The Role of the Main Noncollagenous Domain (NC1) in Type IV Collagen Self-Assembly

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Abstract. Type IV collagen incubated at elevated temperatures in physiologic buffers self-associates (a) via its carboxy-terminal (NC1) domain, (b) via its amino-terminal (7S) domain, and (c) laterally; and it forms a network. When examined with the technique of rotary shadowing, isolated domain NC1 was found to bind along the length of type IV collagen to four distinct sites located at intervals of ~100 nm each. The same 100-nm distance was observed in domain NC1 of intact type IV collagen bound along the length of the collagen molecules during initial steps of network formation and in complete networks. The presence of anti-NC1 Fab fragments in type IV collagen solutions inhibited lateral association and network formation in rotary shadow images. During the process of self-association type IV collagen develops turbidity; addition of isolated domain NC1 inhibited the development of turbidity in a concentration-dependent manner. These findings indicate that domain NC1 of type IV collagen plays an important role in the process of self-association and suggest that alterations in the structure of NC1 may be partially responsible for impaired functions of basement membranes in certain pathological conditions.

Materials and Methods

Preparation of Type IV Collagen

The source of type IV collagen was the Engelbreth-Holm-Swarm (EHS) tumor (16) grown subcutaneously in mice which were rendered lathyritic by the addition of 0.25% β-aminopropionitrile fumarate (Sigma Chemical Co., St. Louis, MO) in their drinking water. Type IV collagen was isolated by a modification of previously described methods (4, 12). Briefly, the tissue was extracted first with 3.4 M NaCl in 0.05 M Tris-HCl, pH 7.4, containing 1 mM EDTA, 50 μg/ml phenylmethylsulfonyl fluoride (PMSF), and 50 μg/ml chloromercuribenzoate, then with 2 M guanidine-HCl (Sigma Chemical Co.) in 0.05 M Tris, pH 7.4, containing the same protease inhibitors and, finally, with 2 M guanidine in 0.05 M Tris-HCl, pH 7.4, containing 2 mM diithiothreitol (DTT) and protease inhibitors. The protein was precipitated with 10% NaCl and the pellet was dissolved in 4 M urea (Schwarz/Mann Div., Cleveland, OH), 0.05 M Tris-HCl, pH 8.6, with protease inhibitors; the solution was then dialyzed against the same buffer overnight at 4°C, and it was incubated with DEAE-cellulose for 12 h at 4°C. The unbound fraction was dialyzed against 2 M guanidine in 0.05 M Tris-HCl, pH 7.4, containing 2 mM DTT and protease inhibitors. Isolated type IV collagen was centrifuged at 60,000 rpm for 90 min to clear aggregates >50S and it was stored on ice. It was found to contain <0.1% type I collagen by the technique of rotary shadowing, a finding which was corroborated previously by ELISA inhibition assays (26). The concentration of type IV collagen was determined by amino acid analysis.

Preparation of Domain NC1 of Type IV Collagen

The main noncollagenous domain (NC1) was isolated from type IV collagen by the use of bacterial collagenase (CLSPA, CooperBiomedical Inc., Malvern, PA) according to published procedures (22, 24). Type IV collagen was centrifuged at 60,000 rpm for 20 min at 3°C to remove large aggregates, it was dialyzed exhaustively against 0.05 M Tris-HCl, pH 7.4, containing...
0.2 M NaCl and 2 mM CaCl₂, and it was incubated with collagenase (substrate/enzyme ratio, 50:1) at 37°C for 24 h. The solution was then centrifuged at 13,000 rpm for 30 min to remove aggregated material, and the supernatant was concentrated with Aquacide IIA (Calbiochem-Behring Corp., La Jolla, CA), dialyzed against 0.2 M NH₄HCO₃, pH 8.5, and chromatographed on a Sephacryl S-300 (2.5 x 95 cm) column equilibrated in the same buffer. Dimeric NC1 eluted as a distinct peak with a Kᵥ 0.430, and its purity was tested by electrophoresis on a 10% SDS polyacrylamide gel (13), and by rotary shadowing, as published previously (22, 24).

