THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 45, Issue of November 8, pp. 28509–28515, 1996

Printed in U.S.A.

Different Collagenase Gene Products Have Different Roles in Degradation of Type I Collagen*

(Received for publication, May 25, 1996, and in revised form, August 19, 1996)

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Vertebrate collagenases, matrix metalloproteinases (MMPs), cleave type I collagen at a single helical locus. We show here that rodent interstitial collagenases (MMP-13), but not human fibroblast collagenase (MMP-1), cleave type I collagen at an additional aminotelopeptide locus. Collagenase cDNAs and chimeric constructs in pET-3d, juxtaposing MMP-13 sequences amino-terminal to the active site in the catalytic domain and MMP-1 sequences carboxyl-terminal and vice versa, were expressed in *Escherichia coli*. Assays utilized collagen from wild type (+/+) mice or mice that carry a targeted mutation (r/r) that encodes substitutions in α1(I) chains that prevent collagenase cleavage at the helical locus. MMP-13 and chimeric molecules that contained the MMP-13 sequences amino-terminal to the active site cleaved (+/+) collagen at the helical locus and cleaved cross-linked (r/r) collagen in the aminotelopeptide (β components converted to α chains). Human MMP-1 and chimeric MMP-1/MMP-13 with MMP-1 sequences amino-terminal to the active site cleaved collagen at the helical locus but not in the aminotelopeptide. All activities were inhibited by TIMP-1, 1,10-phenanthroline, and EDTA. Sequences in the distal two-thirds of the catalytic domain determine the aminotelopeptide-degrading capacity of MMP-13.

When bone and other connective tissues are remodeled, components of the extracellular matrix are degraded and removed, and new components are synthesized and deposited. Three main classes of enzymes have been implicated in the degradation of collagen, the most abundant component of the extracellular matrix. The first includes members of the matrix metalloproteinase (MMP)[1] family, the second, lysosomal cysteine proteinases such as cathepsins B and L which cleave collagens in the telopeptide domain, and the third, serine proteinases such as plasmin, generated through the plasminogen activator system (1–6). Plasmin and cathepsin B have been implicated in the activation of the MMP zymogens (7). It is likely that the MMPs have a role in the physiological resorption of collagen in the uterus, in embryonic development and postnatal remodeling, and in pathological processes such as local invasion by malignant tumors, resorption of periodontal structures in periodontal disease, or the destruction of joints in rheumatoid arthritis (1–5).

The MMPs or matrixins are members of a large subfamily of proteinases and have many common structural features (2, 3, 5). Included in the MMP subfamily (8) are genes that encode at least three collagenases, three stromelysins and several gelatinases among which are cell-bound forms with a transmembrane domain (9). Although there is considerable conservation of amino acid sequences and sequence motifs among the human MMPs, only the collagenases ("fibroblast" collagenase (MMP-1 or collagenase-1), "neutrophil" collagenase (MMP-8 or collagenase-2), and the rodent-type interstitial collagenase which is homologous to the recently described human collagenase-3 or MMP-13 (10)) can cleave native, undenatured, "interstitial" collagens (types I, II, III, and X) within the triple helical domain at neutral pH (1–7). Other MMPs such as the stromelysins do not cleave native collagens in the helical domain; data indicate that the carboxyl-terminal domain of collagenase is a major determinant of substrate specificity (11). In type I collagen, the cleavage occurs between Gly^{775}/Ile^{776} in the α1(I) chain and Gly^{775}/Leu^{776} in the α2(I) chain, three-fourths the distance from the amino terminus yielding a larger (A) fragment and a smaller (B) fragment (12).

Our strategy to understand the role of collagenases has been to generate mutations in the mouse *Col1a-1* gene that encode resistance to collagenase cleavage in the helical domain of type I collagen and test them by transfection into Mov13 cells that have a block in endogenous transcription of *Col1a-1* due to a retroviral insertion (13). Recently, we introduced one of these mutations that encodes a double substitution of Pro for Gln^{774} and Ala^{777} into the endogenous *Col1a-1* gene (14, 15) via homologous recombination in embryonic stem cells. We observed that the mice carrying this mutation (r) developed normally

* This work was supported in part by National Institutes of Health Research Grants AR-03564 and TR-AR-07258 (to S. M. K.), HD-05291 (to J. J. J.), and HL-41484 (to R. J.) and by Grant 3.4522.91F of the Belgian Fonds de la Recherche Scientifique Médicale and a Grant from the Belgian Programme on Interuniversity Poles of Attraction, Prime Minister's Office, Federal Services of Scientific, Technical and Cultural Affairs (to Y. E.). 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.

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‖ Supported in part as Research Fellows of the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture.

‡‡ Supported in part as an Established Investigator of the Arthritis Foundation.

[1] The abbreviations used are: MMP, matrix metalloproteinases; PCR, polymerase chain reaction; TIMP, tissue inhibitor of metalloproteinases; bp, base pair(s); APMA, *p*-aminophenylmercuric acetate; PAGE, polyacrylamide gel electrophoresis; CLH, recombinant human fibroblast (MMP-1) collagenase; CLM, mouse interstitial collagenase.

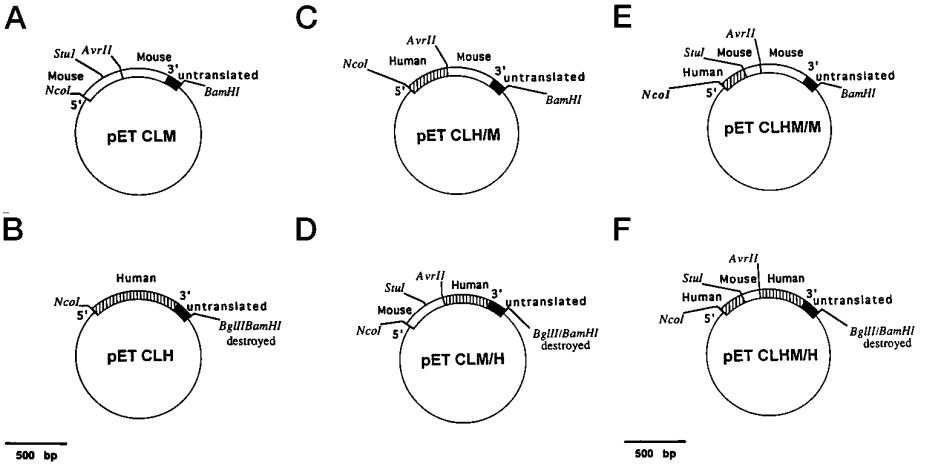

FIG. 1. **Diagram of the structure of mouse interstitial collagenase (*CLM*) and human fibroblast (MMP-1) collagenase (*CLH*) cDNAs and chimeric constructs in the pET-3d expression vector.**

