The Noncollagenous Domain 1 of Type X Collagen
A NOVEL MOTIF FOR TRIMER AND HIGHER ORDER MULTIMER FORMATION WITHOUT A TRIPLE HELIX*

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(Received for publication, September 22, 1998, and in revised form, April 28, 1999)

In this study, we test the hypothesis that the carboxyl noncollagenous (NC1) domain of collagen X is sufficient to direct multimer formation without a triple helix. Two peptides containing the NC1 domain of avian collagen X have been synthesized using a bacterial expression system and their properties characterized. One peptide consists only of the NC1 domain, and the other is a chimeric molecule with a noncollagenous A domain of matrilin-1 fused to the N terminus of NC1. The NC1 peptide alone forms a 45-kDa trimer under native conditions, suggesting that NC1 contains all the information for trimerization without any triple helical residues. This trimeric association is highly thermostable without intermolecular disulfide bonds. This indicates that the NC1 domain contributes to the remarkable structural stability of collagen X. Chemical cross-linking of the NC1 trimer results in a series of varying sized multimers, the smallest of which is a trimer. Therefore the NC1 trimer is sufficient to form higher order multimers. The chimeric A-NC1 peptide forms a homotrimer by itself, and a series of heterotrimers with the NC1 peptide via the NC1 domain. Thus the NC1(X) domain directs multimer formation, even in a noncollagenous molecule.

Type X collagen is expressed specifically by hypertrophic chondrocytes at the transition of cartilage to bone during endochondral bone formation (1, 2). Type X collagen is essential for the structural organization of the matrix preceding calcification (3, 4), occurring in two forms: one is a pericellular matrix (5), which probably represents a multimeric form of type X collagen itself (6); the other is in association with type II collagen containing fibrils where it also interacts with proteoglycans (7, 8). This interaction may be critical for the compartmentalization of matrix components during development (9).

The importance of type X collagen during bone development is evidenced by its mutations in a human limb disorder Schmid metaphyseal chondrodysplasia (10). The NC1 domain of human and avian type X collagen. Also, all the mutants within the NC1 domain mutated in the SMCD patients are conserved between human and avian a1(X). These data, when taken together, suggest that the NC1 domain of type X from different species plays a similar or identical roles, such as in the assembly of type X collagen.

It has been proposed that the NC1 of type X is important for the triple helical assembly of the collagen, similar to the function of the NC1 domains from fibrillar collagens. The NC1 domain is a homotrimer and multimerin, a massive protein found in platelets and endothelial cells (25). Multimerin contains a homologous NC1 domain at their C termini (Fig. 1). The collagenous molecules include a1(X); a1 and a2 chains of type VIII collagen (18); a, b, and c chains of C1q, the first component of the complement system (19); three plasma proteins that are associated with mammalian hibernation, HP-20, -25, and -27 (20); an adipose-specific protein that is dysregulated in obesity, AdipoQ (Acrp30) (21, 22); and an inner ear-specific protein (23). The noncollagenous molecules include two chains of precerebellin, which are expressed specifically in cerebellum during neurogenesis and synapse formation (24); and multimerin, a massive protein found in platelets and endothelial cells (25). Multimerin consists of a series of varying sized disulfide-linked multimers, the smallest of which is a homotrimer (26). The functions of NC1 in these noncollagenous molecules are not known.

In this study, we have analyzed the functions of the NC1 domain by expressing and characterizing two noncollagenous peptides containing the NC1 domain from avian a1(X). The peptide comprised only of the NC1 domain, is sufficient to form a trimer and a series of higher order multimers of this trimeric unit. The other peptide comprised of the NC1 domain plus a...
noncollagen A domain from cartilage matrix protein (matrilin 1) at the N terminus, forms homotrimers by itself, and a series of heterotrimers with the NC1 peptide. These data suggest that the NC1 domain of collagen X functions as a novel nucleation site for trimer and multimer formation, both for collagenous and noncollagenous molecules.

