Binding of Laminin to Type IV Collagen: A Morphological Study

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ABSTRACT A mixture of laminin and type IV collagen was analyzed by rotary shadowing using carbon/platinum and electron microscopy. Laminin was found to form distinct complexes with type IV collagen: one site of interaction is located 140 nm from the COOH-terminal, noncollagenous (NC1) domain and the other is located within the NH_2-terminal region. The isolated NC1 fragment of type IV collagen does not appear to interact with laminin, while pepsin-treated type IV collagen, which lacks the NC1 domain, retains its ability to form complexes with laminin. Analysis of the laminin-type IV complexes indicates that laminin binds to type IV collagen via the globular regions of either of its four arms. This finding is supported by experiments using fragment P1 of laminin which lacks the globular regions and which does not bind to type IV collagen in a specific way. In addition, after heat-denaturation of laminin no specific binding is observed.

It is now well established that type IV collagen (1, 2) and laminin (3, 4) are important basement membrane components. Histochemical studies have indicated that both macromolecules are found exclusively in basement membranes and that they may occupy distinct regions of these structures (5, 6). Furthermore, it has been suggested that in the extracellular matrix of the Engelbreth-Holm-Swarm EHS[1] tumor, which is considered to be a basement membrane–like matrix (7), type IV collagen and laminin are present in equimolar amounts (8). However, it is unknown how these two molecules, as well as others found in basement membranes, are organized at the molecular level. The presence of these two macromolecules in all basement membranes raises the possibility that they may interact in specific ways.

Only few reports have addressed the question of the laminin-type IV collagen interaction. Kleinman et al. (9) suggested the existence of such an interaction on the basis of an increase in turbidity when type IV collagen and laminin extracted from the EHS tumor matrix were incubated together. However, the methods used give little insight into the nature of the interaction. Engvall and Ruoslahti (10), on the other hand, in solid-phase assays, have failed to observe a specific association between type IV collagen and laminin isolated from various sources, including the EHS tumor. In their study, however, laminin was not used in concentrations higher than 10 μg/ml; therefore, the possibility of weak interactions could not be excluded. In view of these incomplete and, to some extent, conflicting data, we sought answers, using the rotary shadowing method, to the following questions: is there a specific association between laminin and type IV collagen which could be demonstrated at the molecular level, and if so, can we identify specific domains of the two macromolecules involved in this interaction?

MATERIALS AND METHODS

Type IV Collagen: Type IV collagen was isolated according to the method of Kleinman et al. (11), from EHS tumor grown subcutaneously in mice rendered lathyritic by adding 0.25% β-aminoproprionitrile to the drinking water. Briefly, following homogenization, the tumor was extracted with 3.4 M NaCl in 50 mM Tris-HCl (pH 7.4), containing 1 mM EDTA, 50 μg/ml phenylmethylsulfonyl fluoride (PMSF), 50 μg/ml *p*-chloromercuribenzoic acid, and 0.5 mM diisopropyl-fluorophosphate, and was centrifuged at 10,000 rpm for 30 min. The pellet was washed five more times with the same buffer and the final residue was extracted with 1.7 M NaCl in 50 mM Tris-HCl (pH 7.4) with 1 mM EDTA, 50 μg/ml PMSF, 50 μg/ml *p*-chloromercuribenzoic acid (Buffer A), pelleted, and extracted (for 24 h) with 2 M guanidine-HCl in 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 50 μg/ml PMSF, and 50 μg/

[1] *Abbreviations used in this paper:* DTT, dithiothreitol; EHS, Engelbreth-Holm-Swarm; NC1, COOH-terminal, noncollagenous domain 1 of type IV collagen; PMSF, phenylmethylsulfonyl fluoride.

