

A Targeted Mutation at the Known Collagenase Cleavage Site in Mouse Type I Collagen Impairs Tissue Remodeling

Xin Liu,* Hong Wu,* Michael Byrne,‡ John Jeffrey,‡ Stephen Krane,‡ and Rudolf Jaenisch*

*Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142; ‡Department of Medicine, Harvard Medical School and Medical Services (Arthritis Unit), Massachusetts General Hospital, Boston, Massachusetts 02114; †Department of Pharmacology and Toxicology, Albany Medical College, Albany, New York 12208

Abstract. Degradation of type I collagen, the most abundant collagen, is initiated by collagenase cleavage at a highly conserved site between Gly₇₇₅ and Ile₇₇₆ of the $\alpha 1(I)$ chain. Mutations at or around this site render type I collagen resistant to collagenase digestion in vitro. We show here that mice carrying a collagenase-resistant mutant Colla-1 transgene die late in embryogenesis, ascribable to overexpression of the transgene, since the same mutation introduced into the endogenous Colla-1 gene by gene targeting permitted normal development of mutant mice to young adulthood. With increasing age, animals carrying the targeted mutation developed marked fibrosis of the dermis similar to that in human scleroderma. Postpartum involution of the uterus in the mutant mice was also impaired, with persistence of collagenous nodules in the uterine wall. Al-

though type I collagen from the homozygous mutant mice was resistant to cleavage by human or rat fibroblast collagenases at the helical site, only the rat collagenase cleaved collagen trimers at an additional, novel site in the nonhelical N-telopeptide domain. Our results suggest that cleavage by murine collagenase at the N-telopeptide site could account for resorption of type I collagen during embryonic and early adult life. During intense collagen resorption, however, such as in the immediate postpartum uterus and in the dermis later in life, cleavage at the helical site is essential for normal collagen turnover. Thus, type I collagen is degraded by at least two differentially controlled mechanisms involving collagenases with distinct, but overlapping, substrate specificities.

TYPE I collagen is among the most abundant components of the extracellular matrix of many tissues, particularly in skin, tendons, ligaments, uterus, large blood vessels and bone. The helical trimeric molecules of type I collagen comprise two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain, encoded by two separate genes, Colla-1 and Colla-2, respectively (50). In mice, type I collagen is first synthesized in the mesenchymal stroma of the head, heart and somites at day 8 of gestation (E8) and its production continues throughout development and postnatal life (27). It has been demonstrated that type I collagen is critical for bone development (9, 10), hematopoiesis (29, 37), integrity of the vascular system (29) and for mesenchymal-epithelial induction in organogenesis (6).

The collagen content of different tissues during development and in adult animals is tightly regulated by coordinated processes of synthesis and degradation (2, 8, 57). Failure to maintain an equilibrium between synthesis and degradation leads to diverse human connective tissue disorders characterized by excessive resorption (e.g. os-

teoporosis or inflammatory joint diseases) or excessive deposition (e.g. pulmonary fibrosis or scleroderma) (32, 39). The degradation of type I collagen requires the action of specific collagenases since native, triple-helical molecules are resistant to attack by proteolytic enzymes at 37°C and neutral pH. The collagenases are members of a family of proteinases, the metalloproteinases or matrixins, all of which contain a catalytic zinc-binding domain that includes the sequence motif HEXXH where the Glu (E) acts as a catalytic base. The peptide bonds between residues Gly₇₇₅ and Ile₇₇₆ of the $\alpha 1(I)$ chain and Gly₇₇₅ and Leu₇₇₆ of the $\alpha 2(I)$ chain are the only sites in native type I collagen molecules known to be cleaved by collagenases from various species including enzymes isolated from human, bovine, pig and rabbit. These cleavage sites are conserved in collagens from amphibians to mammals and are similar in types I, II, and III collagens (8). Cleavage of the collagens at this specific site leads to the production of a three-quarter length helical fragment (TC^A) and a one-quarter length helical fragment (TC^B) (51). The activity of collagenases released from cells is under the regulation of proteolytic collagenase activators and specific inhibitors known as TIMPs (tissue inhibitors of metalloproteinases) (34, 35).

Address all correspondence to R. Jaenisch, The Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142. Ph.: (617) 258-5186. Fax: (617) 258-6505.

It has been assumed that the various metalloproteinases play an important role in development of collagenous structures by altering morphogenetic tissue interactions (6, 8). The evidence supporting this role is largely circumstantial and is based on the specific expression patterns of metalloproteinases or specific inhibitory proteins during particular stages of development (8). Recently, it has been possible to obtain more direct evidence for the role of metalloproteinases in transgenic mice which overexpress stromelysin-1, a proteinase which degrades components of basement membranes. Expression of the transgene was associated with altered branching patterns of the mammary ducts and aberrant expression of β -casein as well as whey acidic protein (48).

We have used a genetic approach to obtain direct evidence for the role of type I collagen degradation in development and disease. Our strategy has involved the generation of a mutant substrate resistant to enzyme cleavage. In previous experiments, we introduced five different mutations into the collagenase cleavage domain of the Col1a-1 gene and demonstrated that substitution of a Pro for Ile₇₇₆ or the stabilization of the helix by substitutions of Pro for Gln₇₇₄ and Ala₇₇₇ at P₂ and P₂' rendered native collagen produced by cells in culture resistant to collagenase digestion (58).

In the present study, we introduced one of the mutations (mutation IV with a stabilized triple helix) into the mouse genome using two different approaches. Transgenic mice carrying multiple copies of the mutant transgene died during gestation. In contrast, mice carrying the mutation in the endogenous Col1a-1 gene developed normally and displayed no obvious abnormalities as young adults. Beginning at approximately seven months of age, mutant mice began to develop thickened skin and patchy hair loss due to dermal fibrosis. In addition, postpartum involution of the uterus in mutant females was impaired. While the mutant collagen was completely resistant to cleavage at the known cleavage site by the human and rat enzymes, rat fibroblast collagenase recognized an additional cleavage site COOH-terminal to the N-telopeptide crosslink. Our results suggest that the ability of rodent collagenase to cleave at this additional NH₂-terminal locus permits normal remodeling during development and early postnatal life, but that cleavage at the helical site is required for subsequent remodeling of the extracellular matrices in tissues such as those in skin and the postpartum uterus in which type I collagen is particularly abundant.

Materials and Methods

Materials

Pepsin, (2,858 U/mg; Cooper Biomedical), trypsin, TPCK, and soybean trypsin inhibitor were purchased from Worthington Biochemical Corporation (Freehold, NJ).

p-Aminophenylmercuric acetate (APMA)¹ was purchased from Sigma Chemical Company (St. Louis, MO).

Generation of Transgenics

Transgenics were derived as described previously (59). A linearized ge-

1. Abbreviations used in this paper: APMA, p-aminophenylmercuric acetate; ES, embryonic stem.

omic DNA fragment that included about 17 kb of coding region and 3.7 kb of 5' and 3' flanking sequences of the Col1a-1 gene carrying mutation IV (58) was microinjected into the pronucleus of fertilized FVB eggs. The eggs were incubated overnight and two-cell embryos were transferred to the oviduct of pseudopregnant females.

