Interaction of Collagen α1(X) Containing Engineered NC1 Mutations with Normal α1(X) in Vitro

IMPLICATIONS FOR THE MOLECULAR BASIS OF SCHMID METAPHYSEAL CHONDRODYSPLASIA*

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Collagen X is a short-chain homotrimeric collagen expressed in the hypertrophic zone of calcifying cartilage. The clustering of mutations in the carboxyl-terminal nonhelical NC1 domain in Schmid metaphyseal chondrodysplasia (SMCD) suggests a critical role for NC1 in collagen X structure and function. In vitro collagen X DNA expression, using T7-driven coupled transcription and translation, demonstrated that although α1(X) containing normal NC1 domains can form electrophoretically stable trimers, engineered SMCD NC1 missense or premature termination mutations prevented the formation of electrophoretically stable homotrimers or heterotrimers when co-expressed with normal α1(X). To allow the detection of more subtle interactions that may interfere with assembly but not produce SDS-stable final products, we have developed a competition-based in vitro co-expression and assembly approach. Our studies show that α1(X) chains containing SMCD mutations reduce the efficiency of normal α1(X) trimer assembly, indicating that interactions do occur between mutant and normal NC1 domains, which can impact on the formation of normal trimers. This finding has important implications for the molecular pathology of collagen X mutations in SMCD. Although we have previously demonstrated haploinsufficiency as one in vivo mechanism (Chan, D., Weng, Y. M., Hocking, A. M., Golub, S., McQuillan, D. J., and Bateman, J. F. (1998) J. Clin. Invest. 101, 1490–1499), the current study suggests dominant interference is also possible if the mutant protein is expressed in vivo. Furthermore, we establish that a conserved 13-amino acid aromatic motif (amino acids 589–601) is critical for the interaction between the NC1 domains, suggesting that this region may initiate assembly and the other NC1 mutations interfered with secondary interactions important in folding or in stabilizing the assembly process.

The collagens are an extensive protein family defined by the characteristic triple helix motif formed from three α-chain subunits and the ability to associate into precise extracellular supramolecular assemblies (1, 2). Collagen X is a short-chain collagen expressed in the hypertrophic zone of calcifying cartilage during skeletal development and bone growth (3–6). The α1(X) homotrimer consists of three distinct protein domains, a short triple helix (COL1) flanked by a small amino-terminal nonhelical NC2 domain and a larger, more conserved, nonhelical carboxyl-terminal NC1 domain. By analogy with the fibrillar collagens, the NC1 domain would be expected to associate and initiate trimerization (7, 8). In vitro expression and assembly studies have also shown that collagen X trimers can form rapidly via NC1 interactions, which are stable to the dissociative conditions of SDS-polyacrylamide gel electrophoresis (PAGE) in the presence of urea (9, 10). These strong NC1 interactions are likely to be largely hydrophobic in nature (8, 11).

The critical importance of the NC1 domain in collagen X molecular assembly and function has also been highlighted by the characterization of mutations in patients with Schmid metaphyseal chondrodysplasia (SMCD) (MIM 156500) (12, 13). Although these mutations include amino acid substitutions, nonsense mutations and deletions resulting in predicted protein truncation, in vitro expression, and assembly studies suggested that a common molecular defect is that the SMCD mutations compromise NC1 association and prevent the formation of stable collagen X homotrimers (9, 10). These data suggested that SMCD resulted from a functional haploinsufficiency of assembly-competent α1(X). The haploinsufficiency model was further supported by the characterization of an SMCD premature termination mutation that led to mutant mRNA instability and a total absence of the α1(X) mutant allele mRNA in patient growth plate cartilage tissue (14). However, although the in vitro assembly data with other SMCD NC1 mutations demonstrated that the formation of electrophoretically stable NC1 mutant homotrimers was prevented, these experiments did not exclude the possibility that weaker or transient interactions may occur that could impact on the formation of the collagen X trimer. Indeed, trace amounts of heterotrimer assembly were detected with some α1(X) NC1 missense mutants expressed in vitro (10), suggesting that some NC1 mutations may allow mutant/normal heterotrimer assembly or interfere with the efficiency of normal α1(X) assembly and could thus exert a dominant negative effect on collagen X assembly.