Preparation of Anti-NC1 Antibodies and Their FAB Fragments

Isolated NC1 in complete Freund's adjuvant was injected subcutaneously in female New Zealand rabbits. The animals received a total of three injections (100 μg per injection) at 2-wk intervals. 2 wk after the last injection, the animals were bled and the antiserum was tested by ELISA and was found to be equally reactive with domain NC1 and intact type IV collagen. With the same type of assay, this antiserum reacted minimally with pepsin-treated type IV collagen which lacks domain NC1. Monovalent Fab fragments were prepared in order to avoid the formation of large aggregates of type IV collagen by intact, divalent anti-NC1 IgG. Fab fragments were produced by papain digestion of the IgG fraction of the antiserum (8). The IgG fraction was precipitated from the antiserum by 50% ammonium sulfate, the pellet was dissolved in 0.1 M sodium phosphate buffer, pH 7.0, and was dialyzed extensively against the same buffer. Subsequently, cysteine (at a final concentration of 10 mM), EDTA (at a final concentration of 2 mM), and papain (Sigma Chemical Co.) at a substrate/enzyme ratio of 50:1 were added to the solution. The mixture was incubated at 37°C for 16 h with stirring and the digestion was terminated by adding chloromercurobenzoate at a final concentration of 1 mM. The solution was dialyzed against distilled H₂O containing 1 mM chloromercurobenzoate and it was centrifuged at 12,000 g for 30 min. The supernatant was passed over a Sepharose 4B-NC1 affinity column, the column was washed with PBS, and the bound fraction was

Figure 1. Binding of isolated domain NC1 to type IV collagen. Type IV collagen (350 μg/ml) was incubated in the presence of isolated NC1 (10 μg/ml) in PBS, at 35°C for 60 min, and a sample of the mixture was used for rotary shadowing. On images from metal replicas, isolated domain NC1 binds to several sites along the length of type IV collagen, at 100 nm (A, B, and H), 200 nm (D, F, and G), 300 nm (E) distal to the carboxy-terminal NC1 domain of intact collagen molecules, at the amino-terminal region (B and F), or at various other sites (C and F). Often more than one NC1 globule is found per collagen molecule (B, C, F, and H). Closed triangles point to domain NC1 of intact type IV collagen molecules; arrows point to isolated NC1 bound to collagen. Bar, 100 nm.
eluted with 1 M acetic acid containing 0.15 M NaCl. The eluted peak which contained the anti-NC1 Fab fragments was tested by electrophoresis on a 12% SDS polyacrylamide gel (13). Anti-NC1 Fab fragments were dialyzed against PBS and stored at -20°C until further use.

**Production of Anti-BSA Fab Fragments**

Rabbit anti-BSA serum was generously provided by H. Furthmayr (Yale University) and anti-BSA Fab fragments were produced by papain digestion of the IgG fraction of the antiserum, as mentioned above. After the end of the digestion, the mixture was passed over a Sepharose 4B-BSA affinity column, and the bound fraction containing anti-BSA Fab fragments was eluted, dialyzed against PBS, and stored as mentioned above.

**Turbidity Measurements**

Type IV collagen and isolated noncollagenous NC1 domain were dialyzed against PBS overnight at 4°C and were centrifuged at 40,000 rpm for 20 min at 3°C to remove aggregated material. Mixtures of the above components were incubated for various time intervals in pre-warmed quartz cuvettes at 35°C (maintained with a water jacket) and the change in absorbance at 360 nm was followed over time with a Beckman spectrophotometer.

**Rotary Shadowing**

Type IV collagen and domain NC1 were dialyzed in PBS overnight at 4°C. Each protein was centrifuged at 40,000 rpm for 20 min to remove large aggregates. Solutions of type IV collagen alone, or mixtures containing type IV collagen and isolated domain NC1 or anti-NC1 Fab fragments or anti-BSA Fab fragments were incubated at 35°C for 1 h; then they were added to a solution containing 50% glycerol in 0.15 M NH4HCO3, pH 7.8, at a final concentration of 5-10 μg/ml, and the samples were sprayed on freshly cleaved mica sheets. The mica sheets were then placed on a rotary stage and shadowed under vacuum with a mixture of 95% platinum-5% carbon in a Balzers apparatus (Balzers Union, Hudson, NH) (4, 19). The replicas were floated in distilled H2O, placed on 300-mesh uncoated copper grids, and examined with a Phillips 300 transmission electron microscope operating at 60 kV.

To evaluate in a quantitative way the binding of isolated domain NC1 to type IV collagen, association events were photographed at a magnification of 26,930 and prints were made at a final magnification of 403, 950. Measurements were then performed on the prints using a Zeiss videoplan computer with a digitizer measuring tablet attachment and a (Y)l videoplan program. We measured the distance from domain NC1 of the type IV collagen molecule to the site of binding of the isolated NC1 globule and expressed this value as a ratio, by dividing it by the total length of type IV collagen.