and had no abnormalities as young adults, yet the type I collagen extracted from the skin and tendons of homozygotes was not cleaved either by rat or human fibroblast collagenase at what was previously considered to be the single site in the helical domain. After approximately 3–6 months of age, the mice carrying the mutation did begin to display thickened skin with dermal fibrosis and nodules of collagen in the uterus of postpregnant females. We then found that rat interstitial collagenase (16, 17) which has 97% amino acid sequence identity with the mouse (18) made an additional cleavage in the aminotelopeptide region carboxyl-terminal to the putative cross-linking Lys residue. We show here that the capacity to cleave in the aminotelopeptide is a property of the rodent interstitial collagenases which have only ~50% amino acid sequence identity with the human MMP-1. Our studies of expressed recombinant chimeric molecules of the mouse (MMP-13) and human (MMP-1) interstitial collagenases indicate that this property is determined by amino acid sequences in the distal two-thirds of the catalytic domain of the mouse collagenase extending to the Zn^{2+}-binding domain. Cleavage at the aminotelopeptide site by collagenases that contain these sequences in the catalytic domain could contribute to remodeling of type I collagen-containing tissues during embryonic development and postnatal life. Additional cleavage at the helical site is necessary for remodeling later in adulthood in both physiological and pathological conditions.

EXPERIMENTAL PROCEDURES

Materials—Tosylphenylalanyl chloromethyl ketone-treated trypsin, soybean trypsin inhibitor, and pepsin (2858 units/mg, Cooper Biomedical) were purchased from Worthington Biochemical Corp. (Freehold, NJ). *p*-Aminophenylmercuric acetate (APMA) was purchased from Sigma. Restriction endonucleases and ligases were purchased from New England Biolabs (Beverly, MA).

Preparation of Collagens and Collagenase Assays—Collagens were extracted from the skin or tail of wild type (+/+) and mutant (r/r or r/−) mice by digestion with pepsin, 1 mg/ml, in 0.5 M acetic acid at 0–4 °C for 2–4 days, or without pepsin but with aprotinin, leupeptin, and pepstatin at 5 μg/ml and phenylmethylsulfonyl fluoride at 100 μg/ml in order to enrich for cross-linked components and prevent nonspecific proteolytic degradation at the nonhelical ends of the molecules during extraction (15). Samples were obtained from mice homozygous (r/r) for this mutation and in some experiments from the r/− (null) progeny of heterozygous (r/+) mice back-crossed into Mov13. Acid- and acid/pepsin-extracted collagens were dialyzed against collagenase buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, and 1 μM $ZnSO_4$) just prior to incubation. Collagen was also extracted from samples of human newborn foreskin in 0.5 M acetic acid, without pepsin. The insoluble residue in the extracts was removed by centrifugation, and the collagen content of an aliquot portion was determined by measurement of hydroxyproline concentration following acid hydrolysis (19). The pepsin was inactivated by neutralization with NaOH, and the solution was dialyzed against 0.5 M acetic acid overnight at 4–5 °C and finally brought to a final concentration of 0.02% sodium azide for storage at 4–5 °C (15, 20).

Incubation with collagenase was carried out for various periods up to 26 h at 20 °C in a constant temperature water bath, and the reaction was stopped by the addition of EDTA to a final concentration of 50 mM; the samples were stored at 4–5 °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 0.25% Coomassie Blue in 20% methanol, 10% acetic acid and destained in 20% methanol, 10% acetic acid. The human collagenase preparations used here were partially purified from medium conditioned by cultured rheumatoid synovial fibroblasts as described previously (21). An additional preparation of human fibroblast collagenase, MMP-1, was a gift of Dr. Michael Lark (Merck, Sharp and Dohme, Rahway, NJ). Rat collagenase was purified from medium conditioned by postpartum rat uterine cells as described previously (16). Mouse interstitial collagenase from mouse bone explant cultures was prepared and immunoaffinity-purified as described (22). A purified sheep fibroblast collagenase preparation was a gift of Dr. Hideaki Nagase (University of Kansas, Kansas City, KA). Recombinant TIMP-1 was a gift from Dr. Howard Welgus (Washington University, St. Louis, MO). Latent collagenase was activated with trypsin, 5 μg/ml for 10 min at 20 °C, and the trypsin was inactivated with 10-fold excess soybean trypsin inhibitor (13); alternatively, activation was with APMA, 1 mM, added at the beginning of the incubation (23, 24). 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.

Amino-terminal Peptide Sequencing—Collagen from the skin of homozygous mutant (r/r) mice was extracted as described in 0.5 M acetic acid without pepsin. One-half of the sample was incubated in collagenase buffer with recombinant mouse fibroblast collagenase for 18 h at 20 °C; the other half of the sample was incubated in buffer alone. Approximately 150 μg 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 Corp.). The proteins corresponding to "α1(I)" chains were cut out, and peptide sequencing was performed as described (15, 25).