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ml *p*-chloromercuribenzoic acid (buffer B). The extract was pelleted and the insoluble material was re-extracted with buffer B containing 2 mM dithiothreitol (DTT). After centrifugation, the supernatant from this extract was dialyzed in buffer A until a precipitate formed which was pelleted, and the pellet was solubilized in 50 mM Tris-HCl (pH 8.6) containing 4 M urea, 2 mM DTT, 1 mM EDTA, 50 μg/ml PMSF, and 250 mM NaCl (buffer C). The solution was incubated for 3 h with DEAE cellulose (Whatman Inc., Clifton, NJ) equilibrated with buffer C. The unbound fraction was dissolved in buffer B containing 2 mM DTT, and was then dialyzed once more into buffer A. The precipitate was pelleted and dissolved again in buffer B containing 2 mM DTT. The solution was centrifuged to clear aggregates larger than 35S and the supernatant was stored on ice.

Laminin: Laminin was extracted according to the method of Timpl et al. (3). EHS tumor was homogenized and washed five times and the final pellet was extracted for 24 h with buffer A as described above. After centrifugation, the supernatant was brought to 3.4 M NaCl by adding solid NaCl. The precipitate was collected by centrifugation, solubilized in 2 M urea, 50 mM Tris-HCl (pH 8.6), with 1 mM EDTA and 50 μg/ml PMSF, and was incubated for 3 h with DEAE cellulose equilibrated with the same buffer. The unbound fraction was dialyzed against 1 M $CaCl_2$ in 50 mM Tris-HCl (pH 7.4), with 50 μg/ml PMSF (buffer D) and chromatographed on an Agarose A 1.5 m column (2.5 × 90 cm) (Bio-Rad Laboratories, Richmond, CA), equilibrated with the same buffer. Laminin eluted close to the void volume. It was dialyzed against 0.4 M NaCl in 50 mM Tris-HCl (pH 7.4) and 50 μg/ml PMSF (buffer E), concentrated with aquacide II-A (Calbiochem-Behring Corp., San Diego, CA) and stored at −196°C.

Heat-denatured Laminin: Heat-denatured laminin was produced by incubation of native laminin in 120 mM NaCl, 10 mM sodium phosphate (pH 7.4) at 90°C for 5 min.

Fragment P1 of Laminin: Fragment P1 of laminin was prepared according to the method of Rhode et al. (12) with the following modifications. Purified laminin at a concentration of 700 μg/ml or lower was dialyzed against 10% acetic acid (pH 2.2) and was incubated with pepsin (Worthington Biochemical Corp., Freehold, NJ), using an enzyme to substrate ratio of 1:15. The incubation was carried out at 15°C for 8 h. The reaction was stopped by addition of solid Tris base to pH 5.0. The digest was then dialyzed extensively against buffer D and fragment P1 was isolated by chromatography on an Agarose A 5 m column (2.5 × 90 cm) (Bio-Rad Laboratories) equilibrated with the same buffer. It was dialyzed against buffer E and stored at −70°C.

Pepsin-treated Type IV Collagen: Pepsin-treated type IV collagen, lacking the COOH-terminal globular domain, was prepared as previously described (13). Isolated type IV collagen was extensively dialyzed against 0.1 M acetic acid, then pepsin (Worthington Biochemical Corp.) was added at an enzyme to substrate ratio of 1:50 and the reaction was allowed to proceed for 70 min at 28°C. It was stopped either with pepstatin or with solid Tris base, and the digest was dialyzed into 2 M urea in 50 mM Tris-HCl (pH 7.4), containing 1 mM DTT, 1 mM EDTA and 5 mM glycine; the digest was then centrifuged for 1 h at 40,000 rpm. The supernatant was purified by chromatography on a Sephacryl S-1000 column (5 × 95 cm). The pepsin-treated, monomeric type IV collagen peak was concentrated with aquacide II-A, dialyzed into buffer B containing 2 mM DTT, and stored on ice.

NC1: Noncollagenous (NC1) domain of type IV collagen was prepared as described by Timpl et al. (14). Purified type IV collagen was extensively dialyzed against 0.2 M NaCl in 50 mM Tris-HCl (pH 7.6) containing 2 mM $CaCl_2$, and was digested with chromatographically purified bacterial collagenase (CLSPA) (Worthington Biochemical Corp.) at an enzyme to substrate ratio of 1:100, at 37°C for 48 h. The digest was then centrifuged at 20,000 rpm for 30 min, dialyzed against 0.2 M NH_4HCO_3 (pH 8.5), and then applied onto a Sephacryl S-300 column equilibrated with the same buffer. The dimeric NC1 peak exhibited a $K_{av} = 0.433$. It was concentrated with aquacide II-A and stored at −20°C.