The following criteria were applied in order to include association events in the measurements: (a) the whole type IV collagen molecule should be clearly traced from its carboxyl to its amino end; (b) its length should be 410 ± 40 nm; and (c) the binding of added NC1 should be clearly visualized.

The results from these measurements were used to construct a histogram in which the absolute frequency of binding events was plotted against the length of the type IV collagen molecule, divided into 20 equal segments (>20 nm per segment). When intermediate or complete forms of assembly of type IV collagen were examined, the distance between NC1 globules was measured directly, and histograms were made in which the absolute frequency of binding events was plotted against length, divided into 20-nm segments.

**Results**

Isolated, dimeric domain NC1 was visualized by rotary shadowing as a globule 10-12 nm in diameter, as described previously (22, 24). Intact type IV collagen, EHS-derived, was 80-90% dimeric, and appeared as two flexible rods connected by a single globule corresponding to domain NC1. The average length of the monomeric form was 410 nm.

When isolated domain NC1 (final concentration, 10 μg/ml) was co-incubated with type IV collagen (final concentration, 350 μg/ml) at 35°C for 60 min in PBS, it was found by rotary shadowing to bind along the length of the collagen molecule (Fig. 1). Often more than one NC1 globule was observed to be bound (Fig. 1, B, C, F, and H). When a histogram of the binding events was constructed, as mentioned in Materials and Methods, four peaks were observed, approximately one every 100 nm, at intervals of about one fourth the length of the type IV collagen molecule (Fig. 2). When examined by statistical analysis, with the goodness of fit of the Poisson distribution, the binding was found to be nonrandom at a statistically significant level (P < 0.001) and to have an ordered distribution (27). Bound NC1 was usually easily discernible, because the NC1 domain of native intact collagen was located in the middle of well-resolved dimers and the rodlike portion of each monomer emanated from the center of this globular domain. In contrast, added NC1 was bound to the rodlike portion of type IV collagen, usually via the edge of the globule (Fig. 1). Because isolated domain NC1 was generated by enzymatic digestion of type IV collagen, the possibility existed that sticky ends were created and that the observed binding, although repeated at regular intervals, could be due to nonspecific sticking. To eliminate this possibility, we determined whether this binding occurred in intact type IV collagen. Type IV collagen at 300 μg/ml was incubated in PBS at 35°C for 1 h, and was then examined by rotary shadowing. Under these conditions, it self-associated end-to-end and laterally as well, to form intermediate complexes and an irregular polygonal network (26). In this instance, we observed that the NC1 globules of intact type IV collagen dimers which were not treated enzymatically also appeared to bind to the rodlike part of collagen. This binding was apparent both in intermediate forms of self-association and in completely formed networks. When the distance between NC1 globules was measured in intermediate forms of assembly (Fig. 3), a prominent peak was observed at 100 nm, followed by another peak at 200 nm and a minor peak at 300 nm. No peak was observed at 400 nm (Fig. 4). The reduction and disappearance of peaks at 300 and 400 nm, respectively, can be explained by the difficulty of tracing the total length of individual type IV collagen.
molecules all the way from the carboxy- to the aminoterminal end when assembly occurs (Fig. 3).

In completely formed networks, it is impossible to trace the whole length of individual type IV collagen molecules, but the distance between neighboring NCI globules can be traced easily (see Fig. 6 B). When this distance was measured, a single peak was observed at 100 nm (Fig. 5).

It appears therefore that domain NCI binds to four sites along the length of type IV collagen molecules with an apparent periodicity of ~100 nm.

Furthermore, we observed that in intermediate forms of assembly, binding events between domain NCI and the rod-like part of collagen were accompanied by lateral association between adjacent molecules. Lateral assembly was evident due to the increased thickness of associated collagen molecules (Fig. 3, A–C). The question then arises, whether lateral association is a consequence of the binding of NCI to collagen or whether it is initiated by other binding events.

