Type IX Collagen NC1 Domain Peptides Can Trimerize in Vitro without Forming a Triple Helix*

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Synthetic peptides of the three chains of type IX collagen consisting of the carboxyl-terminal end of the COL1 domain and the complete NC1 domain were characterized by circular dichroism spectroscopy and analyzed for their ability to assemble into trimers. In vitro association and oxidation result in disulfide-linked oligomers as shown by molecular sieve chromatography and SDS-polyacrylamide electrophoresis. Whereas the individual peptides show a tendency to self-associate, when an equimolar amount of the three peptides was oxidized, a heterotrimer of the three chains was observed. This heterotrimer is recognized by a monoclonal antibody against the disulfide-linked NC1 domain of chicken type IX collagen, indicating the correct formation of the disulfide bonds. Circular dichroism measurements show that under the association conditions used, a triple helix does not form between the chains. These results indicate that these peptides contain all the necessary information for chain selection and assembly.

Vertebrate connective tissues rely on the utilization of an assortment of at least 19 types of collagen molecules (over 30 distinct gene products) to achieve their specific architectures and biomechanical properties. The biosynthesis of the collagen triple helix requires the assembly of the appropriate combination of three chains from the extensive selection of collagen polypeptides typically produced by connective tissue cells. From studies of the homologous group of "interstitial" collagens (types I, II, III, V, and XI), it was determined that this function resides in the carboxyl-terminal propeptides (1-3). For example, chondrocytes in fetal cartilage synthesize three interstitial collagenous polypeptides, α1(II), α1(XI), and α2(XI). Of the 10 possible trimers, the carboxyl-terminal propeptides direct the formation of only two predominant species, which are observed in this tissue, type II collagen, α1(II)3, and type XI collagen, [α1(IX)2α2(IX)]=α1(IX) (4). Association of the chains via their carboxyl-terminal propeptides prior to triple helix formation satisfies a further requirement, the alignment or registration of the three collagenous domains in the appropriate one residue stagger.

It is not known whether the carboxyl-terminal propeptides direct the assembly of other collagens, some of which have substantially smaller carboxyl-terminal propeptide domains than the interstitial collagens. Types IX, XII, and XIV comprise the fibril-associated collagens with interrupted triple helices, which are a subgroup of the collagen family (5). Type IX collagen, the paradigm of this subgroup, is assembled from three genetically distinct chains, each of which contains three collagenous domains (COL1-COL3) and four noncollagenous domains (NC1-NC4). The molecule is covalently associated with the surface of interstitial collagen fibrils in cartilage (6), where it plays a role in maintaining the long term structural integrity of this tissue (7). Of the 10 possible molecules that could be formed by these three chains, only one, the [α1(IX)2α2(IX)]=α1(IX) heterotrimer is found in cartilage (8). The carboxyl-terminal noncollagenous domains (NC1) of these chains are only 20–28 amino acid residues long, including two cysteine residues that form three interchain disulfide bonds. In order to further test the model, that triple helix formation is mediated by an association of the carboxyl-terminal noncollagenous domains, the NC1 domains of each chain of type IX collagen were synthesized and tested for the ability to direct the association of the appropriate heterotrimer.

Materials and Methods

Peptide Synthesis and Purification—Peptides were synthesized using Fmoc (N-(9-fluorenly)methoxycarbonyl) chemistry on a Milligen/Biosearch 9050 peptide synthesizer. Cleavage and deprotection were performed in trifluoroacetic acid, thioanisole, ethanethiol, and anisole (90:5:3:2, v/v, 2 h, room temperature), which was thoroughly degassed with nitrogen before mixing with the peptide resin to ensure that cysteine residues remained reduced after the removal of the trityl protection groups. The cleaved peptide was separated from the resin by filtration through a medium sintered glass filter. The filtrate was precipitated overnight in ethyl ether at 4 °C and recovered by centrifugation for 10 min at 3,000 × g. The soft pellet was dissolved in 20 volumes of water, degassed, and lyophilized. The dried peptide was suspended in 1% trifluoroacetic acid and purified on a Vydac C-18 reversed-phase HPLC column with a mobile phase of 0.1% trifluoroacetic acid and a linear 60-min gradient of 0–75% acetonitrile. Chromatography runs were recorded with a diode array detector and analyzed with the Millennium 2000 software (Waters). Peaks that eluted between 30 and 50% acetonitrile were pooled and lyophilized. The peptide was dissolved (2 mg/ml) in 50 mM N-ethylmorpholineacetate, pH 8.3, and cysteine residues were reduced with 50 mM dithiothreitol for 2 h at 55 °C under nitrogen. The pooled products were rechromatographed and characterized by amino acid analysis, Edman sequencing, and mass spectrometry. From a theoretical yield of 500 mg, approximately 150 mg of each peptide were pure and used for association experiments.

