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BINDING DOMAIN FOR LAMININ ON TYPE IV COLLAGEN

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Binding of type IV collagen to laminin was studied by attaching one member of the ligand pair to a solid phase. When laminin was bound to a solid phase, type IV collagen exhibited saturable binding. Digestion of type IV collagen with high concentrations of pepsin destroyed the laminin binding activity. Type IV collagen was also found to bind to fibronectin but the binding activity was not destroyed by pepsin treatment. Rotary shadowing electron microscopy of the pepsin digested type IV collagen indicated that the carboxy terminal end region of about 100 nm is cleaved. Rotary shadowing electron microscopy studies demonstrate that the carboxy terminal end of type IV collagen has a major laminin binding site.

The basement membrane (BM) is an extracellular matrix which underlies parenchymal cells. The structure and biologic function of the BM may be the result of specific and multiple interactions of its collagenous and noncollagenous constituents. These include laminin (1), type IV collagen (2), heparan sulphate rich proteoglycan (3,4) and entactin (5). By electron microscopy, the laminin molecule appears as a cross-shaped structure with one long arm and three short arms (6). All arms contain end globular domains measuring 7 to 15 nm. In vitro, laminin has been shown to mediate the attachment of epithelial and endothelial cells, including certain types of tumor cells to type IV collagen (7). A laminin receptor has been identified and isolated from murine and human cells and bovine smooth muscle cells (8-11). The receptor has a molecular weight of 67,000 daltons and binds laminin with a high affinity (Kd 2 x 10^{-9} M) (9-11). By electron microscopy type IV collagen has the appearance of a rope-like structure of 360 nm in length with the carboxyterminal end terminating as a prominent globular "knob" and the aminoterminal end with a collagen triple helix enriched with S-S bonds (12,13). The noncollagenous globular domain

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(NC_1) is pepsin sensitive. The aminoterminal end serves as the major cross-linking site of four type IV collagen molecules via disulphide bridges and other covalent crosslinks (7S domain). Apart from the end to end associations, Yurckenko and Furthmayr have recently shown that dimeric type IV collagen forms multimers by lateral associations (14). It has been reported that laminin through its long arm binds to heparin and heparan sulphate (15-17). Kleinmann et al. have shown that when laminin is incubated with type IV collagen, precipitation occurs and this process is enhanced by heparan sulphate proteoglycan (18). In this report we demonstrate that type IV collagen interacts with laminin through its carboxy terminal domain extending about 100 nm.

MATERIALS AND METHODS

Laminin was isolated from mouse Engelbreth Holm Swarm tumor (EHS) as described previously and stored frozen in phosphate buffered saline (1). ^{14}C proline labelled pro-type IV collagen (1200-1800 cpm/µg of protein) was purified from organ cultures of EHS tumor tissue by the method of Tryggvason et al (19). Laminin was coupled to the activated Sepharose 4B (Pharmacia Fine Chemicals) according to the manufacturer's recommendations. Human plasma fibronectin was purchased from Sigma Chemical Company. 8 µm SCWP millipore filters were obtained from Millipore Corporation (Bradford, PA). Pepsin (3000 U/mg protein) was purchased from Worthington Biochemicals. A) Laminin Affinity Chromatography: Neutralized ^{14}C labelled pro-type IV collagen or enzyme digested type IV collagen (in a total volume of 1.0 ml) was incubated for 8 hours at 4°C in a laminin affinity column (1x5 cm) previously equilibrated with 50 mM Tris, 1 mM $CaCl_2$, 3.0 mM $MgCl_2$, 0.15 M NaCl, pH 7.40 (TBS). The column was thoroughly washed with TBS until the radioactivity in the eluate reached the background. Bound material was then eluted with 1.0 M NaCl followed by 8.0 M urea. The radioactivity in each fraction was measured in a Beckman 7800 liquid scintillation counter. In some fractions the proteins were identified by electrophoresis on 5% acrylamide gels and autoradiography. B) Ligand Binding to Nitrocellulose Filters: 8 µm SCWP millipore filters (13 mm in diameter) were placed in 24 well costar dishes and known amounts of laminin was applied in a volume of 20 µl and allowed to bind for 30 min under humidified conditions. The unoccupied protein binding sites were then blocked by incubating the filters in 3% bovine serum albumin (BSA) (2 x 90 min). The residual BSA was washed away with PBS (2 x 5 min). The labelled ligand to be tested for binding was made up to 350 µl in TBS-BSA buffer, added to each filter and incubated overnight at 4°C under mild shaking. After the incubation period, the solution was quickly aspirated and the filters were thoroughly washed (6 x 20 min) to remove the unbound ligand. The filters were solubilized in 10 ml of hydrofluor and counted in a liquid scintillation counter. C) Enzyme Digestion of type IV Collagen: ^{14}C-type IV collagen was incubated with pepsin at various substrate to enzyme ratios. The digestion of type IV collagen was carried out at 4°C for 24 hours. The enzyme activity was arrested by increasing the pH to 7.4 with 1.0 M Tris. 3.0 mM $CaCl_2$, 6.0 mM $MgCl_2$. The digestion products formed from type IV collagen by the enzyme were analyzed on SDS-polyacrylamide gels and autoradiography. D) Other Procedures: Polyacrylamide slab gel electrophoresis on 5% acrylamide slab gels was according to Laemmli's procedure in the presence of 0.5 M urea for better resolution (20). The labelled proteins were fixed in 10% TCA, 50% MeOH for 1 hr and enhanced in autofluor (National Diagnostics) for 2 hr. Fluorography was

