The Globular Domain of the Proα1(I) N-Propeptide Is Not Required for Secretion, Processing by Procollagen N-Proteinase, or Fibrillogenesis of Type I Collagen in Mice*

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The globular domain in the NH2-terminal propeptide (N-propeptide) of the proα1(I) chain is largely encoded by exon 2 of the Col1a1 gene and has been implicated in a number of processes that are involved in the biogenesis, maturation, and function of type I collagen. These include intracellular chain association, transcellular transport and secretion, proteolytic processing of the precursor, feedback regulation of synthesis, and control of fibrillogenesis. However, none of these proposed functions has been firmly established. To evaluate the function of this procollegen domain we have used a targeted mutagenesis approach to generate mice that lack exon 2 in the Col1a1 gene. Mouse lines were established on both a mixed 129 OlaHsd/Sv and C57BL/6 background and a pure 129 OlaHsd/Sv background. Adult mice on the mixed background are normal in appearance and are fertile. To the extent that they have been studied, procollegen synthesis, secretion, and proteolytic processing are normal in these mice, and collagen fibrillogenesis is only slightly altered. However, breeding of heterozygous mutant mice on the 129 background generated homozygous mutants at only 64% of the expected frequency. These findings suggest that although the N-propeptide is not essential for collagen biogenesis in mice it may play some essential role during embryonic development.

Type I collagen is synthesized as a precursor, procollegen, with NH2- and COOH-terminal non-triple helical extensions (N- and C-propeptides) that are released extracellularly by limited proteolysis with procollogen N- and C-proteinases (1–4). The C-propeptide domain of procollegen participates in the association of the two proα1 and one proα2 chains to initiate triple helix formation from the COOH terminus of the protein (5–7).

A number of functions have been proposed for the α1(I) N-propeptide in the biogenesis of type I collagen, including prevention of premature intracellular molecular association and facilitation of transcellular transport and secretion, conversion of procollegen to collagen, regulation of extracellular fibrillogenesis, and feedback regulation of procollagen synthesis. However, none of these functions has been established unequivocally, and some have been questioned. Lee et al. (8) studied the secretion of mutated type I procollagen, generated from human cDNA genes that were transfected into Chinese hamster lung, Mov-13, and COS-7 cells. Whereas wild-type (WT) procollagen was secreted efficiently, proteins lacking either the entire N-propeptide (139 amino acids) or the majority of it (114 amino acids) were secreted poorly from Chinese hamster lung cells. In contrast, the WT and mutant proteins were secreted equally well by Mov-13 and COS-7 cells. Because Chinese hamster lung cells are epithelial-like, whereas Mov-13 and COS-7 cells are fibroblast-like, the failure of the former to secrete a collagen lacking N-propeptides may be unrelated to the structure of the protein. In all cases, triple helical assembly of the transfected gene products occurred normally. Similar experiments indicated that murine proα1 chains (marked by a substitution of Ile for Met-822) with a deletion of exon 2 were secreted normally and without post-translational overmodification by 3T6 cells (9). Exon 2 encodes 65 of the 129 amino acids in the murine α1 N-propeptide, which comprise the "globular" domain of the propeptide, and include all 10 of the conserved cysteines.

A great deal of evidence points to a correlation between synthesis of the molecular chaperone, HSP47, and type I collagen (10, 11). HSP47 has been postulated to promote triple helix formation in the endoplasmic reticulum by binding and stabilizing partially folded triple helical intermediates of procollagen, thus inhibiting the intracellular aggregation and degradation of the precursor and facilitating its transcellular transport and secretion (12, 13). On the other hand, interactions between HSP47 and peptide sequences in the N-propeptide have also been demonstrated (14). The latter observations are supported by studies in which Mov-13 fibroblasts were stably transfected with a control or a mutant Col1a1 gene that lacked exon 2. Comminoprecipitation experiments indicated that binding of HSP47 to the mutant collagen was reduced compared with the control, thus implicating the N-propeptide indirectly in binding to HSP47 (9).