Association Conditions—Freshly reduced peptide was suspended in 20 mM Hepes buffer, pH 7.5, containing 150 mM NaCl to a final concentration of 6 mM. Of the three peptides α2 was the least soluble, setting this as the maximum concentration. Oxygen was purged from...
the association buffer with nitrogen, and all peptide manipulations were performed in a glove box under a constant flow of nitrogen. The peptides were allowed to fold into their own inherent structure for 30 min and then were mixed in equimolar amounts to final concentrations ranging from 0.1 to 2.0 mM. Lipid vesicles were prepared by suspending phosphatidylcholine dissolved in chloroform (5 mg/ml) and purged under nitrogen for 30 min (14). Circular dichroism spectra of each of the three peptides in the reaction mixture were run over tandem Spherogel TSK-2000S molecular sieve HPLC columns (7.5 mm × 50 cm) with a mobile phase of 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The separation was monitored with a diode array detector. Chromatographic traces were extracted, and the peaks were integrated with the Millennium 2000 chromatography management system.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—Aliquots of the mixtures and fractions of chromatography runs were analyzed on duplicate 10-20% Tricine SDS gels (12). One gel of each set was fixed 30 min in 50% methanol, 7% acetic acid, washed twice with 5% methanol, 7% acetic acid, fixed an additional 30 min with 10% glutaraldehyde, washed extensively with water, and then stained with Coomassie Blue and were recorded in a gel scanner from 260 to 180 nm at 25°C. Concentration values for each peptide solution were determined by amino acid analysis. Analysis of the secondary structure was performed with the variable selection method (11).

Molecular Sieve Chromatography—The formation of disulfide-bonded trimers was monitored by analytical molecular sieve HPLC. 25 μl of the reaction mixture were run over tandem Spherogel TSK-2000S molecular sieve HPLC column. Despite their similarity in molecular weight, the peptides also eluted at different acetonitrile concentrations on a reversed-phase HPLC column (data not shown). The circular dichroism spectra of each of the three peptides in the buffer used for association experiments are shown in Fig. 2. The absence of a positive peak at 221 nm in conjunction with a negligible contribution from α-helices indicates that these peptides do not form a triple helix in the chosen buffer conditions at 25°C at a concentration of 2 mM. An analysis of the secondary structural content of the peptides using the variable selection method is given in Table I. The secondary structure of these peptides consists mainly of β-sheets, β-turns, and aperiodic structures with a very low content of α-helix.

The individual peptides at a concentration of 2 mM were allowed to associate in 20 mM Hepes buffer, pH 7.5, containing 150 mM NaCl for 2 h at 25°C under a N2 atmosphere. The solutions were then adjusted to pH 8.5 with Taps and exposed to air. Oxidation was allowed to proceed for 2 h. The resulting association products were then analyzed by molecular sieve
HPLC. Fig. 3 shows the chromatograms for the three peptides. The α1-peptide exhibits the strongest propensity for self-assembly by forming dimers, trimers, and higher aggregates as shown by chromatography and gel electrophoresis (not shown). The α2-peptide shows some trimer formation, whereas the α3-peptide remains mostly monomeric. These experiments serve as a control for the assembly and oxidation of a mixture of the three peptides.

An equimolar mixture of the three peptides was treated in the same manner as the individual peptides, and the chromatogram of the resulting association products is shown in Fig. 4. A new trimer peak emerges, indicating the formation of (α1α2α3). This composition was established by purification on reversed-phase HPLC, followed by reduction and re-chromatography on the molecular sieve column. The peak labeled (α1α2α3) is a new and unique peak and forms only when the peptides are mixed. The other peaks can be accounted for by the self-assembly of the individual peptides as shown in Fig. 3.