Preparation of Collagenase cDNA Constructs and Expression in E. coli—A diagram of the partial structure of mouse interstitial and human fibroblast (MMP-1) collagenase cDNAs in the pET-3d vector (Novagen, Madison, WI) that forms the basis for the construction of chimeric collagenases is shown in Fig. 1. Standard procedures were used to prepare these constructs (26, 27). The mouse interstitial collagenase cDNA (18) was modified to contain a 5′-*Nco*I site at bp 10 and a 3′-*Bam*HI site at bp 1639 to facilitate cloning into the pET-3d expression vector by Lemaître *et al.*[2] The new 5′ sequence, 5′-CATGGATTCAGCTATC-3′, encodes a substitution of Asp for His at amino acid residue 2. The new 3′ sequence that contains the *Bam*HI site is in the untranslated region. This construct is named pET CLM and encodes a peptide of 472 amino acids. The human collagenase (MMP-1) cDNA (28), a gift from Dr. H. J. Rahmsdorf (Karlsruhe, Germany), was used as

[2] V. Lemaître, P. Henriet, and Y. Eeckhout, manuscript in preparation.

template for amplification by PCR (27) with the upstream primer, 5′-AAAGGCCACCATGGACAGCTTTCC-3′, and the downstream primer, 5′-AGGGAACGGATAGATCCCACTGTGG-3′. In this and other PCR reactions, UltraTaq polymerase (Perkin-Elmer) was used. The new 5′-sequence containing the *Nco*I site at bp 66 also encodes a substitution of Asp for His at amino acid residue 2. This PCR fragment contains a naturally occurring *Bgl*II site at bp 1736. The fragment from bp 66–1735, *i.e.* 1670 bp, was ligated into the *Nco*I/*Bam*HI sites of the pET-3d vector and named pET CLH; it encodes a peptide of 469 amino acids. To construct the chimeric molecule with amino-terminal human sequences, we took advantage of an *Avr*II site present in the mouse collagenase (MMP-13) cDNA that is not present in the human MMP-1 cDNA. The human MMP-1 cDNA (above) was used as template for amplification by PCR with the upstream primer, 5′AAAGGCCACCATGGACAGCTTTCC-3′, that contains the *Nco*I site. The downstream primer, 5′-GGGAGAGTCCTAGGGAATGGCCGA-3′, was designed to create a new *Avr*II site in the human MMP-1 sequence at bp 736 and contains a substitution of C for T at bp 735 and A for T at bp 738 that still maintains codons for Ser and Leu, at residues 223 and 224, respectively (*i.e.* residues 228 and 229 in the cDNA sequence of Henriet *et al.* (18)). After incubation with *Nco*I and *Avr*II restriction endonucleases, the resultant human MMP-1 *Nco*I-*Avr*II fragment from bp 66–735 was then exchanged in pET CLM for the mouse *Nco*I-*Avr*II fragment from bp 10–694 and named pET CLH/M. To construct the chimeric molecule with the mouse sequences in the amino-terminal portion, the human MMP-1 cDNA was used as template for amplification by PCR with the upstream primer, 5′-CTCGGCCATTCCCTAGGACTCTCC-3′, that contains a new *Avr*II site as above and the downstream primer, 5′-AGGGAACGGATAGATCCCACTGTGG-3′. The PCR product was cut with *Avr*II at bp 736 and *Bgl*II at bp 1736, and this human MMP-1 *Avr*II-*Bgl*II fragment of bp 736-1735 was exchanged in pET CLM for the mouse *Avr*II-*Bam*HI fragment of bp 695-1638 and named pET CLM/H.

In order to more precisely localize the amino acid sequences that determine the capacity of the collagenases to cleave the aminotelopeptide site, additional constructs were similarly prepared (Fig. 1, *E* and *F*). A *Stu*I site within the codon for Ala^{141} was identified between the *Nco*I and the *Avr*II sites in the pET CLM sequence and was used to further subdivide this region. A new PCR product was synthesized using the 5′-pET CLH primer with the *Nco*I site and a 3′ primer, 5′-GAA GGC TTT CTC TAA GGC CTG GTC CAC ATC-3′, encoding a *Stu*I site with pET CLH as template. The PCR product was trimmed with *Nco*I and *Stu*I and substituted into pET CLM between these same sites to produce the chimeric molecule pET CLHM/M. Similarly, the new PCR product was trimmed and substituted between these two sites in pET CLM/H to produce the chimeric molecule, pET CLHM/H.

Each of the constructs described above and in Fig. 1 was expressed in BL21(DE3) or BL21(DE3)pLysS (Novagen) cells, grown to a density of 0.6 at $A_{600\ nm}$ and then induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (Boehringer Mannheim). After a 3–4-h incubation, the cells were harvested by centrifugation at 2000 × *g*, lysed by sonication, and the supernatant and inclusion body pellet fractions were separated by centrifugation at 30,000 × *g*. The inclusion body fraction was solubilized in 6 M guanidine and renatured by the dropwise addition of 1 volume to 10 volumes of 50 mM Tris·HCl, pH 7.4, containing 150 mM NaCl, 10 mM $CaCl_2$, and 1 μM $ZnSO_4$, by a modification of the procedure of Ye *et al.* (29) designed for expression of stromelysin-1 in *Escherichia coli*. The protein was precipitated with an equal volume of saturated $(NH_4)_2SO_4$ at 2–5 °C, harvested by centrifugation, dissolved in and dialyzed against collagenase buffer, and aliquots of 500 μl were frozen and stored at −70 °C until use.

RESULTS

Rodent Interstitial Collagenases but Not Human Fibroblast (MMP-1) Collagenase Cleave Type I Collagen at the Aminotelopeptide Site—We showed previously that purified rat interstitial collagenase (MMP-13) and human fibroblast collagenase (MMP-1) cleave wild type (+/+) collagen at the helical locus to yield the three-fourths length A^{α} and and one-fourth length B^{α} fragments (15). Although neither collagenase cleaves the collagen extracted from the skin of homozygous collagenase-resistant mice (r/r) at the helical locus, the rat collagenase, but not the human MMP-1, cleaves at a site just down from the aminotelopeptide cross-link. The assays for the latter cleavage are based on the formation of chains migrating similarly to α chains using collagens extracted from the r/r mice that are enriched in cross-links such as β components. The assays are shown schematically in Fig. 2. Cleavage at the helical site is determined by measuring the conversion of α1(I) chains to $A^{\alpha1(I)}$ chains using pepsinized +/+ collagen, which contains few cross-linked (β and γ) components, as substrate. Cleavage at the aminotelopeptide site is determined by measuring the conversion of β components to α1(I) chains using highly cross-linked collagen, extracted with acid, but no pepsin, from r/r mice, as substrate. Protein bands are resolved by SDS-PAGE and quantitated by densitometric analysis of the Coomassie Blue-stained bands. With some preparations of human MMP-1, partially purified from medium conditioned by fibroblasts cultured from synovium derived from patients with rheumatoid arthritis, a low but perceptible rate of cleavage at the aminotelopeptide site was observed (data not shown). Purified sheep and rabbit MMP-1 (~50% amino acid sequence identity with the rat collagenase but ~85–90% identity with the human enzyme and each other), activated with APMA and stromelysin-1, also readily cleaved at the helical site but at low rates or not at all at the amino-terminal site (data not shown). Purified rat collagenase also cleaved the cross-linked components in human, acid-extracted collagen to α chains, based on the relative conversion to A^{β} components and A^{α} fragments (Fig. 3).