Because failure to remove the N-propeptide from procollagen results in formation of abnormal collagen fibrils, both in humans with type VII Ehlers-Danlos syndrome (15) and in animals with dermatosparaxis (16), consideration has been given to the role of the N-propeptide in regulating both NH2-terminal

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* The abbreviations used are: WT, wild-type; ES, embryonic stem; exon 2α1 allele, Col1a1 gene with a deletion of exon 2; PGK-Neo, phosphoglycerate kinase promoter-neomycin resistance gene; PNP, procollagen N-proteinase; RT, reverse transcription.
proteolysis and fibrillogenesis. There is good evidence that the triple helical conformation of collagen is required for efficient proteolysis by procollagen N-proteinase (PNP; 2), but the role of the N-propeptide in the proteolytic event is uncertain. An indication that the N-propeptide is not essential for proteolysis is provided by the demonstration that a recombinant homotrimeric protein, composed of three proα2(I) chains with shortened triple helices, was at least partially cleaved by exogenous PNP (17). Proα2(I) chains naturally lack the sequence encoded by exon 2 in proα1(I). However, these results leave open the possibility that the presence or conformation of the N-propeptide could modulate this proteolytic event.

A feedback regulatory role for the N-propeptide, after its release from procollagen by PNP, was originally suggested by the observation that bovine dermatosparactic fibroblasts, which have a defect in PNP activity, synthesize higher amounts of collagen than control cells (18). Consistent with a feedback inhibitory effect, a bacterial collagenase-resistant fragment of the N-propeptide reduced collagen synthesis when it was added to bovine or human fibroblasts (18). Subsequently, it was shown that this collagenase-resistant peptide specifically inhibited the translation of types I and III mRNA in a cell-free translation system (19, 20) and that the transfection of bovine nuchal ligament cells with a plasmid encoding the N-propeptide selectively reduced endogenous collagen synthesis by these cells (21). The mechanisms responsible for these effects are still not understood. The C-propeptides have also been implicated in feedback regulation of procollagen synthesis (22, 23), but no recent confirmation of this activity has been reported.

In view of the uncertain role of the N-propeptide in the biogenesis and regulation of type I collagen synthesis, we performed a targeted deletion of exon 2 in the Col1a1 collagen gene and generated mice that lacked the NH2-terminal, globular half of the N-propeptide encoded by this exon (Fig. 1). We were encouraged in this endeavor by the realization that in the absence of exon 2, fusion of exons 1 and 3 would preserve the reading frame of the protein. Furthermore, the finding that the protein encoded by a proα1(I) cDNA gene that lacked exon 2 was secreted, at least by fibroblast-like cells, gave us some assurance that the phenotype of the mutant mouse would not be embryonic lethal on the basis of an inability to secrete type I procollagen. Mouse lines were established on both a mixed 129 and C57BL/6 background and on a pure 129 OlaHsd/Sv background. As described in this report, adult mixed background mice that lack the amino acid sequence encoded by exon 2 of the proα1(I) chain are normal in appearance and are fertile. To the extent that these mice have been studied, it would therefore appear that none of the functions ascribed to the N-propeptide has been compromised sufficiently by its absence to prevent adequate synthesis of type I collagen or to hinder its function as a fiber-forming protein. However, homozygous mutant 129 background mice were generated at only 64% of the frequency predicted by Mendelian ratios. The N-propeptide may therefore perform some essential function in embryonic development which is subject to the influence of genetic modifier genes.

**Experimental Procedures**

*Generation and Diagnosis of Mutant Mice—*Murine 129 genomic clones, containing fragments of the Col1a1 gene, were kindly provided by Dr. H. Wu and were assembled to form a 14.2-kb EcoRI fragment. This sequence, and the 13.2-kb EcoRI-SphI exon 23 targeting construct derived from it, are shown in Fig. 2. Exon 2 was deleted by restriction of an appropriate clone of Col1a1 at flanking BamHI and KpnI sites and subsequent ligation of the blunt ended chains, a procedure that recreated a BamHI site in place of the KpnI site. A PGK-Neo expression cassette, flanked by loxp sites, was then inserted in the BamHI site to generate a targeting construct with ~6.2 kb of 5’- and ~6.8 kb of 3’-sequence identity with the endogenous allele. The exon 23 targeting construct also contains a silent mutation within exon 7 which created a new XhoI restriction endonuclease cleavage site but did not change the amino acid sequence of the protein. The generation and utility of the XhoI mutation have been described previously (24, 25).