Collagen X interactions in in vitro expression studies have been assessed by their stability to the denaturing conditions of SDS-PAGE, conditions under which assemblies of other collagen types are not stable. To overcome the obvious limitations of assessing potentially biologically important interactions in this harsh system, we have developed a competition-based approach, which is designed to allow the assessment of such
SMCD Mutations and Collagen α1(X) NC1 Interactions

**TABLE I**

| Primers | Primer sequence (5' to 3') | Primer location | Orientation |
|---------|-----------------------------|-----------------|-------------|
| ter6610 | TGCATGACATCATTAAACATGATCCTT<sup>a</sup> | 1831–1817 | Antisense |
| ter6500 | TGCATGACATCATTAAACATGATCCTT<sup>a</sup> | 2041–2029 | Antisense |
| ter6666 | TGCATGACATCATTAAACATGATCCTT<sup>a</sup> | 2091–2079 | Antisense |
| BX1     | AGGGGTTAAGCAGGACTGGCC     | 1726–1745 | Sense |
| HX1     | TAAAGGAGGCCTCAGGAAAGG     | 1491–1510 | Sense |
| HX6     | CTTTTCAGCTTAAGCTTCTCAAT   | 2235–2216 | Antisense |
| AΔ-1    | GTCTGGTGAGTTAGGATGATGCT<sup>b</sup> | 1654–1655, 1849–1860 | Sense |
| AΔ-2    | GTCTGGTGAGTTAGGATGATGCT<sup>b</sup> | 1860–1849, 1665–1654 | Antisense |
| BΔ-1    | AGGCTAGGAATCCACGGTCATGTTG | 1849–1860, 1990–1911 | Sense |
| BΔ-2    | CACATGCACTGAGTTCCAGGCCCTT | 1911–1990, 1860–1849 | Antisense |
| CΔ-1    | TACACGTTGACCAGCTGCTTCTCTT | 1897–1908, 2096–2160 | Sense |
| CΔ-2    | AGGGAGGCTGCTACGGCTGAAC    | 2109–2098, 1905–1897 | Antisense |

<sup>a</sup> Sequences in bold are the annealing sequences in the PCR reactions.
<sup>b</sup> Sequence assignment numbers are from the transcription start site (33).
<sup>c</sup> This primer contains an overhang sequence with an engineered stop codon (underlined sequence) and an A/II restriction enzyme site.
<sup>d</sup> Sequences shown in italics are the overlapping sequences in secondary PCRs.

SDS-stable assemblies, as well as weaker interactions that may interfere with assembly but not produce SDS-stable final products. We have used this in vitro co-expression and assembly assay to examine in detail the molecular consequences of SMCD mutations and to further dissect the role of NC1 domains in the assembly process. Our studies show that α1(X) chains containing SMCD mutants can interfere with the efficiency of normal α1(X) assembly during in vitro co-expression, demonstrating the potential of these mutations to exert a dominant negative effect if the mutant protein is expressed in vivo. Furthermore, our studies demonstrate that deletion of the 13-amino acid conserved aromatic motif (7) completely abrogates the ability of the α1(X) NC1 to interact with other normal or mutant chains, identifying this domain as a crucial sequence in collagen X assembly.

**EXPERIMENTAL PROCEDURES**

**Construction of α1(X) NC1 Domain Mutations**—The production of full-length α1(X) cDNA expression constructs containing specific SMCD NC1 missense mutations (G618V, Y598D) and a nonsense (premature termination) mutation (Y632X) predicting the production of a truncated protein has been described previously (10). Three additional nonsense mutations at amino acid positions 610, 650, and 666 were generated using PCR to produce sequential protein truncations of the NC1 domain from the carboxyl terminus (see “Results and Discussion”). The mutant primer sets and their relative positions are shown in Table I. Primer sets AΔ-1 and AΔ-2, BΔ-1 and BΔ-2, and CΔ-1 and CΔ-2 were designed to delete regions A, B, and C, respectively. The PCR reactions were carried out as described above. Two independent PCR products were first produced by using HX1 (sense) with primer set 2 (AΔ-2, BΔ-2, or CΔ-2; antisense) and by using HX6 (antisense) with primer set 1 (AΔ-1, BΔ-1 or CΔ-1; sense) with each of the mutant primer sets in the primary round of PCR using pTM1-h10wt (5 ng) as a template. After purification, the appropriate fragment pairs were mixed and used as templates in the second round of overlapping PCR with primers HX1 and HX6. The recombinant mutant PCR fragments were digested with BamHI and NciI, purified, and cloned into corresponding sites within pTM1-h10wt. All mutant constructs were sequenced to ensure there were no PCR errors.