All procedures were carried out at 4°C. All macromolecules and their fragments described above were analyzed by SDS PAGE, rotary shadowing, and amino acid analysis; they yielded data comparable to those previously reported (not shown).

Incubation Conditions: Before each experiment, laminin, type IV collagen, or their proteolytic fragments, were dialyzed against 120 mM NaCl, 10 mM sodium phosphate (pH 7.4), and then centrifuged for 20 min at 40,000 rpm to remove aggregated material. Incubations were carried out at 37°C for 60 min except in the case of pepsin-treated type IV monomeric collagen, which was first incubated at 28°C overnight in order to form tetramers (13) and then co-incubated with laminin for 60 min at 37°C. Equimolar amounts (100 nM) of each macromolecule were used. The concentration of intact and pepsin-treated type IV collagen was determined by amino acid analysis. The concentration of the NC1 fragment of type IV collagen, laminin, and fragment P1 of laminin was determined by the method of Lowry (15). After incubation the sample was diluted with 0.1 M ammonium bicarbonate or ammonium acetate and glycerol to a volume of 1 ml.

Rotary Shadowing: The technique originally described by Shotton et al. (16), as modified by Engel et al. (17), was used. The final protein concentration was 10 μg/ml in 0.1 M ammonium bicarbonate or ammonium acetate and 50% glycerol. A volume of 1 ml was sprayed onto freshly cleaved mica, from a distance of 40 cm. Mica pieces were transferred onto the rotating stage of a Balzers apparatus, where, under a pressure of 2×10^{-5} torr a replica was made by first evaporating a platinum-carbon mixture at an angle of 6°, followed by a second carbon evaporation at an angle of 90°. Replicas were allowed to float on distilled H_2O and were picked up on carbon-coated grids.

Quantitation of Morphological Data: Grids were examined by using a Phillips 300 electron microscope, operating at 60 kV. The percentage of nonpolymerized type IV collagen molecules associated with native laminin, heat-denatured laminin, or fragment P1 of laminin was determined by direct examination of the replicas and counting of the respective macromolecular species. Images printed at a final magnification of 370,000 were analyzed by using a Zeiss videoplan computer with a digitizer measuring tablet attachment and a (Y)1 Videoplan program to determine the site of binding of the three potential "ligands" (native and denatured laminin, P1 fragment) along the length of type IV collagen molecules. The results from these measurements were used to construct histograms in which the "relative frequency" of binding was plotted against the length of the type IV collagen molecule, divided into 20 equal segments. Statistical analysis of the histograms was performed by using the Kolmogorov-Smirnov test (18).

RESULTS

When laminin and type IV collagen were co-incubated at 37°C for 60 min and then examined in rotary shadow replicas, they were observed to form distinct complexes. Fig. 1 shows selected examples of individual complexes in which binding of laminin can be seen at multiple sites along the length of type IV collagen molecules.

To determine whether the binding of laminin occurs randomly along the length of type IV collagen or at preferred sites, measurements were performed on photomicrographs such as those shown in Fig. 1. We measured the distance of the binding of laminin from the COOH-terminal, NCl domain of type IV collagen (Fig. 1, arrowheads) and expressed this value as a ratio, by dividing it by the total length of the molecule. The calculated value is thus independent of variations in total length measurements. Several criteria were used for these measurements: (*a*) the site of binding of laminin to type IV collagen had to be clearly visualized; (*b*) the entire length of the type IV molecule had to be traceable; (*c*) the length of individual type IV molecules had to be 410 ± 40 nm; (*d*) laminin molecules which were located across the type IV collagen were excluded since, being extremely few in number, they could be due to superimposition and not binding.