Generation of Chimeras and Germlines

J1 ES cells carrying the subtle mutations (mutation IV) at the collagenase cleavage site (60) were injected into C57-BL/6 and BALB/c embryos as described (28). Chimeras were identified on the basis of agouti pigmentation in the coat. C57BL/6 and BALB/c chimeras were backcrossed and their agouti offspring were genotyped by Southern blot analysis.

Southern Blot Analysis

Embryo and tail DNAs were prepared according to Laird et al. (26). The DNA was digested with Sph I and electrophoresed in a 0.7% agarose gel. The DNA was denatured, neutralized and transferred onto Hybond nylon membranes (Amersham Corp., Arlington Heights, IL) as described by Sambrook et al. (43). The probe is shown in Fig. 2.

Preparation of Collagens and Digestion with Collagenases

Collagens were extracted from mouse skin, tail, or whole embryos by digestion with pepsin, 50 μ g/ml, in 0.5 M acetic acid at 0–4°C for 2–4 d. In some samples, extraction was carried out under the same conditions, but without pepsin. The insoluble residue was removed by centrifugation and the collagen content of an aliquot portion was determined by measurement of hydroxyproline content following acid hydrolysis (5). The pepsin was inactivated by neutralization with NaOH and the solution was dialyzed against Cl₂ collagenase buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, 10 mM CaCl₂/0.25 M glucose) overnight at 4°C (58). Incubation with collagenase was usually for 18–20 h at 20°C and the reaction was stopped by the addition of EDTA to a final concentration of 50 mM; the samples were stored at 4°C prior to analysis by SDS-PAGE (15). Digestion products were resolved by SDS-PAGE using 5 or 7% acrylamide without reduction and gels were stained with Coomassie blue. The human collagenase preparation used here was partially purified from medium conditioned by cultured rheumatoid synovial fibroblasts as previously described (12). Rat collagenase was purified from medium conditioned by postpartum rat uterine cells as previously described (42). The latent collagenase was activated with trypsin, 5 μ g/ml for 10 min at 20°C and the trypsin inactivated with excess soybean trypsin inhibitor (12); alternatively, activation was with APMA, 1 mM, added at the beginning of the incubation (7, 36). For quantitation of relative protein content of appropriate stained bands after SDS-PAGE, photographs of stained gels were analyzed using the Hewlett Packard (Santa Clara, CA) H-P DeskScanII scanner and the Scan Analysis program (Biosoft, Cambridge, UK) for the Macintosh computer. Cyanogen bromide digestion of collagens was performed as previously described (58) in order to determine the incorporation of the mutated α 1(I) chain. Band 7a (58) was quantitated, after resolution by SDS-PAGE, by densitometry.

NH₂-terminal Peptide Sequencing

Collagen from the skin of mice homozygous for mutation IV was extracted as described, in 0.5 M acetic acid without pepsin. One sample was incubated in collagenase buffer with purified rat fibroblast collagenase for 18 h at 20°C; the other sample was incubated in buffer alone. Approximately 150 μ g of each was loaded into individual wells of a 5% polyacrylamide gel for SDS-PAGE. Proteins were stained with Coomassie blue and blotted onto Immobilon-P (polyvinyl fluoride) membranes (Millipore Corporation, Bedford, MA). The proteins corresponding to " α 1(I)" chains were cut out and peptide sequencing was performed as described (30).

Histological Analysis

Uterus and skin from age and sex matched wild-type and homozygous mutant mice were dissected and fixed in 10% buffered Formalin. They were then placed in successive ethanol and xylene baths and finally embedded in Paraplast Plus (Oxford, UK) using an Autotechnicon embedder (Technicon). The embedded tissues were sectioned to 5 μ m using a

Reichert-Jung microtome and stained with Masson's trichrome as described (3).

Results

Mice Carrying a *Colla-1* Transgene with Mutations at the Collagenase Cleavage Site Die During Late Gestation

To address the role of collagenases in type I collagen turnover in vivo, the murine genomic clone containing substitutions of Pro for Gln₇₇₄ (P₂) and Ala₇₇₇ (P₂') and Met for Ile₇₇₆ (P₁') (Mutant IV, see reference 58, Fig. 1), was microinjected into the pronuclei of FVB mouse embryos. Only a few transgenic animals were recovered: one of 13 live embryos dissected at day 13 and one of 18 animals analyzed at weaning carried the transgene. The latter animal, a male, was bred with FVB females to derive transgenic offspring.

As shown in Table I, none of the 11 offspring that survived to weaning carried the transgene, suggesting that expression of the mutation might result in embryonic lethality. Embryos at different developmental stages were therefore isolated and genotyped by Southern analysis. Transgenic embryos developed normally up to day 14.5 (E 14.5) of gestation. Between E 15 and E 16.5, however, only 2 out of 13 total transgenics were alive and by day 19 there were no live transgenics (Table I). The most obvious phenotypic abnormality of transgenic embryos was the failure to close the abdominal and chest cavities (not shown). These observations suggested that expression of the transgene in the offspring of the founder male interfered with normal embryogenesis. It was likely that a lower gene dosage due to genetic mosaicism (54) might have allowed survival of the founder male. Indeed, of 110 genotyped offspring, only 23 were transgenic, consistent with the founder being a germline mosaic. This was confirmed by comparing the number of the transgene copies in the founder and his transgenic offspring by quantitative Southern analysis (58). While the founder had approxi-

Table I. Genotype of Offspring Derived from Transgenic Founder

Age	Total animal	Transgenics	Viable transgenics
E10.5-14.5	40	6	6
E14.5-16.5	39	13	
E18-19	8	4	
Three weeks	11	0	

mately 20 copies of the transgene in his DNA, his offspring carried more than 70 copies (data not shown).

To directly investigate whether increased mutant gene dosage resulted in augmented transgene expression, the amounts of mutant RNA in founder and offspring were compared. Quantitative S1 analysis (58) revealed that the level of transgene RNA in the founder was approximately 25% of the endogenous *Colla-1* RNA, whereas the level in its transgenic offspring was approximately 150% (data not shown). To determine the composition of type I collagen chains produced in transgenic animals, collagen extracted with pepsin in 0.5 M acetic acid at 4°C was analyzed by SDS-PAGE. As shown in Fig. 1, a significant excess of $\alpha 1(I)$ over $\alpha 2(I)$ chains was noted in the transgenics (lanes 2 and 6) compared to control embryos (lane 4). These results indicate the presence of $\alpha 1(I)$ trimers and are consistent with overexpression of the transgene. To assess whether the collagen was resistant to cleavage, the proteins were incubated with human synovial collagenase. As seen in lanes 1 and 5, type I collagen extracted from the transgenic embryos was highly resistant to cleavage compared to that from the control embryos (lane 3). These results are consistent with those obtained previously where the same mutant construct was introduced into cultured Mov13 fibroblasts (58) and indicate that cleavage-resistant type I collagen was produced from the mutant transgene. Our results do not allow us, however, to conclude that the lethality of the transgenic embryos is due to the presence of the mutant $\alpha 1(I)$ chains rather than the overexpression of the transgene. To resolve this issue, we used targeted mutagenesis through homologous recombination in embryonic stem (ES) cells to introduce the same mutation into the endogenous *Colla-1* gene.