E14TG2a HPRT-embryonic stem (ES) cells derived from 129 OlaHsd mice (a gift from Dr. T. Doetschman) were cultured on neomycin-resistant STO cells in Dulbecco’s modified Eagle’s medium (high glucose, 4.5 g/liter). The medium was supplemented with 15% fetal calf serum (ES-qualified, Invitrogen), 0.1 mM β-mercaptoethanol, 2 mM t-glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin, nonsensetive amino acids (0.1 mM each, Invitrogen), and 1,000 units/ml leukemia inhibitory factor (Invitrogen). Generation of cells containing the exon 23 allele was performed essentially as described previously (26). Briefly, 2 × 107 cells were electroporated with 30 μg of linearized targeting exon 2A DNA and 24 h later were subjected to selection in media containing 400 μg/ml G418. Surviving colonies were picked 8–10 days later and were screened by Southern blotting for correct targeting of the mutation to the locus (Fig. 3). The strategy for screening can be discerned from Fig. 2, which also shows the relevant restriction sites, sizes of the diagnostic DNA fragments, and the fragments used in the preparation of Probes 1 and 2.
Fig. 2. Strategy for the generation of a Col1a1 allele that lacks exon 2. Maps of the 14.2-kb EcoRI/EcoRI fragment of the Col1a1 allele and its derivatives in the exon 2ΔNeo and exon 2A alleles are shown. The PXG-Neo expression cassette, flanked by the loxP sites, is represented by an arrow in the targeting construct and in the exon 2ΔNeo allele. The exon 2A allele, with a single loxP sequence 5' to the BamHI site, was generated from the exon 2ΔNeo allele by Cre recombination-mediated deletion of the PGK-Neo expression cassette. The exons downstream from exon 7 are omitted for the purpose of clarity. The sizes, in kb, of the restriction fragments used for genotype diagnosis by Southern analysis are shown. The locations of the fragments from which Probes 1 and 2 were derived are also shown. B, BamHI; E, EcoRI; K, KpnI; S, SphI; X, XhoI; Xb, XbaI.

Fig. 3. Southern analysis of genomic DNA extracted from ES cells. DNA was restricted with BamHI and XhoI, fractionated on a 0.8% agarose gel, and hybridized with Probe 1 (Fig. 1). Lanes 1–3, DNA from three correctly targeted ES cell clones (clones 145, 252, and 111); lane 4, DNA from a WT clone. The sizes of the hybridized bands, in kb, are shown and correspond to the sizes predicted from the restriction map presented in Fig. 2.

Transfection of COS Cells and PCR Analysis—COS cells were cultured in Dulbecco’s modified Eagle’s medium, high glucose, supplemented with 10% fetal bovine serum (Invitrogen), 2 mM l-glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin, and nonessential amino acids (1X each). 1.5 × 10^6 cells were transfected with 20 μg of DNA using an Invitrogen Cell-Porator (800 microfarads, 150 V). Stable transfecants were selected in media containing G418 (800 μg/ml), which was added 24 h after electroporation. For the transfection studies shown in Fig. 5, XbaI-EcoI fragments of the WT and exon 2A alleles were cloned into the pcDNA3 expression vector (Invitrogen). The XbaI site is located 5’ to the translation initiation codon, and the EcoI site is located in exon 10. The exon 2A construct also contained the XhoI mutation in exon 7 (Fig. 5A). The Stratascript RT-PCR kit was utilized to identify mRNA derived from expression of the pcDNA3 constructs. Conditions recommended by the manufacturer were followed. 300 ng of primer P3’ (5’-GCTAGTCGACACTGATCAGGAAGCAAAGTTTCCTC-3’) and P1’ (5’-CCGGGCTTGCCAGCTTCCCCCATCATC-3’) (Fig. 4A) amplify a 550-bp fragment of genomic DNA from the WT and a 257-bp fragment from the exon 2A allele. Southern analysis was performed on BamHI-XhoI digests of DNA with Probe 1.
and tail were harvested from male mice at 8 weeks of age. Tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for light microscopy. Electron microscopy was performed as described previously (26). Briefly, the shaved dermis was fixed in half-strength Karnovsky’s fixative in cacodylate buffer and postfixed in osmium tetroxide. After dehydration, the sample was stained en bloc with uranyl acetate and infiltrated with epoxy. Sections (90–110 nm) were stained with lead citrate and saturated uranyl acetate and examined with a JEOL 1200 EXII transmission electron microscope.