The results of our measurements are shown in Fig. 2*A*. In this histogram, two peaks are observed, one at a distance of ~140 nm from NCl and the other within the NH_2-terminal region of type IV collagen. Statistical analysis indicates that the binding at these two sites is significant ($P < 0.05$, cf. Fig. 2*A*). In half of the observed complexes, native laminin (Fig. 2*A*) binds to type IV collagen at either 140 nm distal to the NC1 domain (Fig. 1, *A*, *C–G*) or at the NH_2-terminal region (Fig. 1, *C*, *I–K*). On the other hand, in a considerable number (50%) of the laminin-type IV collagen complexes, in which binding occurred at different locations, the apparent binding could be due to nonspecific association or random distribution of the two molecules on the mica surface. The relatively high number of such events is not entirely surprising. In other rotary shadowing studies, the mapping of a monoclonal an-

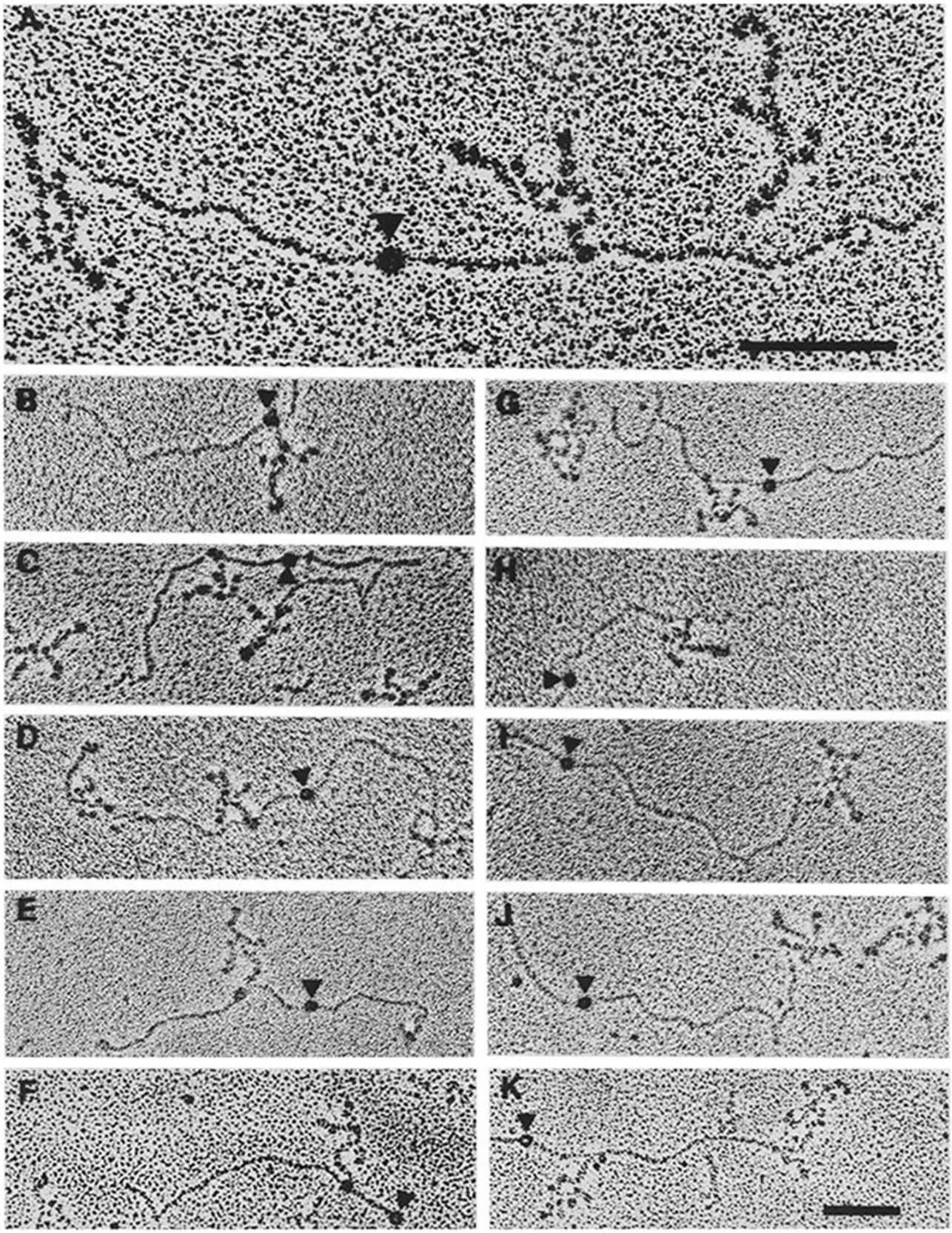

FIGURE 1 Images obtained by rotary shadowing of mixtures of equimolar amounts of laminin and type IV collagen, incubated for 60 min at 37°C. Laminin binds to type IV collagen with its globular domains. The binding occurs mainly either at ~140 nm distal to the NC1 domain (*A*, *C–G*, *K*) or at the NH_2-terminal region (*C*, *I–K*). Binding at other sites is also observed (*A*, *B*, *H*). Arrowheads point to the NC1 domain. Bar, 100 nm.

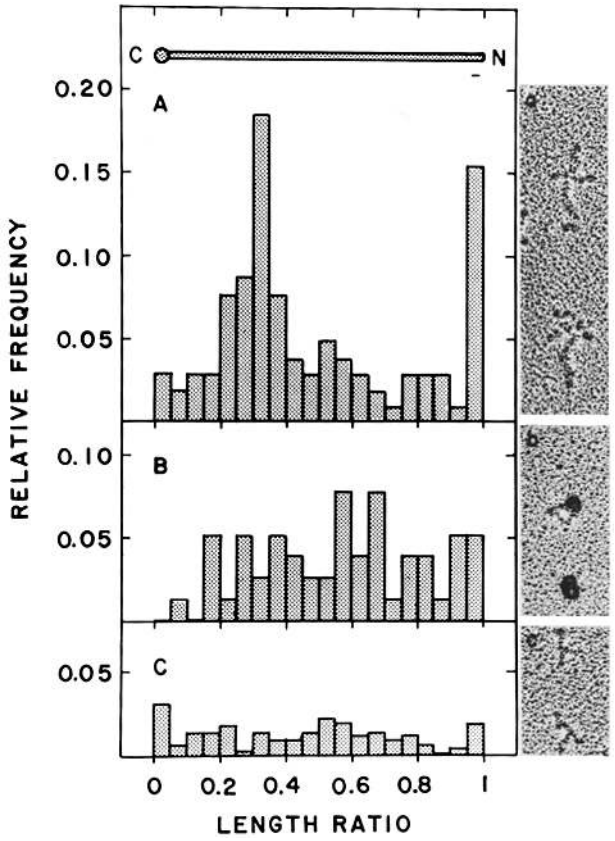

FIGURE 2 Histogram of the relative frequency distribution of binding of potential ligands to segments of the type IV collagen molecule shown diagrammatically on the top (COOH-terminal, *left*; NH_2-terminal, *right*). Distribution of the binding of native laminin (*A*), denatured laminin (*B*), and