**In Vitro Cell-free Transcription and Translation**—Cell-free transcription/translation was performed as described previously in the presence or absence of canine pancreatic microsomal membranes (Promega) using the TNT T7 polymerase coupled transcription and translation system (Promega) (9, 10). The conditions were modified by reducing the amount of added plasmid DNA (25–50 ng), and the reaction volume of 6.25 μl was labeled with 5 μCi of translation grade l-<sup>35</sup>S-methionine (1000 Ci/mmol, NEN Life Science Products). Co-translation experiments were performed in the presence of microsomal membranes by aliquoting 5.25 μl from a master mix containing 25 ng/25 μl of control plasmids, which were either the wt full-length α1(X) or an assembly-competent helix deletion (helix Δα1(X) reporter construct (10). To these reactions, 1 μl containing 0, 6.25, 12.5, or 25 ng of NC1 mutant plasmids or a luciferase control plasmid (Promega) were added prior to transcription and translation at 30 °C for 90 min. Reactions were terminated by adding 40 μl of sample buffer (10 mM Tris/HCl, pH 6.8, containing 2% SDS (w/v), 2 mM urea, 10 mM dithiothreitol, and 20% sucrose (w/v)) (9). In some experiments, a sample buffer with milder denaturation properties was used (10 mM Tris/HCl, pH 6.8, containing 0.5% SDS (w/v) and 20% sucrose (w/v)) (9).

In some experiments, a sample buffer with milder denaturation properties was used (10 mM Tris/HCl, pH 6.8, containing 0.5% SDS (w/v) and 20% sucrose (w/v)). Samples were incubated at room temperature for 10 min prior to electrophoresis on a 7.5% SDS-polyacrylamide gel at 4 °C for 16 h in the absence of urea. Radioactive bands were imaged and quantified using a PhosphorImager (Molecular Dynamics).

**RESULTS AND DISCUSSION**

Although the characterization of collagen X mutations in SMCD clearly demonstrates that it is an important component in cartilage development and growth, the precise molecular role of type X in the hypertrophic cartilage extracellular matrix remains elusive (12). Detailed ultrastructural analysis of cartilage from CD10a1-null mice revealed subtle changes in the normal distribution of proteoglycans and cartilage matrix vesicles in the growth plate, suggesting that the collagen X matrix plays a role in the organization, extracellular matrix structure during remodeling and mineralization (18). The molecular interactions that initiate collagen X assembly and facilitate the formation of the functional extracellular assemblies are not yet well characterized. The definition of the effects of SMCD mutations on protein assembly in vitro has provided information...
FIG. 1. Schematic representation of the protein products of normal and mutant human collagen X cDNA constructs. The signal peptide (open box), the amino-terminal globular domain (NC2), the triple helical domain (COL1), and the conserved carboxyl-terminal domain (NC1) of the normal (wt) collagen X protein product are shown diagrammatically. Helix A is an in-frame collagen X helix deletion of 283 amino acids of the COL1 (10). Site-directed mutations were created in the NC1 domain (see Experimental Procedures), and the expected mutant protein products are shown relative to the normal (wt) precursor chain. Arrows indicate the position of the two amino acid substitutions, Y598D and G618V (9, 10). Constructs labeled ter@666, ter@650, ter@632, and ter@610 are nonsense mutations that sequentially remove portions of region C, terminating the NC1 at amino acids 666, 650, 632, and 610, respectively. Three large in-frame NC1 deletions were also produced removing region A (ΔA, amino acids 525–584), region B (ΔB, amino acids 589–601), and region C (ΔC, amino acids 605–667). Region B represents the conserved aromatic motif described by Brass et al. (7). Three regions with localized missense mutations in patients with SMCD are numbered B, c1, and c2.

on homotrimer assembly and identified the NC1 domain to be critical for the initiation of assembly (9, 10), but the precise motifs within the NC1 domain that drive this process are not known. Furthermore, because SMCD is a heterozygous dominant disorder, important information is also missing about the effect of mutant collagen X expression on the assembly and function of the normal collagen X expressed in the growth plate cartilage.