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**FIG. 4. Genotyping of exon 2Δ mice.** Panel A, schematic representation of the first 10 exons in the Col1a1 and exon 2Δ alleles. The positions of the P1 and P1’ primers used to amplify genomic DNA sequences by PCR are shown. The gap in the chimeric 1-2 intron in the exon 2Δ allele represents the replacement of exon 2 with loxP and some adjacent sequence (see “Experimental Procedures” and Fig. 6A). Ea, EagI; X, XhoI. Panel B, results of a PCR analysis of WT (+/+), heterozygous (+/−), and homozygous exon 2Δ (−/−) mice. DNA was fractionated on a 2% agarose gel. The 550-bp band is indicative of the WT allele, and the 257-bp band of the exon 2Δ allele.

**FIG. 5. The chimeric intron 1-2 is spliced correctly in transcripts from the exon 2Δ allele.** Panel A, schematic representation of the first 10 exons in the Col1a1 and exon 2Δ alleles. The positions of the P3 and P3’ primers used to amplify cDNA sequences by RT-PCR are shown. For additional details, see the legend to Fig. 3. Ea, EagI; X, XhoI. Panel B, 2% agarose gel electrophoresis of RT-PCR products from transfected COS cells. Lane 1, WT cells; lane 2, cells transfected with a fragment of a Col1a1 allele with a large deletion in intron 1; lanes 3 and 4, cells transfected with DNA from each of two independent clones (clones 145 and 252) expressing the exon 2Δ allele; lane 5, cells transfected with pcDNA3 vector; lane 6, molecular weight markers. The sizes in bp of the markers and the two amplified bands are shown.
**Targeted Deletion of Exon 2 of the Murine Col1a1 Gene**

**Extraction of Collagen from Skin**—Total body skin was harvested from mice with an average weight of 12 g. Mice were divided into four experimental groups (male/WT, female/WT, male/exon 2a, and female/exon 2a), with 5–11 animals per group. Skin was completely shaved, removed from the animal, and weighed. A fraction was set aside for determination of total hydroxyproline and the remainder was minced finely and incubated in 30 volumes of phosphate-buffered saline overnight at 4 °C with stirring. Tissue was harvested by centrifugation at 10,000 × g for 15 min and suspended in 30 volumes of 0.5 M acetic acid. Extraction was performed overnight at 4 °C with stirring. An aliquot of acetic acid-extracted collagen was then subjected to acid hydrolysis by incubation at 110 °C for 16 h in 6 N HCl. The hydrolysate was neutralized with NaOH, and hydroxyproline was quantified by the addition of chloramine T to a final concentration of 0.017 M and incubation at 25 °C for 20 min. The reaction was stopped by the addition of perchloric acid to 0.8 M, color developed with 4% p-dimethylaminobenzaldehyde, and absorbance measured in a spectrophotometer at 557 nm. The amount of collagen in experimental samples was calculated by comparison with a hydroxyproline standard curve.

**Protein Synthesis by Dermal Fibroblasts in Culture**—Skin fibroblasts were isolated from age- and sex-matched WT and exon 2Δ mice as described previously (28). Cells were grown to confluence, washed with phosphate-buffered saline, and incubated at 37 °C for 24 h in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 μM N-ethylmaleimide. The conditioned medium was harvested, 1.2 mg/ml N-ethylmaleimide added, the medium was then divided into two equal parts. One aliquot was digested for 2 h at 37 °C with 9.6 units of highly purified collagenase (Worthington, CLSPA). The remaining aliquot was incubated in the absence of collagenase. Proteins were harvested by trichloroacetic acid precipitation, washed in 2% trichloroacetic acid followed by 95% ethanol, and dissolved in SDS loading buffer containing dithiothreitol. Incorporation of [3H]proline into proteins secreted into the conditioned medium was measured by scintillation counting, and the proteins were resolved by 7.5% SDS–polyacrylamide gel electrophoresis and autoradiography.

**Analysis of Excisional Skin Wounds**—Three-month-old sex-matched mice were subjected to excisional wounds as described previously (29). Briefly, wounds were made with 6-mm punches on four WT and four exon 2Δ mice under Avertin anesthesia. Two mice of each genotype were killed at 7 and 14 days, and the wounds were harvested with a rim of ~2 mm of unwounded tissue. Wound tissues were placed in porous holders to prevent curling, fixed in 10% formalin, embedded in paraffin, and sectioned. Five-μm sections were stained with hematoxylin and eosin or with Sirius Red.