The area of the (α1α2α3) peak was determined by integration and when compared with that of the starting material constitutes an approximately 10% yield of correctly cross-linked heterotrimer. However, the (α1α2α3) peak consists of more than 95% of the newly formed molecules.

Purified (α1α2α3) obtained from HPLC sieve chromatography was analyzed by circular dichroism spectroscopy at 25 °C (Fig. 5). The absence of a positive peak around 221 nm, in

**TABLE I**

Secondary structure content of the type IX collagen peptides

| Peptide | H | A | P | T | O |
|---------|---|---|---|---|---|
| α1      | 0.07 | 0.18 | 0.0 | 0.29 | 0.46 |
| α2      | 0.04 | 0.28 | 0.0 | 0.24 | 0.44 |
| α3      | 0.05 | 0.30 | −0.2 | 0.21 | 0.43 |
As a negative control glass beads were used to account for the rough endoplasmic reticulum membrane and phosphatidyl choline vesicles. Phosphatidyl choline vesicles were chosen because 60% of the naturally occurring lipids in association reactions were carried out in the presence of phosphatidyl choline vesicles. Phosphatidyl choline vesicles were chosen because 60% of the naturally occurring lipids in the rough endoplasmic reticulum membrane are phosphatidyl choline. As a negative control glass beads were used to account for the rough endoplasmic reticulum membrane.

conjunction with a low α-helical content, indicates that the triple helix has not formed under these conditions. At 5 °C the spectrum indicates that at lower temperatures triple helix formation is possible. At this temperature the disulfide bonds stabilize the triple helix as indicated by the positive peak at 221 nm. The reduced heterotrimer would have a lower thermal stability of the triple helix, and therefore formation of the triple helix prior to oxidation is unlikely.

The structure of the heterotrimer formed was further investigated using a monoclonal antibody specific for the disulfide linked type IX collagen NC1 domain. The antibody reacts only with nonreduced type IX collagen on Western blots, and electron micrographs of antibody-antigen complexes after rotary shadowing indicate that the epitope is located within the NC1 domain (Fig. 6). Products from the individual peptide associations and the three chain association reaction were resolved on a 10–20% Tricine SDS-polyacrylamide gel and analyzed on a corresponding Western blot as shown in Fig. 7. No immunoreactivity was found with the individual peptide associations, indicating that the epitope recognized by the antibody comprises more than one chain. All the peaks from Fig. 4 were also analyzed with the monoclonal antibody. Only the heterotrimer peak showed reactivity with the antibody. Similar gels were used to study the kinetics of association (data not shown). A substantial amount of the heterotrimer is already formed after 30 min. The gel in Fig. 7 shows the 2 and 20 h time points. According to immunoblots, the amount of trimer formed seems to increase up to 4 h, and then the formation of higher aggregates becomes more prevalent.

The process was shown to be concentration-dependent over a range from 0.5 to 2 mM peptide concentration. Increasing concentrations of peptides leads to increasing amounts of trimers but also an increased amount of higher aggregates. Under these conditions, protein disulfide isomerase did not facilitate heterotrimer formation but clearly decreased the formation of higher aggregates (data not shown). To test whether trimer formation of these peptides is facilitated by the presence of lipids, association reactions were carried out in the presence of phosphatidyl choline vesicles. Phosphatidyl choline vesicles were chosen because 60% of the naturally occurring lipids in the rough endoplasmic reticulum membrane are phosphatidyl choline. As a negative control glass beads were used to account for the rough endoplasmic reticulum membrane.

FIG. 5. Circular dichroism spectra of purified heterotrimer. The circular dichroism spectra of purified α1, β2, γ3 trimer peptide was recorded at 25 °C (dotted line) and at 5 °C (solid line) in 20 mM Hepes buffer, pH 7.5. The inset shows a 16% Tricine SDS gel of the purified heterotrimer under nonreducing conditions (lane 1), reducing conditions (lane 2), and molecular mass markers (lane 3). The molecular weight markers are the same as in Fig. 7.

