Site-directed Mutagenesis of Human Type X Collagen

EXPRESSION OF α1(X) NC1, NC2, AND HELICAL MUTATIONS IN VITRO AND IN TRANSFECTED CELLS*

(Received for publication, January 2, 1996, and in revised form, March 11, 1996)

Danny Chan, Yi Ma Weng, Anne M. Hocking, Sue Golub, David J. McQuillan, and John F. Bateman

From the Orthopaedic Molecular Biology Research Unit, Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Parkville, Victoria 3052, Australia

Type X collagen is a short chain collagen expressed in the hypertrophic zone of calcifying cartilage during skeletal development and bone growth. The α1(X) homotrimer consists of three protein domains, a short triple helix (COL1) flanked by nonhelical amino-terminal (NC2) and carboxyl-terminal (NC1) domains. While mutations of the NC1 domain result in Schmid metaphyseal chondrodysplasia, which suggests a critical role for this protein domain, little biochemical detail is known about type X collagen synthesis, secretion, and the mechanisms of molecular assembly. To study these processes, a range of mutations were produced in human α1(X) cDNA and the biochemical consequences determined by in vitro expression, using T7-driven coupled transcription and translation, and by transient transfection of cells. Three NC1 mutants, which were designed to be analogous to Schmid mutations (1952delC, 1963del10, and Y598D), were unable to assemble into type X collagen homotrimers in vitro, but the mutant chains did not associate with, or interfere with, the efficiency of normal chain assembly in co-translations with a normal construct. Expression in transiently transfected cells confirmed that mutant type X collagen assembly was also compromised in vivo. The mutant chains were not secreted from the cells but did not accumulate intracellularly, suggesting that the unassociated mutant chains were rapidly degraded. In-frame deletions within the helix (amino acid residues 72–354) and the NC2 domain (amino acid residues 21–54) were also produced. In contrast to the NC1 mutations, these mutations did not prevent assembly. Mutant homotrimers and mutant-normal heterotrimer were formed in vitro, and the mutant homotrimers formed in transiently transected cells had assembled into pepsin-stable triple helical molecules which were secreted.

Type X collagen is a “short chain” collagen with a restricted pattern of expression, confined to the terminally differentiated hypertrophic chondrocytes of growth plate cartilage during endochondral bone growth (1–4), fracture repair (5), and as a consequence of joint degeneration in osteoarthritis (6). The type X collagen molecule is a homotrimer of three α1(X) chains encoded by a condensed gene (COL10A1) of three exons, one of which (exon 3) codes for the majority of the polypeptide chain including the entire triple helical domain (7, 8). The α1(X) homotrimer consists of three distinct protein domains. The short triple helical domain, COL1 (amino acids 57–519), containing eight imperfections in the Gly-Y-X triplet repeat sequence is flanked by a small nonhelical globular NC2 domain at the amino terminus (amino acids 19–56) and a larger more conserved nonhelical carboxyl-terminal NC1 domain (amino acids 520–680) (7). However, the specific restricted distribution and transient synthesis of type X collagen has meant that, relative to the more abundant and well studied collagen types, little is known about its synthesis, secretion, and mechanisms of molecular assembly. The chains are synthesized with an amino-terminal signal peptide which is proteolytically removed from the prevα1(X) chains during synthesis (7, 9). This appears to be the only post-translational proteolytic processing event, since both the NC2 and NC1 domains, which are thought to be important for intermolecular interactions and the formation of extracellular supramolecular assemblies, are retained on extracellular type X collagen (10).

Sequence comparisons of the carboxyl-terminal NC1 domain of type X collagen with the C-propeptides of fibrillar collagens demonstrated a conserved cluster of aromatic residues within a 130-amino acid domain with a marked hydrophobicity profile similarity (11). The role of the carboxyl-terminal propeptide domain of interstitial collagens in initiating intracellular α-chain selection, assembly, and helix formation is well established, and thus by analogy the α1(X) NC1 domain is likely to play a similar crucial role in type X assembly. Further evidence for the importance of the NC1 domain comes from studies on patients with Schmid metaphyseal chondrodysplasia (SMCD, MIM 156500), a mild autosomal disorder of the osseous skeleton resulting from cartilage growth plate abnormalities, where a range of type X collagen mutations have now been identified. All of the mutations so far detected are localized in the NC1 domain and include amino acid substitution mutations C591R (12), G595E, Y598H (13), Y598D, L614P (14), N617K (13), G618V (9), L644R and D648G (13); frameshift mutations 1952delC (12), 1952delCC (15), 1952del13 (16), 1963del10 (17), 2004delT (13), and 2088delCT (12); premature termination mutations Y628X and W651X (15).

For one of these NC1 mutations (G618V), in vitro expression was used to demonstrate that the mutation prevented in vitro type X collagen assembly (9), providing direct evidence for the

* This work was supported by grants from the National Health and Medical Research Council of Australia and the Royal Children's Hospital Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Center for Extracellular Matrix Biology, Albert B. Alkek Institute of Biosciences and Technology, Texas A & M University, Houston, TX 77030-3303.

‡ To whom correspondence should be addressed: Dept. of Paediatrics, University of Melbourne, Royal Children's Hospital, Parkville, Victoria 3052, Australia. Fax: 61-3-9345-6668; E-mail: bateman@cryptic.rch.unimelb.edu.au.