**RESULTS**

**Generation and Genotyping of Exon 2Δ Mice**—The strategy for the generation of a Col1a1 allele that lacks exon 2 is described under “Experimental Procedures” and summarized in Fig. 2. Because our experience with the targeting of other genes indicated that the introduction of a phosphoglyceraldehyde-thymidine kinase cassette into the targeting vector and negative selection with gancyclovir gave us at best a 2–5-fold enrichment in correctly targeted ES cell clones, we dispensed with this additional step. G418-resistant, homologously recombined clones containing the exon 2Δneo allele were identified by Southern blot analysis of BamHI-digested DNA. As illustrated in Fig. 3, mutant clones produced a band of 6.3 kb and WT clones a band of 7.8 kb when hybridized with Probe 1B. Hybridization of EcoRI-XhoI digests of DNA from correctly targeted ES cell clones with Probe 2 provided assurance that the structure of the disrupted Col1a1 gene was as expected; targeted clones produced a band of 8.8 kb and WT clones a band of 13.4 kb (data not shown).

Chromosomal spreads were performed on several correctly targeted clones to confirm a normal karyotype, and two independently derived ES cell clones with a favorable morphology in culture (clones 145 and 252) were injected into blastocysts. These blastocysts were then transplanted into the uterine horns of pseudopregnant mice. Several high percentage chimeras were obtained from each clone, and these were bred with both C57BL/6 and 129SvJ mice. Coat color and genetic screening were used to determine germline transmission of the mutation. However, repeated mating of these heterozygous animals failed to produce homozygous exon 2Δneo mice. This failure most likely reflected silencing of the Col1a1 promoter by the PGK-Neo transcription unit (30), which would result in embryonic lethality of homozygous mutant mice. Accordingly, heterozygous mutant mice were bred with cytomegalovirus-Cre-expressing mice and offspring tested for the presence or absence of Cre and Neo by PCR. In almost all cases Cre-positive mice were Neo-negative, reflecting removal of the floxed PGK-Neo cassette by Cre recombinase (data not shown). Subsequent mating of these mice produced homozygous exon 2Δ mice, as judged by both PCR (Fig. 4B) and Southern blot analysis (data not shown). Breeding of mixed background (129 OlaHsdSv × C57BL/6) heterozygous mice produced WT, heterozygous, and homozygous exon 2Δ animals in the expected Mendelian ratio of 1:2:1. However, breeding of pure 129 OlaHsd/Sv background heterozygous mice consistently produced fewer than predicted homozygous mutant animals. This finding was equally true for mutant mice generated from ES cell clones 145 and 252. Of 176 offspring that were genotyped at 4 weeks of age, we identified 56 WT, 92 heterozygous, and 28 homozygous exon 2Δ mice. Thus, homozygous mutant mice were generated at only 64% of the expected frequency. There was no indication that potentially exon 2Δ mice died after birth.

**Transcripts from the Exon 2Δ Allele, Containing a Chimeric Intron 1-2, Are Spliced Correctly**—To determine whether the absence of exon 2 in the Col1a1 gene affected the biosynthesis or function of type I collagen, it was first important to establish that the resulting chimeric intron 1-2 was efficiently spliced. Otherwise, retention of the intronic sequence could lead to nonsense-mediated degradation of heteronuclear RNA (31). We therefore transfected COS cells with a construct containing the normal sequence of exons in the Col1a1 gene or with a construct lacking exon 2. Amplification of a reverse transcribed WT transcript with primers P3 and P3′ (Fig. 5) should generate a 428-bp fragment, whereas amplification of a transcript with a deletion of the exon 2 sequence should generate a 233-bp fragment, provided that splicing of the chimeric intron 1-2 occurred normally. As shown in Fig. 5, a fragment of the size predicted by splicing of exon 1 to exon 3 (233 bp) was detected with RNA encoding the mutant collagen sequence, whereas RNA encoding the intact proα1(I) sequence produced the expected band of 428 bp.