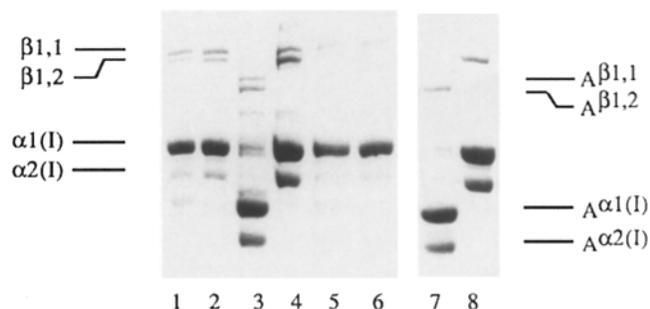


Figure 1. Effects of human fibroblast collagenase on collagens from wild-type and transgenic embryos. Proteins were extracted from E14 embryos from a litter. Lanes 1, 2, 5, 6: Transgenics; lanes 3 and 4: a wild-type litter mate. Samples in lanes 7, 8 were collagens from mouse tail as control. Samples in lanes 1, 3, 5, and 7 were incubated with trypsin-activated human collagenase for 18 h at 20°C (sufficient collagenase was used to digest 25 μ g collagen); samples in lanes 2, 4, 6, and 8 were incubated in buffer without collagenase.

Mice Carrying Targeted Subtle Mutations at the Collagenase Cleavage Site are Viable

The same mutation as described above was introduced into the endogenous *Colla-1* gene of ES cells using the Hit-and-Run procedure (20, 60). The mutant allele of the *Colla-1* gene was named *Colla1^{tm1 Jae}* according to the nomenclature rules (11). Chimeric mice, derived from C57/BL6 or BALB/c blastocysts injected with targeted ES cell clones were derived and backcrossed to C57/BL6 or BALB/c mice. The agouti offspring were genotyped at weaning by Southern blot analysis to identify germline transmission of the mutation. Heterozygous *Colla1^{tm1 Jae}* mice were viable and, when intercrossed, produced wild-type, heterozygous and homozygous offspring at a normal Mendelian ratio based on Southern blot analysis (Fig. 2 and Table II). No gross abnormalities were observed in mutant mice through approximately three to six months of age.

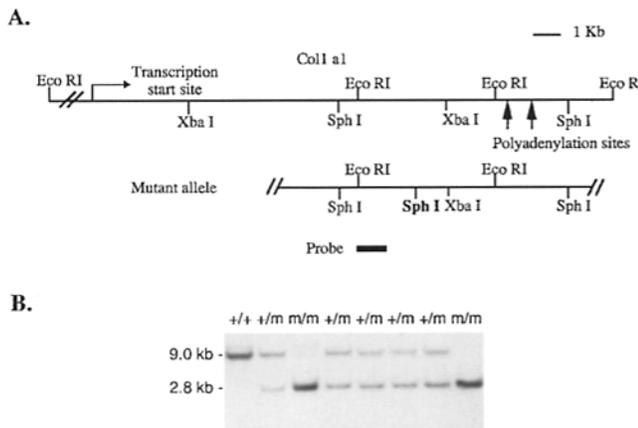


Figure 2. Restriction map and southern blot analysis of the fl from heterozygous $Col1a1^{tm1Jae}$ breeding. (A) Restriction map of a 23.5-kb genomic DNA fragment covering $Col1a-1$ gene. Mutation at the coding sequences for the collagenase cleavage site created a new $Sph I$ site which was indicated with bold letters. (B) A representative Southern blot analysis of DNA samples from offspring of heterozygous mutant mice. DNAs were digested with $Sph I$ and the Southern blots were hybridized with the probe showed in A. Wild-type allele, 9 kb; mutant allele, 2.8 kb.

Mutant Mice Develop Skin Alterations and Show Reproductive Impairment

At approximately seven months of age, homozygous male $Col1a1^{tm1Jae}$ mice developed skin abnormalities consisting of thickening and roughening and associated with patchy hair loss and small ulcerations. Comparison of a section of skin from a ten month homozygous mutant male (Fig. 3 B) with that of skin from an age-matched control mouse (Fig. 3 A) revealed that the dermis from the mutant mice was significantly thicker than that from control mice and was filled with dense collagen fibers. The collagen fibers were irregular in form and penetrated deeply into the hypodermis. The overall increased thickness of the skin in the homozygous $Col1a1^{tm1Jae}$ mice extending from the epidermis to the muscular layer was accounted for by the increase in thickness of the dermis. Analysis of pepsin digests of mutant skin protein by SDS-PAGE and delayed reduction indicated that ~95% of the collagen was type I collagen (data not shown). Homozygous female and heterozygous male and female mice developed similar but milder skin abnormalities compared to those of homozygous males at the same age (data not shown).

Mutant females revealed a striking reproductive impairment. While wild-type females produce an average of four to five litters with a mean litter size of six pups during a seven months breeding period in our laboratory, both heterozygous and homozygous mutant females produced a significantly reduced number of litters which also tended to be small (Table III). It is well established that the col-

Table II. Genotype of Offspring Derived from Heterozygous Parents

Age	Wild-type	Heterozygous	Homozygous
3 weeks (n = 97)	23	51	23

lagen content of the uterus, which increases markedly during pregnancy, decreases strikingly within a few days after parturition due to degradation by collagenases (24, 56). When the postpartum uteri of mutant females were compared to those of wild-type females, a striking difference was seen: instead of a tube-like structure with a smooth outer surface, characteristic of normal postpartum uteri, the uteri of mutant mice were filled with nodules. Cross sections of these uteri showed that the nodules consisted of collagen fibers and these fibers occupied much of the endometrium and myometrium of the uterine wall (Fig. 4, A–D). SDS-PAGE and analysis by delayed reduction of the pepsin digests of uterine protein showed that ~95% of the collagen was type I collagen (data not shown). Collagen accumulation in the uteri of heterozygous $Col1a1^{tm1Jae}$ mice was less pronounced compared to homozygous $Col1a1^{tm1Jae}$ mice but was clearly different from wild-type females (data not shown). In virgin $Col1a1^{tm1Jae}$ mice, small accumulations of collagen in the myometrium of the uterus were occasionally observed.

The results described so far indicate that animals carrying the collagenase-resistant mutation develop normally to adulthood. Severe abnormalities, explainable by impaired collagen degradation, became manifest with increasing age or the challenge of intense collagen resorption.

