The Function of the NC1 Domains in Type IV Collagen*

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At its C terminus, the collagen IV molecule bears a globular NC1 domain, to which two functions have been assigned. In the macromolecular network of collagen IV, two molecules are connected via their NC1 domains, which form a hexameric complex, stabilized by intermolecular disulfide bonds. In addition, the NC1 domains are thought to be responsible for chain selection and assembly. In order to understand the role of the NC1 domains during these steps, hexameric complexes were isolated and further investigated. SDS-polyacrylamide gel electrophoresis and Western blot revealed disulfide-linked α1(IV)NC1 and α2(IV)NC1 homodimers but no heterodimers. The hexamers were dissociated at low pH, separated into monomers and dimers, and submitted to reconstitution experiments. Only α1(IV)NC1 dimers were able to reconstitute a hexameric complex. α1(IV)-NC1 and α2(IV)NC1 monomers as well as the α2(IV)NC1 dimers showed only a low tendency to form complexes. It is assumed that during formation of the collagen IV network, lateral aggregation of the molecules via the triple helical domains brings the C termini of two molecules into close vicinity and that subsequently the weak interactions observed between the NC1 subdomains provide the correct alignment for a disulfide exchange. It is, however, questionable whether the low affinity between the NC1 subdomains alone is sufficient for chain assembly and alignment of the α(IV) chains before molecule formation.

The main collagenous constituent of basement membranes is type IV collagen (1, 2). Its molecules bear a globular NC1 domain at their C termini, and their triple helical region is characterized by frequent interruptions with non-triple helical segments, which provide them with a high flexibility (3). The macromolecular structure of collagen IV is a network, in which the molecules are connected via like ends (4). At the N terminus, the triple helical end regions of four molecules overlap by 25 nm, and this arrangement is covalently connected by intermolecular disulfide bonds and lysine-derived aldime bonds, characteristic for collagens (5). At the C terminus, the NC1 domains of two molecules become aggregated to form a hexameric complex, which is also stabilized by intermolecular disulfide bonds (6, 7). In addition, the triple helical domains of the molecules are aligned laterally, and, in the electron microscope, superhelices between two or three molecules have been observed (8).

Presently, six different α(IV) chains, α1(IV) to α6(IV), are known (9, 10). Their genes are arranged head-to-head in three pairs to form the transcription units COL4A1-A2, -A3-A4, and -A5-A6, which apparently give rise to three isoforms in the protein level (11-14). The isoform with the chain composition [α1(IV)]2α2(IV) is ubiquitous to all basement membranes, whereas the [α3(IV)]2α4(IV) isoform is mainly restricted to the glomerular basement membrane and has also been found in less abundance in some other basement membranes (10). Whether the α5(IV) and α6(IV) chains also form a common molecule is unclear. Both chains are present in several tissues. However, α5(IV) is mainly expressed in kidney, whereas the highest expression of α6(IV) has been observed in esophagus and lung (14).

Comparison of the amino acid sequences of α(IV) chains of different species, from nematodes to mammals, including the recently characterized isoforms in man, revealed a particularly well conserved primary structure of the NC1 domain, indicating an important general function for this domain (2). Two functions have been associated with the NC1 domains. Their crucial role in linking two molecules via their C-terminal ends in the macromolecular network is obvious. In addition, the NC1 domains are thought, by analogy with the C-terminal propeptides of the fiber-forming collagens, to be responsible for chain selection and assembly before an intact triple helical molecule can be generated.

In order to understand the role of the NC1 domains during these two steps, hexameric NC1 complexes were isolated from human placenta after collagenase treatment and, after dissociation at low pH, were separated into NC1 monomers and cross-linked dimers (6, 7). The monomeric and dimeric NC1 domains were submitted to reconstitution experiments at physiological pH and ionic strength (6).

MATERIALS AND METHODS

Isolation of Hexameric, Dimeric, and Monomeric NC1 Domains—The hexameric NC1 domain was isolated from human placenta similarly to the two-step collagenase procedure described previously (6). The first collagenase treatment of formic acid-washed tissue (1000 units of type IV collagenase per kg of wet tissue) was carried out in 30 mM Tris-HCl, pH 7.6, containing 10 mM CaCl2 at 20°C for 24 h. The second collagenase treatment (2 units/mg of protein) was performed at 37°C for 16 h. The digest was stopped with EDTA, dialyzed against 50 mM ammonium carbonate, and lyophilized. For final purification, the protein was dissolved in 1 M CaCl2, 0.50 mM Tris-HCl, pH 7.4, and passed over an agarose A-1 column.

