPCK, tosyl-L-phenylalanylchloromethane

antibodies binding to the pepsin-resistant region, the antibodies were further purified over an affinity column of 5 mg pepsin-treated type IV collagen, 1 mg P1-P2 fragments and 2 mg pepsinized lens capsule collagen bound to 5 ml of CNBr-activated Sepharose-4B.

2.3. *Proteolytic modification of antigens*

Chymotrypsin-treated pro-type IV collagen was prepared by incubating a solution of [^{14}C]proline-labeled pro-type IV collagen with a high concentration (0.1%) of chymotrypsin (approximate enzyme/substrate ratio of 100/1) 4 h at 25°C in 50 mM Tris-HCl, 10 mM $CaCl_2$, 0.2 M NaCl, pH 7.4. The reaction was stopped by adding an equal volume of 0.5% TPCK and storing the mixture at 4°C. This material showed in SDS-PAGE a double band pattern with an apparent M_r-value approx. 5% lower than the original (fig.3A).

Pepsin-treated type IV collagen was prepared by incubating overnight at 4°C [^{14}C]proline-labeled pro-type IV collagen with 0.1% (final concn.) pepsin in 0.5 M acetic acid. The reaction was stopped by neutralizing the solution.

Native pepsin fragments P1 and P2 from mouse EHS tumor type IV collagen were generously supplied by Dr P. Fietzek (Piscataway, NJ).

7 S collagen [10] was generously supplied by Dr S. Dixit (Memphis, TN). This material was further reduced and alkylated as follows: 1 mg was solubilized in 1 ml of 8 M urea, 50 mM Tris, pH 8 at 4°C. Then DTT was added to a final concentration of 20 mM and the solution was incubated at 37°C for 4 h and α-iodoacetamide was added to final concn. of 80 mM. After 1 h at room temperature the mixture was extensively dialyzed in 10 mM acetic acid and then in 1% BSA in PBS. By gel electrophoresis, in reduced form this material contained a family of components ranging in M_r between 200 000–250 000.

Collagenase-treated pro-type IV collagen was prepared as in [11] by incubating 30 000 cpm of [^{14}C]-proline-labeled pro-type IV collagen for 16 h at 37°C with 100 U of collagenase Form III (Advanced Biofactures) in 0.4 ml of 50 mM Tris-HCl, 10 mM $CaCl_2$, 0.2 M NaCl, 5 mM NEM, pH 7.4. The reaction was stopped by adding 50 μl of 0.3 M EDTA.

CNBr peptides of pro-type IV collagen were prepared as in [12,13].

2.4. *Radioimmunoassays*

Binding reactions between labeled pro-type IV collagen and the antibodies in the presence or absence of competitors were undertaken in tubes containing 100 μl of 1% BSA in PBS and 100 μl of antiserum diluted in normal rabbit serum; after 30 min of incubation at 4°C with 100 μl of competitors (0.1 mg/ml–10 ng/ml) diluted in 1% BSA in PBS, 100 μl of labeled pro-type IV collagen (3000–5000 cpm) were added and the mixture incubated overnight at 4°C. Immunocomplexes were then separated from free antigen by precipitation with a second antibody (100 μl of 50 μl/ml goat anti-rabbit IgG, light and heavy chains; Cappel). After 2 h incubation at room temperature, 1 ml of cold saline solution was added and the mixture was then centrifuged at 1600 × *g* for 30 min. The precipitate was solubilized in 10 ml of Ultrafluor (National Diagnostic) and analyzed in a scintillation counter. Nonspecific precipitation of labeled antigen was determined using either normal rabbit serum or 1% BSA in PBS and was found to be approx. 10% of the total radioactivity added.

2.5. *Immunofluorescence*

Frozen sections (7–10 μm) of human tissue including amnion, thyroid, kidney, breast and skin were hydrated in PBS. Antiserum (approx. 50 μg/ml of protein) was applied and the section incubated for 60 min in a humidified chamber at room temperature. After extensive PBS washing fluorescine-conjugated goat anti-rabbit antibody (Cappel) was applied. Following a 30 min incubation at room temperature and additional PBS washing, the sections were coverslipped over 1/9 glycerol/PBS and examined with a Leitz epifluorescence Ortholux 2 microscope. Appropriate controls were prepared using preimmune rabbit serum and other non-reacting antisera.