fragment P1 (C). Statistical analysis demonstrates that only the distribution of native laminin (*A*) is statistically significant ($P < 0.05$). Both denatured laminin (*B*) and fragment P1 (C) are randomly distributed ($0.5 < P < 0.25$). On the right side of the figure, individual examples of rotary shadowing images of native laminin (*a*), denatured laminin (*b*), and P1 fragment (*c*) are shown.

tibody to a specific epitope on type IV collagen exhibited 30% nonspecific binding (19), even though the binding affinity of such an antibody for its antigen may well be orders of magnitude higher than that of laminin for type IV collagen.

We decided to determine the domain(s) of laminin involved in the binding of type IV collagen. In almost all of the images, the binding occurred at the free ends of laminin, involving presumably the globular domains (Fig. 1). Although in many instances it was not possible to identify unequivocally which arm (short lateral, short opposite to long, or long) was mediating the binding, preliminary observations indicate that the long arm of laminin might be preferentially involved in the laminin-type IV collagen binding.

The possibility that the end globular domains of laminin participate in the association of laminin with type IV collagen was investigated by using fragment P1 of laminin, which lacks the entire long arm and the globular domains of the short arms (12, 17) (Fig. 2*c*). When this fragment was co-incubated with intact type IV collagen, it was associated with type IV collagen to a considerably lesser extent than laminin, and the distribution was random (Fig. 2*C*). These data strongly suggest that the end globular domains of laminin are required for association with type IV collagen.