A Novel Collagenase Cleavage Site at the NH_2 -terminus of $\alpha 1(I)$ Chain

To confirm that $Col1a1^{tm1Jae}$ mice produce collagenase-resistant collagen, pepsin-resistant proteins were isolated from tails of wild-type, heterozygous and homozygous mice and digested with human synovial fibroblast collagenase prior to SDS-PAGE. As shown in lanes 1 and 2 of Fig. 5 A, the $\alpha 1(I)$ and $\alpha 2(I)$ collagen chains from wild-type mice were totally cleaved by collagenase into the expected $A^{\alpha 1(I)}$ and $A^{\alpha 2(I)}$ (TC^A) and $B^{\alpha 1(I)}$ and $B^{\alpha 2(I)}$ (TC^B) fragments (the TC^B fragments migrated further than the TC^A and are not shown here in Fig. 5 A, or in Fig. 5, B and C). In contrast, collagens extracted from hemizygous mutant mice ($Col1a1^{tm1Jae}/Mov 13$, for Mov 13 see reference 18), which express only the mutant but not the wild type $\alpha 1(I)$ chain, were completely resistant to digestion (lanes 7 and 8). Collagens extracted from the tail of the chimeric founder mouse and from a heterozygous $Col1a1^{tm1Jae}$ mouse were partially resistant to collagenase digestion (lanes 3–6). The extent of cleavage of the collagen from these heterozygous animals was 26% as calculated by densitometric scanning. Quantitation by cyanogen bromide cleavage (58) revealed that the distribution of mutant and wild-type $\alpha 1(I)$ chains was approximately equal in the collagen extracted from the heterozygous animal (data not shown). This suggests that a heterotrimer of type I collagen containing a single mutated $\alpha 1(I)$ chain is resistant to collagenase cleavage, consistent with a dominant negative effect of the mutation.

The observation that the collagen in homozygous $Col1a1^{tm1Jae}$ mice was completely resistant to collagenase degradation raised the question whether other mechanisms of degradation were responsible for the massive collagen turnover characteristic of embryonic development. The results described in Figs. 1 and 5 A were based upon

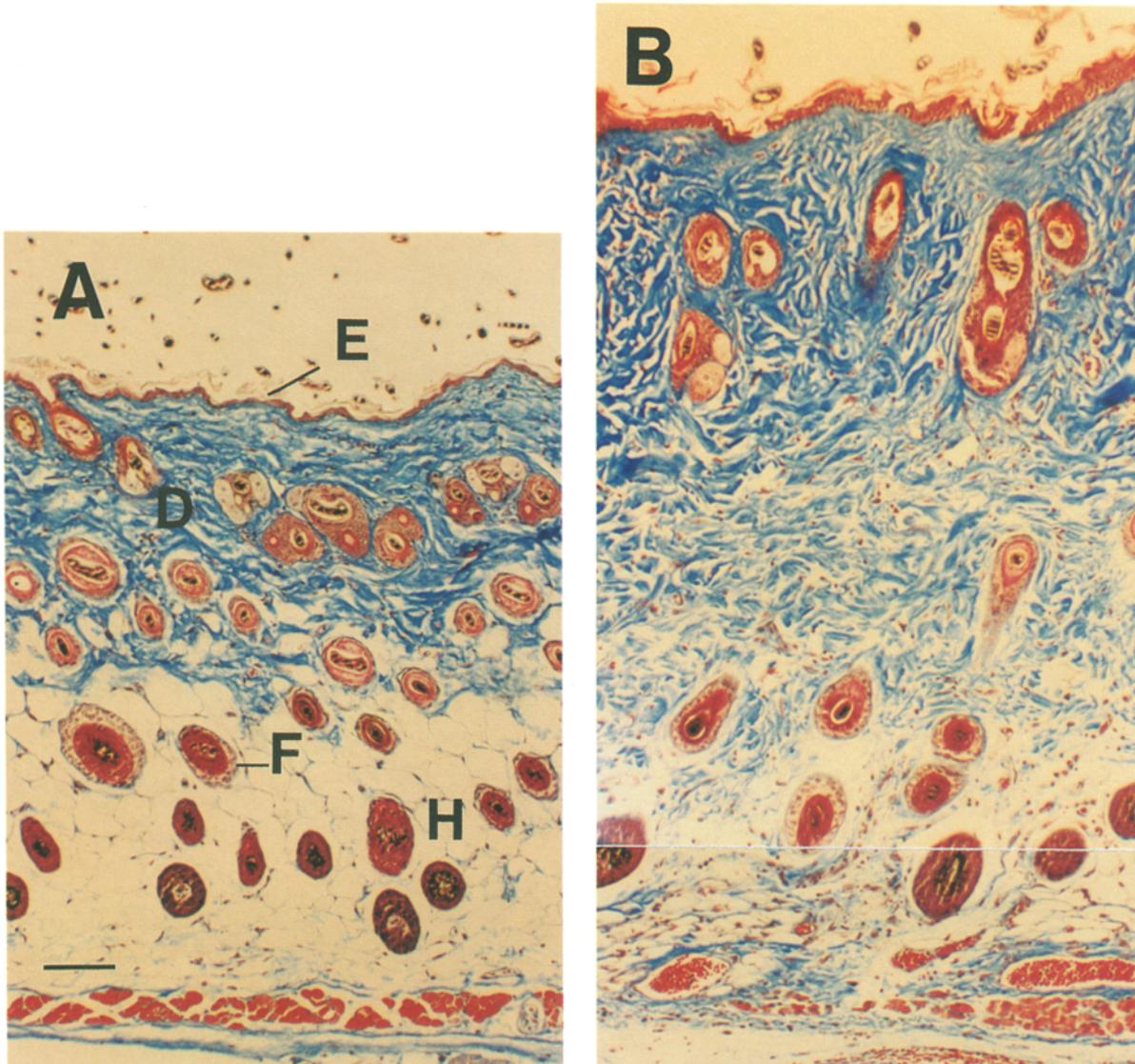


Figure 3. Histological analyses of the skin from 11-mo-old male wild-type and homozygous *Col1a1^{tm1Jae}* mice. (A) Wild-type skin. The epidermis (E), dermis (D), hypodermis (H), and hair follicle (F) are indicated. The collagen fibers are stained blue with Masson's trichrome. (B) Homozygous mutant skin. The magnification is the same as A. Bar, 0.1 mm.

digestion with human interstitial collagenase, which was the form of collagenase used in our initial study (58). To test whether the mutant collagen was also resistant to cleavage by rodent enzymes, collagens extracted from mu-

tant and control mice were incubated with rat collagenase obtained in highly purified form (40). As seen in Fig. 5 B, the rodent enzyme cleaves wild-type I collagen at a similar position as the human enzyme (lanes 2 and 3). Digestion of mutant collagen isolated from hemizygous mutant mice revealed, however, an additional cleavage site at the amino end of collagen, which is not recognized by the human enzyme. This conclusion was based on the observation that the content of the $\beta^{1,1}$ and $\beta^{1,2}$ cross-linked dimer components from mutant mice was dramatically reduced after digestion with rat collagenase (lanes 3 and 6) and that the expected $A^{\beta^{1,1}}$ and $A^{\beta^{1,2}}$ cleavage products normally seen after digestion of wild-type collagen with the human enzyme (lane 2) were absent. Instead, the intensity of bands corresponding to $\alpha 1(I)$ and $\alpha 2(I)$ chains increased significantly, suggesting that the $\beta^{1,1}$ and $\beta^{1,2}$ components from mutant collagen were digested by rat collagenase to fragments that co-migrated with $\alpha 1(I)$ and $\alpha 2(I)$ collagen