2 The abbreviations used are: SMCD, Schmid metaphyseal chondrodysplasia; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; bp, base pair(s); wt, wild type.
Site-directed Mutagenesis of Human Type X Collagen

role of the NC1 domain in assembly. To further study the biochemical role of the NC1 domain, and to address the question whether the common molecular defect in SMCD is compromised type X collagen molecular assembly due to NC1 mutations, we have produced three NC1 mutations, duplicating the spectrum of mutations defined in patients, 1952delC (NC1ΔC), 1963del10 (NC1Δ10), and Y598D, and expressed these in vitro and by transient transfection of cells.

The biochemical consequences of mutations in the type X collagen helix and in the NC2 are also not known since naturally occurring mutations in these protein domains have not yet been defined. Transgenic mice have been generated expressing a chick type X collagen with a helical deletion (18). These express a more severe spondyloepiphyseal dysplasia phenotype which was attributed to the dominant negative effect of the mutant chick α1(X) associated with endogenous mouse α1(X) chains although the association of mutant:normal heterotrimers necessary for this dominant effect was not directly demonstrated. In this study an in-frame helix deletion (helixΔ), removing 283 helical amino acid residues, and a deletion mutation, removing the entire NC2 domain (NC2Δ), were constructed and expressed in vitro and in vivo. These site-directed mutagenesis studies were designed to determine the contribution of the NC1, NC2, and helical domains on type X collagen heterotrimer and on mutant:normal heterotrimers molecular assembly and secretion.

EXPERIMENTAL PROCEDURES

Construction of a Full-length Human Type X Collagen cDNA Containing the 3'-Untranslated Region—The production of a full-length α1(X) cDNA construct, pTM1-h10, in a T7-driven expression vector suitable both for in vitro transcription-translation and transient expression in cells using the vaccinia/T7 phage system (19) has been described previously (9). The 3'-untranslated region and a polyadenylation site was amplified using a reverse transcription-PCR kit (Perkin-Elmer) from oligo(dT)-primed cDNA produced from hypochromic chondrocyte mRNA (9). A 1546-bp fragment was amplified using primers (BX1) 5’-CAGGGGTAAACAGGAATGCC-3’ (1726–1745) and (HX1) 5’-TT-GTGTGACTGAAAACGCTTGA-3’ (3256–3242). An additional sequence containing a Sall restriction enzyme site (sequence underlined) was added to primer HX14 for subsequent cloning purposes. The amplified fragment was digested with Sall and purified by agarose electrophoresis, recovered by electroelution, and cloned into the Sall and SalI site of pUC19. A 1406-bp region of the 3'-untranslated region (2211–3256) was released from a positive clone by digestion with unique restriction enzymes Sall and SalI and cloned into pTM1-h10 (9) to produce a full-length human type X cDNA (pTM1-h10wt) containing the 3'-untranslated region including the first polyadenylation site.

Construction of an In-frame Helix Deletion of Type X Collagen—An in-frame helix deletion, 310delB (helixΔ) was constructed within the COL1 domain in plasmid pTM1-h10wt. Unique restriction sites XhoI and NcoI were utilized to remove a 853-bp fragment. The digested plasmid was purified by agarose electrophoresis, recovered by electroelution, the resultant overhang sequences were filled in with Klenow, and blunt ends were ligated to produce the plasmid pTM1-helixΔ. This procedure removed the amino acid residues 72–354, a total of 283 amino acids within the amino-terminal region of the triple helical domain of human type X collagen (Fig. 1). Within the normal helical domain, there are eight interruptions of the Gly-X-Y inter-ruption resulting from the blunt ligation of codons for Arg19 to His30.

Site-directed Mutagenesis of the NC1 Domain—Three human type X collagen NC1 Schmid metaphyseal chondrodysplasia mutations (Fig. 1) were produced using splicing by overlap extension PCR (20). The mutant primer sets and their relative positions are shown in Table I. Primer set NC1ΔC-A and NC1ΔC-B introduce a single base deletion of cysteine 1952, 1962delC (12); primer set NC1Δ10-A and NC1Δ10-B introduce a 10-base pair deletion, 1963del10 (17); while primer set

2 The base pairs in the COL10A1 coding sequence are numbered from the transcription start site (33).
3 Amino acids are numbered from the translation start site of pre-α1(X) (7).

Y598D-A and Y598D-B introduce a single base substitution of T1086G and changed the amino acid tyrosine 598 to aspartate, Y598D (14). The PCRs were carried out in 20 μl of 10× Tris/HCl, pH 8.0, containing 3 μg MgCl2, 50 μM KCl, 0.2 μM dNTPs, and 0.75 μl of each of the primers. The polymerase chain reactions were carried out in capillaries using the FTS-1 thermal sequencer from Corbett Research (Melbourne, Australia) over 26 cycles. Cycle one was performed at 96°C for 2 min, 62°C for 1 min, and 72°C for 1 min, then followed by 25 cycles at 96°C for 10 s, 62°C for 15 s, and 72°C for 20 s. The reaction was terminated at 72°C for 1 min. Two independent PCR products were first produced using BX1 (sense) with primer B (antisense) and primer A (sense) with HX6 (antisense) of each of the mutant primer sets in the primary round of PCR using pTM1-h10wt (5 ng) as a template. The amplified fragments were purified by agarose electrophoresis and recovered using Genedoc (BIO 101 Inc., Vista, CA) and subjected to a second round of overlapping PCR with primers BX1 and HX6. The recombinant mutant PCR fragments were purified, cloned, and sequenced to ensure that the correct mutations were introduced and that there were no PCR errors. A PflMI and NsiI fragment containing the mutations were first cloned into a genomic type X clone pMC2N (9), and then introduced into pTM1-h10wt via Ncol and NsiI sites.