**DNA Sequence Analysis Confirms the Absence of Exon 2—Genomic DNA sequence analysis of a PCR-amplified fragment, produced with a forward primer in intron 1 upstream from the BamHI site used to generate the deletion of exon 2 and a reverse primer in intron 5, confirmed the formation of a chimeric intron 1-2 (Fig. 6A). Homologous recombination constitutes a BamHI site in place of the Kpnl site in intron 2. The 34-bp lacP sequence, boxed in Fig. 6A, which is inserted between the intron 1 and 2 sequences, is flanked by a 29-bp 5’-sequence derived from bacteriophage and a 25-bp 3’-sequence of undetermined origin. Identical results were obtained from the sequences of DNA derived from exon 2Δ mice generated from either ES cell clone 145 or 252.**

The evidence obtained for correct splicing of the chimeric intron 1-2 (Fig. 5) was confirmed by DNA sequence analysis of the PCR-amplified product of reverse transcribed RNA extracted from lungs of mutant and control mice. Thus, in contrast to the WT sequence, the exon 2Δ allele does in fact encode a protein chain in which the amino acid sequence in exon 1 is linked directly to that in exon 3 (Fig. 6B). The bridging codon composed of 1 base from exon 1 and 2 bases from exon 3
The Phenotype of Exon 2 Δ Mice Is Apparently Normal—Mice lacking the 65 amino acids encoded by exon 2 appear normal on inspection and are fertile. The skin is not abnormally stretchable, and there is no indication of unusual fragility of tissues. Examination of the dermis by light microscopy revealed a normal appearance and organization of collagen fibers as determined by staining with hematoxylin and eosin and Sirius Red (data not shown). There were no significant differences in the collagen content of skin or in the quantity of collagen that was extractable with 0.5 M acetic acid from the skin of young mutant and WT mice (Table 1). These findings provide a strong indication that covalent cross-links are formed normally in the absence of the protein sequence encoded by exon 2. Finally, the time course of healing of 6-mm excisional skin wounds was normal in exon 2 Δ mice, and histological examination of the wound beds at day 7 and day 14 revealed no abnormalities (data not shown). These experiments and those described below were performed with mixed background mice.

Electron Microscopy of Dermal Collagen Fibers—Preliminary transmission electron microscopic studies of dermis revealed a slight increase in the average diameter of collagen fibrils and a tendency for subtle irregularity in fibril contour in sections from exon 2 Δ mice. The observed changes were patchy in distribution and were most evident in the deep dermis adjacent to the adipose layer.

The Absence of Exon 2-encoded Amino Acids in the Pro1(I) Chain Does Not Appear to Inhibit the Processing of Procollagen by PNP—The absence of the 65 amino acids encoded by exon 2 nevertheless leaves an N-propeptide of 64 amino acids, which must be released by PNP if the collagen α1(I) chain is to acquire its normal NH2-terminal sequence (Fig. 1). Analysis of acetic acid-extracted dermal collagen by SDS-acrylamide gel electrophoresis indicated that the migration of α1(I) chains derived from WT and exon 2 Δ type I collagens was the same (Fig. 7A). The presence of an additional mass of 6.6 kDa in the exon 2 Δ α1(I) chains should have been detected as a reduced distance of migration under this circumstance. It is of interest that the exon 2 Δ collagen preparation examined in Fig. 7A showed an increase in α1(III) chains compared with that from WT mice. The biological significance of this observation is not known.

However, the increase could reflect a compensatory up-regulation of the Col3a1 gene and a corresponding increase in formation of the α1(III) N-propeptide, which is very similar in amino acid sequence to the α1(I) N-propeptide.

It is possible that the truncated N-propeptide on exon 2 Δ α1(I) chains could have been released by limited proteolysis by extraneous proteases during extraction of the collagen from skin. Indirect evidence that such proteolysis does not occur was provided by NH2-terminal sequence analysis of isolated exon 2 Δ α1(I) chains. Automated amino acid sequence analysis of such chains, after transfer from SDS-acrylamide gels to polyvinylidene difluoride membranes, indicated a blocked NH2-terminus (data not shown). Proteolysis of pN-α1 collagen chains by PNP normally occurs at a Ser-Gln bond, and the NH2-terminal Gln characteristically cyclizes to 2-pyrrolidone 5-carboxylic acid, which cannot be removed by the Edman reaction. It therefore seems likely that exon 2 Δ α1(I) chains contain an NH2-terminal Gln that is generated normally by PNP.