FIG. 6. Mapping of the type IX monoclonal antibody epitope to the NC1 domain containing intact disulfide bonds. A, SDS-polyacrylamide gel and immunoblot of a mixture of cartilage collagens including type II, type XI, and type IX collagen (sample 1) and type IX collagen with chondroitin sulfate chains (sample 2). Lanes 1, 3, 5, and 7 contain sample 1; lanes 2, 4, 6, and 8 contain sample 2. Lanes 1 and 2 are from the stained gel of nonreduced samples. Lanes 5 and 6 are from a gel of reduced samples. Lanes 3, 4, 7, and 8 are the corresponding immunoblots. B, rotary shadowed images of type IX molecules with the NC4 domain at the left. C, rotary shadowed images of the type IX collagen-antibody complex, again with the NC4 domain at the left.

FIG. 7. 10–20% Tricine gels and immunoblot of type IX Collagen NC1 peptides. A, gels of type IX NC1 peptide associations. Lanes 1–6 are 2-h associations; lanes 7–12 are 20-h associations. Lane 13 contains marker proteins with the corresponding molecular masses in kDa indicated on the right-hand side. Lanes 1–3 and lanes 7–9 are associations with a concentration of 1 mM peptide. Lanes 4–6 and lanes 10–12 are associations with a concentration of 2 mM peptide. Lanes 1, 4, 7, and 10 are associations in buffer (B); lanes 2, 5, 8, and 11 are associations in presence of phosphatidyl choline vesicles (V); and lanes 3, 6, 9, and 12 are associations in presence of glass beads (G). B, immunoblot of the samples in gel A with the monoclonal antibody against the NC1 domain of type IX collagen. Lanes 1–3 correspond to lanes 4–6 of A, and lanes 4–6 correspond to lanes 10–12 of A. The arrow indicates the position of the trimers, and the molecular masses of the marker proteins are indicated on the right side of A.
for general surface effects. At shorter time points the amount of heterotrimer was reduced in the presence of phosphatidyl cholines, but at longer time points there was an increased amount of heterotrimer found. The monoclonal antibody identifies a band with a molecular mass of approximately 12 kDa, which is the correct theoretical molecular mass for (α1α2α3). The antibody does not recognize other higher molecular mass bands or oxidation products formed by individual peptides. Because of the specificity of the monoclonal antibody, these experiments demonstrate that the correct disulfide bonds are formed in this in vitro association.

**DISCUSSION**

A single cell will simultaneously synthesize different types of collagens. How does the cell select the three appropriate chains for assembly into a triple helical molecule? The mechanism of chain selection and association has been the focus of many studies (1–3, 23–25). The information for chain selection and association of interstitial collagens was shown to reside in the carboxyl-terminal propeptides. During biosynthesis the nascent constituent chains of these collagens become partially hydroxylated at proline and lysine residues. Only after synthesis of the carboxyl-terminal propeptides do chains associate. The carboxyl-terminal propeptides become interchain disulfide-linked, and triple helix formation proceeds concurrently from the carboxyl-terminal end toward the amino-terminal end. Hydroxylation of proline residues continues until the triple helix is formed. There is evidence that folding of the triple helix is catalyzed by peptidyl-prolyl cis-trans isomerases (3, 26–28). The carboxyl-terminal propeptides of the interstitial collagens are highly conserved, but further studies are required to determine which residues determine the selection of the appropriate chains. In this study, we investigated whether this mechanism of association also applies to fibril-associated collagens with interrupted triple helices. The small size of the carboxyl-terminal noncollagenous domains of the constituent chains of type IX collagen makes it possible to investigate this question with synthetic peptides. Our results indicate that the chain selection and association of type IX is determined by the NC1 domain. The results with the peptides that were synthesized without the triple helical segments indicate that these sequences do not have to be in a triple helical conformation. This is in contrast to experiments which indicate, however, that these sequences do not have to be in a triple helix conformation. This is in contrast to experiments which indicate, however, that these sequences do not have to be in a triple helical conformation. These results with the NC1 domains to come together and/or form the NC1 domain. Triple helix formation however is not the driving force for this interaction.