Construction of an In-frame Deletion of the NC2 Domain—Splicing by overlap extension PCR was also used to produce a NC2 1572del102 mutation (NC2Δ). This deletion removes most of the NC2 domain except the first two amino acids (residues 19 and 20) and the last two amino acids (residues 55 and 56) of the NC2 domain (Fig. 1). The signal peptide (residues 1–18) remains unaltered. The plasmid pTM1-h10wt (5 ng) was used as a template for the primary round of PCR with primer pairs pTM1-1 (sense)/NC2A8 (antisense) and NC2A8 (sense)/HX10 (antisense) (Table I) to generate the two independent fragments with overlapping sequences. The second round PCR reaction was carried out with primers pTM1-1 and HX10 and 5 ng of each of the purified fragments from the primary PCR reactions. The recombinant fragment was purified and cloned into Bluescript SK II (+) and sequenced to confirm the deletion. A 362-bp fragment containing the NC2 deletion was released from the Bluescript SK II (+) by digestion with KpnI and XhoI and ligated to a 957-bp XhoI-KpnI fragment (305–1262) from pTM1-h10wt. After digestion with KpnI, the expected 1319-bp fragment was purified and reintroduced into pTM1-h10wt via KpnI sites.

2 The base pairs in the COL10A1 coding sequence are numbered from the transcription start site (33).
3 Amino acids are numbered from the translation start site of pre-α1(X) (7).

FIG. 1. Schematic representation of the protein products of normal (wt) and mutant human type X collagen cDNA constructs. Site-directed mutations were created in the COL1 and NC1 domains of type X collagen (see "Experimental Procedures" for details). The location of the mutations and the expected protein products are shown relative to the normal (wt) preα1(X) chain. NC1ΔC is a single base deletion of cytosine 1952 introducing a frameshift at amino acid residue 620 and a premature termination at 621; NC1Δ10 is a 10-base pair deletion, introducing a nonsense amino acid sequence (filled box) from residue 623 and a premature termination at 671; Y598D and G618V are amino acid substitutions of tyrosine 598 to aspartate and glycine 618 to valine, respectively; helixΔ is an in-frame helix deletion of 283 amino acids of the COL1 domain, and NC2Δ removes the NC2 domain. The signal peptide (open box) is unaffected in all mutations generated.

ViroTranscription and Translation—Plasmids for cell-free transcription and translation were purified using ethidium bromide/CsCl2 centrifugation. The ethidium bromide was removed by several extractions with isopropanol equilibrated with NaCl-saturated 10× Tris-HCl, pH 8.0, 1.0 mM EDTA and dialyzed against milli-Q water. The plasmids were translated using the TNT T7 polymerase coupled transcription and translation system (Promega) as described previously in a reaction volume of 12.5 μl (9). Routinely, 0.25 μg of plasmid was used per reaction, and 2.5 μl of the final reaction are required for gel electrophoresis. In some experiments, canine microsomal membranes (Promega) were also
Site-directed Mutagenesis of Human Type X Collagen

Normal and mutant PCR primers for site-directed mutagenesis using strand overlap extension PCR

Table I

| Primes | Primer sequence (5′ to 3′) | Primer location |
|--------|----------------------------|-----------------|
| NC1α10-A | GGCACCCTGGTAAAGTGATACACCAAG | 1948-1962, 1974-1990 |
| NC1α10-B | CTTCGGTGATTCATCATTAACAGGCGGCCC | 1990-1974, 1962-1948 |
| NC1αC-A | GTGATGAAAGAGCAGGCCCTGATGAAATCAC | 1937-1952, 1953-1967 |
| NC1αC-B | GTGATCATACAGGCGCCCTTATGACCA | 1967-1953, 1951-1937 |
| Y598D-A | CCAGAAATGACGATTTTTCATACC | 1876-1900 (1888) |
| Y598D-B | GTGATGAAATGACGATTTTTCATACC | 1900-1876 (1888) |
| BX1 | CAGGGGGTAACAGGAACTGCC | 1726-1745 |
| HX6 | CTTTTCAGCCTACCTCCATA | 2235-2216 |
| NC2α-A | GTGCATGGAGGTTGTAAGGGAGGCA | 142-156, 259-273 |
| NC2α-B | TGGCTCTCCTGCTTAACACACCTCCATGAAC | 273-259, 156-142 |
| pTM1-1 | TATAGATACACTGGCAAG | 1711-1190 |
| HX10 | TTTTGTCACTTCCTACCCAG | 660-641 |