To answer this question, we used anti–NCI Fab fragments to block this domain and examined the effects on the assembly of type IV collagen by rotary shadowing. Under control conditions, type IV collagen (350 μg/ml) was incubated at 35°C in PBS for 1 h in the presence of ~15 M excess anti–BSA Fab fragments (60 μg/ml) and was examined by rotary shadowing. Intermediate forms of assembly (Fig. 6 A) and areas containing the polygonal network (Fig. 6 B) were present in 80% of the fields examined. Lateral associations were evident in both stages of assembly. The remaining 20% contained randomly oriented collagen molecules. However, when type IV collagen at 350 μg/ml was incubated under the same conditions in the presence of 15 M excess anti–NCI Fab fragments (60 μg/ml), the NCI globule was not observed to bind along the length of the collagen molecule. In this instance, no lateral association or network was observed (Fig. 6, C–D). Instead, randomly oriented molecules (Fig. 6 C) and various aggregates (Fig. 6 D) were seen in 90% of the fields examined. Only 10% of the fields contained initial stages of assembly. Thus, it appears that when the binding
Figure 6. Effect of anti-NC1 Fab fragments on type IV collagen lateral association and network formation. Rotary shadow images obtained from type IV collagen (350 μg/ml) incubated at 35°C for 60 min in PBS, in the presence of anti-BSA Fab (A and B), or anti-NC1 Fab (C and D) fragments. The concentration of Fab fragments in both cases was 60 μg/ml. Type IV collagen incubated in the presence of anti-BSA Fab fragments was associated laterally (A) and formed an extensive polygonal network (B). The presence of anti-NC1 Fab fragments in incubated type IV collagen solutions inhibited both lateral association (C) and network formation (D) although aggregates of various forms were observed (D). Bar, 200 nm.

Figure 7. Turbidity of type IV collagen incubated under control conditions and in the presence of isolated domain NC1. Type IV collagen (200 μg/ml) was incubated at 35°C for 30 min, in PBS, either alone (solid circles) or in the presence of isolated domain NC1 at 50 μg/ml (open triangles), and at 100 μg/ml (open circles). The presence of domain NC1 considerably suppressed the development of turbidity, in a concentration-dependent manner.

Discussion

In this report we describe a novel type of association between...
the main noncollagenous NC1 domain of type IV collagen and the rodlike part of collagen. This association appears to be important for lateral assembly of collagen and formation of the irregular polygonal network which has been described elsewhere (26). To our knowledge, this is the first study which reveals a specific function of domain NC1 other than the binding to itself which results in the formation of dimeric collagen molecules (22, 24).

We present evidence that domain NC1 binds at intervals of 100 nm along the length of type IV collagen. This 100-nm periodicity was observed in the binding of both isolated and native domain NC1, during the process of self-assembly. In a previous study, the distance between adjacent NC1 globules of the complete network was measured and two peaks were observed, one at 170 nm and a second, smaller peak at 300 nm (26). We have no obvious explanation for this discrepancy although in the latter instance, the conditions used for incubation of type IV collagen were different. The temperature used during the incubation was lower (28°C instead of 35°C) and the buffer (PBS) also contained 100 μM DTT and 100 μM EDTA. Furthermore, the buffer used for the technique of rotary shadowing was ammonium acetate instead of ammonium bicarbonate and most of the experiments were done in the absence of glycerol. The possibility exists that one or more of these different experimental conditions could account for the differences we observe in the periodicity of the binding of domain NC1 to type IV collagen. Nevertheless, even under these conditions, a substantial number of measurements were observed at ~100, 200 and 300 nm (Fig. 5 of reference 26). The nature of the binding site which apparently is repeated every 100 nm is unknown. Type IV collagen differs from most interstitial collagens because it contains interruptions of the triplet Gly-X-Y sequence which reveals a specific function of domain NC1 other than the binding of domain NC1, during the process of self-assembly. In a previous study, the distance between adjacent NC1 globules of the complete network was measured and two peaks were observed, one at 170 nm and a second, smaller peak at 300 nm (26). We have no obvious explanation for this discrepancy although in the latter instance, the conditions used for incubation of type IV collagen were different. The temperature used during the incubation was lower (28°C instead of 35°C) and the buffer (PBS) also contained 100 μM DTT and 100 μM EDTA. Furthermore, the buffer used for the technique of rotary shadowing was ammonium acetate instead of ammonium bicarbonate and most of the experiments were done in the absence of glycerol. The possibility exists that one or more of these different experimental conditions could account for the differences we observe in the periodicity of the binding of domain NC1 to type IV collagen. Nevertheless, even under these conditions, a substantial number of measurements were observed at ~100, 200 and 300 nm (Fig. 5 of reference 26). The nature of the binding site which apparently is repeated every 100 nm is unknown. Type IV collagen differs from most interstitial collagens because it contains interruptions of the triplet Gly-X-Y sequence which has been observed that pepsin-treated type IV collagen, which is cleaved to produce a smaller structure deprived of domain NC1, cannot self-assemble laterally. In fact, it can only associate via the amino-terminal domain to form small intermediate forms, up to tetramers, which have a spider-like appearance (22, 26). These data taken together strongly suggest that the binding of domain NC1 which is repeated every 100 nm along the length of type IV collagen is required for lateral association and network formation.