MATERIALS AND METHODS
Construction and Expression of Recombinant NC1 and A-NC1—For the NC1 construct, a DNA fragment corresponding to the amino acid sequence of the NC1 domain from avian α(1)(X) (Thr513 to Ile674) was amplified by polymerase chain reaction from a plasmid pNY3116 (12, 27). The polymerase chain reaction primers (5'-GCG CGC GAA TCG CAG AAT GGT TAT ACG AAG GTT AT-3') contained a XbaI and a HindIII site at the respective 5' and 3' end. The fragment was then cloned into a pQE32 (type IV) (Qiagen, Santa Clarita, CA) to carry a six histidine-tag at its N terminus. For the A-NC1 construct, a cDNA fragment (582 base pairs) of the A2 domain from avian cartilage matrix protein (CMP) was amplified from a mini-CMP cDNA (28) with a pair of primers carrying XbaI and a SPE site upstream of the NC1 insert in the expression vector. This ligation created a chimeric molecule with the A2 domain at its 5' end and the NC1 domain at its 3' end. Both cDNA constructs were sequenced to confirm the correct reading frame and no spontaneous point mutations. The recombinant plasmids were transfected into competent E. coli DH5α. The expression of the His-tagged peptides alone sufficient to form a trimer? Question 2 (Q2): Is the NC1 domain sufficient to form trimers? Question 3 (Q3): Is the NC1 domain sufficient to direct trimer formation of a noncollagenous molecule? Question 4 (Q4): Can the NC1 peptide and the A-NC1 peptide form heterotrimers via the NC1 domain? A2, the second A domain in CMP, CC, the coiled-coil domain in the A domain of CMP, gradient buffers over the same period of time (8 h). After refolding, the peptide was eluted with imidazole elution buffer (400 mM imidazole, 500 mM NaCl, 20% glycerol, 20 mM Tris-HCl, 1 mM PMSF, pH 7.4). For co-refolding of the NC1 and A2-NC1 peptides, equal amounts of the bacterial cultures expressing the NC1 and A2-NC1 peptides were mixed. The peptide mixture was purified and refolded on a solid phase, identical to the procedure for a single peptide described above. The concentration of total protein was determined with BCA protein assay reagent kit (Pierce).

 SDS-Polyacrylamide Gel Electrophoresis and Cross-linking Assay—Cross-linking was carried out with BS3 (Bis(sulfosuccinimidyl)suberate) (Pierce), a water-insoluble, homobifunctional-N-hydroxysuccinimide ester analog ( spacer arm length 11.4 Å). 10 µM of the refolded NC1 peptides were mixed with various concentration of BS3 and incubated at room temperature with a gentle shake for 1 h followed by adding Tris-HCl buffer (final concentration: 50 mM) to stop the reaction. The samples were loaded on a 10% SDS-PAGE for electrophoresis. For reducing conditions, samples were mixed with 5X SDS gel loading buffer containing 15% β-mercaptoethanol, 15% SDS, 1.5% bromphenol blue, and 50% glycerol. For nonreducing conditions, the 4X loading buffer contains 16% SDS and 1% bromphenol blue. Sometimes the nonreducing buffer contains 2 mM urea. The presence of urea in loading buffer does not affect dissociation properties of NC1 peptides. Samples were boiled for 10 min before loading when required. Gels were stained with Coomassie Blue.

 Matrix-assisted Laser Desorption Ionization Mass Spectrometry—Refolded NC1 peptides (300 µg/ml in 500 mM NaCl, 20% glycerol, 500 mM NaHPO4, pH 7.4) were prepared for mass spectrometry by the dried droplet method in which the protein solution and a solution of sinapinic acid matrix (0.5 µl each) were mixed and air-dried. The crystals were then rinsed with 10 µl of 0.1% trifluoroacetic acid. Spectrum was obtained with STR Perseptive Biosystems (Framingham, MA). It primarily shows the monomeric NC1 (20,554 Da), with small trimer was obtained with STR Perseptive Biosystems (Framingham, MA). It primarily shows the monomeric NC1 (20,554 Da), with small fragments correspond to the NC1 domain (Fig. 2A) with the NC2 domain at its N terminus. Both cDNA constructs were sequenced to confirm the correct reading frame and no spontaneous point mutations. The recombinant plasmids were transfected into competent E. coli DH5α. The expression of the His-tagged peptides was confirmed by SDS-PAGE. For recombining conditions, samples were mixed with 5X SDS gel loading buffer containing 15% β-mercaptoethanol, 15% SDS, 1.5% bromphenol blue, and 50% glycerol. For nonreducing conditions, the 4X loading buffer contains 16% SDS and 1% bromphenol blue. Sometimes the nonreducing buffer contains 2 mM urea. The presence of urea in loading buffer does not affect dissociation properties of NC1 peptides. Samples were boiled for 10 min before loading when required. Gels were stained with Coomassie Blue.