To dissociate the hexameric NC1 complex into dimeric and monomeric NC1, the NC1-containing fraction from the agarose A-1 eluate was dialyzed against 0.1 M ammonium formate, pH 3, and subsequently applied to a column (1 × 30 cm) of Superose 12 and separated by elution with the same buffer. The fractions containing dimers and monomers were stored at −20°C.

SDS-PAGE and chromatographic separation did not give evidence for the presence of α1(IV), α4(IV), α5(IV), and α6(IV)NC1 domains in the hexameric complex isolated from placenta. Also, Western blot anal-

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1 The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.
gel electrophoresis under nonreducing conditions (Fig. 1). Un-}

collagenase procedure, was submitted to SDS-polyacrylamide

NC1 complex, isolated from human placenta with the two-step

heights of peaks in the sedimentation profiles. For equilibrium experi-

ments were performed at a rotor speed of 56,000 rpm. Proportions

20°C in 12-mm Epon double-sector cells in an AnD rotor. Velocity

Optima XL-A analytical ultracentrifuge (Beckman Instruments) at

after electrophoretic separation and transfer to nitrocellulose mem-

with incomplete Freund’s adjuvant. Immunoblotting was performed

acrylamide containing 0.8% bisacrylamide.

performed as described previously (16).

hexamers.

determined in short column runs according to the method of Van Holde

ualmolarmasses, but in other cases weight averagemolarmasses were

rotor center. Rotor speeds between 9,000 rpm and 22,000 rpm were

was measured at 280 nm, and the molarmass was
determined from ln

A versus r

is the distance from the

r

the presence of intramolecular disulfide bonds connecting the

chains within a collagen IV monomer via the α1(IV)NC1 domains.

their formation after end-to-end aggregation of
dimers and monomers (Fig. 3).

Reconstitution Experiments—These experiments were car-

out to test the affinity of monomeric and dimeric NC1 domains

for each other at neutral pH and their ability to form

ordered hexameric and trimeric complexes. Native hexamers,

monomers in formate buffer, of pH 3, and their ability to form

formed complexes and the presence of nonassociated mono-

were dialyzed for 16 h against a phosphate buffer, pH 7.4, and

from the Superose 12 chromatography were dialyzed for 16 h at 4°C

against 0.1 M NaCl, 0.05 M sodium phosphate buffer, pH 7.4, and

filtered through a membrane with 0.45-nm pore diameter (polyvinyli-

dene difluoride, Millipore). 200 μl of each fraction, with a protein

concentration of 150–200 mg/ml, were separated on a column (1 × 30

cm) of Superose 6, by elution with the same phosphate buffer, pH 7.4,

using a flow rate of 0.3 ml/min. The individual fractions containing

hexameric, dimeric, and monomeric NC1 domains were analyzed by

SDS-PAGE.

ElectronmicroscopyoftheNC1domainsafterrotaryshadowingwas

electron microscopy and Electron Microscopy—Sedimen-
tation velocity and equilibrium experiments were performed with an

Optima XL-A analytical ultracentrifuge (Beckman Instruments) at

20°C in 12-mm Epon double-sector cells in an AnD rotor. Velocity

experiments were performed at a rotor speed of 56,000 rpm. Proportions

of differently sedimenting materials were estimated from the relative

heights of peaks in the sedimentation profiles. For equilibrium experi-

ments, absorbance A was measured at 280 nm, and the molarmass was
determined from InA versus r^2 plots where r is the distance from the

rotor center. Rotor speeds between 9,000 rpm and 22,000 rpm were

selected for best resolution. In cases where there was only little con-
tamination of the major species, it was possible to estimate the individ-

ual molar masses, but in other cases weight average molar masses were
determined in short column runs according to the method of Van Holde

and Baldwin (15). An average partial specific volume of υ = 0.73 ml/g

was used, which was based on the amino acid composition of NC1

hexamers.

Electron microscopy of the NC1 domains after rotary shadowing was

performed as described previously (16).