Table III. Number of Litter and Offspring Raised by Wild-type, Heterozygous, and Homozygous *Col1a1^{tm1Jae}* Female

Genotype	Number of litter	Litter size
Wild-type (n = 5)	4.4 ± 1.1	5.9 ± 1.8
Heterozygous (n = 10 females)	2.2 ± 0.9	4.4 ± 1.6
Homozygous (n = 5 females)	1.4 ± 0.9*	4.7 ± 1.5

Breeding period: 7 months.

Data expressed as mean ± SEM.

*Differs from wild-type, paired student *t*-test, $P < 0.05$.

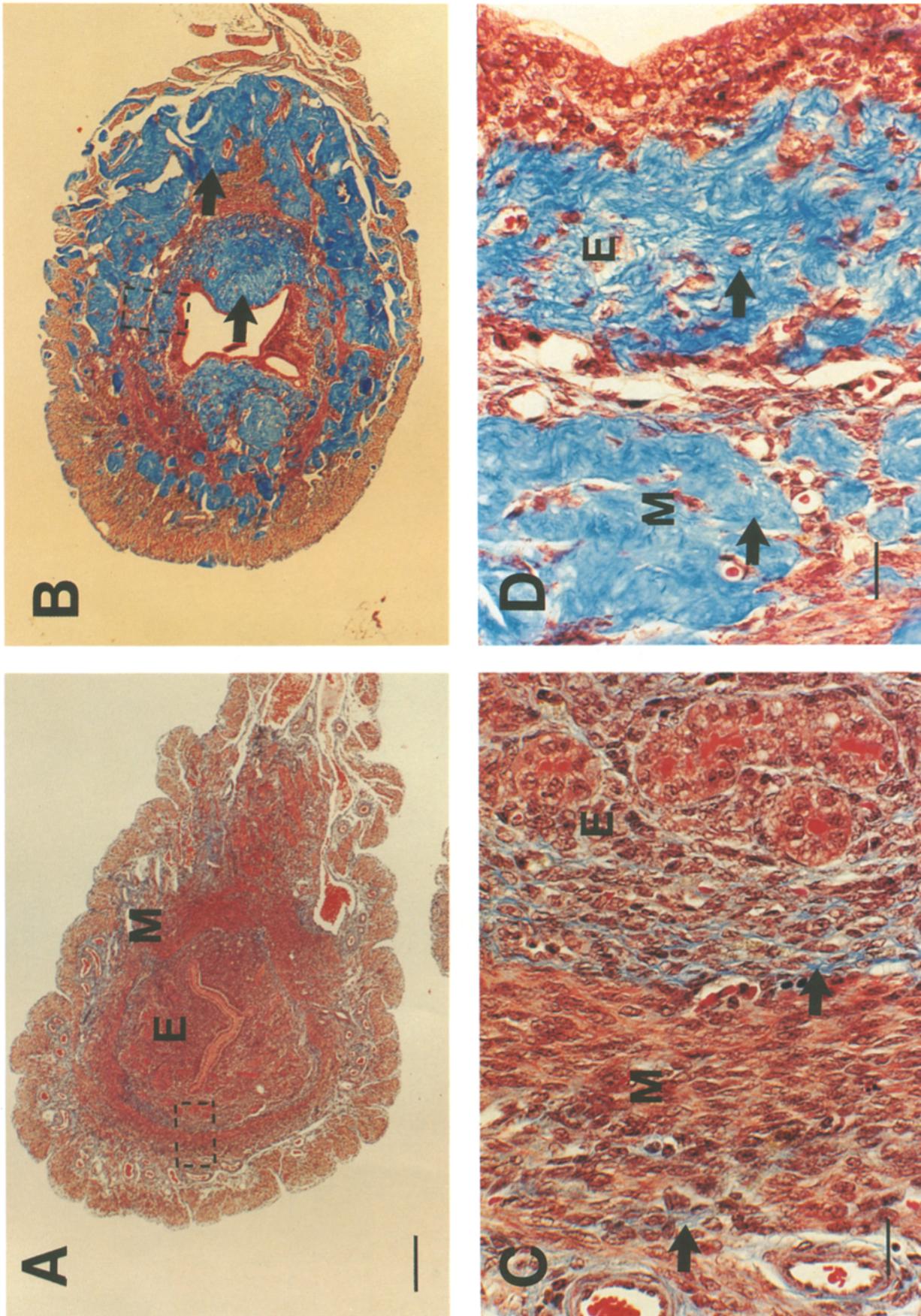


Figure 4. Histological analyses of 15-d-post partum uteri from wild-type and homozygous *Coll1a1^{tm1.1ac}* mice. The myometrium (M), and endometrium (E) are indicated. (A) Cross-section of a uterus from a postpregnant wild-type mouse. (B) Cross-section of an uterus from a postpregnant homozygous mutant mouse. Arrows point to the collagen accumulation. (C) A higher magnification of the boxed area in A. (D) A higher magnification of the boxed area in B. Arrows point to the collagen fibers that are stained blue with Masson's trichrome (see Materials and Methods). Bars: (A and B) 0.1 mm; (C and D) 0.025 mm.