To explore the consequences of NC1 mutations on collagen X homotrimer assembly and assembly with normal chains to form heterotrimers, a range of SMCD mutations was studied by co-expression and assembly in vitro. In addition to point mutations G618V and Y598D (9, 10), which are likely to be expressed at the protein level in vivo, mutations were generated, that resulted in the in vitro production of truncated α1(X) chains terminating at amino acids 610, 650, and 666. In conjunction with a previously identified nonsense mutation, ter@632 (14), these termination mutations allowed us to explore the possible effect of sequentially truncated proteins on collagen X assembly to address whether NC1 microdomains important for assembly are identified by the concentration of SMCD amino acid substitution mutations at residues 589–601, 614–618, and 644–652 (Fig. 1).

To study the contribution of other NC1 domains in assembly, three in-frame deletions (ΔA, ΔB, and ΔC) were produced (Fig. 1). Region A (amino acids 525–584) represents a variable region that differs between species (19), in which no mutations have been identified. Regions B (amino acids 589–601) and C (amino acids 604–667) are conserved regions in the NC1 domain containing SMCD mutations. Region B also corresponds to the conserved 13-amino acid aromatic motif proposed by Brass et al. (7) to have functional importance in trimer association.

In vitro transcription and cell-free translation demonstrated that the mutant plasmid constructs generated in the current study (ter@610, ter@650, ter@666, ΔA, ΔB, and ΔC) were translated into α1(X) chains of molecular weight consistent with the introduced premature terminations or deletions when compared with the normal (wt) α1(X) chains (Fig. 2). In the presence of canine microsomal membranes, translocation of pre-α1(X) chains into the microsomes was demonstrated by the removal of the signal peptide, which resulted in smaller α1(X) chains for wt α1(X) chains and helix A α1(X) chains (Fig. 2) and for all the mutant α1(X) chains (data not shown). This efficient translocation of normal and several SMCD α1(X) mutations (Y598D, G618V, Y632X, 1952delC, and 1963del10) has been demonstrated previously (10). Under these conditions the wt α1(X) chains and helix A α1(X) chains containing normal NC1 domains assembled efficiently into heterotrimers that were electrophoretically stable (Fig. 2) (9, 10). In contrast, all the α1(X) chains containing NC1 mutations (Y598D, G618V, Y632X, ter@666, ter@632, ter@650, ter@666, ΔA, ΔB, and ΔC) did not form electrophoretically stable mutant heterotrimers (Fig. 2). In addition, co-translational expression studies also demonstrated that all these NC1 mutant chains also did not associate stably with chains containing a normal NC1 domain to form heterotrimers that were detectable electrophoretically (Figs. 5–7). To determine whether the relatively harsh denaturing conditions of the preparation for electrophoresis (2% SDS and 2 M urea)
masked the detection of mutant \( \alpha 1(X) \) assembly, the analyses were repeated using sample loading buffer containing 0.5% SDS and no urea under nonreducing conditions. Care was taken to ensure that the samples were kept at room temperature or below during preparation and electrophoresis. Even under these mild sample preparation conditions no electrophoretically stable mutant homotrimer or mutant/normal heterotrimer formation was detected (data not shown). These results are also consistent with previous translation experiments carried out with other SMCD NC1 mutant \( \alpha 1(X) \) chains (1952delC and 1963del10) (10).