RESULTS
Construction of Two Peptides Containing the NC1 Domain and Experimental Design—To characterize the function of the NC1(X) domain, two molecules containing this domain were created. The first peptide, NC1, consists of the C-terminal 162 amino acid residues of α1(X), and thus comprises the entire NC1 domain (Fig. 2A). This peptide is devoid of any triple

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**FIG. 1. Schematic of known members of the NC1(X) domain family.** 1) Collagenous molecules: α1(X), α1(VIII), α2(VIII), CIqA, CIqB, CIqC, hibernation-associated proteins (HP): HP-20, HP-25, and HP-27, AdipoQ (Acrp30), and inner ear specific protein. 2) Noncollagenous molecules: two forms of preceellarin: one with a transmembrane domain, and one without a transmembrane domain, and multimerin.

**FIG. 2. Construction of the two molecules containing the NC1 domain and experimental strategy.** Question 1 (Q1): Is the NC1 domain alone sufficient to form a trimer? Question 2 (Q2): Is the NC1 domain sufficient to form trimers? Question 3 (Q3): Is the NC1 domain sufficient to direct trimer formation of a noncollagenous molecule? Question 4 (Q4): Can the NC1 peptide and the A-NC1 peptide form heterotrimers via the NC1 domain? A2, the second A domain in CMP; CC, the coiled-coil domain at the C terminus of CMP.
helical (Gly-X-Y) residues, and thus can be used to test whether the NC1 domain alone is sufficient to form a trimer and higher order multimers.

To test whether the NC1 domain is sufficient to direct trimer formation of a noncollagenous molecule, the second peptide, A-NC1, is comprised of two domains: the NC1 domain at the C terminus and the entire A2 domain (194 amino acid residues) of avian CMP at the N terminus (Fig. 2B). Previous studies (29) have used a mini-CMP, which comprises a heptad-repeat domain at the C terminus and the A2 domain at the N terminus (Fig. 2B), to demonstrate that the heptad-repeat domain forms a three-chain coiled-coil thereby facilitating the trimer formation of the molecule. This trimer then is covalently stabilized by the intermolecular disulfide bonds involving the two cysteines at the N terminus of the heptad repeat (29). The chimeric peptide construct that we designed deleted the coiled-coil domain and the cysteine residues, and replaced these with the X-NC1 domain. If the NC1 domain functions as the coiled-coil domain, it would direct trimer formation of the A-NC1, as does the coiled-coil in mini-CMP. Finally, the two peptides, A-NC1 and NC1, would be refolded together in vitro. This is to test whether they form heterotrimers via the NC1 domain. The number of the co-refolding products would confirm their trimeric states (i.e. four products are expected for the formation of trimers) (Fig. 2C).

**Bacterial Synthesis and Assembly of a Native NC1 Peptide into a Trimeric Form**—We observed that *E. coli* containing a His-tag bacterial expression vector encoding the NC1 domain assembled the newly synthesized peptides into a trimeric form under native conditions. Upon induction by isopropylthio-β-D-galactoside, *E. coli* expressed a 20-kDa peptide (Fig. 3A). This is the molecular mass predicted from the amino acid sequence of the peptide, and also of the NC1 monomer from authentic type X collagen (30). The expressed His-tagged NC1 peptide, when affinity purified from cytoplasm using Ni-NTA resin under native conditions, produced a His-tag protein that migrated on SDS-PAGE with a molecular mass of 45 kDa (Fig. 3B, lane 2). This apparent molecular weight is the same as that of the NC1 trimer isolated by bacterial collagenase digestion of authentic type X collagen from avian hypertrophic chondrocytes (30). The NC1 trimer is 45 kDa instead of 60 kDa, because of its compact conformation (30). Additional evidence for a trimeric form will be presented later. Some of the 45-kDa band was shifted to 20-kDa monomer after heating at 100 °C in a SDS-containing buffer (Fig. 3B, lane 3). This suggests that the native NC1 peptide alone is sufficient to form a trimer.

**In Vitro Folding**—Analysis of the intracellular location of the recombinant protein within the bacteria indicated that less than 10% of the protein was in a soluble form; more than 90% of the recombinant protein was insoluble in inclusion bodies (data not shown). To isolate large quantities of the recombinant protein for further analysis, the insoluble fraction was purified under denaturing conditions. The denatured peptide existed as a 20-kDa monomer and a 40-kDa putative dimer. However, no 45-kDa trimeric form was visible (Fig. 3B, lane 4).