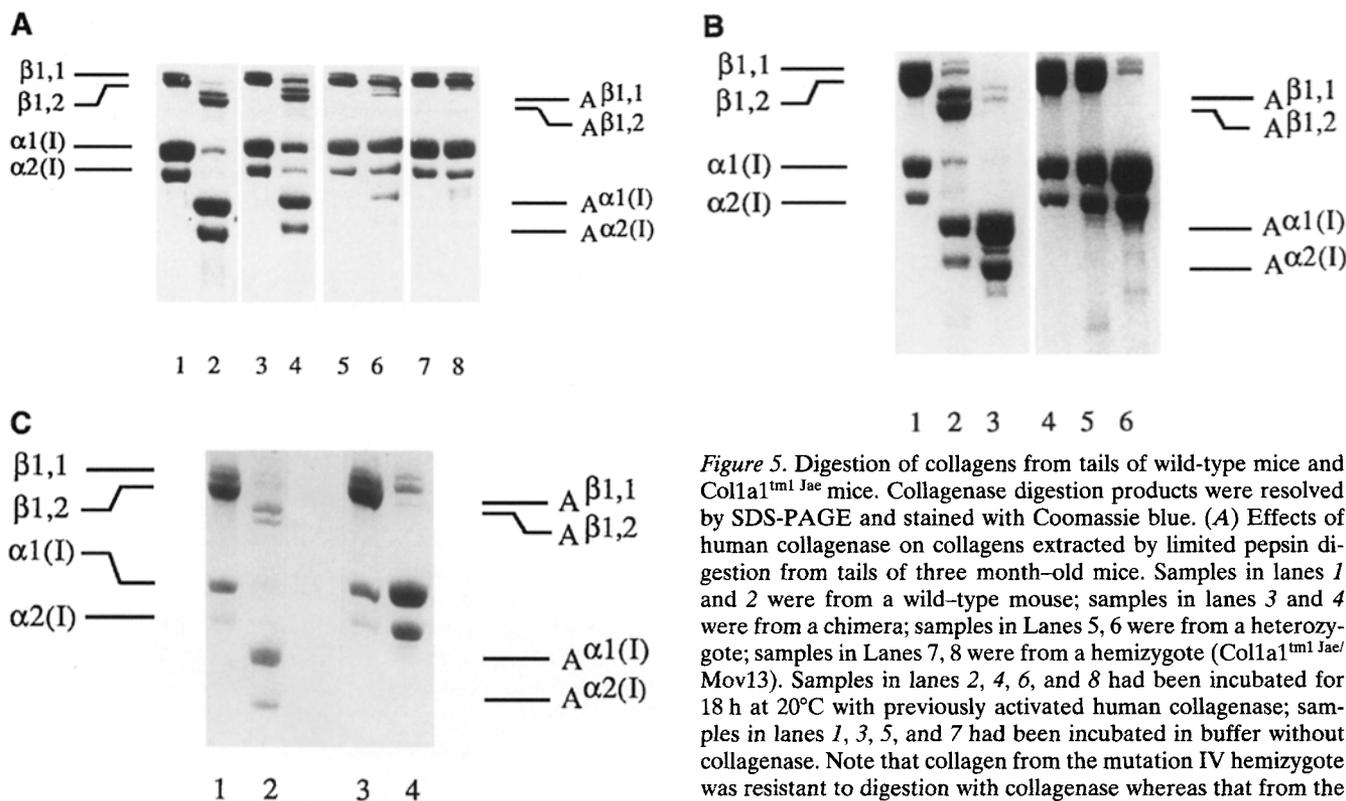


Figure 5. Digestion of collagens from tails of wild-type mice and *Col1a1^{tm1 Jae}* mice. Collagenase digestion products were resolved by SDS-PAGE and stained with Coomassie blue. (A) Effects of human collagenase on collagens extracted by limited pepsin digestion from tails of three month-old mice. Samples in lanes 1 and 2 were from a wild-type mouse; samples in lanes 3 and 4 were from a chimera; samples in Lanes 5, 6 were from a heterozygote; samples in Lanes 7, 8 were from a hemizygote (*Col1a1^{tm1 Jae}/Mov13*). Samples in lanes 2, 4, 6, and 8 had been incubated for 18 h at 20°C with previously activated human collagenase; samples in lanes 1, 3, 5, and 7 had been incubated in buffer without collagenase. Note that collagen from the mutation IV hemizygote was resistant to digestion with collagenase whereas that from the wild-type was completely digested under the same conditions.

(B) Effects of human and rat collagenase on collagens extracted in 0.5 M acetic acid without pepsin from tails of 3-mo-old mice. Samples in lanes 1–3 were from the wild-type mouse and samples in lanes 4–6 were from the mutation IV hemizygous mouse shown in A. Samples in lanes 1, 4 had been incubated in buffer without collagenase; samples in lanes 2, 5 had been incubated with human collagenase as in A; samples in lanes 3, 6 had been incubated with rat collagenase, 6.7 $\mu\text{g/ml}$. All samples had been incubated at 20°C for 18 h. Note that the human collagenase converted the β components of the wild-type collagen to A^β and the α chains to A^α fragments, whereas the rat collagenase appeared to convert the β components as well as the α chains to A^α fragments. In contrast, whereas neither the human nor the rat collagenase cleaved the α chains of the collagen containing mutation IV, the rat collagenase converted the β components of the collagen to α chains but the human collagenase did not appreciably diminish the concentration of the β components. (C) Effects of rat collagenase on collagens extracted in 0.5 M acetic acid without pepsin from skin of 3-mo-old mice. Samples in lanes 1, 2 were from a wild-type mouse; samples in lanes 3, 4 were from a homozygous *Col1a1^{tm1 Jae}* mouse. Samples in lanes 1, 3 had been incubated without collagenase; samples in lanes 2, 4 had been incubated with rat collagenase, 6.7 $\mu\text{g/ml}$. Samples had been incubated for 18 h at 20°C. These results on the collagenase digestion of crosslinked collagen from the mouse homozygous for mutation IV are similar to those in B using collagen from the hemizygous mouse.

chains. Similar results were also obtained with collagen isolated from homozygous *Col1a1^{tm1 Jae}* mice (Fig. 5 C). These results suggested that the rodent enzyme recognizes a novel cleavage site located carboxy-terminal to the crosslinking site.

To more accurately map the novel NH_2 -terminal collagenase cleavage site, mutant type I collagen was digested with rat collagenase and the products were resolved by SDS-PAGE and transferred to Immobilon-P membranes. The appropriate bands were excised for amino acid sequence analysis and the results of the digested and undigested samples were compared. The NH_2 -terminal sequence of the undigested $\alpha 1(\text{I})$ chain was TyrAspGlu-LysSer whereas that of the digested $\alpha 1(\text{I})$ chain was ValSerValProGlyVal. Comparison with the peptide sequence encoded by the nucleotide sequence of Exons 6 through 10 of the mouse *Col1a-1* gene (Breindl, M., unpublished data) places the NH_2 -terminal rodent enzyme-specific cleavage site between a Gly and Val, four residues preceding the start of the major helix and five residues C-terminal to the putative cross-linking Lys (Fig. 6). We

conclude that the rat fibroblast collagenase recognizes two different sites in type I collagen: (a) a cleavage site in the collagen helical domain between residues 775 and 776 of the $\alpha 1(\text{I})$ chain which is also recognized by all other collagenases so far tested; (b) a second cleavage site COOH-terminal to the lysine-derived cross-link in the N-telopeptide of the $\alpha 1(\text{I})$ chain.