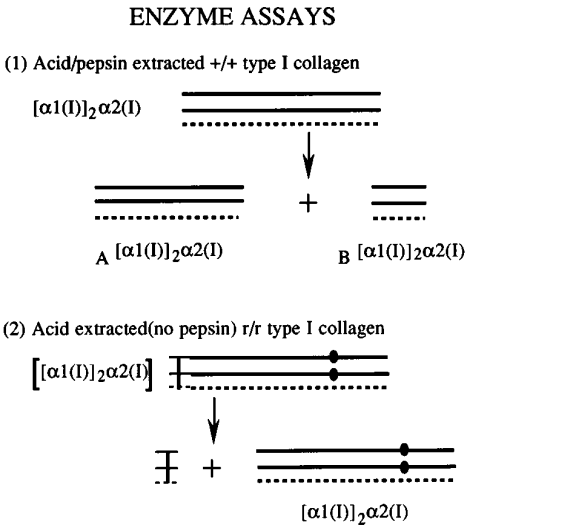

FIG. 2. **A scheme for the collagenase assays.** To measure cleavage at the helical site (*1*), the substrate is type I collagen extracted from +/+ mouse skin and/or tail with pepsin in acetic acid. This substrate contains relatively few cross-linked (β and γ) components. To measure cleavage at the aminotelopeptide site (*2*), the substrate is type I collagen, relatively enriched in cross-linked components, extracted from r/r or r/− mouse skin or tail with acetic acid in the absence of pepsin. Following incubation for the stated period at 20 °C, the reaction is stopped with excess EDTA. For the measurement of cleavage at the helical site, the conversion of α1(I) chains of +/+ type I collagen to $A^{\alpha1(I)}$ fragments is quantitated, and, for measurement of cleavage at the aminotelopeptide site, the conversion of the β components of r/r or r/− collagen to α1(I) chains is quantitated. The latter substrate, which cannot be cleaved at the helical site, is relatively enriched in cross-linked components since extraction in 0.5 M acetic acid was carried out in the absence of pepsin. The position of the mutated amino acid sequences in the α1(I) chains that confer collagenase resistance is indicated by ●.

Purified mouse collagenase acted on the collagens extracted from +/+ and r/r mice in a manner indistinguishable from that of the rat enzyme (Fig. 4). The aminotelopeptide cleavage activity is therefore a property shared by the rodent fibroblast collagenases but not by human MMP-1 or the related enzymes from other species. Nevertheless, the human type I collagen is a substrate for this cleavage by the rodent MMP-13 collagenase.

Effects of Inhibitors on Collagenase Cleavage at Helical and

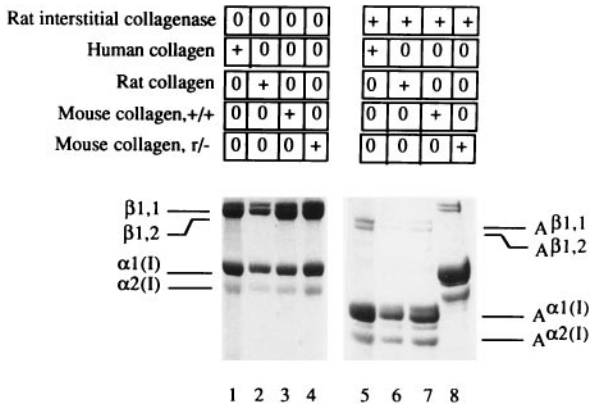

FIG. 3. **Digestion of collagens from human, rat, and mouse by purified rat interstitial collagenase.** *Lanes 1* and *5*, acid-soluble human foreskin collagen; *lanes 2* and *6*, acid-soluble rat tail tendon collagen; *lanes 3* and *7*, acid-soluble collagen from +/+ mouse skin; *lanes 4* and *8*, acid-soluble collagen from skin of the progeny of +/r mice back-crossed into Mov13 mice (r/−). Purified rat interstitial collagenase was incubated for 18 h at 20 °C, as in *A* with samples shown in *lanes 5–8*. Buffer without collagenase was incubated with samples shown in *lanes 1–4*. It can be seen that the β components of the collagens from normal human, rat, and mouse were converted predominantly to A^{α} fragments rather than B^{α} fragments or, as in the case of the collagen from r/− mice, to α chains.

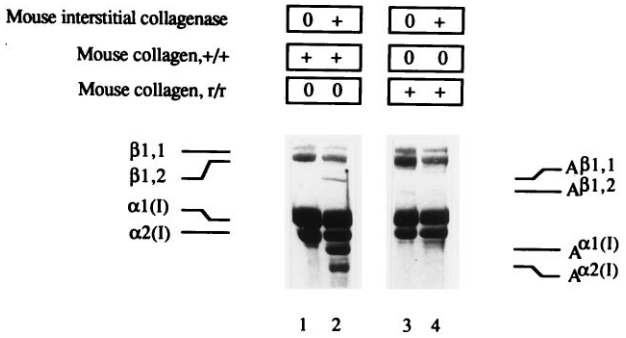

FIG. 4. **Effects of purified mouse interstitial collagenase on cleavage at the helical and aminotelopeptide sites in mouse type I collagen.** Collagens (50 μg) were incubated with 0.1 unit of purified mouse interstitial collagenase for 18 h at 20 °C as in Fig. 3, and reaction products were analyzed by SDS-PAGE (7.5% acrylamide) and quantitated by densitometry. Using densitometric quantitation of appropriate bands, it was determined that the collagenase converted ~37% of the α1(I) chains of +/+ type I collagen to $A^{\alpha 1(I)}$ fragments (helical cleavage) and ~34% of the β components of r/r collagen to α1(I) chains (aminotelopeptide cleavage).