After heat-denaturation laminin was still recognizable in rotary shadow replicas as dense, circular structures with one or two short, curved arms (Fig. 2*b*), and after co-incubation with type IV collagen it appeared to be occasionally associated with type IV collagen. However, this association was found to be nonspecific by using the same statistical analysis which was used for the binding of native laminin or fragment P1 (Fig. 2*B*). Hence, the native structure of laminin is required for specific binding with type IV collagen. Heat-denatured type IV collagen or its individual chains cannot be visualized with the technique of rotary shadowing (H. Furthmayr, unpublished observations). However, it is reasonable to assume that the native structure of type IV collagen is also required since the two binding sites appear to be localized within triple-helical regions of the molecule.

When pepsin-treated, monomeric type IV collagen is incubated at 28°C, it self-associates via the NH_2-terminal regions to form dimers, trimers, and tetramers (13). In preliminary experiments, laminin was seen to bind to preformed tetramers of pepsin-treated type IV collagen, mainly at the position corresponding to the site closer to the NC1 region (cf. Fig. 1*A*, Fig. 3, *A* and *B*), but not to the NH_2-terminal region of

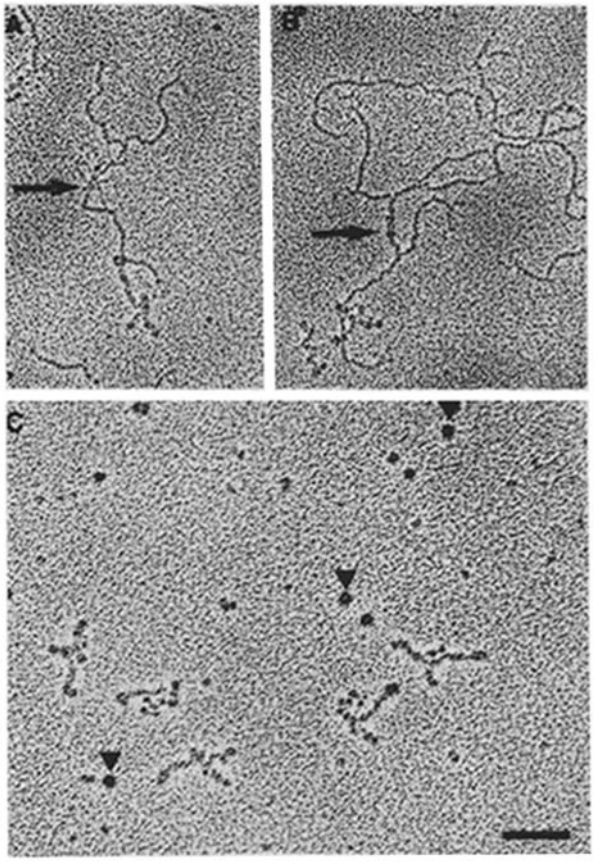

FIGURE 3 Binding of laminin to oligomeric, pepsin-treated type IV collagen, missing its NC1 globular domain. Laminin is seen to interact with a trimer (*A*) and a tetramer (*B*) which are formed by association of monomers at their NH_2-terminal ends (arrows). Laminin is bound at ~140 nm from the opposite (COOH-terminal) end but not at the site which was observed at the NH_2-terminal end. No binding of laminin to collagenase-derived NC1 domains of type IV collagen (arrowheads) is observed (*C*). Bar, 100 nm.

type IV collagen. It is possible that the affinity of binding of laminin to the NH_2-terminal site of type IV collagen is altered when type IV collagen self-associates to form tetramers.

The fact that removal of the NC1 domain of type IV collagen does not abolish laminin binding (Fig. 3, *A* and *B*) is not surprising. This was indicated already in Fig. 2*A*, since binding at this region was not above background. It is further supported by direct experiments in which isolated fragment NC1 does not appear to interact with laminin (Fig. 3*C*).