Discussion

Embryonic Lethality of Transgenics Carrying a Collagenase-resistant Type I Collagen Gene

It has been well established that vertebrate collagenase initiates degradation of native collagen by cleavage at a highly conserved sequence motif present in types I, II, and III collagens in species as diverse as man and *Xenopus*. Consistent with the conclusion that collagenase recognizes a single site in native collagen, point mutations of the mouse $\alpha 1(\text{I})$ collagen chain such as mutation IV which would be predicted to affect cleavage and stabilize the tri-

N-TERMINAL SEQUENCE OF MOUSE $\alpha 1(I)$ COLLAGEN

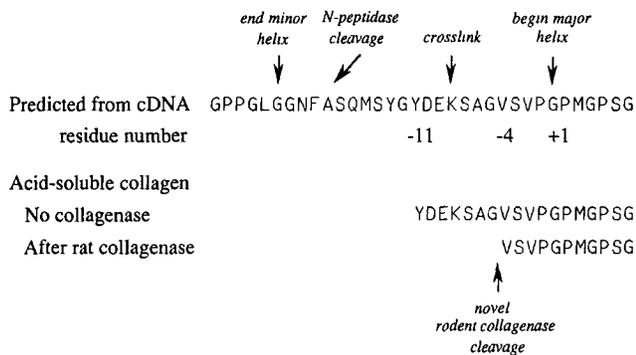


Figure 6. A novel cleavage site near the NH₂ terminus of the $\alpha 1(I)$ chain. A partial NH₂-terminal sequence of pro $\alpha 1(I)$ chain predicted from the cDNA sequence and the NH₂-terminal sequence of $\alpha 1(I)$ chain with or without rat collagenase digestion were presented. The amino acid residues are numbered as such; the glycine (G) at the beginning of the major helix is +1. Several other sites in this region are indicated with arrows.

ple helix around the cleavage site render the protein resistant to degradation (58). Transgenic mice carrying mutation IV in multiple copies indeed produced type I collagen highly resistant to cleavage by collagenase, indicating that the mutation had the predicted effect in vivo. With the exception of the founder, animals carrying the transgene invariably died. Previous experiments had established that the transgene constructs carried all known regulatory elements required for tissue specific expression (unpublished data), suggesting that ectopic expression of the transgene was not the cause of lethality. The S1 analysis as well as protein analysis indicated that the transgene was highly expressed, resulting in the accumulation of cleavage-resistant type I collagen. The ratio of $\alpha 1(I)/\alpha 2(I)$ peptide chains of type I collagen from transgenic mice varied from 3:1 to 15:1 compared to 2:1 in the collagen from wild-type mice, consistent with the accumulation of $\alpha 1(I)$ homotrimers (Fig. 1). Overexpression of collagen and the deposition of type I homotrimers lacking $\alpha 2(I)$ chains, rather than expression of a mutant collagen, may, therefore, be the primary explanation for the embryonic lethal phenotype of the transgenics. This conclusion was confirmed by the lack of a lethal phenotype in mutant mice carrying the same mutation in the endogenous gene, as discussed below. We have observed occasionally that transgenic embryos overexpressing type I collagen from a wild-type *Colla-1* transgene died prenatally with a phenotype similar to that observed with the mutant transgene described here (unpublished data). These observations support the notion that embryonic lethality may be caused by overexpression of the transgene rather than by the mutation introduced.

Attempts have been made to analyze the function of a particular gene in development by expressing a mutant form of the protein in transgenic mice (dominant negative mutations: reference 22). Our results emphasize that caution must be used in interpreting the data in transgenics carrying mutant transgenes since the phenotypes may not always be due to the expression of the mutant form of the

protein but may also be due to overexpression or ectopic expression of the transgene. This caveat is particularly relevant when the stoichiometry of a multimeric protein such as collagen is altered by overexpression of one component of the multimer (23, 38, 41, 47, 49).

Mice Producing Mutant Collagen From *Colla1*^{tm1 Jae} Develop Normally to Adulthood

In contrast to the transgenic mice discussed above, mice generated by gene targeting technology carrying the same mutation in their endogenous *Colla-1* gene developed normally to young adulthood and only later displayed alterations compatible with impaired collagen turnover. Type I collagen isolated from homozygous *Colla1*^{tm1 Jae} mice was completely resistant to cleavage by human interstitial synovial collagenase, confirming that the mutation had the predicted effect in vivo. Only 26% of collagen extracted from heterozygous mutants was cleaved by the human collagenase, suggesting that the mutation acted in a dominant negative manner.

Our observations that homozygous *Colla1*^{tm1 Jae} mice developed normally to adulthood posed an interesting problem: how could type I collagen turnover be accomplished during embryogenesis in the absence of a functional collagenase cleavage site? The identification of the second site at the N-telopeptide domain of the protein, that is recognized by rodent fibroblast collagenase but not by the human enzyme, suggests the possibility that cleavage at this previously unrecognized site could be sufficient to achieve collagen degradation during embryonic development. Cleavage at this site alone, however, seems not to be adequate to assure full equilibrium between collagen synthesis and degradation during later life. Our results are consistent with the concept that enzymes with different specificity utilizing two distinct cleavage sites are involved in collagen degradation. The two sites may be of different significance for type I collagen degradation in prenatal and postnatal life.

Tissue Remodeling in the Adult Depends on a Functional Collagenase Cleavage Site between Amino Acid Residues 775 and 776

As mutant mice aged, they developed a number of alterations which reflected their inability to degrade type I collagen during tissue remodeling. In the skin, the alterations consisted of thickening of the dermis, hair loss, and patchy ulcerations. Histochemical analysis revealed substantial accumulation of collagen in the dermis, indicating the importance of collagen turnover in the skin of the adult mouse. It had been shown previously that cultured human skin produces large amounts of collagenase, but the significance of this observation for the in vivo situation had not been established (4). The accumulation of collagen in the dermis from mutant mice suggests, however, that type I collagen is actively turned over even in adult skin. Clearly, the degradation of type I collagen in the adult depends critically on the presence of a functional collagenase cleavage site between Gly₇₇₅ and Ile₇₇₆. In contrast, cleavage at the newly defined N-telopeptide site by rat collagenase or another, as yet unidentified proteinase, may be sufficient for collagen degradation during embryonic development

and in the young adult. It is interesting to note that the alterations in the skin of mutant mice resemble those seen in the skin of patients with scleroderma (thickening of the dermis and accumulation of irregular collagen fibers; references 32, 39). This raises the possibility that mutations at the collagenase cleavage site of type I collagen contribute to the dermal fibrosis in some patients with scleroderma.

The second major defect caused by the mutant collagen became evident in the impaired reproductive ability of mutant females. In mammals, the uterine collagen mass changes considerably during pregnancy and postpartum involution (16, 17, 33, 55). In mice, the total uterine collagen mass increases up to 20-fold during pregnancy, and after parturition the uterus rapidly recovers to prepregnant size. This postpartum involution is accomplished within the first two days after giving birth and involves transcriptional activation of the collagenase gene and increased release of collagenase extracellularly followed by the massive degradation of most of the collagen deposited in the uterus within a few days (44, 45). In mutant females, degradation of collagen was severely disturbed, leading to the accumulation of nodules in the uterine wall. These nodules consisted of large collagen aggregations, reflecting the impaired collagen degradation during the postpartum period. The maintenance of a high collagen content in the postpartum uterus is presumably responsible for the reduced number of litters and decreased litter size of mutant females. Our results suggest that the massive degradation of collagen at the time of parturition is critically dependent on the presence of a functional cleavage site between Gly₇₇₅ and Ile₇₇₆. Our observations that there is a diffuse as well as nodular pattern to the collagen deposition in the mutant postpartum uterus suggests that the intensity of the normal resorptive process initiated at the time of parturition is not uniform throughout the uterus.