**REFERENCES**

1. Fessler, J. H., and Fessler, L. I. (1978) Annu. Rev. Biochem. 47, 129–162
2. Prokop, D. J., Berg, R. A., Kivirikko, K. I., and Uitto, J. (1978) in Biochemistry of Collagen (Ramachandran, G. N., and Reddi, H., eds) pp. 163–273, Plenum Publishing Corp., New York
3. Engel, J., and Prokop, D. J. (1991) Annu. Rev. Biophys. Biophys. Chem. 20, 137–152
4. Morris, N. P., and Bächinger, H. P. (1987) J. Biol. Chem. 262, 11345–11350
5. Shaw, L. M., and Olsen, B. R. (1991) Trends Biochem. Sci. 16, 191–194
6. Eyer, D. R., and Wu, J. (1995) J. Rheumatol. 22, 82–85
7. Fässler, R., Schneegerg, P. N. J., Daumans, J., Shinya, T., Muragakai, Y., McCarthy, M. T., Olsen, B. R., and aenschen, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5070–5074
8. Frewton, B. G., and Mayne, R. (1994) in Extracellular Matrix Assembly and Structure (Yurchenco, P. D., Birk, D. E., and Meacham, R. P., eds) pp. 129–170, Academic Press, New York
9. Hwang, C., Simskey, A. J., and Lodish, H. F. (1992) Science 257, 1496–1502
10. Lambert, N., and Freedman, R. B. (1983) Biochem. J. 213, 213–235
11. Compton, L. A., Mathews, C. K., and Johnson, W. C. (1987) J. Biol. Chem. 262, 13039–13043
12. Schägger, H., and von Jagow, G. (1987) Anal. Biochem. 168, 368–379
13. Watt, S. L., Luntrum, G. P., McDonough, A. M., Keene, D. R., Burgeson, R. E., and Morris, N. P. (1992) J. Biol. Chem. 267, 20093–20099
14. Saki, L. Y., Keene, D. R., and Engvall, E. (1986) J. Cell Biol. 103, 2499–2509
15. Saki, L. Y., and Keene, D. R. (1994) Methods Enzymol. 245, 29–52
16. Lozano, G., Ninomiya, Y., Thompson, H., and Olsen, B. R. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4050–4054
17. Ninomiya, Y., van der Rest, M., Mayne, R., Lozano, G., and Olsen, B. R. (1985) Biochemistry 24, 4223–4229
18. Brevet, R. G., Ojitsuka, M. V., van der Rest, M., and Mayne, R. (1992) Eur. J. Biochem. 205, 443–449
19. Heidenmann, E., and Rohn, W. (1981) Adv. Polym. Sci. 34, 143–203
20. Muragakai, Y., Kimura, T., Ninomiya, Y., and Olsen, B. R. (1989) Eur. J. Biochem. 192, 703–708
21. Elima, K., Mutasara, M., Kallio, J., Perala, M., Eerola, I., Garofalo, S., DeCondebrugge, B., and Vuorio, E. (1992) Biochem. Biophys. Acta 1130, 78–80
22. Brevet, R. G., Wood, B. M., Ren, X-Z., Gong, Y., Tiller, G. E., Warnan, M. L., Lee, B. H., Horton, W. A., Olsen, B. R., Baker, J. R., and Mayne, R. (1995) Genomics 30, 329–335
23. Doege, K. J., and Fessler, H. J. (1986) J. Biol. Chem. 261, 8924–8935
24. Middleton, R. B., and Bulled, N. J. (1993) Biochem. J. 296, 511–517
25. Lee, J. F., and Bulled, N. J. (1994) Biochim. Biophys. Acta 1213, 2435–2436
26. Schmid, F. X., Mayer, L. M., Mücke, M., and Schönbrunner, E. R. (1993) Adv. Protein Chem. 44, 25–66
27. Bächinger, H. P., Morris, N. P., and Davis, J. (1993) Am. J. Med. Genet. 45, 152–162
28. Steinmann, B., Bruckner, P., and Superti-Furga, A. (1991) J. Biol. Chem. 266, 1299–1303
29. Mazorana, M., Graff, H., Sergeant, A., and van der Rest, M. (1993) J. Biol. Chem. 268, 3029–3032
30. Labudette, L., and van der Rest, M. (1993) FEBS Lett. 320, 211–214
31. Riehle, A., Engel, J., Ludvig, A., and Kühn, K. (1995) J. Biol. Chem. 270, 23790–23794

**Assembly of Type IX Collagen Peptides**

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