Aminotelopeptide Sites—In order to be certain that the proteolytic conversion of β components to α chains by the preparations of purified rat interstitial collagenase used here is due to a MMP and not to a contaminating proteinase other than a MMP, 20 or 50 mM EDTA to bind the Ca^{2+} or 1 mM 1,10-phenanthroline to chelate Zn^{2+} was added to the reaction mixture; addition of either of these reagents resulted in complete inhibition of proteolytic activity at both the helical and aminotelopeptide sites (Fig. 5*A*). Furthermore, as is shown in Fig. 5*B*, these cleavages were both inhibited completely by the addition of recombinant human TIMP-1 at 30 μg/ml. These results further indicate that the amino-terminal cleavage activity is ascribable to a MMP.

Collagenolytic Activity of Recombinant Human and Mouse Chimeric Collagenases—Since preparations of purified human MMP-1 that cleaved at the helical site in collagen from +/+ mice did not cleave at the amino-terminal site or did so at a low rate, we assayed recombinant human fibroblast (MMP-1) and mouse interstitial collagenases and chimeric molecules of these collagenases to determine which broad domains are responsible

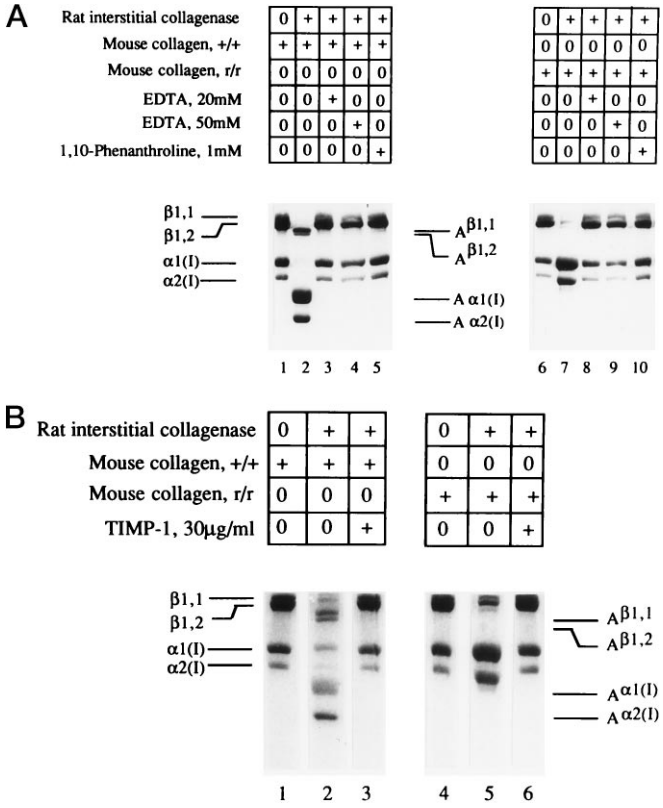

FIG. 5. **Effects of collagenase inhibitors on cleavage at the helical and aminotelopeptide sites in mouse type I collagen.** *A*, samples of collagen (500 μg/ml) extracted with pepsin in 0.5 M acetic acid from the skin of +/+ mice (*lanes 1–5*) or in 0.5 M acetic acid in the absence of pepsin from r/r mice (*lanes 6–10*) and dialyzed *versus* Tris·HCl, pH 7.5, NaCl, $CaCl_2$ were incubated with or without purified rat interstitial collagenase, EDTA, or 1,10-phenanthroline as shown. The incubation period was for 18 h at 20 °C, and the products were analyzed by SDS-PAGE. Note the parallel inhibition by EDTA and 1,10-phenanthroline of collagenase cleavage at the helical and aminotelopeptide sites, measured as described in Figs. 2–4. *B*, collagens from the skin of +/+ (*lanes 1–3*) and r/r mice (*lanes 4–6*) were incubated with or without rat interstitial collagenase, in the absence (*lanes 2* and *5*) or presence (*lanes 3* and *6*) of 30 μg/ml recombinant human TIMP-1 as shown. Note complete inhibition by TIMP-1 of collagenase cleavage at the helical (*lane 4*) and aminotelopeptide sites (*lane 6*) of type I collagen.

for the aminotelopeptide proteinolysis.

The constructs pET CLM, pET CLH, pET CLM/H, and pET CLH/M (Fig. 1, *A-D*) were expressed in *E. coli*, and enzymatic activity was assayed in the supernatant and inclusion body fractions, the latter after extraction with 6 M guanidine and renaturation in Ca^{2+}/Zn^{2+}-containing collagenase buffer. Enzyme activity, measured after activation with APMA, was usually present in the inclusion body fraction after renaturation rather than the supernatant fraction. The experiments described below include only those using the extracted renatured inclusion body fraction.

We found that all preparations had collagenase activity of varying potency assessed by cleavage at the helical site using the collagen extracted with pepsin from +/+ mice as substrate. As shown in Fig. 6, the recombinant human (MMP-1) collagenase (CLH) which cleaved at the helical site had negligible activity toward the aminotelopeptide site, whereas the recombinant mouse enzyme (CLM) cleaved at both sites. In contrast to the recombinant human (MMP-1) collagenase (CLH), the relative initial rate of cleavage by the mouse enzyme (CLM) at the aminotelopeptide site relative to the helical site, in the assay shown in Fig. 6, was calculated to be ~0.43. The chimeric collagenase that contained the human sequence amino-terminal to the Zn^{2+}-binding site at the active site in the catalytic