3. Results

Antisera from rabbits immunized with whole proα1(IV) and proα2(IV) chains (fig.1, insert A) contained antibodies directed against multiple antigenic determinants, including determinants resistant to bacterial collagenase digestion. Immunoprecipitation (fig.1) of the labeled pro-type IV collagen chains was markedly reduced but not abolished by chymotrypsin in pepsin treatment. Bacterial collagenase treatment of the antigen reduced precipitation to approx. 60% of the radioactivity precipitated with the untreated antigen. The existence of multiple antigenic determinants was confirmed by the immunoprecipitation of

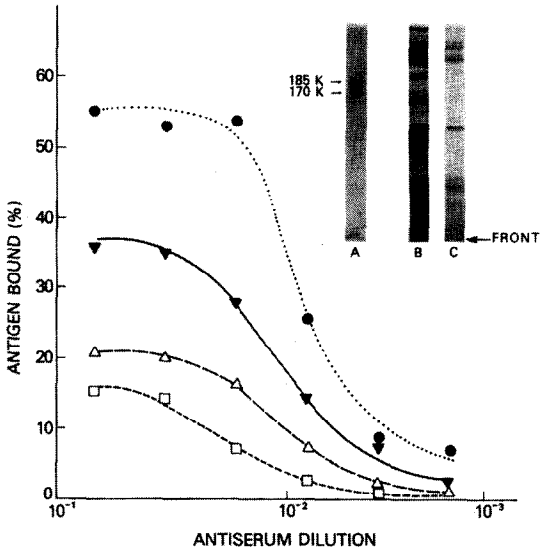

Fig.1. Binding of [^{14}C]proline-labeled pro-type IV collagen by antiserum to native pro-type IV collagen. The antigen was native (•), bacterial collagenase treated (▼), pepsin treated (△) and chymotrypsin treated (□). Insert: (A) the native pro-type IV collagen used as immunogen (M_r 170 000–185 000 proα1(IV) and proα2(IV) chains) run in 5% SDS-PAGE; (B) CNBr peptide pattern of the immunogen, and (C) the immunoprecipitate of the CNBr peptides using the antiserum, run in 12% SDS-PAGE.

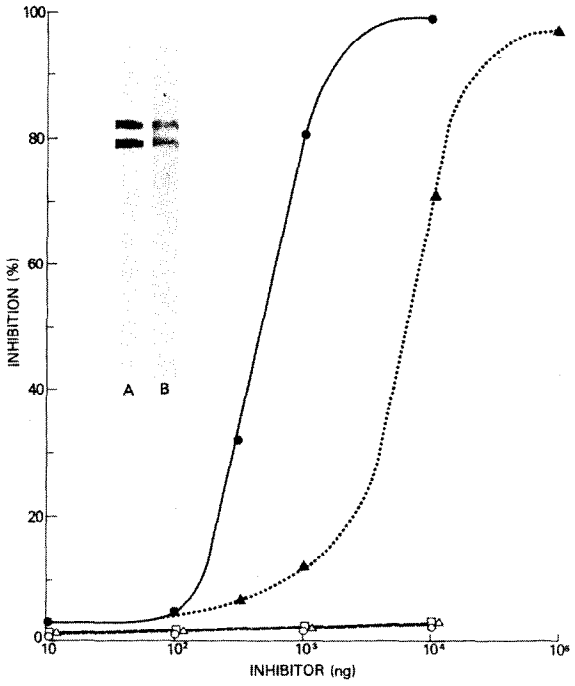

Fig.2. Inhibition of binding of [^{14}C]proline-labeled native pro-type IV collagen (insert A) by affinity purified antibodies. The competitors shown are: unlabeled native pro-type IV collagen (•), reduced and alkylated 7 S collagen (▲), lens capsule collagen, C1, C and D chains (○), P1 and P2 pepsin fragments of EHS tumor pro-IV (△) and laminin (□). Insert B: autoradiography of the immunoprecipitate of proα1(IV) and proα2(IV) obtained without competitors, run in 5% SDS-PAGE.