A third manifestation of the mutation was seen in bone remodeling. Preliminary observations suggest that, while overall bone development was normal in Colla1^{tm1} Jae mice, deformities of the tibia and increased deposition of trabecular and cortical bone in femurs and tibias were frequent after the age of 6 mo, suggesting that bone remodeling may also be affected (unpublished observations).

Type I Collagen Degradation Is Initiated at Two Distinct Cleavage Sites

The discovery of a novel cleavage site recognized by the rat fibroblast collagenase but not the known human, bovine, porcine or rabbit enzymes provides new insights into the mechanisms of collagen turnover in development and disease (summarized in Fig. 7). Previously it was thought that the major mechanism of degradation of native collagen involves cleavage between Gly₇₇₅ and Ile or Leu₇₇₆, a highly conserved sequence recognized by all interstitial collagenases. We have shown here that rat collagenase cleaves, in addition, the type I heterotrimer in the N-telopeptide region of the $\alpha 1(I)$ chain between a Gly-Val bond four residues before the sequence Gly-Pro-Met, the first triplet of the major collagen helix. Consistent with this specificity is the observation that several collagenases cleave a variety of susceptible peptide bonds in noncollagenous proteins, including a Gly-Val bond in the human

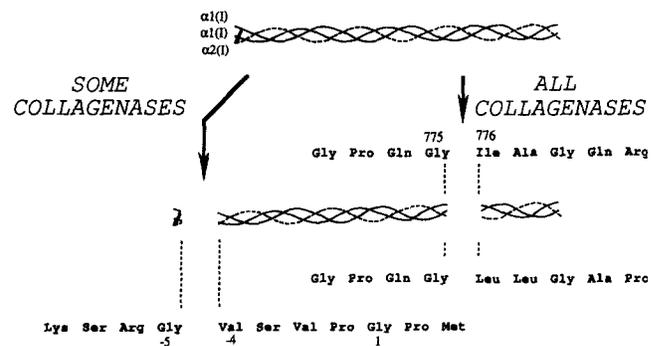


Figure 7. Proposed scheme for collagenase cleavage of type I collagen. Type I collagen is a helical trimeric molecule consisting of two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain. The position of interchain crosslinks that involve modified Lys side chains, at residue -5, in the N-telopeptide region between the $\alpha 1(I)$ chains or between one $\alpha 1(I)$ chain and the $\alpha 2(I)$ chain is indicated. These modified Lys residues would also be involved in intermolecular crosslinks in collagen fibrils and fibers. Degradation of type I collagen requires the cleavage by collagenases at defined sites in the molecule. All collagenases so far described recognize a cleavage site in the helical portion of the molecule, as shown, between amino acid residues 775 and 776. Some collagenases, such as the rodent fibroblast collagenases, recognize an additional site in the N-telopeptide region and would cleave between the Gly at residue -5 and Val at -4 as demonstrated in this report (Fig. 6).

pregnancy zone protein (8, 46). Proteinases, including metalloproteinases other than the collagenases so far described (see below), might also function in the degradation of collagen by cleaving the proteins in the nonhelical domains. For example, stromelysin has been shown to cleave another interstitial collagen, type II collagen, at sites in the N-telopeptide region (61).

The novel cleavage site recognized by the rodent enzyme would have been difficult to detect using the wild-type type I collagen as a substrate. In our experiments, the use of animal tissues which contain crosslinked collagen as a source of mutant collagen facilitated the identification of the novel cleavage site. Digestion of wild-type but not the mutant type I collagen by the human enzyme cleaved the $\beta^{1,1}$ and $\beta^{1,2}$ cross-linked dimers to the $A^{\beta 1,1}$ and $A^{\beta 1,2}$ products. In contrast, the rodent enzyme, cleaving COOH-terminal to the cross-link, further digested the $A^{\beta 1,1}$ and $A^{\beta 1,2}$ dimers to $A^{\alpha 1}$ and $A^{\alpha 2}$ and cleaved $\beta 1,1$ and $\beta 1,2$ from mutant mice to fragments comigrating with $\alpha 1(I)$ and $\alpha 2(I)$ chains (Fig. 5). In previous studies with rat collagenase, the NH_2 -terminal cleavage site was not detected since the substrate used contains little crosslinked collagen and the digestion did not reach completion (42, 51).

While our studies were in progress, the mouse fibroblast collagenase gene was cloned and sequenced (21). Sequence comparison of the mouse and rat collagenases revealed 97% identity at the amino acid level whereas both differ significantly from the human neutrophil and fibroblast collagenases as well as the porcine, rabbit and bovine fibroblast enzymes (52–53% amino acid sequence identity, references 14, 19, 53). Recently, we have found that mouse collagenase, similar to the rat enzyme, cleaves type I collagen at the NH_2 -terminal site (Krane, S., M. Byrne, Y. Eeckhout, P. Henriot, V. Lemaitre, X. Liu, H. Wu, R. Jaenisch, manuscript in preparation) suggesting that the ro-

dent enzymes represent a class of collagenases with different specificities than those of the previously characterized mammalian enzymes. It has just been reported that during murine embryonic development, expression of this collagenase is not detected by *in situ* hybridization until E15(31). At that time expression is limited to cells in the developing skeleton such as hypertrophic chondrocytes, osteoblasts and endothelial cells. In view of these findings and those described in this paper, the action of other proteinases must also be necessary for the soft tissue remodeling during embryonic growth. A new enzyme with 86% amino acid sequence identity to the rodent collagenases has recently been cloned from a human breast carcinoma library (13). This enzyme likely represents a human homologue of the rodent enzymes and would therefore be expected to cleave type I collagen at the NH₂-terminal as well as the helical sites. Another enzyme, the human 72-kD gelatinase (type IV collagenase), has been shown recently to cleave helical collagens at the same Gly₇₇₅/Leu₇₇₆ locus as the collagenases if the preparations are free of TIMP (1). It is not yet known whether this enzyme could cleave in the N-telopeptide region. Therefore, the enzymes that are responsible for the collagen resorption during embryonic remodeling remain to be determined. The normal development of the mutant mice to adulthood suggests that cleavage at the NH₂-terminal site is one possibility to account for normal type I collagen degradation during embryogenesis and early adulthood. The pathological changes seen in older animals, however, suggest that cleavage between Gly₇₇₅ and Ile₇₇₆ is essential for rapid collagen turnover in the adult.

The phenotype of the mice has some resemblance to human scleroderma. It will therefore be of major interest to investigate whether similar mutations around the helical collagenase cleavage site occur in humans. It is also important to establish the role of the novel N-telopeptide cleavage during embryogenesis and postnatal life. Mice carrying mutations at this site should provide an excellent tool for further investigating type I collagen turnover in physiological and pathological remodeling of the extracellular matrix during embryogenesis and adulthood.

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