The denatured peptide was tested for its ability to undergo refolding in solution by a step wise dilution of denaturants (see "Materials and Methods"). The resulting material formed an insoluble precipitate that upon SDS-PAGE analysis was still the monomeric peak at 20.6 kDa, with small dimer and trimer bands on gels, was actually a trimer, matrix-assisted laser desorption ionization mass spectrometry was performed. The refolded peptides were subject to dissociation process by removing salts from the solution. The resulting spectrum primarily showed the monomeric peak at 20.6 kDa, with small dimer and trimer peaks at 41.1 and 61.7 kDa, respectively. Thus, the refolded NC1 was a trimer with a molecular mass of 61.7 kDa.

**Thermal Stability of the NC1 Trimer**—To determine the thermal stability of the NC1 domain, the refolded NC1 peptides were incubated at different temperatures (70, 80, 90, and 100 °C) before analysis by SDS-PAGE. The trimers were resistant to thermal denaturation up to 90 °C under reducing conditions (Fig. 5). At 90 °C, the majority of the NC1 domain still remained as trimers (Fig. 5). Only at 100 °C were the trimers separated completely into monomers (Fig. 5).

To determine whether disulfide bonds were involved in the thermal stability of the NC1 domain, the NC1 trimers were

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**Fig. 3.** Expression of the NC1(X) peptide in *E. coli*. *A*, expression of recombinant NC1(X) was induced by 2 mM isopropylthio-β-D-galactoside (lane 1); negative control without induction (lane 2). The arrows point to the induced 20-kDa peptide. Cell lysates were heated for 10 min before loading. *B*, NC1(X) was expressed and purified under native and denatured conditions. The NC1 peptides were purified with His-tag affinity chromatography, and quantified by a BCA protein assay reagent kit (Pierce). SDS-PAGE analysis was performed with an equal amount of purified peptides loaded in each lane under reducing conditions. Lane 1, molecular weight marker; lanes 2 and 3, the NC1 peptide purified under native conditions; lane 4, the NC1 peptide purified under denatured conditions (see "Methods and Material" for details). As indicated, the peptides were either heated for 10 min at 100 °C or without heating prior to loading.

**Fig. 4.** In vitro refolding of denatured NC1(X) peptide. Puriﬁed NC1(X) peptide in a buffer containing 8 M urea was subjected to renaturation by a step wise dilution of the denaturant. The refolding process was performed with denatured NC1 peptides either in solution (lanes 2 and 3, liquid phase refolding), or immobilized on a column (lanes 4 and 5, solid phase refolding). Conditions such as the length of folding time and solutions used in each step were identical between liquid and solid phase refolding. As indicated, the samples were either heated for 10 min at 100 °C or without heating prior to electrophoresis under reducing conditions. Lane 1, molecular weight markers.
incubated in the absence of reducing reagents. Similar to the results under reducing conditions, the trimers became completely separated only at 100 °C (Fig. 5). Thus, disulfide bonds were not involved in the thermal stability of the NC1 trimer.

**Multimeric Forms of NC1 by Cross-linking**—To further characterize the association of the NC1 peptides, we performed a cross-linking experiment with a cross-linker BS3. The spacer arm length of BS3 is 11.4 Å. Therefore, neighboring peptides whose distance is equal to or less than 11.4 Å will be covalently cross-linked by BS3. The refolded NC1 peptides were incubated in a series of solutions with different concentrations of BS3 (Fig. 6). In a solution with an equal ratio of cross-linker to NC1, a ladder of bands appeared above the trimer (Fig. 6, arrows in the middle panel). The smallest of this series of bands was the 45-kDa NC1 trimer. Therefore, the NC1 trimer formed higher order multimers in solution. After heating to 100 °C, the cross-linked sample included, in the least, monomers (20 kDa), dimers (40 kDa), and trimers (60 kDa) (Fig. 6, arrows in the right panel). The unheated native NC1 trimer migrated at the 45-kDa position faster than a heated denatured trimer. This is probably because of the compact configuration of the native peptides. With excessive cross-linker, (1,000 times of that of NC1), the NC1 peptides remained as a high molecular polymer under both heating and nonheating conditions (Fig. 6, arrowheads).

**Trimer Formation of the Chimeric A-NC1 Peptide**—To test whether the NC1 domain facilitates trimer formation of a non-collagenous molecule, a chimeric A-NC1 peptide was purified under denatured conditions and refolded on a solid phase. With SDS-PAGE performed without heating, this material contained three forms (Fig. 7). The smallest was the 40-kDa form, the predicted molecular mass of an A-NC1 monomer. The second was 100 kDa, consistent with a native trimeric form following refolding (see below). The third was 200 kDa, twice the molecular mass of the trimer and most probably a hexamer. After heating to 100 °C, both of the multimeric forms shifted to the monomeric 40-kDa form (Fig. 7).