In brief, 5 \times 10^5 UMR 106-01, 1.8 \times 10^5 CV1 or 3T3-A31 cells were cultured for 24 h in DMEM containing 10% (v/v) fetal calf serum and 0.25 mM sodium ascorbate. Cells were infected with a T7 polymerase-expressing vaccinia virus vTF7-3 (30 plaque-forming units/cell) for 30 min in 0.5 ml serum-free DMEM, followed by transfection with 2 µg of plasmid which had been complexed with 15 µl of Lipofectamine (Life Technologies, Inc.) for 30 min at room temperature. Transfections were carried out in 1 ml of serum-free DMEM for 6 h at 37°C in a 5% CO₂ incubator. The transfection medium was removed and replaced with 1.0 ml of serum-free DMEM, containing 0.25 mM sodium ascorbate and 10 µCi of [3H]-proline and incubated for 8 or 18 h at 37°C. At the appropriate time point, the medium fractions were removed, and the cell layers were scraped into 1 ml of 50 mM Tris/HCl, pH 7.5, containing 0.15 mM NaCl, 5 mM EDTA, 10 mM N-ethylmaleimide, and 0.1 mM phenylmethylsulfonyl fluoride. The cells were lysed by repeated freezing and thawing. The labeled proteins were recovered from both the medium and cell fractions by 75% (v/v) ethanol precipitation and analyzed by 7.5% (v/v) SDS-polyacrylamide gel electrophoresis. In some experiments, type X collagen was detected by Western blotting and probed with a type X collagen specific antibody (23), generously provided by Dr. Gary Gibson (Henry Ford Hospital, Detroit, MI).

RESULTS

In Vitro Transcription and Cell-Free Translation of Normal and Mutant Plasmids—In vitro transcription and cell-free translation demonstrated that all the mutant plasmids were translated into preα1(X) chains of molecular weight consistent with the introduced mutations (Fig. 2, lanes 1-6). While no molecular weight change was observed for the Y598D amino acid substitution (Fig. 2, lane 4), both the single base deletion of cysteine 1952 (NC1αC) and the 10-base pair deletion (NC1α10) of the NC1 domain resulted in shortened preα1(X) chains (Fig. 2, lanes 2 and 3) when compared with the normal (wt) preα1(X) chain of 680 amino acids (Fig. 2, lane 1). Both of these mutations produced codon frame shifts causing premature chain terminations at amino acid 621 for NC1αC and 673 for NC1α10. Similarly, the electrophoretic migrations of proteins translated from the in-frame deletion mutations, helix A and NC2α, were consistent with the expected sizes of 397 and 646 amino acids, respectively (Fig. 2, lanes 5 and 6).

Analysis of the Nonhelical NC1 and NC2 Domains by Bacterial Collagenase Digestion—The site-directed mutations in the NC1 and NC2 domains were confirmed by the analysis of these domains following bacterial collagenase digestion of preα1(X) chains synthesized in the absence of microsomes. The deletion of the NC2 domain in the NC1α10 mutant was clearly demonstrated by its absence in the collagenase digest (Fig. 3, lane 6). The NC1 domains from the NC1αC and NC1α10 mutants were also markedly reduced in size as expected (Fig. 3, lanes 2 and 3), whereas the electrophoretic migrations of the NC1 and NC2 domains from the Y598D and helix A mutants were unaffected (Fig. 3, lanes 1 and 5).
While the preα1(X) chains produced do not spontaneously assemble into multimers during in vitro translation, assembly could be induced by incubation in the Ca^{2+}-containing buffer used for collagenase digestion (9). A significant proportion of the wt α1(X), helixΔ α1(X), and NC2α α1(X) chains could be induced to assemble into multimers, as evidenced by the presence of multimers of the collagenase-released NC1 domains (Fig. 3, lanes 4–6), however, the mutant NC1 domains were present only as monomers (lanes 1–3). This is consistent with our previous report which demonstrated that a NC1 G618V mutation prevented in vitro multimer assembly (9).

In Vitro Chain Assembly Induced by Translation with Microsomes—The ability of the mutant type X collagen chains to associate into trimers in vitro was studied further by translation in the presence of canine microsomal membranes. Translation of the normal and mutant preα1(X) chains into the microsomes was demonstrated by the removal of the signal peptide which resulted in the smaller α1(X) chains for all the plasmids translated (Fig. 2, lanes 7–12). As shown in previous studies (9), under these translation conditions the normal human type X collagen associated into a trimeric component (Fig. 2, lane 11). The helixΔ α1(X) and NC2Δ α1(X) chains also formed trimers in vitro (Fig. 2, lanes 12 and 7), whereas the NC1 mutants, Y598D (Fig. 2, lane 8), NC1Δ10 (Fig. 2, lane 10), and NC1ΔC (Fig. 2, lane 9) were unable to form trimers in vitro.

An important functional consideration is whether the mutant α1(X) chains are able to associate with normal α1(X) into heterotrimers. To determine this, normal and mutant plasmids were co-transcribed and translated in the presence of microsomes. Co-translation of the normal transcript and the helixΔ transcript, which contained a helical deletion but a normal NC1 domain, showed that in addition to the trimeric components of each product, two additional multimer bands labeled αa and βb were also observed (Fig. 4, lane 1). The relative electrophoretic migrations of these suggested that band a represented heterotrimers containing two normal α1(X) chains and one helixΔ α1(X) chain, while band b was the heterotrimer containing one normal and two helixΔ α1(X) chains. The efficient formation of helixΔ heterotrimers with the full-length normal α1(X) chains demonstrated an important practical use of the helixΔ construct as a protein reporter to assess the ability of the mutants to assemble into heterotrimers in further co-translation experiments. If the mutants can assemble with the helixΔ chains, which are shorter but contain a normal NC1 domain, the stoichiometry of these heterotrimers can be readily determined by assessing the electrophoretic migration of the multimers.