performed with the dried gels after exposing to Kodak Xomat films. Rotary shadowing electron microscopy of type IV collagen and pepsin digested type IV collagen was according to the previously described procedures (6,23). In order to visualize laminin and type IV collagen complexes, equal amount of proteins were incubated overnight at 4°C and observed by electron microscopy. The measurements of the length of the molecules was obtained in a Hewlett Packard 9840 digitizer (Fort Collins Co.).

RESULTS AND DISCUSSION

Type IV collagen binding to laminin was first studied by incubating ^{14}C labelled pro-type IV collagen in a laminin affinity column followed by elution with 8.0 urea. The data are shown in fig. 1. In three separate experiments, a substantial amount (up to 50% of the total applied) of type IV collagen bound to the solid phase laminin. The bound type IV collagen was not eluted with 1.0 M NaCl indicating that the binding affinity of these two molecules is quite high. Inclusion of 8.0 M urea in the buffer eluted all the type IV collagen bound to the laminin (fig. 1). Scatchard analysis indicated that the

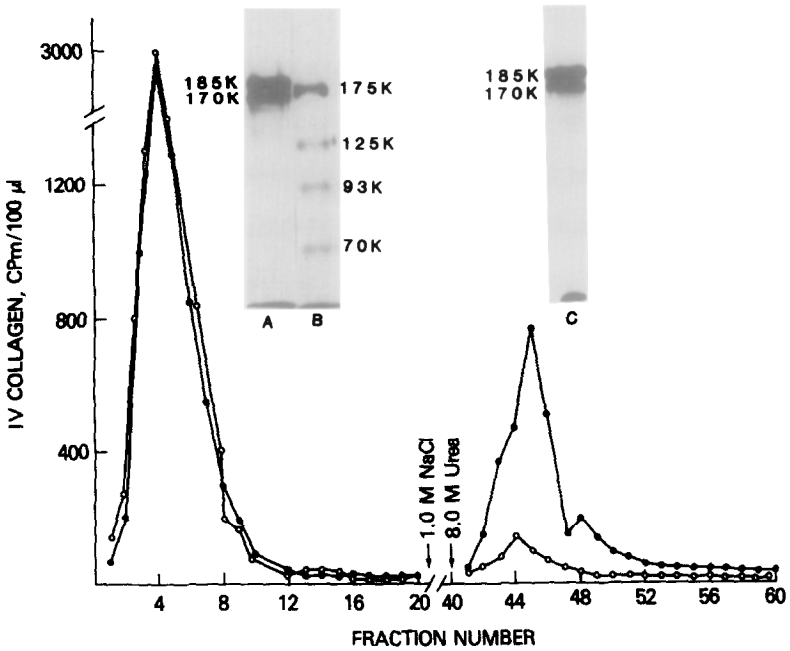

Figure 1: Binding of ^{14}C-type IV collagen to laminin immobilized on CNBR activated Sepharose 4B. Neutralized type IV collagen was soaked over night in the column and the unbound fraction was washed with 50 mM Tris, 1 mM $CaCl_2$, 3.0 mM $MgCl_2$ 0.15 M NaCl pH 7.4. The Bound fraction was eluted successively with 1.0 M NaCl and 8.0 M urea. (●-●) ^{14}C-type IV collagen, (o-o) pepsin digested type IV collagen. Note pepsin digested type IV collagen did not bind to laminin while a significant amount of ^{14}C-type IV collagen is bound to laminin and is eluted with 8.0 M urea but not with 1.0 M NaCl. (A) Autoradiogram of ^{14}C-type IV collagen (B) pepsin digested ^{14}C-type IV collagen (C) ^{14}C-type IV collagen eluted with 8M urea.