To demonstrate that the 100-kDa form was, in fact, an A-NC1 trimer, denatured A-NC1 peptide was refolded together with the NC1 peptide. If both the A-NC1 and the NC1 peptides form trimers, co-refolding of these two peptides should result in four products, the 100 kDa (A-NC1)2, the 45 kDa (NC1)2, and two heterotrimers (A-NC1)2NC1 and A-NC1(NC1)2, between 100 and 45 kDa (Fig. 2). The molecular mass of the (A-NC1)2(NC1) should be above 72.5 kDa (the average of the molecular weights of the two heterotrimers), and the molecular mass of the A-NC1(NC12), is expected to be below 72.5 kDa.

Upon analysis by SDS-PAGE, the co-refolded material shows the predicted four products (Fig. 8). Thus, the A-NC1 peptide and the NC1 peptide can form heterotrimers via the NC1 domain.

**DISCUSSION**

In collagens, it is well established that triple helix formation is initiated at the carboxyl-terminal end, where the individual chains are maintained in register by interchain disulfide links (31). In some collagens (e.g. homotrimer type XII collagen), disulfide bond formation requires triple helix and thus will not occur in the absence of prolylhydroxylase (32). Formation of the type IX collagen NC1 heterotrimers also requires some adjacent triple helical segments, although in this case, a triple helical conformation is not necessary (33). The trimeric NC1 domain of avian type X collagen is unique in that it does not contain interchain disulfide bonds. Another puzzling fact is that a homologous NC1(X) domain has been found at the C terminus of some molecules that do not contain any triple helical sequences at all (Fig. 1).

We have demonstrated here that peptides of the NC1 domain of type X are sufficient to form trimers without adjacent triple helical segments. This suggests that the NC1 domain alone is sufficient for chain association and proper trimeric assembly.
The NC1(X) forms an extraordinary stable trimer without any disulfide bonds. The trimeric interaction of the NC1 is resistant to denaturing conditions of SDS-PAGE, as is that of the native molecule (30). Under such conditions noncovalently linked subunits of most molecules would be dissociated. For example, the CMP trimer, which is held together by a C-terminal coiled-coil, is dissociated into monomers under denaturing conditions if disulfide bonds are not present (29). In contrast, we have shown that, when the coiled-coil domain of CMP is replaced by the NC1 domain, the NC1 domain is able to hold the mini-CMP trimer together under such reducing conditions.

The noncovalent association of the NC1(X) is highly thermostable. Only at 100 °C did the trimer become completely dissociated. It was known that the intact native type X collagen can be dissociated only by boiling in the presence of detergents (30). Our data suggest that the NC1 domain is responsible for this remarkable stability of collagen X. The stability of the NC1 may also contribute to the rapid renaturation of collagen X after thermal denaturation. The denaturation temperature (Tm) of the helical structure of avian collagen X is 47 °C (30). It has been found previously that, after thermal denaturation at 55 °C, more than 60% of the helical structure from intact type X was reformed after 40 min. However, without the NC1, the collagenous domain renatured 15% at most, even after 24 h (30). Our data have shown that the NC1 peptide remains as a trimer under thermal denaturation up to 90 °C. This suggests that at a denaturing temperature between 47 °C and 90 °C the NC1 domain would still hold trimers together, thus maintaining proper chain registration and allowing rapid renaturation of the adjacent triple helix.

In vitro refolding of NC1 suggests that to achieve proper trimeric assembly of the molecule, premature interchain association has to be prevented. It is possible that a chaperon may perform such a function in vivo, as does immobilization on a solid phase in vitro. This “chaperon” hypothesis is consistent with the recent finding that protein disulfide isomerase, a noncollagenous molecule with a homologous NC1 domain at its C terminus, is not known. In the light of the present data, it will be intriguing to test whether precerebellin forms trimers and higher order multimers during synapse formation.

The structural features within the NC1(X) domain which direct trimer and multimer formation remain to be determined. Computer analysis of the secondary structure of the NC1(X) indicates that the NC1 peptide may be a novel structural entity comprised of multiple β sheets and loops. The elucidation of the three-dimensional structure of this domain will rely on x-ray crystallography and mutational analysis of the peptide.

Acknowledgment—We thank Phyllis LuValle for providing the plasmid pYN3116, A. Daniel Jones for performing mass spectra, and Tom Linsenmayer for critical reading of the manuscript.

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