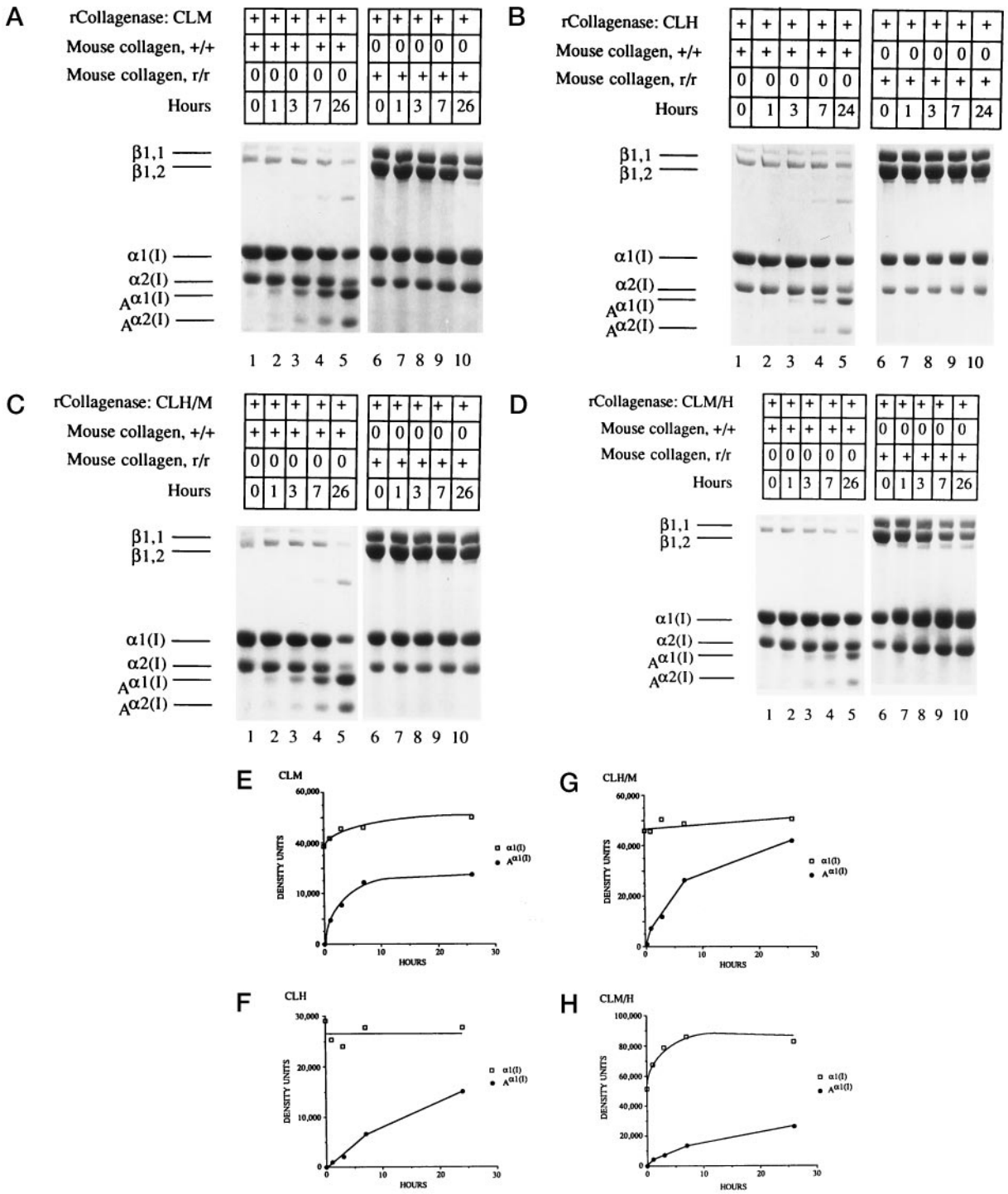

FIG. 6. **Effects of recombinant mouse interstitial and human fibroblast (MMP-1) collagenase and chimeric enzymes on cleavage at the helical and aminotelopeptide sites in mouse type I collagen.** The cDNAs for these collagenases were inserted into the pET-3d vector (Fig. 1) and expressed in *E. coli* as described under "Experimental Procedures." The collagenases were solubilized from the inclusion body fraction and renatured in buffer containing Zn^{2+} and Ca^{2+} as described. The time course of conversion of α1(I) chains of +/+ type I collagen to $A^{\alpha 1(I)}$ fragments (helical cleavage) and conversion of the β components of r/r collagen to α1(I) chains (aminotelopeptide cleavage) at 20 °C as described in Figs. 2–5 was quantitated. Photographs of the gels are shown in *A–D*. Densitometric quantitation of appropriate bands in arbitrary "density units" as a function of time of incubation is shown in *E–H*. *CLM*, mouse interstitial collagenase; *CLH*, human fibroblast (MMP-1) collagenase; *CLH/M*, human fibroblast (MMP-1) collagenase amino-terminal to the Zn^{2+}-binding site and mouse interstitial collagenase carboxyl-terminal; *CLM/H*, mouse interstitial collagenase amino-terminal to the Zn^{2+}-binding site and human fibroblast (MMP-1) collagenase carboxyl-terminal.

domain (CLH/M) had properties similar to those of the human MMP-1 collagenase (CLH), *i.e.* negligible activity toward the aminotelopeptide site. The chimeric collagenase with the mouse sequence amino-terminal to the Zn^{2+}-binding site (CLM/H) retained the activity of the mouse interstitial collagenase (CLM) toward the aminotelopeptide site. All preparations tended to lose activity on storage, but some stored preparations of the CLM/H collagenase tended to lose activity toward the helical site after weeks of storage at 2 °C but partially retain activity toward the aminotelopeptide site (data not shown).

In order to further localize the sequences in the collagenase

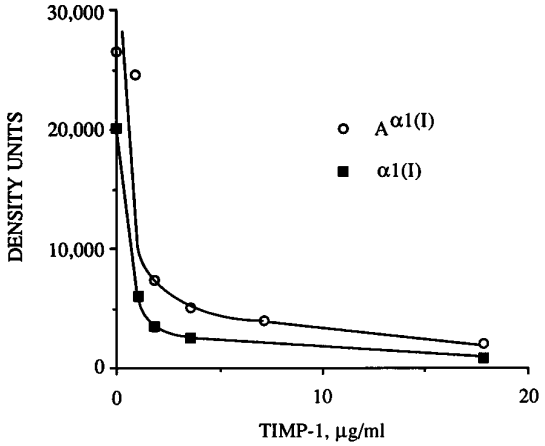

FIG. 7. **Effects of TIMP-1 on cleavage by recombinant mouse collagenase of type I collagen at helical and aminotelopeptide sites.** Sufficient expressed recombinant mouse interstitial collagenase (CLM) was incubated for 24 h at 20 °C to digest ~30% of wild-type(+/+) collagen in the absence of TIMP-1. In this experiment, the same amount of collagenase was also incubated under these conditions with +/+ collagen and r/r collagen and TIMP-1 at the concentrations indicated. Cleavage at the helical site was quantitated after SDS-PAGE as shown in Figs. 3–7 by measuring conversion of α1(I) chains of +/+ type I collagen to $A^{\alpha 1(I)}$ fragments and cleavage at the aminotelopeptide site by conversion of the β components of r/r collagen to α1(I) chains.