Skin from WT and Exon 2 Δ Mice Does Not Contain Increased Amounts of Collagen Fibers—If feedback inhibition of collagen synthesis by released N-propeptides occurred in culture, one would predict that fibroblasts prepared from exon 2 Δ skin might demonstrate an increase in synthesis and secretion of type I procollagen and procollagen-collagen intermediates in culture. However, as shown in Fig. 7B, there was no difference in the amount and pattern of collagenase-sensitive bands visible on SDS-acrylamide gels of...
culture medium from WT and exon 2Δ cells. The ~6-kDa difference between pNα1 chains produced by the two cell types would not be visible in this gel. In a representative experiment, scintillation counting of equal aliquots of conditioned media, normalized for cell number, yielded 1,386 cpm for WT cells and 1,335 cpm for exon 2Δ cells. We therefore conclude that feedback inhibition of procollagen synthesis by the N-propeptide does not occur in mouse fibroblasts cultured under the conditions described in this work. However, we have not established that effective levels of N-propeptide were produced by WT fibroblasts during the 24-h labeling period of this experiment, and it is also possible that the truncated N-propeptide, generated in the absence of exon 2 (see Fig. 1), still has some effect.

**DISCUSSION**

We have used gene targeting followed by removal of the selectable neomycin-resistance gene with the Cre-loxP recombinase system, to generate proα1(I) chains with a deletion of the exon 2-encoded 65 amino acids that comprise 87% of the NH2-terminal globular domain of the N-propeptide (Fig. 1). Because there was a potential for incomplete or incorrect splicing of the chimeric intron 1-2 that would result from deletion of exon 2, we first ascertained that this intron was spliced correctly in a model system in which a minigene, containing the existence of the expected mutation was then verified by DNA sequence analysis of both genomic and cDNA from mutant mice. Exon 2Δ-type I procollagen was secreted normally as judged by the level of incorporation of [3H]proline into bacterial collagenase-sensitive proteins in the culture medium of dermal fibroblasts and by the pattern of these proteins on SDS-acrylamide gels. The truncated N-propeptide was also cleaved normally by PNP, as suggested by the normal migration of α1 chains obtained from mutant acetic acid-extractable dermal collagen on SDS-acrylamide gels and by the expected blocked NH2 terminus of these chains. Stabilization of fibrous collagen by covalent cross-links occurred normally, as determined by quantification of the extractability of dermal collagen with acetic acid in mutant and WT mice. Collagen fibers appeared to be normal by light microscopy, and fibrils were only slightly altered, as determined by electron microscopic examination in exon 2Δ mice. The course of excisional skin wound healing was also normal in these animals. Finally, there was no evidence that the ability of the N-propeptide to regulate procollagen synthesis, if it occurs under these circumstances, was compromised by the lack of its globular NH2-terminal domain.

The interpretation of initial studies of the structure and function of type I procollagen was complicated by a failure to distinguish clearly between the roles of the N- and C-propeptides (for a review of the early literature, see Ref. 32). As research progressed, it became evident that association, alignment, and facilitation of triple helix formation during molecular assembly were properties that should be ascribed to the C- rather than to N-propeptides (5, 7). However, assumptions were then made that the N-propeptides might function to in-
hibit intracellular fibrillogenesis and to participate in the appropriate lateral aggregation and packing of collagen molecules during the formation of extracellular fibrils. These assumptions may have had their origins in observations that dermatosparastic calves, which are deficient in PNP activity (33) and therefore retain the N-propeptide on a large proportion of a1 chains, show abnormalities in collagen fibril formation (16). Subsequently, a role for the N-propeptide of type I procollagen in regulation of extracellular fibril growth was deduced from immunoelectron microscopic visualization of the propeptide exclusively in association with thin fibrils in human skin (34). Procollagen intermediates containing either N- or C-propeptides (pN- and pC-collagen) could also be demonstrated in chick tendons, both during development and postnatally by immunofluorescence and SDS-acrylamide gels, although there was no indication that these intermediates were involved in regulation of fibrillogenesis (35). Our studies of exon 2Δ dermal fibroblasts show an apparently normal rate of secretion of type I procollagen. This finding argues against an absolute requirement for the globular domain of the N-propeptide in the inhibition of intracellular collagen fibril formation. However, the detection of mildly abnormal collagen fibrils in the dermis of exon 2Δ mice supports the earlier suggestion of Fleischmajer and colleagues (34, 35) that the N-propeptide plays a role in the modulation of extracellular collagen fibrillogenesis.