Co-expression of the helixΔ α1(X) reporter construct and the NC2Δ α1(X) construct (Fig. 4, lane 8) showed that the NC2Δ mutation allowed assembly of the mutant chain into homotrimers and heterotrimers (lanes 8, labeled as c and d, respectively). Molecular weight analysis again indicated that band c represented heterotrimers containing two NC2α and one helixΔ chains, while band d was a heterotrimer containing one NC2Δ and two helixΔ chains.

In contrast, co-translation of normal α1(X) (wt) with NC1ΔC or NC1Δ10 transcripts showed no evidence of any heterotrimer formation (Fig. 4, lanes 4 and 5). Similarly, no heterotrimers were observed when these two NC1 mutant transcripts were co-translated with helixΔ (Fig. 4, lanes 2 and 3). Mutant transcripts with single amino acid substitutions within the NC1 domain (G618V, Y598D) were also co-translated with the helixΔ reporter to determine if association of normal and mutant NC1 domains occurred in vitro (Fig. 4, lanes 6 and 7). The predominant multimeric component in each co-translation was the helixΔ α1(X) trimer and the two mutants were unable to form homotrimers in vitro. However, a faint band corresponding to species b (Fig. 4, lane 1) was evident with both the G618V and Y598D (Fig. 4, lanes 6 and 7) and was better demonstrated in an overexposure (Fig. 4, lanes 9 and 10). This species corresponds to a mutant:normal heterotrimer containing one mutant NC1 α1(X) chain and two reporter helixΔ α1(X) chains. This species was a very minor product (<1%), demonstrating that heterotrimer formation was extremely inefficient. There was no evidence of trimers containing two or three mutant chains.

Transient Expression of Normal and Mutant Type X Collagen in Transfected Cells—Transient transfection of the normal (wt), helixΔ and NC2Δ plasmids into UMR 106-01, which produce no endogenous type X collagen, demonstrated that these
Site-directed Mutagenesis of Human Type X Collagen

Fig. 5. Expression of normal, helix A, and NC2Δ type X collagen constructs in transiently transfected UMR 106-01 cells. Transient transfections and biosynthetic labeling was performed as described under "Experimental Procedures." [3H]Proline-labeled collagens in the cell (C) and medium (M) fractions were analyzed on a 7.5% (w/v) SDS-polyacrylamide gel (panel b). The samples were denatured at 60 °C (panels a and c) or at 100 °C (panel b) for 10 min prior to electrophoresis. Collagens in the medium fraction were also analyzed after limited pepsin digestion as described under "Experimental Procedures" (panel c). The pepsinized forms of the α1(X) and helix A α1(X), α1(I), and helix A α1(X)p, and the α1(I) and α2(I) chains of type I collagen are indicated. UT designates the lane containing the labeled products produced by untransfected cells.

Fig. 6. Expression of G618V, NC1ΔC, NC1ΔC, and Y598D type X collagen constructs in transiently transfected UMR 106-01 cells. [3H]Proline-labeled collagens in the cell (C) and medium (M) fractions were analyzed on a 7.5% (w/v) SDS-polyacrylamide gel (panel b). The samples were denatured at 60 °C for 10 min prior to electrophoresis. The samples were also analyzed after limited pepsin digestion as described under "Experimental Procedures" (panel c). The arrows in panel b correspond to the positions of the expected mutant type X collagen products. The position of the expected pepsinized type X α1-chains (α1(X)p) in panel c is also indicated. Panel a is an immunoblot of the samples from panel b probed with a type X collagen-specific antibody. The migration positions of the control and mutant α1(X) chains, pepsinized products α1(X)p, and the α1(I) and α2(I) chains of type I collagen are indicated.

A very different pattern emerged when the plasmids containing the NC1 mutations (G618V, Y598D, NC1ΔC, and NC1Δ10) were transiently expressed in UMR 106-01 cells (Fig. 6). The level of expression of the mutant proteins was consistently lower (Fig. 6, panel b) than the control, helix A, and NC1ΔC α1(X) chains expressed under identical conditions in the same cells (Fig. 5). The faint mutant α1(X) chain monomeric components were difficult to distinguish from other protein bands within the cell fraction (Fig. 6, panel b) but were identified by immunoblotting (Fig. 6, panel a). The most striking observation was the absence of type X collagen in the medium fraction (Fig. 6, panel a), indicating that the mutant collagen was not secreted by the UMR 106-01 cells. The intracellular mutant α1(X) chains were not resistant to pepsin digestion (Fig. 6, panel c) demonstrating that they were unable to form correctly assembled triple helical type X collagen. Intracellular mutant chains were more evident in short term labeling of the transfected UMR 106-01 cells. Extension of the labeling period from 8 to 18 h resulted in a decrease in the amount of mutant α1(X) chain detected, suggesting that the unassembled NC1 mutant α1(X) chains were unstable and degraded intracellularly (data not shown).