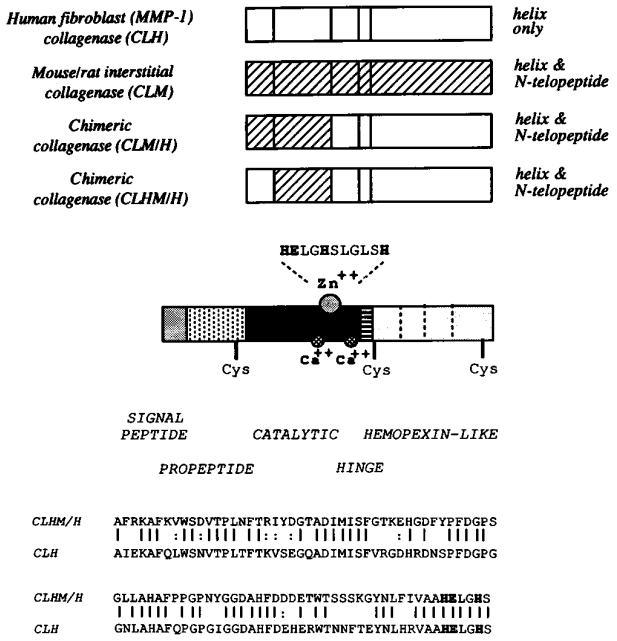

FIG. 8. **Schematic representation of domains in collagenases that determine cleavage of type I collagen at helical and aminotelopeptide sites.** Domains are depicted according to Birkedal-Hansen *et al.*(5). The amino acid sequences of the Zn^{2+}-binding site in the catalytic domain with the conserved residues (*bold*) are indicated. The *Avr*II site in the DNA used to construct the chimeric molecules corresponds to the junction of the codons for Ser and Leu codons in the Zn^{2+}-binding site. The human fibroblast (MMP-1) collagenase (*open areas*) cleaves only at the helical site, whereas the rat and mouse interstitial collagenases (*cross-hatched areas*) cleave at both the helical and aminotelopeptide sites. Recombinant chimeric human collagenases containing substitutions of sequences of the distal two-thirds of the mouse catalytic domain cleave at both sites. An alignment of sequences in the *cross-hatched area* of CLHM/H and those of CLH are shown at the *bottom* of the figure. Identical (|) and conserved (:) amino acids are indicated.

that determine the capacity to cleave at the aminotelopeptide site, the other two constructs, pET CLHM/M and pET CLHM/H (Fig. 1, *E* and *F*) were also expressed in *E. coli*. Active enzyme was extracted with 6 M guanidine from the inclusion body fraction and renatured as described for the other enzymes. We found that these chimeric molecules also cleaved at both the aminotelopeptide and helical sites similarly to the CLM and CLM/H collagenases (data not shown). These results indicate that the sequences that determine the capacity for the amino-terminal cleavage by the mouse interstitial collagenase extend from the distal two-thirds of the catalytic domain (corresponding to the position of the *Stu*I site in the cDNA) to the Zn^{2+}-binding site in that domain (corresponding to the position of the *Avr*II site in the CLM cDNA).

In order to determine the site in the aminotelopeptide domain where the recombinant mouse fibroblast collagenase cleaves, the CLM enzyme was incubated with collagen extracted without pepsin from r/r mice, and, following SDS-PAGE, the products were transferred to Immobilon-P membranes. The band migrating in the position of α1(I) chains was excised, and the amino-terminal sequences were determined. We found that the recombinant mouse collagenase cleaved in the aminotelopeptide region of type I collagen at the same site (between Gly and Val four residues before the Gly-Pro-Met triplet at the beginning of the major helix) as that cleaved by purified rat collagenase (data not shown).

Activity of all of the recombinant enzymes toward the helical site as well as the aminotelopeptide site in type I collagen was inhibited by TIMP-1 similar to the inhibition of the activity of preparations purified from fibroblast-conditioned medium described in Fig. 5. As shown in Fig. 7, increasing concentrations of TIMP-1 inhibited helical and aminotelopeptide cleavage by the recombinant collagenase, CLM, to approximately the same extent. More than 80% inhibition of both activities of the recombinant CLM/H enzyme was also observed at concentrations of TIMP-1 above 2 μg/ml (data not shown).

DISCUSSION

We show in this paper that mouse and rat interstitial collagenases previously thought to cleave at a single locus in the helical region of type I and other interstitial collagens have the capacity for cleavage in the aminotelopeptide region, distal to the putative cross-linking modified Lys residue. We first identified this additional site of cleavage with purified rat interstitial collagenase, using as substrate type I collagen extracted from tissues of mice that express an engineered mutation in both alleles of the *Col1a-1* gene that encodes resistance to cleavage at the helical site (14). The rat and mouse interstitial collagenases have ~97% sequence identity at the amino acid level (18). As we have demonstrated here, purified, as well as recombinant, mouse interstitial collagenase also cleaves type I collagen at the aminotelopeptide site. The cleavage at both sites is susceptible to inhibition by EDTA and 1,10- phenanthroline and by the specific inhibitor, TIMP-1, arguing that cleavage at this site is carried out by a MMP and not some other contaminating proteinase. As expected from the amino acid sequence similarity between human and mouse type I collagen, our results demonstrated that the rodent interstitial collagenases cleave at an aminoteleopeptide site in human as well as rodent acid-soluble collagens. The aminotelopeptide sequence of the human α1(I) chain (GenBank) is Lys-Ser-Thr-Gly-Gly-Ile-Ser-Val-Pro-Gly-Pro-Met, very similar to that of the mouse, Lys-Ser-Ala-Gly-Val-Ser-Val-Pro-Gly-Pro-Met. We predict that the site of aminotelopeptide cleavage in the human α1(I) chain would be between Gly and Ile rather than between Gly and Val that we demonstrated in the mouse α1(I) chain by amino acid sequence analysis (15). Our assays have all been performed using soluble collagen at 20 °C. Effects of collagenases on fibrillar r/r collagens have not yet been studied.