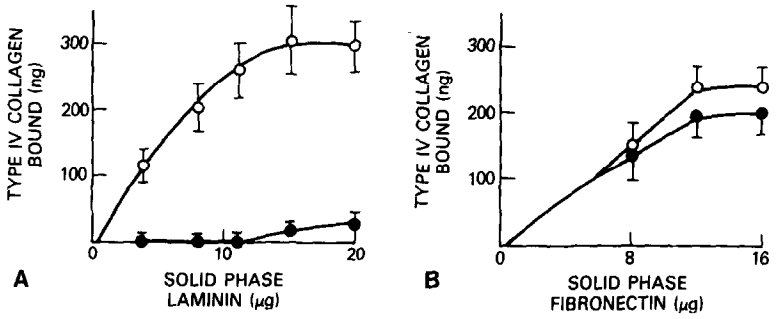

Figure 2: Differential binding of ^{14}C-type IV collagen (o-o) and pepsin treated type IV collagen (•-•) to (A) laminin and (B) fibronectin. The details of the experiment are given in materials and methods. Note that the pepsin digested type IV collagen did not bind laminin but retains 100% binding to fibronectin.

binding constant is in the order of 5.3 x 10^{-7}M (r=0.91). SDS-PAGE electrophoresis and autoradiography of the eluted material showed the presence of the two type IV collagen alpha chains of Mr 185,000 and 170,000 daltons after reduction. In contrast to the whole type IV collagen molecule, the pepsin digested type IV collagen did not bind to the laminin affinity resin (fig. 1).

We also studied the binding of type IV collagen to SCWP 8 μM (13 mm diameter) millipore filters coated with laminin. We have used these filters as matrices to study the lectin binding domains on the laminin molecule (21). When a series of concentrations of laminin were bound to the filters, the application of 16 to 20 μg of laminin on the filter was in the plateau range. Under these conditions, 300 ng of labelled type IV collagen bound to the immobilized laminin (fig. 2A). Type IV collagen also bound to immobilized fibronectin, but the plateau range was from 12 to 16 μg of fibronectin on the filter. Fibronectin, however, bound only 200 ng of labelled type IV collagen (fig. 2B). This confirms the previous observation that type IV collagen preferentially binds laminin compared to fibronectin. Fibronectin binds denatured type IV collagen much better (three times that of native) while laminin binds only native collagen.

In fig. 2, the binding activity of pepsin digested type IV collagen to laminin or fibronectin was shown. Digestion of type IV collagen with pepsin destroyed both chains producing a major 175 K polypeptide chain presumably derived from 185 K chain (fig. 3B and C). However, at an enzyme to substrate ratio of 1:1, the 175 K polypeptide chain is further degraded (fig 3D). In order to study the laminin

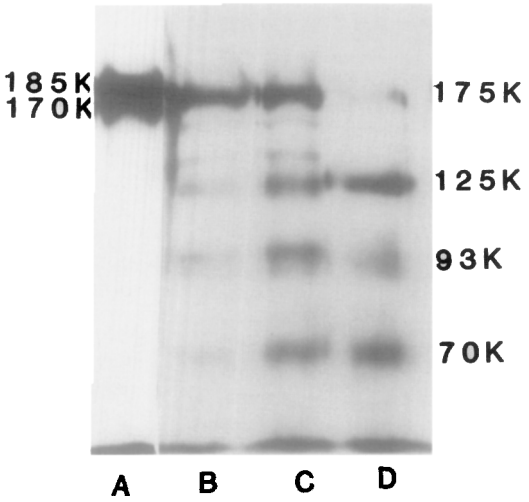

Figure 3: Digestion of ^{14}C-type IV collagen by pepsin. (A) ^{14}C-type IV collagen was digested with pepsin at an enzyme to substrate ratio of (B) 1:20 (C) 1:5 (D) 1:1. The digestion products were reduced in presence of 2-mercaptoethanol, separated on 5% polyacrylamide slab gels and identified by autoradiography.

binding activity of pepsin digested type IV collagen, we have chosen the enzyme to substrate ratio of 1:5, as much of the 175 K polypeptide is preserved and the 170 K polypeptide is degraded (fig. 3C). The data in fig. 2 on the binding of pepsin digested type IV collagen shows that the binding activity to laminin is totally destroyed, while 100% of the fibronectin binding activity is retained. These data clearly indicate that type IV collagen has separate binding domains for laminin and fibronectin. The binding site for laminin is pepsin sensitive under the conditions chosen.