Although these data suggested that the likely molecular defect in SMCD is a reduction of collagen X because of the inability of the mutant chain to assemble, it is possible that some NC1 mutations may allow mutant/normal heterotrimer assembly (10) or through weak and/or transient interactions interfere with the efficiency of normal \( \alpha 1(X) \) assembly and thus exert a dominant negative effect on collagen X assembly. To address this issue, the \textit{in vitro} expression and assembly system was developed further to determine whether any measurable interaction occurred between normal and NC1 mutant \( \alpha 1(X) \) chains by determining if the presence of mutant chains altered the ability of chains containing normal NC1 domains to trimerize. In these studies the accurate measurement of the extent of normal NC1 trimerization is critically dependent on the electrophoretic discrimination of the mutant chains from \( \alpha 1(X) \) chains with normal NC1 domains. For the truncation mutations this could be achieved by co-translation with full-length wt \( \alpha 1(X) \) as a trimerization reporter. For single amino acid substitutions, co-translation with full-length wt \( \alpha 1(X) \) as a trimerization reporter is not informative as the mutant and wt \( \alpha 1(X) \) chains cannot be electrophoretically resolved. For these mutations, an \( \alpha 1(X) \) with an internal helical deletion (helix\( \Delta \)) was used as a trimerization reporter because it has been shown to form homotrimers with the same efficiency as wt \( \alpha 1(X) \) chains and trimerize with wt \( \alpha 1(X) \) to form heterotrimers that are electrophoretically distinguishable (Fig. 2) (10).

To maximize the detection of NC1 sequence-specific interactions and diminish any possible contribution of collagen helix sequences to the \textit{in vitro} assembly process, the co-assembly experiments were performed using microsomes from pancreatic cells that do not produce collagen. These microsomes are deficient in prolyl hydroxylase, and thus post-translational proline hydroxylation and triple helix formation does not occur (20).

To determine the linear range of \textit{in vitro} \( \alpha 1(X) \) synthesis and assembly, increasing amounts of normal \( \alpha 1(X) \) plasmid were performed in the nonlinear, saturated range of plasmid concentration. The competency of the transcription and translation system to support a linear \( \alpha 1(X) \) synthesis and assembly process was tested in a system containing canine microsomal membranes (see "Experimental Procedures"). The \( [\text{35S}] \) methionine-labeled products were analyzed on a 7.5% SDS-PAGE (panel A), and bands corresponding to the trimers and monomers were quantified after exposing the gel to a storage phosphor screen. The amount of product translated and assembled into the trimer (in arbitrary units (au)) was expressed relative to the amount of plasmid DNA (12.5–150 ng) in each reaction (panel B).
and mutant homotrimers were not secreted from the cells (10). The fate of these mutant chains was not determined, but it was assumed that they were degraded intracellularly by cellular collagen quality control mechanisms, such as those that prevent the secretion of unassembled collagen I in osteogenesis imperfecta (21–23), leading to a collagen X deficiency in the extracellular matrix. Because collagen X with NC1 mutations can interact with normal NC1 in vitro and compromise the efficiency of trimerization of the normal a1(X), it is possible that the functional levels of collagen X expression in SMCD are reduced to less than that predicted from a haploinsufficiency model. Although our data demonstrate that in this SMCD patient (14), the mutant allele is functionally null, and the pathology results from collagen X haploinsufficiency. Thus we would predict that in other cases of SMCD resulting from nonsense mutations, the mutant mRNA would be subjected to a similar degradation process in vivo, leading to collagen X haploinsufficiency. However, the extent of this degradation may be mutant-specific, because there are examples of nonsense mutations leading to only partial mutant degradation (24, 25, 27, 28). As a result some truncated a1(X) expression may occur, raising the possibility of a partial dominant negative phenotype superimposed on the reduced mutant allele expression because of nonsense-mediated mRNA decay. Missense mutations of the NC1 may also result in haploinsufficiency of a1(X) by preventing assembly and targeting the mutant protein for breakdown. However, our data suggest that the missense mutant a1(X) chains also interact with normal

Fig. 4. Co-expression of normal or helixA collagen X and luciferase cDNA. 25 ng of the wt collagen X or helixA collagen X reporter cDNA was co-transcribed and translated with increasing amounts (0–25 ng) of luciferase DNA (see “Experimental Procedures”). The [35S]methionine-labeled products were analyzed by 7.5% SDS-PAGE (panel A), and the identities of the reporter monomer and trimer and luciferase products are indicated. Bands corresponding to the reporter trimers and monomers were quantified after exposing the gel to a storage phosphor screen, and the trimer/monomer ratio was expressed as a percentage of reporter a1(X) trimerization when no luciferase DNA was added to the transcription/translation (panel B).