RESULTS

Separation of Monomeric and Dimeric NC1—The hexameric

NC1 complex, isolated from human placenta with the two-step

collagenase procedure, was submitted to SDS-polyacrylamide
gel electrophoresis under nonreducing conditions (Fig. 1). Under

the special conditions used, 200 V for 1 h, the NC1 dimers

were resolved into four bands. Western blot analysis, using

α1(IV)NC1- and α2(IV)NC1-specific antisera, revealed that the
two upper bands consist of α1(IV)NC1 dimers, whereas the two

bands with higher electrophoretic mobility represent α2(IV)-

NC1 dimers (Fig. 1). Hybrid dimers between α1(IV)NC1 and

α2(IV)NC1 were not observed.

The hexameric complex was dissociated at pH 3, and the

resulting mixture of monomeric and dimeric NC1 domains was

chromatographed on a Superose 12 column. The two peaks,

containing dimers and monomers, respectively, were divided

into smaller fractions and submitted to SDS-PAGE (Fig. 2). It

is striking that for both the dimers and the monomers,

α1(IV)NC1 eluted earlier than the α2(IV)NC1 domains. There

are fractions which contain only α1(IV)NC1 dimers and others

with mainly α2(IV)NC1 dimers. Densitometric analysis of gels

from seven different preparations revealed that 79% of the

α1(IV)NC1 and 54% of the α2(IV)NC1 domains were cross-

linked to form dimers, and that the two bands observed for each

type of dimer were of equal intensity. There is no evidence for

the presence of intramolecular disulfide bonds connecting the

α1(IV) chains within a collagen IV monomer via the α1(IV)NC1

domains. Their formation after end-to-end aggregation of
dimers is unlikely in view of the results of Siebold et al. (7) but

cannot be excluded completely.

Reconstitution Experiments—These experiments were car-

out to test the affinity of monomeric and dimeric NC1 domains

for each other at neutral pH and their ability to form

ordered hexameric and trimeric complexes. Native hexamers,

α1(IV)NC1 dimers, α2(IV)NC1 dimers, and a mixture of α1-

(IV)NC1 and α2(IV)NC1 monomers in formate buffer, of pH 3,

were dialyzed for 16 h against a phosphate buffer, pH 7.4, and

finally chromatographed on a Superose 6 column to analyze for

reformed complexes and the presence of nonassociated mono-

mers and dimers (Fig. 3).

The hexamers reconstituted from the unseparated mixture of

monomers and dimers showed the same composition as in

the native complex (Fig. 3A). From the isolated dimers and

monomers, only the α1(IV)NC1 dimer was able to reform a
hexameric complex (Fig. 3B). The hexameric fraction observed after reconstitution of α2(IV)NC1 dimers is due to a small amount of α1(IV)NC1 dimers still present in the preparation (Fig. 3C). The α1(IV)NC1 dimers are, however, also able to incorporate other NC1 constituents that are present during the reconstitution procedure. This is shown in Fig. 3D, where hexamers reconstituted from α1(IV)NC1 dimers in the presence of α1(IV)NC1 and α2(IV)NC1 contained monomers, and it is notable that α2(IV)NC1 was preferentially incorporated. A mixture of α1(IV)NC1 and α2(IV)NC1 monomers showed no tendency to aggregate (Fig. 3E).

Under the chromatographic conditions used, separation of tetrameric, trimeric, and dimeric NC1 complexes was only partially achieved, so that any such complexes which may have formed would have been overlooked. The preparations were therefore further investigated in the analytical ultracentrifuge. The data obtained confirmed on the whole the results described in Fig. 3. In Table I, the values of the dominating materials are underlined but for dimers and mixtures of monomers, smaller and/or larger species were also visible. Their fractions were estimated from the sedimentation profiles. The sedimentation coefficients and molar masses are in good agreement with previously determined values (6).

Monomeric NC1 did not form hexamers, although a slight tendency to form aggregates could be seen clearly. For the α1 dimers, 80% recovery of intact hexamers was observed. The α2(IV) dimer fraction showed 30% hexamers. This is apparently due to contamination with α1(IV) dimers (see Fig. 3C).

The hexameric complexes reconstituted from a native NC1 mixture or from isolated α1(IV)NC1 dimers were investigated after rotary shadowing in the electron microscope (Fig. 4). Comparison of the reconstituted material with the native hexameric complex did not reveal obvious differences. Hexamers, however, can easily be distinguished from monomers and dimers.