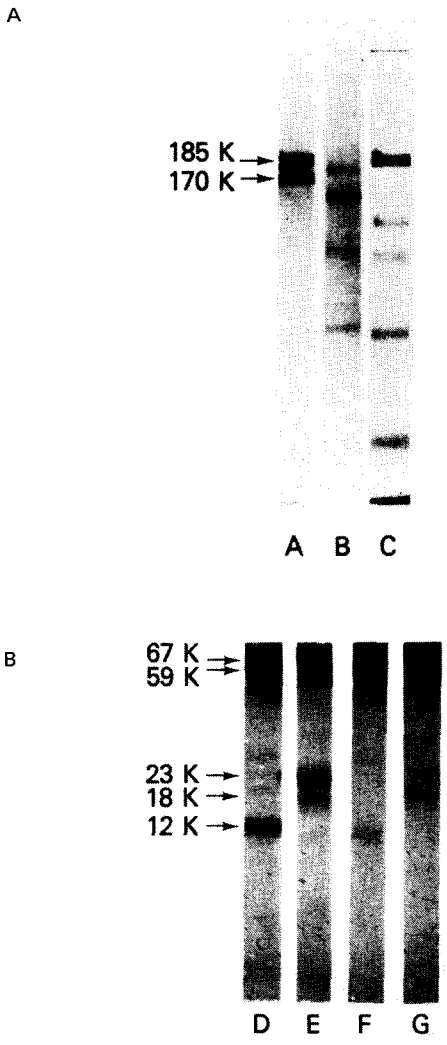

Fig.3. (A) 5% SDS-PAGE autoradiography of [^{14}C]proline-labeled pro-type IV collagen: A, native form; B, following chymotrypsin digestion; C, following pepsin digestion. (B) 12% SDS-PAGE autoradiography of the same substrate as (A): D, after bacterial collagenase digestion; E, the same following DTT reduction; F, immunoprecipitate, with the purified antibodies of the bacterial collagenase digestion; G, the same following DTT reduction.

CNBr-derived peptides from the pro-IV chains (fig.1, inserts B,C).

The antiserum was further purified through an affinity column containing bound pepsinized lens capsule type IV collagen composed of C1, C and D chains [14], P1 and P2 fragments and pepsin-treated type IV collagen from EHS sarcoma. The eluted unbound antibodies were tested for their specificity using competition radioimmunoassay. As shown in fig.2 immunoprecipitation of the tracer antigen was competitively inhibited by whole pro-type IV chains and by reduced and alkylated 7 S collagen. Bacterial collagenase-resistant fragments compete for the tracer with an efficiency about 5-times higher than the reduced and alkylated 7 S (data not shown). No competition is shown by unreduced 7 S collagen, P1 in P2 chains [15,16] from pepsinized EHS tumor pro-IV, nor by pepsinized lens capsule collagen, types I, III or V collagen, fibronectin or laminin. Labeled laminin was not precipitated by the antibodies.

The antibodies precipitated both proα1(IV) and proα2(IV) chains and the ratio of the 2 did not vary at different antibody dilutions (fig.2, inserts A,B). Immunoprecipitation studies were also performed on bacterial collagenase digests of pro-IV chains. As shown in fig.3B at least 5 major fragments of bacterial collagenase digest are identified by 12% SDS-PAGE. Two major disulfide bound-containing components are estimated to be of M_r 67 000 and 59 000 without reduction and 23 000 and 18 000 after reduction. Another major component migrates with a M_r-value of 12 000 before reduction; after reduction this component migrates with the front. Two minor components are also present which are not affected by reduction. All of the major components resistant to bacterial collagenase are precipitated by the antibodies.

The affinity purified antibodies to the bacterial collagenase resistant determinants were used to study the localization of this antigen in tissue basement membranes. Using indirect immunofluorescence technique the antibodies reacted with amnion (fig.4), thyroid, breast and skin basement membranes.