The defective assembly and secretion of the NC1 mutant α1(X) was best demonstrated in a transfection of CV1 cells with the normal and G618V mutant α1(X) (Fig. 7), and confirmed the results obtained in transfections of UMR 106-01 cells. The normal (wt) chains were fully assembled into trimers intracellularly, more than half of which were secreted into the medium during the labeling period (Fig. 7, panel a). Pepsin digestion demonstrated that the secreted type X trimer was stable to pepsin digestion and thus triple helical (Fig. 7, panel b). However, pepsin digestion of the intracellular trimeric type X collagen revealed that only a portion of this assembled collagen was helical. This may reflect delayed helix formation in these cells due to inadequate machinery to deal with the high levels of α1(X) from T7-driven cytoplasmic overexpression, or it may result from inherent differences between the rates of NC1-driven assembly and subsequent helix formation of type X collagen. The expression of the G618V mutant in CV1 demonstrated that the mutant α1(X) chains were present as unassembled monomeric chains which were not secreted. However, pepsin digestion of cell and medium samples from transfected CV1 (Fig. 7, panel b) resulted in the detection of a very faint pepsin-resistant band in the cell fraction, with similar molecular weight to the normal chain, indicating that in this experiment a very small proportion of the intracellular G618V chains assembled into stable helical molecules. In other transfections of UMR 106-01 cells, overexposure of the fluorograms demonstrated the Y598D and NC1Δ10 mutant chains also form a minute amount of pepsin stable molecules, but this was even less efficient than the G618V mutant. No pepsin-resistant components were detected for the NC1ΔC chains in any transfection experiments (data not shown).

While these data clearly demonstrated that in multiple experiments the assembly and secretion of the NC1 α1(X) was severely compromised in transiently transfected cells, some assembly and secretion was observed in one experiment. UMR
controversial. To gain insight into the protein domains which the hypertrophic chondrocyte extracellular matrix remains Xcollagenbiosynthesisandassembly,and its functional role in transiently transfected CV1 cells.

![Image](75x590 to 278x732)

**FIG. 7.** Expression of the G618V construct type X collagen in transiently transfected CV1 cells. [3H]Proline-labeled collagens in the cell (C) and medium (M) fractions were analyzed on a 7.5% (w/v) SDS/polyacrylamide gel (panel a). The samples were denatured at 60 °C for 10 min prior to electrophoresis. Collagens were also analyzed after limited pepsin digestion as described under “Experimental Procedures” (panel b). The migration positions of the control and mutant α1(X) chains, pepsinized products α1(X)α1(X)α1(X)α1(X), are indicated. UT designates the labeled products from untransfected cells. An uncharacterized pepsin-resistant band present in untransfected and transfected CV1 cells is indicated (I).

106-01 cells transiently transfected with NC1 mutants secreted trace amounts of pepsin resistant α1(X) G618V chains, and even smaller amounts of α1(X)Y598D and α1(X)NC1Δ10, after 18 h of labeling. There was no evidence of any secretion of the NC1ΔC mutant chains (data not shown).

**DISCUSSION**

Despite intense interest, relatively little is known about type X collagen biosynthesis and assembly, and its functional role in the hypertrophic chondrocyte extracellular matrix remains controversial. To gain insight into the protein domains which control type X collagen trimer assembly and helix formation, mutations were produced in the α1(X) noncollagenous amino-terminal NC2 domain, the carboxy-terminal NC1 domain, and the collagenous triple helical COL1 domain using overlap extension PCR. To assess the effects of these mutations on type X collagen assembly and helix formation, the mutant constructs were expressed in vitro using T7-driven coupled transcription and translation, and in transiently transfected cells.

Consistent with our previous finding of a NC1 G618V substitution in a patient with SMCD (9), the three additional mutations of the NC1 domain which represent the spectrum of naturally occurring mutations in SMCD, similarly prevented the in vitro assembly of the mutant α1(X) chains into trimers. While no detectable homotrimer was evident from these NC1 mutants in vitro, it was of considerable importance to test whether the mutant chains can associate with normal NC1 domains and interfere with normal chain assembly, since the analogous NC1 mutations in SMCD are heterozygous. To test this, an α1(X) protein reporter chain construct was produced which contained a normal NC1 domain but an in-frame helix deletion (helixΔ) allowing discrimination of heterotrimers containing the reporter by a molecular weight shift on electrophoresis. Assembly analysis of these chains with the helical deletion (helixΔ) have resulted in a number of important observations. First, the efficient association of the helixΔ chains into trimers during cell-free translation further demonstrates the crucial role of the NC1 domain in this in vitro assembly process which appears to be unaffected by the partial removal of helical sequences. Co-translation with normal α1(X) chains resulted in the formation of helixΔ α1(X) and normal α1(X) heterotrimers of all possible chain configurations. The ability of the helixΔ chain to freely associate with normal type X collagen α1(X) chain suggests that heterozygous helical mutations would result in the generation of aberrant type X collagen heterotrimers and exert a “dominant negative” effect in vivo, with the biochemical consequences of increased intracellular degradation and matrix disruption similar to that seen with type I collagen helix mutations in osteogenesis imperfecta (24).