Fig. 5. Co-expression/interaction analysis of collagen X containing a normal NC1 domain with chains containing single amino acid substitutions in the NC1 domain. 25 ng of the trimer reporter (helixA collagen X) was co-transcribed and translated with increasing amounts (0–25 ng) of mutant (G618V or Y598D) collagen a1(X) cDNA (see “Experimental Procedures”). The [35S]methionine-labeled products were analyzed by 7.5% SDS-PAGE (panel A), and the identity of the helixA reporter monomer and trimer are indicated. Bands corresponding to the trimers and monomers were quantified after exposing the gel to a storage phosphor screen, and the trimer/monomer ratio was expressed as a percentage of reporter a1(X) trimerization when no mutant DNA was added to the transcription/translation (panel B). Each data point is an average of at least four experiments, and the standard error is less than two standard deviations (not shown on the graph).
α1(X) chains and thus may exert a dominant negative effect further reducing the secretion and supramolecular assembly of functional collagen X in the growth plate matrix. Perhaps when the effects of a spectrum of SMCD mutations are evaluated in vivo, the molecular pathology will be comparable with that of collagen I in osteogenesis imperfecta where the defects range from haploinsufficiency in mild dominant forms to dominant negative mutations, which compromise matrix assembly in the more clinically severe forms (23).

Because SMCD mutations compromise collagen X assembly in vitro, the localization of the mutations in the NC1 domain may provide us with important clues on the molecular basis of collagen X association. For example, the three regions in which SMCD amino acid substitutions are localized (Fig. 1) are likely to represent the domains critical for assembly, possibly a series of microinteracting regions involved in a sequential or cooperative multidomain folding and assembly of the collagen X NC1 domain. The nonsense mutation cDNA constructs were used in in vitro expression (where nonsense-mediated mRNA decay does not occur) to produce sequential truncations of the NC1 domain at amino acid residues 610, 632, 650, and 666 to experimentally address the role of these regions in assembly. Specifically, ter@650 and ter@666 preserve all three microregions with localized SMCD amino acid substitutions, whereas ter@632 would preserve the first two of these regions, and ter@610 would only contain the conserved aromatic motif (Fig. 1). Interestingly, all truncated α1(X) chains interfered with wt α1(X) assembly to a similar extent (Fig. 6) within the detection limitations of this competition assay, suggesting that the truncated α1(X) chains retained a common NC1 region responsible for the in vitro interaction. Based on theoretical considerations a conserved aromatic motif (amino acids 589–601) has been proposed as the putative site of NC1-NC1 interaction (7), and this is consistent with our data because the common NC1 sequence in all these truncated α1(X) chains is the aromatic motif.

To directly test the role of the conserved aromatic motif in assembly, a construct was produced where this sequence was deleted from the otherwise normal NC1 (BΔ construct). In vitro assembly studies with the BΔ protein demonstrated that not only was it unable to associate into mutant homotrimers in

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**Fig. 6.** Co-expression/interaction analysis of collagen X containing a normal NC1 domain with chains containing nonsense mutations in the NC1 domain. Collagen X (wt) trimer reporter (25 ng) was co-transcribed and translated with increasing amounts (0–25 ng) of mutant (ter@666, ter@650, ter@632, and ter@610) collagen α1(X) cDNA (see “Experimental Procedures”). These nonsense mutations result in sequential truncations of the NC1 domain from the carboxyl terminus in the protein products expressed in vitro. The [35S]methionine-labeled products were analyzed by 7.5% SDS-PAGE (panel A), and the identities of the wt reporter monomer and trimer are indicated. Bands corresponding to the trimers and monomers were quantified after exposing the gel to a storage phosphor screen, and the trimer/monomer ratio was expressed as a percentage of reporter α1(X) trimerization when no mutant DNA was added to the transcription/translation (panel B). Each data point is an average of at least four experiments, and the standard error is less than two standard deviations (not shown on the graph).