To compare the frequency of complex formation, we performed measurements on over 200 molecules in each case by direct examination of the replicas; from these data we calculated the relative frequency of complex formation as the percentage of nonpolymerized type IV molecules associated with native laminin, heat-denatured laminin, or fragment P1 of laminin. This restriction was necessary, because under our experimental conditions type IV collagen and laminin form aggregates by themselves (13, 20) which have to be excluded from the analysis since they are not well resolved. The relative frequency was 30% for laminin, 18% for heat-denatured laminin, and 7% for fragment P1 of laminin. These figures may not be directly comparable since laminin has the ability to self-associate resulting in a lower effective concentration, while heat-denatured laminin and fragment P1 of laminin do not aggregate (20).

DISCUSSION

In this report we describe for the first time the in vitro association of laminin with type IV collagen, visualized with the technique of rotary shadowing. We have observed that the end globular domains of laminin bind preferentially at two sites to the type IV collagen molecule. The interaction at these sites requires native, intact laminin, since denaturation or removal of the globular domains of laminin reduces its binding to background levels. Our data suggest binding of at least one of the globular domains of laminin to regions of type IV collagen which are located within the triple-helical, collagenous part of the molecule. The detailed structural requirements, however, remain to be determined since the binding sites might coincide with regions of the molecule that contain short interruptions of the triple helical conformation (21).

In general, a simple binding interaction observed in vitro could be operationally defined as specific if it demonstrates saturability, if it can be competed for by the appropriate component, and if it exhibits topographical specificity. In our system we have strong indications that the binding is topographically specific, because of the distinct localization of native laminin binding sites along the type IV collagen molecule. Factors such as the high background observed with the rotary shadowing, the ability of both laminin and type IV collagen to self-associate even at low concentrations, and the possible weakness of the interaction between these two macromolecules (see below), taken together, make it extremely difficult to use this technique for demonstrating saturability. Furthermore, competition of the specific binding will have to await the unequivocal identification and isolation of the domains involved in the binding.

The association between laminin and type IV collagen has been controversial. Kleinman et al. (9) have reported that mixtures of these macromolecules, when incubated at 35°C, developed substantial turbidity. However, the possibility exists that the increased turbidity is due to a catalytic effect of one macromolecule on the polymerization of the other. Engvall and Ruoslahti (10) have failed to detect binding of laminin to type IV collagen in solid-phase assays. Their failure could have occurred because they used very low concentrations of laminin. They concluded that if such a binding exists, it should be very weak.

We have attempted to demonstrate laminin-collagen interactions by a number of other techniques. In velocity sedimentation experiments we were unable to observe a laminin-type IV collagen interaction. Sucrose could have been responsible for this failure. In solid-phase assays and radioimmunoassays, although substantial binding could be observed, we were unable to saturate the binding of laminin to type IV collagen even at the highest concentrations which could be achieved in vitro. This failure suggests either non-ideal conditions or nonspecific "sticking", or it can be explained on the basis of the behavior of the proteins themselves in solution. We have shown recently that laminin, as well as type IV collagen, can self-assemble to form distinct oligomeric structures (13, 20).

The difficulties in demonstrating conclusively interactions between laminin and type IV collagen suggest that their in vitro interaction might be weak. If this is true, could these interactions, nevertheless, be important for basement membranes? We suggest that, in vivo, the following factors could allow such weak interactions to contribute to the formation of the basement membrane network. (*a*) Laminin might interact more strongly with components of basement membranes other than type IV collagen, like heparan sulfate (8), nidogen, entactin, or other yet unknown molecules and with cell surface receptors (22, 23). (*b*) Laminin binding to type IV collagen might be stabilized eventually by the formation of cross-links known to develop between other interstitial components with time (24). In support of this possibility is the report by Wewer et al. (25) that laminin could not be extracted from placental basement membranes with salt treatment, i.e., with the protocol used for extraction from the EHS tumor. (*c*) Type IV collagen and laminin have been observed to self-associate and form polymers in vitro (13, 20). It is not yet known whether such polymers exist in vivo. If they exist, then multiple weak binding sites could interact in a positively cooperative way to result in the anchoring of laminin to the basement membrane skeleton provided by type IV collagen.

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