This suggestion is supported by results from a transgenic mouse model heterozygous for a deletion in the type X collagen helical domain (18). The transgenic mice expressed a more severe clinical phenotype with skeletal deformities consistent with a spondyloepiphyseal dysplasia phenotype.

Co-expression of the reporter helixΔ and the NC1 mutant constructs demonstrated that the NC1 mutations prevented in vitro heterotrimer formation. The data indicated that the NC1 deletion mutations, which either truncated (NC1ΔC) or added abnormal carboxy-terminal sequences (NC1Δ10), totally prevented heterotrimer assembly. On the other hand, point mutations allowed the incorporation, albeit inefficiently, of one mutant NC1 chain into the trimeric assembly with two normal NC1 domains. These results suggest that amino acid substitution mutations compromise assembly by introducing more subtle changes to NC1 protein structure which can be partially accommodated in trimeric assemblies containing two normal NC1 domains.

While the experiments expressing the protein by transcription and translation provided important information on the effect of these NC1 mutations on assembly in vitro, cellular collagen assembly mechanisms are infinitely more complex, with coordinated intracellular post-translational processing as well as possible interactions with accessory proteins which may regulate both normal assembly and secretion and the fate of the mutant α1(X) chains. To address these problems, normal and mutant α1(X) was expressed in transiently transfected cells. Expression studies in UMR 106-01 cells, a collagen-producing osteoblast-like cell line, indicated that chains with normal NC1 domains (wt α1(X), helixΔ α1(X), and NC2Δ α1(X)) can be assembled and secreted efficiently by the cells. The formation of pepsin resistant components indicated that these assembled trimers were post-translationally hydroxylated and assembled into stable triple helical molecules (25).

Transient expression of the mutant NC1 plasmids in transfected cells confirmed the results of the in vitro expression and assembly experiments, demonstrating that all the NC1 α1(X) mutations resulted in a biochemical phenotype where assembly and secretion were severely compromised. This result was consistent in collagen-producing cells (UMR 106-01 and 3T3-A31) and cells which produce little or no collagen (CV1). The NC1 region of type X collagen contains a sequence domain of approximately 130 amino acid residues including a conserved cluster of aromatic acids which has a hydrophobicity profile of marked similarity to that of the carboxy-terminal propeptides of the fibrillar collagens. Mutations in the NC1 domain which disturb this aromatic cluster, or change NC1 protein folding such that hydrophobic surface interactions are impaired, would be expected to result in the observed failure to trimerize.

The mutant NC1 collagen retained within the cell was in an unassembled, pepsin-sensitive form. Taken together with the consistently lower levels of protein expression seen with the mutant constructs compared to control construct transfected in parallel, these results suggest that the mutant collagen is unstable and rapidly degraded intracellularly. Such an intracellular degradative “quality control” mechanism for type I collagen with structural mutations has been described (26–28). While the vast majority of the NC1 mutant α1(X) chains were retained within the cell and degraded, in one experiment a trace level of mutant assembly and secretion was detected.
While these preliminary studies are not totally conclusive, the data suggest that there may be subtle biochemical heteroge-
neities in NC1 mutations. In general, amino acid substitutions in the NC1 domain showed a limited ability to assemble,
whereas mutations causing frameshifts and premature termina-
tions had a more severe effect on the protein-protein inter-
actions involved in assembly. These results have possible im-
lications for the molecular pathology of type X collagen muta-
tions and begin to address the apparent contradictions be-
tween the phenotypes of the type X null mice which have little or no abnormal skeletal phenotype (29) and the NC1
mutations that result in SMCD. Previous studies have suggested that the SMCD mutations completely prevent as-
sembly and exclude the mutant allele a1(X) product leading to
a haploinsufficiency (9), at apparent odds to the reported find-
ings in the type X null transgenic mice. The data presented
here suggest that while a1(X) haploinsufficiency due to com-
promised assembly, secretion, and intracellular breakdown is
likely to be the major defect in SMCD, it is possible that some
NC1 mutations may result in the assembly of trace amounts
of mutant: normal heterotrimers and/or mutant: mutant homotri-
mers which may be secreted and further disrupt the type X
collagen matrix.

These transient transfection studies provide the first direct
information on how cells handle mutant type X collagen. It is
important, however, to recognize some possible limitations of
the vaccinia-based transfection protocol. Vaccinia virus infec-
tion leads to cell death, and it is possible that during these
expression studies some cell lysis may have occurred resulting
in the unphysiological release of some type X collagen. To
decipher this important point and determine if mutant collagens
are secreted from the cells, it is crucial that these cells are
stably transfected with the mutant constructs and that assem-
by and secretion be studied in detailed kinetic experiments.

The role of the a1(X) amino-terminal NC2 domain in assem-
bling was also explored by site-directed mutagenesis. Removal of
the NC2 domain did not prevent NC2a homotrimer formation
or affect the ability of the mutant chains to associate with
normal a1(X) into heterotrimers in cell-free translations or into
heterotrimers in transiently transfected cells. While NC2a mu-
tant containing heterotrimers were secreted from the cells, the
efficiency of this will need to be accurately quantified in pulse-
chase experiments of stable transfectants to determine if this
domain plays a subtle role in regulating assembly and the
efficiency of secretion. Recent data suggest that type X collagen
can form several supramolecular assemblies, either as fine
pericellular filaments, in association with type II collagen
fibrils (30), or as a hexagonal lattice (10). The NC2 domain is
likely to play an important role in the formation and stabili-
zation of these structures, and mutations of the NC2 could be
expected to have a severe effect on the structure and stability of
these assemblies. It has also been suggested that the hexagonal
lattice is stabilized, in part, by lateral overlapping of the type X
collagen helical domains (10), and helical mutations should
thus also compromise these supramolecular structures.