**Fig. 7.** Co-expression/interaction analysis of collagen X containing a normal NC1 domain with chains containing in-frame deletions within the NC1 domain. A trimer reporter (25 ng of wt or helixΔ collagen X cDNA) was co-transcribed and translated with increasing amounts (0–25 ng) of mutant (ΔA, ΔB, or ΔC) collagen α1(X) cDNA (see “Experimental Procedures”). The [35S]methionine-labeled products were analyzed by 7.5% SDS-PAGE (panel A), and the identities of the wt, helixΔ reporter monomers and trimers are indicated. Bands corresponding to the trimers and monomers were quantified after exposing the gel to a storage phosphor screen, and the trimer/monomer ratio was expressed as a percentage of reporter α1(X) trimerization when no mutant DNA was added to the transcription/translation (panel B). Each data point is an average of at least four experiments, and the standard error is less than two standard deviations (not shown on the graph).
vitro (Fig. 2), as was the case with all NC1 mutations, but more importantly it had no effect on the efficiency of normal α1(X) chain assembly (Fig. 7). Thus the removal of this specific NC1 domain completely abrogated the ability of the α1(X) chains to associate in vitro, directly demonstrating the importance of this sequence motif in assembly. In contrast, the more global deletions of the variable region (ΔA), a domain also common among the truncated α1(X) chains, and region C (ΔC), where most of the SMCD mutations are localized, maintained the ability to interact with normal NC1 domains, reducing the efficiency of normal NC1 trimerization in co-translation assembly to a similar extent as the SMCD mutations (Fig. 7).

These studies provide the first biochemical evidence supporting the proposal by Brass et al. (7) that the conserved aromatic motif is critical for assembly, and it seems likely that this region represents the initial point of interaction necessary for the formation of a stable collagen X NC1 trimer. It is of interest that a point mutation in this conserved domain (Y598D) allowed some interaction with normal NC1 domains (Fig. 5), although the strength of the interaction was dramatically reduced and no electrophoretically stable trimers were formed. This ability to interact transiently or weakly with normal NC1 appears to be the common observation for all SMCD mutations studied, suggesting that the mutations disrupted or removed microinteracting regions critical in the folding or alignment of discontinuous motifs into a functional unit. Similarly, our data also suggest that other regions of the NC1, including the variable region (Fig. 1, region A1), participate in the final interaction or perhaps allow the NC1 to attain a three-dimensional structure necessary for productive interactions. Based on these studies we propose that the assembly events of type X collagen may perhaps allow the NC1 to attain a three-dimensional structure necessary for productive interactions in experimental systems, which more closely recreate the in vitro cellular environment, will be required before the molecular pathology of the disease can be fully explained.