### DISCUSSION

The hexameric NC1 complex represents the connecting element between the C termini of two collagen molecules incorporated in the macromolecular network of collagen IV (4). The complex is stabilized by intermolecular disulfide bonds between two like NC1 domains (7). Only α1(IV)NC1 and α2(IV)NC1 homodimers but no α1-α2 heterodimers have been found. Striking is the occurrence of two species for the α1(IV)NC1 as well as for the α2(IV)NC1 dimers, which may be explained by two sets of intermolecular disulfide bonds. These different sets may possibly originate from the fact that each NC1 domain consists of a tandem repeat of two homologous domains, and disulfide exchange could be between either the first or second of these repeats (7).

From earlier investigations (6), it is known that the compact and stable hexameric complex dissociates at low pH into its monomeric and dimeric constituents and that readjustment to neutral pH leads to reformation of the complex, whereby all constituents, dimers and monomers, are again incorporated. It was therefore surprising that the aggregation behavior of the separated monomers and dimers was so different. At neutral pH, only the α1(IV)NC1 dimers were able to reform a hexameric complex, which could not be differentiated from the native complex in the electron microscope. The α1(IV)NC1 and α2(IV)NC1 monomers, as well as the α2(IV)NC1 dimers, showed only a slight tendency to aggregate. Weak noncovalent interactions, as revealed by ultracentrifugation analysis, may however be of decisive importance for the disulfide exchange reaction which leads to stable hexameric complexes connecting collagen IV molecules at their C termini (4–7)....
because dissociation of the final complex is rendered impossible by the disulfide bridges. It is obvious that the disulfide exchange between two \( \alpha 1(IV) \) NC1 domains is accompanied by a drastic alteration of their aggregation behavior. In the presence of a mixture of \( \alpha 1(IV) \)NC1 and \( \alpha 2(IV) \)NC1 monomers, mainly the latter were incorporated into the hexamers formed by \( \alpha 1(IV) \)NC1 dimers, indicating a tendency to restore the native ratio of \( \alpha 1(IV) \) to \( \alpha 2(IV) \) chains.

The observation that monomeric NC1 domains have no strong affinity for one another correlates with the behavior of monomeric collagen IV molecules secreted into the medium of collagen IV synthesizing cells (18). The molecules in solution tend to form dimers and tetramers via their N termini. Dimers connected by the C termini have not been observed. Such dimers, however, can be extracted from the cell layer, where a minority of collagen IV molecules have already been laid down to form a network. We assume that lateral aggregation of the molecules with their triple helical domains may help to align the NC1 domains, so that disulfide exchange and subsequent formation of stable hexamers can occur. A putative model is given in Fig. 5. In the first step, two molecules aggregate at their C-terminal regions in an antiparallel manner. The NC1 domains have not yet found a partner. Subsequently, a third molecule attaches, bringing two C-terminal ends into close vicinity, so that the weak interaction between the NC1 domains is sufficient to form complexes in the correct arrangement for disulfide formation (2). Another possibility could be an end-to-end alignment as an intracellular event, which may be facilitated by chaperones. However, this appears to be very unlikely. In collagen IV synthesizing cells in culture, all intracellular modifications including chain assembly, occur in an ordered manner. There is no reason why just the end-to-end aggregation should be impaired, so that in culture monomeric molecules are secreted instead of dimers.

It was demonstrated previously for dimers of collagen IV connected by their NC1 domains (19) and for procollagen IV (20) that refolding of the collagen triple helix after thermal denaturation started at the C terminus. For collagen I it was established that the C-terminal propeptides of two \( \alpha 1(IV) \) and one \( \alpha 2(IV) \) chains form a heterotrimeric complex, which has to be stabilized by interchain disulfide bonds before folding of a triple helix can occur (21–24). It is questionable whether the NC1 domains in type IV collagen have a similar recognition function because of their low affinity to each other. Also, no interchain disulfide bonds have been observed by which three NC1 domains, which had formed a complex, would be stabilized. One could speculate that intramolecular disulfide bridges between NC1 domains are not formed since they would interfere with the formation of the hexameric NC1 complex necessary for the organization of the macromolecular network of collagen IV. The mechanism responsible for selection of the \( \alpha 1(IV) \) and \( \alpha 2(IV) \) chains in a molecule is also unknown, and further work is needed to elucidate the specificity of chain recognition.
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