REFERENCES

1. Apte, S. S., and Olsen, B. R. (1993) Matrix 13, 165–179
2. Schmid, T. M., and Linsenmayer, T. F. (1983) J. Biol. Chem. 258, 9504–9509
3. Kielty, C. M., Kwan, A. P. L., Holmes, D. F., Schor, S. L., and Grant, M. E.
(1985) Biochem. J. 227, 545–554
4. Nerlich, A. G., Kirsch, T., Wiest, I., Betz, P., and von der Mark, K. (1992)
263, 275–281
5. Grant, W. T., Wang, G. J., and Ballian, G. (1987) J. Biol. Chem. 262, 9844–9849
6. Reichenberger, E., Aigner, T., von der Mark, K., Stoss, H., and Bertling, W.
(1991) Dev. Biol. 146, 562–572
7. Thomas, J. T., Cresswell, C. J., Rash, B., Nicolai, H., Jones, T., Solomon, E.,
Grant, M. E., and Bood-Handford, R. P. (1991) Biochem. J. 280, 617–623
8. LuValle, P., Ninomiya, Y., Rosenblum, N. D., and Olsen, B. R. (1986) J. Biol.
Chem. 263, 18378–18385
9. Chan, D., Cape, W. G., Rogers, J. G., and Bateman, J. F. (1995) J. Biol. Chem.
270, 4558–4562
10. Kwan, A. P. L., Cummings, C. E., Chapman, J. A., and Grant, M. E. (1991) J.
Cell Biol. 114, 597–604
11. Brass, A., Kadler, K. E., Thomas, J. T., Grant, M. E., and Bood-Handford, R. P.
(1992) FEBS Lett. 303, 126–128
12. McIntosh, I., Abbott, M. H., Warman, M. L., Olsen, B. R., and Francomano, C.
A. (1994) Hum. Mol. Genet. 3, 303–307
13. Bonaventure, J., Channinade, P., and Maroteaux, P. (1995) Hum. Genet. 96, 58–64
14. Wallis, G. A., Rash, B., Sweetman, W. A., Thomas, J. T., Super, M., Evans, G.,
Grant, M. E., and Bood-Handford, R. P. (1994) Am. J. Hum. Genet. 54, 169–178
15. McIntosh, I., Abbott, M. H., and Francomano, C. A. (1995) Hum. Mutat. 5, 121–125
16. McIntosh, I., Hamosh, A., and Dietz, H. C. (1990) Nat. Genet. 4, 219
17. Dhamanavaram, R. M., Elbersen, M. A., Peng, M., Kirson, L. A., Kelley, T. E.,
and Jimenéz, S. A. (1994) Hum. Mol. Genet. 3, 507–509
18. Jacono, O., LuValle, P. A., and Olsen, B. R. (1993) Nature 365, 56–61
19. Rich, D. P., Anderson, M. P., Gregory, R. J., Cheng, S. H., Paul, S., Jefferson, R. A.,
McCann, J. D., Klinger, K. W., Smith, A. E., and Welsh, M. J. (1990) Nature 347, 358–363
20. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989)
Gene (Amst.) 77, 61–68
21. Middleton, R. B., and Bulleid, N. J. (1993) Biochem. J. 296, 511–517
22. Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986) Proc. Natl. Acad.
Sci. U. S. A. 83, 8122–8126
23. Chan, D., Cape, W. G., Chow, C. W., Mundlos, S., and Bateman, J. F. (1995) J.
Biochem. 270, 1747–1753
24. Byers, P. H. (1993) In Connective Tissue and Its Heritable Disorders. Molecular,
Genetic, and Medical Aspects (Royce, P. M., and Steinmann, B., eds) pp.
317–350, Wiley-Liss, New York
25. Berg, R. A., and Prockop, D. J. (1973) Biochem. Biophys. Res. Commun. 52, 115–120
26. Bateman, J. F., Mascara, T., Chan, D., and Cape, W. G. (1984) Biochem. J. 217, 103–115
27. Chessor, S. D., and Byers, P. H. (1993) J. Biol. Chem. 268, 18226–18233
28. Lameande, S. R., Chessor, S. D., Golub, S. B., Byers, P. H., Chan, D., Cole, W.
G., Silence, D. O., and Bateman, J. F. (1995) J. Biol. Chem. 270, 8642–8649
29. Rosati, R., Horan, G. S. B., Piner, G. J., Garfola, S., Keene, D. R., Horton, W.
A., Vuorio, E., de Crombrugghe, B., and Behringer, R. R. (1994) Nat. Genet. 5, 129–135
30. Schmid, T. M., and Linsenmayer, T. F. (1990) Dev. Biol. 138, 53–62
31. Reichenberger, E., Beier, F., LuValle, P., Olsen, B. R., von der Mark, K., and
Bertling, W. M. (1992) FEBS Lett. 311, 305–310