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REFERENCES
1. Kielty, C. M., Hopkinson, I., and Grant, M. E. (1993) in Connective Tissue and Its Heritable Disorders. Molecular, Genetic, and Medical Aspects (Royce, P. M., and Steinmann, B., eds) pp. 103–147, Wiley-Liss, Inc., New York
2. Bateman, J. F., Lamande, S. R., and Ramshaw, J. A. M. (1996) in Extracellular Matrix: Molecular Components and Interactions (Comper, W. D., ed) pp. 23–67, Harwood Academic Publishers, Amsterdam
3. Kielty, C. M., Kwan, A. P., Holmes, D. F., Schor, S. L., and Grant, M. E. (1985) Biochem. J. 227, 545–554
4. Agte, S. S., and Olsen, B. R. (1993) Matrix 13, 165–179
5. Schmid, T. M., and Linsenmayer, T. F. (1983) J. Biol. Chem. 258, 9504–9509
6. Nielson, L. G., and Rochester, C. T., Jr. (1993) J. Biol. Chem. 268, 9402–9407
7. Brass, A., Kudler, K. E., Thomas, J. T., Grant, M. E., and Boot-Handford, R. P. (1992) FEBS Lett. 303, 126–128
8. Barber, R. E., and Kwan, A. P. (1996) Biochem. J. 320, 479–485
9. Chan, D., Cole, W. G., Rogers, J. G., and Bateman, J. F. (1995) J. Biol. Chem. 270, 4558–4562
10. Chan, D., Weng, Y. M., Hocking, A. M., Golub, S., McQuillan, D. J., and Bateman, J. F. (1996) J. Biol. Chem. 271, 15366–15372
11. Schmid, T. M., and Linsenmayer, T. F. (1984) Biochemistry 23, 553–558
12. Chan, D., and Jacenko, O. (1998) Matrix Biol. 17, 169–184
13. Wallis, G. A., Rash, B., Sykes, B., Bonaventure, J., Maroteaux, P., Zabel, B., Wynne-Davies, R., Grant, M. E., and Boot-Handford, R. P. (1996) J. Med. Genet. 33, 450–457
14. Chan, D., Weng, Y. M., Graham, H. K., Sillence, D. O., and Bateman, J. F. (1998) J. Clin. Invest. 101, 1490–1499
15. McIntosh, I., Abbotti, M. H., and Franchomano, C. A. (1995) Hum. Mutat. 5, 121–125
16. McIntosh, I., Abbotti, M. H., Warman, M. L., Olsen, B. R., and Franchomano, C. A. (1994) Hum. Mol. Genet. 3, 303–307
17. Horton, R. M., Hunt, H. D., Ho, S. N., Pallen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 61–68
18. Kwan, K. M., Pang, M. K., Zhou, S., Cowan, S. K., Kong, R. Y., Pfordte, T., Olsen, B. R., Sillence, D. O., Tam, P. P., and Cheah, K. S. (1997) J. Cell. Biol. 136, 459–471
19. Elima, K., Kerola, I., Rosati, R., Mutasaranta, M., Garofalo, S., Peralta, M., de Crombrugghe, B., and Vuorio, E. (1993) Biochem. J. 299, 247–253
20. Middleton, R. B., and Bulleid, N. J. (1993) Biochem. J. 296, 511–517
21. Bateman, J. F., Mascara, T., Chan, D., and Cole, W. G. (1984) Biochem. J. 217, 103–115
22. Bateman, J. F., Chan, D., Lamande, S., Mascara, T., and Cole, W. G. (1988) Ann. N.Y. Acad. Sci. 543, 95–105
23. Byers, P. H. (1993) in Connective Tissue and Its Heritable Disorders. Molecular, Genetic, and Medical Aspects (Royce, P. M., and Steinmann, B., eds) pp. 317–550, Wiley-Liss, Inc., New York
24. Maquart, L. E. (1995) RNA (N.Y.) 1, 453–465
25. Willing, M. C., Deschenes, S. P., Slavton, R. L., and Roberts, J. E. (1996) Am. J. Hum. Genet. 59, 799–809
26. Carter, M. S., Dowsk, J., Morris, P. L., Shi, N., Schor, S. L., and Wilkinson, M. F. (1995) J. Biol. Chem. 270, 28995–29003
27. Zhang, Z. X., Wakamatsu, N., Mules, E. H., Thomas, G. H., and Gravel, R. A. (1994) Hum. Mol. Genet. 3, 139–145
28. Masahiro, Y., Murakami, A., Weeler, R. G., Kennaugh, N. W., Clark, L., Shiono, T., and Inanaga, G. (1992) Am. J. Hum. Genet. 51, 81–91
29. Lees, J. F., and Bulleid, N. J. (1994) J. Biol. Chem. 269, 24354–24360
30. Lees, J. F., Tasab, M., and Bulleid, N. J. (1997) EMBO J. 16, 908–916
31. Shapiro, L., and Scherer, P. E. (1998) Cell 90, 315–328
32. Thomas, J. T., Cresswell, C. J., Rash, B., Nicolai, H., Jones, T., Solomon, E., Grant, M. E., and Boot-Handford, R. P. (2001) Biochem. J. 360, 617–623
33. Reichenberger, E., Beier, F., Luvalle, P., Olsen, B. R., von der Mark, K., and Bertling, W. M. (1992) FEBS Lett. 311, 305–310
34. McLaughlin, S. H., and Bulleid, N. J. (1998) Biochem. J. 331, 793–800

3 S. R. Lamande and J. F. Bateman, unpublished data.