The turbidity experiments further indicate a possible role for domain NC1 in type IV collagen self-assembly. Type IV collagen has been described as raising turbidity readily when incubated at elevated temperatures in physiologic buffers (26). Co-incubation of type IV collagen with isolated domain NC1 suppressed the development of turbidity depending upon the concentration of added NC1 (Fig. 7). Interestingly, this competition by added NC1 for binding and subsequent formation of turbid aggregates indicates that only one site for binding to the rodlike part of collagen exists per NC1 globule. If two or more binding sites were available, this would facilitate the association of neighboring type IV collagen molecules and the formation of aggregates and would thus result in increased turbidity compared to that of control collagen. The suggestion that only one site exists per NC1 globule is reinforced by morphological findings which indicate that the maximal observed thickness of laterally associated type IV collagen molecules corresponds to three strands (26). Indeed, if four binding sites exist in the rodlike part of each collagen molecule for domain NC1 (Fig. 2) and if all are occupied by only one NC1 globule each, the result would be a staggered arrangement with three strands in its thicker portions.

It is exciting to begin to understand the reason why type IV collagen is not enzymatically cleaved like other interstitial collagens. Maintenance of procollagen peptides could serve several important functions, including the assembly of type IV collagen in a polygonal network. This network would provide a scaffolding and could contribute to the sieving properties of basement membranes.

It would be important to determine the binding site in the sequence of domain NC1 which is responsible for the initiation of lateral assembly of type IV collagen. In fact, the whole amino acid sequence of the NC1 domain of the α1 chain has been deciphered from cDNA clones for the human (17) and murine (15) NC1. There are several striking observations. First, the two sequences, 229 amino acids long each, are nearly identical, indicating a possible conservation of this domain. Second, the sequence shows pronounced homology between the first and second parts of the structure (15, 17). It is exciting to determine if conservation of the amino acid sequence of this domain exists between various species.

The whole amino acid sequence of domain NC1 contains five lysines. These residues are important because they are commonly involved in cross-linking and they are known to become chemically modified in diabetes. Under diabetic conditions, when the levels of glucose in the plasma are abnormally high, glucose binds non-enzymatically to the ε-amino group of lysine (14). Non-enzymatic glucosylation occurs in proteins with slow turnover rates such as albumin (6), hemoglobin (3), crystallins (20), fibronectin (21), etc., in diabetic conditions.

In preliminary experiments, non-enzymatically glucosylated domain NC1 was tested for binding to type IV collagen by turbidimetry and was found to cause minimal or no decrease of turbidity. In contrast, control NC1 maintained the ability to suppress turbidity substantially (23) as mentioned previously in this report. This effect could be due to modified lysesins. This would indicate that at least one lysine residue could participate in the binding site or be very close to it. In diabetes, the glomerular basement membrane becomes leaky to plasma proteins, an indication that the sieve formed...
by basement membrane components became defective. It is tempting to speculate that in vivo non-enzymatic glucosylation of domain NC1 of collagen is related to aberrant leakage of proteins, since the binding needed for lateral assembly and network formation is defective. Furthermore, domain NC1 appears to be the main antigen in patients with Goodpasture syndrome, in which auto-antibodies to this domain of collagen are most commonly observed (25). As a result, immune responses are directed specifically against domain NC1 of the glomerular basement membrane, and cause proteinuria. It remains to be determined how these antibodies which are directed specifically against domain NC1 affect the structure of basement membranes in such a way as to cause leakage of plasma proteins in the urine.

In diabetes, Goodpasture syndrome and other nephrotic syndromes (i.e., Alport's syndrome) proteinuria could be related in part to abnormal functions of domain NC1. It is possible that chemical or other modifications of this domain and antibodies directed against it could affect not only network assembly but also binding to other components of basement membranes such as nidogen (entactin), which has been reported to bind to domain NC1 and to other basement membrane proteins as well (7).

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