In order to localize the pepsin sensitive region of type IV collagen we visualized the structures of type IV collagen and pepsin digested type IV collagen by rotary shadowing electron microscopy and shown in fig. 4. Acid extracted type IV collagen is visualized as two major species of particles. The majority of the molecules are connected at their globular ends as dimers (fig. 4A). Occasionally monomeric molecules were present in the preparation. The pepsin digested type IV collagen lacked the dimeric structures and existed predominantly as single-stranded species (fig. 4B). The average length of the type IV collagen molecule is close to 358 nm $\pm$ 12 S.D. (50 molecules measured). In contrast, the pepsin digested type IV collagen measured only 255 nm $\pm$ 20 (70 molecules measured) indicating

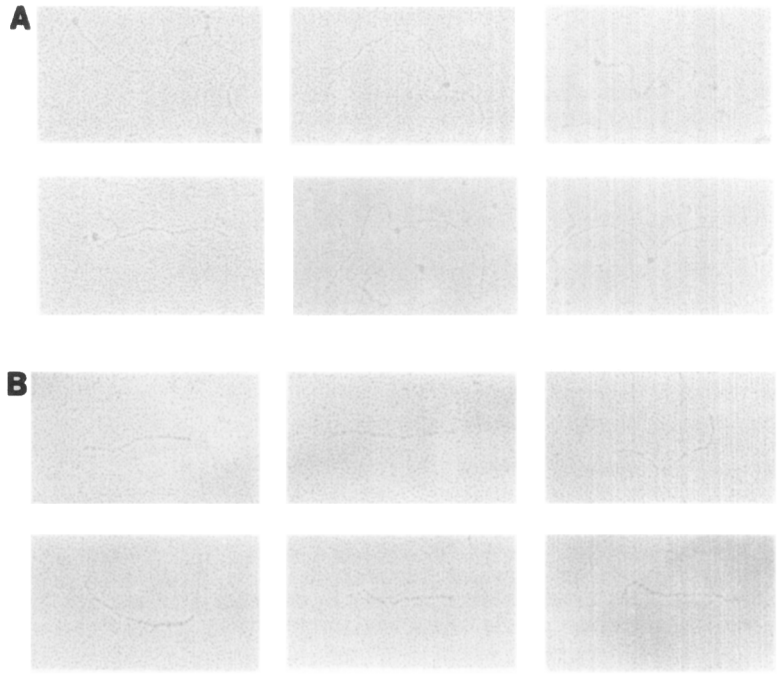

Figure 4: Rotary shadowing electron microscopy of intact and pepsin digested ^{14}C labled type IV collagen (A) type IV collagen was mainly visualized as dimers. The 7S domain is clearly visualized in some of the micrographs. (B) Pepsin digested type IV collagen is mainly visualized as monomeric molecules containing the 7S domain but lacking the globular domain.

that a 100 nm region from the globular end is cleaved by the high concentrations of pepsin employed (fig. 4B). Binding of laminin molecules on type IV collagen near the globular domain is demonstrated in Fig 5. From several micrographs, we conclude that more than one laminin molecule binds to the dimeric form of type IV collagen near its globular end extending about 100 nm.

Binding of type IV collagen to laminin may play an important role in cell adhesion and organization of basement membranes. Woodley et al., have previously reported that laminin preferentially binds to type IV collagen over other collagens and predicted that laminin and fibronectin have separate binding domains on type IV collagen (22). Our previous studies with purified protease derived fragments of laminin indicated that end globular domains on the laminin three short arms are essential for laminin to interact with type IV collagen (8,23,24). The present studies indicate that native type IV collagen binds to both laminin and fibronectin. Pepsinization or denaturation of type IV collagen abolishes laminin but not

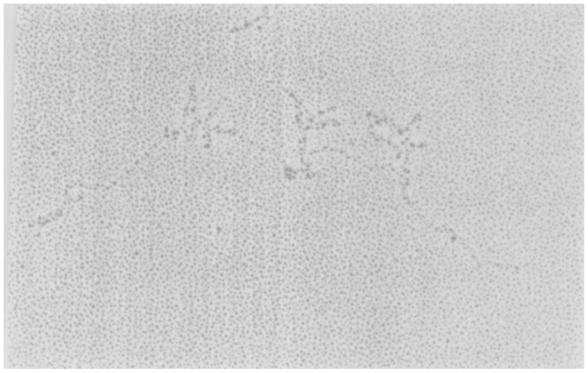

Figure 5: Electron micrographs showing laminin molecules binding near the carboxy terminal globular domain of type IV collagen.

fibronectin binding. These findings have implications for specific interactions of laminin and type IV collagen in establishing the basement membrane architecture.

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