Perhaps the most puzzling property of the N-propeptide has been its purported ability, after its release by PNP, to function as a feedback regulator of procollagen synthesis. Support for such a function was provided initially by the observation that isolated N-propeptide, produced from dermatosparastic calf skin collagen by bacterial collagenase digestion, reduced collagen synthesis by bovine fibroblasts in culture and that dermatosparastic bovine fibroblasts, which are deficient in PNP activity, synthesized more collagen in culture than control cells (18, 36). Subsequently, it was shown that bovine N-propeptide was capable of selectively inhibiting the translation of collagen-enriched mRNA in cell-free reticulocyte lysate systems (19, 20), whereas chick N-propeptide reduced procollagen mRNA levels in human fibroblasts (37). More recently, Fouser et al. (21) transfected a metallothionein-human N-propeptide minigene into fetal calf ligament cells and observed a selective reduction in type I collagen synthesis in these cells. Because the minigene lacked a signal peptide sequence, the translation product was retained in the cytosol, and this was shown by staining with a guinea pig antibody to a neoepitope encoded by the minigene. However, in attempts to follow up on these observations we have been unable to confirm that human rN-propeptide, produced in Escherichia coli and in insect cells, was capable of inhibiting collagen synthesis by a variety of human fibroblasts. On the other hand, when COS cells were stably transfected with a construct expressing the N-propeptide, synthesis of the protein ceased after only a few passages, despite continued transcription of the transfected gene, as judged by the mRNA level. A possible interpretation of the latter experiments, which would be in accord with the findings of Fouser et al. (21), is that endogenously produced N-propeptide inhibited its own synthesis. Although the contradictory results of in vitro experiments remain unresolved, the findings in this study, which are subject to the reservation that compensatory mechanisms might substitute for the modulatory feedback effects of the N-propeptide, do not support a physiological role for the globular domain of the N-propeptide in the regulation of collagen synthesis.

If the globular domain of the N-propeptide does not function critically in any of the steps in collagen synthesis or fibrillogenesis, what role does it play in vertebrate biology? It seems unlikely that a highly conserved domain of 65 amino acids can be deleted from type I procollagen, or any other protein, without some serious functional consequence. It has been proposed recently that the type II collagen N-propeptide is involved in regulation of chondrogenesis by interaction with bone morphogenetic protein 2 and/or transforming growth factor α (38). Exon 2 of the Col2a1 gene, which also encodes the globular domain of the type II N-propeptide, is alternatively spliced in a pattern that may be developmentally significant (39–43). The spacing of the 10 cysteines in the N-propeptide and the positions of several other amino acids are conserved not only in the α1 N-propeptides of types I, II, and III procollagens, but also in thrombospondins 1 and 2, Drosophila short gastrulation protein, and its ortholog, Xenopus chordin. Short gastrulation protein antagonizes the dorsalizing effects on pattern formation of decapentaplegic in Drosophila (44, 45) and chordin, the ventralizing effects of bone morphogenetic protein 4 in Xenopus (46). Short gastrulation protein and chordin therefore play important roles in the formation of the dorsal-ventral axis. It is of interest that Xenopus type IIA procollagen mRNA, which contains the exon 2 sequence, has dorsalizing activity when microinjected into Xenopus embryos and that the exon 2 cysteine-rich sequence is required for this activity (46). It therefore seems possible that the type I collagen N-propeptide could play an analogous role in osteogenesis and in developmental processes in other type I collagen-containing tissues, to the role proposed for the type II collagen N-propeptide in chondrogenesis. In exon 2Δ mice, this putative function might be provided in part by compensatory up-regulation of the Col3a1 gene. The failure of homozygous exon 2α mice to be generated in the expected proportion from the mating of heterozygous mutant 129 background mice supports a role for the N-propeptide in some critical process during embryogenesis. Genetic modifier genes in this background, possibly relating to the ability to increase type III collagen synthesis, could enable a fraction of exon 2Δ mice to survive.

On the other hand, a multiple sequence alignment of the 13 available cysteine-rich domains that are encoded by exon 2 of the Col1a1 gene indicates that the amino acid sequences in exon 2 of the Col1a1 gene indicates that the amino acid sequences in mice and rats diverge more from the homologous sequences in other mammals than do sequences from more evolutionarily distant species, such as chicken, trout, Xenopus, and newt (data not shown). This is not true for a similar alignment of the cysteine-rich domains in type II procollagens. It is therefore possible that the globular domain of the α1(I) N-propeptide fulfills a specialized function in rodents.

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The Globular Domain of the Procα1(I) N-Propeptide Is Not Required for Secretion, Processing by Procollagen N-Proteinase, or Fibrillogenesis of Type I Collagen in Mice

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