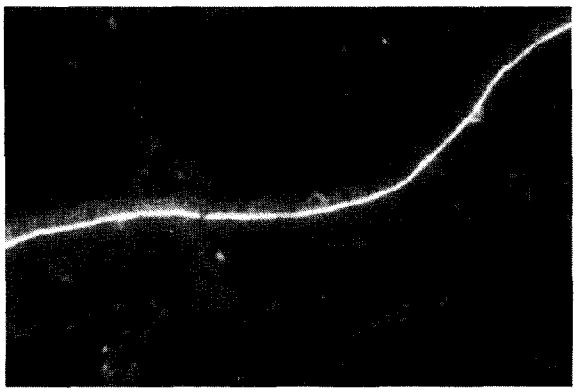

Fig.4. Indirect immunofluorescence of whole basement membrane from fresh human amnion showing intense localization with antibodies to collagenase-resistant terminal regions of native pro-type IV collagen (87.5×).

4. Discussion

Type IV collagen can be isolated from tissue rich in basement membrane either by pepsin or acetic acid extraction: The former method extracts a family of collagenous fragments ranging in M_r-value from 15 000–140 000 [3], the latter method yields 2 chains of 140 000 and 160 000 [15]. Biosynthetic data have more recently shown that type IV collagen is synthesized as chains of 170 000–185 000 M_r. As an approach to studying the actual molecular form of type IV collagen in the tissue we have developed antibodies to the bacterial collagenase-resistant end-regions of the 170 000–185 000 M_r chains.

Type IV collagen has previously been shown to contain multiple antigenic determinants both in the helical and non-helical collagenase-resistant regions [16]. In the present study, rabbit antiserum produced against the 2 pro-IV chains also recognizes multiple antigenic determinants, as shown in fig.1 where at least 8 pro-IV CNBr peptides are immunoprecipitated with the antiserum. In order to estimate the location of these determinants the antigen was subjected to enzyme treatments. Chymotrypsin treatment reduced the antibody binding to a greater degree than pepsin or bacterial collagenase (fig.1). Clark et al. (personal communication) showed that chymotrypsin treatment of the native parietal yolk sac pro-IV molecules yields a 5–10% reduction in the M_r-value of both chains. We have confirmed this findings using the EHS sarcoma-derived pro-type IV (fig.3A). Thus a major antigenic determinant is chymotrypsin sensitive and does not reside on a major central structural portion of pro-IV collagen.

To further study these determinants the antiserum was processed in an affinity column to remove all antibodies directed against the pepsin-resistant domains.

Table 1
Immunoprecipitation of [^{3}H]mannose-labeled type IV collagen using affinity purified antibodies

Substrate	Precipitate (cpm)	% of control
Control pro-type IV collagen[a]	2350	100
After bacterial collagenase treatment[b]	2338	100
After chymotrypsin treatment[c]	335	14

[a] The background using preimmune serum was approx. 25% of the control precipitate
[b] The total amount of antigen radioactivity was 5000
[c] EDTA and TPCK used to stop the reactions 2 and 3, respectively, were included in the controls

The resulting antibodies precipitated both pro-IV chains and such precipitation was not competitively inhibited by pepsinized or chymotrypsin-treated type IV collagen. Moreover, bacterial collagenase-resistant fragments of the molecules were precipitated by these antibodies (fig.3B). These fragments were derived from the pro-IV chains since we verified that the labeled antigen did not contain contaminating non-collagenous labeled proteins such as laminin [17]. The bacterial collagenase-resistant fragments obtained are similar as in [18]. These investigators described a single bacterial collagenase-resistant peak on 8% agarose, with an apparent M_r-value of 70 000; after reduction the peak shifted to a lower value. They also noted additional smaller M_r components. The pattern of the present material in SDS-PAGE consists of 2 fragments in the range of M_r 59 000 and 67 000 which are decreased to 18 000 and 23 000 after reduction. This decrease to 1/3 in M_r-value is consistent with 2 disulfide-bounded tripeptides. The present study also identifies a major 12 000 M_r bacterial collagenase-resistant fragment which migrates with the front after reduction. If the 2 chains are in separate molecules as in [19] then the present antibodies must recognize at least 1 region in each molecule to precipitate both chains.

Radioactive proteins were precipitated from mannose-labeled pro-IV chains before and after treatment with bacterial collagenase (table 1). It is known for other collagens that mannose is present only in the globular terminal region of pro-collagen [20,21]. Assuming an analogous distribution of mannose for pro-type IV collagen, the present data suggest the existence of collagenase-resistant sugar containing terminal regions in both proα1(IV) and proα2(IV).

The recently characterized 7 S basement membrane collagen without reduction shows less than 5% competition for these antibodies. After reduction and alkylation 7 S competes completely at a concentration 15-times greater than native pro-type IV. This result is in agreement with the hypothesis that 7 S is a domain of type IV collagen in which 4 triple helical molecules are held together by disulfide bridges and other covalent cross-links [5].

The purified antibodies specific for the terminal regions of pro-IV reacted specifically with the basement membrane in a wide variety of tissues (fig.4). These results support previous studies which propose that type IV collagen retains non-collagenous terminal regions within the tissue basement membrane matrix [1]. Whether or not the molecular form of type IV collagen may vary in different tissue basement membranes during various pathologic conditions [22] remains to be determined. The present antibodies may be useful to address this question.

Acknowledgements

We thank Dr G. R. Martin and Dr L. Varesio for helpful discussions.

References

[1] Minor, R. R., Clark, C. C., Strause, E. L., Koszalka, T. R., Brent, R. L. and Kefalides, N. A. (1976) J. Biol. Chem. 251, 1789–1794.
[2] Tryggvason, K., Gehron-Robey, P. and Martin, G. R. (1980) Biochemistry 19, 1284–1289.
[3] Bornstein, P. and Sage, H. (1980) Annu. Rev. Biochem. 49, 957–1004.
[4] Schwartz, D. and Veis, A. (1978) FEBS Lett. 85, 326–332.

[5] Kühn, K., Wiedemann, H., Timpl, R., Risteli, J., Dieringer, H., Voss, T. and Glanville, R. W. (1981) FEBS Lett. 125, 123–128.
[6] Nagai, Y., Lapiere, C. M. and Gross, J. (1966) Biochemistry 5, 3123–3130.
[7] Chung, E., Rhodes, R. K. and Miller, E. J. (1978) Biochemistry 17, 3442–3448.
[8] Hoffer, D. E., Adelman, B. C., Gentner, G. and Gay, S. (1976) Immunology 30, 249–259.
[9] Engvall, E. and Ruoslahti, E. (1977) Int. J. Cancer 20, 1–5.
[10] Risteli, J., Bachinger, H. P., Engel, J., Furthmayr, H. and Timpl, R. (1980) Eur. J. Biochem. 108, 239–250.
[11] Sherr, C. J., Taubman, M. B. and Goldberg, B. (1973) J. Biol. Chem. 248, 20, 7033–7038.
[12] Dixit, S. N. and Kang, A. H. (1979) Biochemistry 18, 5686–5692.
[13] Dixit, S. N. and Kang, A. H. (1980) Biochemistry 19, 2692–2696.
[14] Kresina, T. F. and Miller, E. J. (1979) Biochemistry 18, 3089–3097.
[15] Timpl, R., Martin, G. R., Bruckner, P., Wich, G. and Wiedemann, H. (1978) Eur. J. Biochem. 84, 43–52.
[16] Timpl, R., Glanville, R. W., Wick, G. and Martin, G. R. (1979) Immunology 38, 109–116.
[17] Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J. M. and Martin, G. R. (1979) J. Biol. Chem. 254, 9933–9937.
[18] Clark, C. C. and Kefalides, N. A. (1978) in: Biology and Chemistry of Basement Membranes (Kefalides, N. A., ed), pp. 287–298, Academic Press, New York.
[19] Gehron-Robey, P. and Martin, G. R. (1981) Coll. Res. 1, 27–38.
[20] Clark, C. C. and Kefalides, N. A. (1976) Proc. Natl. Acad. Sci. USA 73, 34–38.
[21] Guzman, N. A., Greves, P. N. and Prockop, D. J. (1978) Biochem. Biophys. Res. Commun. 84, 691–698.
[22] Madri, J. A. and Furthmayr, H. (1980) Hum. Pathol. 11, 4, 353–366.