Our observations using the chimeric collagenases indicate

that determinants of proteolytic specificity toward the aminotelopeptide site in type I collagen are located in the distal two-thirds of the catalytic domain extending to the Zn^{2+}-binding site in the rodent interstitial collagenase molecule as shown schematically in Fig. 8. Although, as shown in Fig. 8, there is ~64% amino acid sequence similarity in this region of the mouse MMP-13 and human MMP-1 collagenases, substituting this domain of the mouse sequence confers aminotelopeptide cleavage activity on the human enzyme.

Nevertheless, in all chimeric molecules that we constructed, preservation of the carboxyl-terminal hemopexin-like domain maintained proteolytic activity toward the helical cleavage site. These results are consistent with previous reports indicating that the carboxyl-terminal hemopexin-like domain that has the capacity for collagen binding is essential for proteolytic activity toward triple helical interstitial collagens (11). Recombinant neutrophil collagenases in which the catalytic domains are preserved but contain engineered mutations encoding carboxyl-terminal truncations still retain nonspecific proteinase activity but do not attack helical collagens (31). Furthermore, exchange of the hemopexin-like domain of stromelysin-1, which does not cleave helical collagens, for that of the human fibroblast collagenase (MMP-1) results in a proteinase that also does not cleave helical collagens even though the chimeric enzyme retains the capacity to bind collagen (30). In our experiments, however, where we substituted the mouse amino-terminal domain for the human sequence, without carboxyl-terminal truncation, the capacity to cleave at the aminotelopeptide site was retained.

Our data on the effects of collagenases on cleavage of triple helical collagen that contains the targeted mutations in the α1(I) chains around the helical cleavage site indicate that all three chains in the type I heterotrimer must have the proper cleavable sequences (13, 15). Even a single mutated α1(I) chain prevents the cleavage of the other two +/+ chains (*i.e.* another α1(I) chain and an α2(I) chain). The K_m for the triple helical type I collagen molecules had been shown to be several orders of magnitude lower than that for the corresponding gelatins (32, 33). It is not known whether cleavage at the aminotelopeptide site also depends on the helical conformation of downstream sequences. Based on the results shown in Fig. 6, *A*, *D*, *E*, and *H*), cleavage at the aminotelopeptide appeared to reach a plateau sooner than at the helical site. This could possibly be explained if the K_m for cleavage at the aminotelopeptide is higher than that for cleavage at the helical site. Alternatively, native-type collagen itself might stabilize the helical activity.

A homologue of the rat and mouse fibroblast/osteoblast MMP-13 collagenases cloned from a human breast carcinoma cDNA library (10) and now called collagenase-3 or MMP-13, has ~86% sequence identity to the rat and mouse collagenases but is structurally different from the human MMP-8 and MMP-1 collagenases (52–53% amino acid sequence identity). We have obtained preliminary results indicating that recombinant human collagenase-3 cleaves at the aminotelopeptide site similar to the rat and mouse interstitial collagenases.[3] In view of our observations on the r/r mutant mouse phenotype (15), we suggest that the human collagenase-3 might also function similarly during embryonic development and subsequent remodeling. Cleavage by collagenase-3 or possibly other proteinases at the aminotelopeptide site may be sufficient for remodeling of type I collagen-containing extracellular matrices during this period, particularly in skeletal tissues where the enzyme is predominantly expressed during development (34, 35). Under other circumstances in organs where there is a requirement for rapid collagen degradation during a brief window of time, *e.g.* in the uterus immediately postpartum, degradation at the helical site becomes critical. In addition, during aging there is increased formation of stable intermolecular cross-links in collagen (30, 36). Solubilization of this mature collagen and further degradation would require proteolysis at both the aminotelopeptide and helical sites. Thus, the increased collagen content of the dermis and possibly other organs in the r/r mice as they age could be accounted for, at least in part, by the greater resistance to proteolytic digestion of the older, more cross-linked polymers.

[3] J. P. Witter, S. M. Krane, M. H. Byrne, C. López-Otín, and M. B. Goldring, unpublished observations.

REFERENCES

1. Alexander, C. M., and Werb, Z. (1989) *Curr. Opin. Cell Biol.* **1,** 974–982
2. Woessner, J. F. J. (1991) *FASEB J.* **5,** 2145–2154
3. Murphy, G., and Docherty, A. J. (1992) *Am. J. Respir. Cell. Mol. Biol.* **7,** 120–125
4. Krane, S. M. (1993) in *Arthritis and Allied Conditions. A Textbook of Rheumatology* (McCarty, D. J., and Koopman, W. J. eds) pp. 763–779, Lea & Febiger, Philadelphia
5. Birkedal-Hansen, H., Moore, W. G. I., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., Decarlo, A., and Engler, J. A. (1993) *Crit. Rev. Oral. Biol. Med.* **4,** 197–250
6. Burleigh, M. C. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed) pp. 285–309, Elsevier, Amsterdam
7. Eeckhout, Y., and Vaes, G. (1977) *Biochem. J.* **166,** 21–31
8. Bode, W., Gomis-Rüth, F. X., and Stöcker, W. (1993) *FEBS Lett.* **331,** 134- 140
9. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994) *Nature* **370,** 61–65
10. Freije, J. M. P., Díez-Itza, I., Balbín, M., Sánchez, L. M., Blasco, R., Tolivia, J., and López-Otín, C. (1994) *J. Biol. Chem.* **269,** 16766–16773
11. Murphy, G., Allan, J. A., Willenbrock, F., Cockett, M. I., O'Connell, J. P., and Docherty, A. J. P. (1992) *J. Biol. Chem.* **267,** 9612–9618
12. Gross, J. (1981) in *Cell Biology Extracellular Matrix* (Hay, E. D., ed) pp. 217–258, Plenum Press, New York
13. Wu, H., Byrne, M. H., Stacey, A., Goldring, M. B., Birkhead, J. R., Jaenisch, R., and Krane, S. M. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5888–5892
14. Wu, H., Liu, X., and Jaenisch, R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 2819–2823
15. Liu, X., Wu, H., Byrne, M., Jeffrey, J., Krane, S., and Jaenisch, R. (1995) *J. Cell Biol.* **130,** 227–237
16. Roswit, W. T., Halme, J., and Jeffrey, J. J. (1983) *Arch. Biochem. Biophys.* **225,** 285–295
17. Quinn, C. O., Scott, D. K., Brinckerhoff, C. E., Matrisian, L. M., Jeffrey, J. J., and Partridge, N. C. (1990) *J. Biol. Chem.* **265,** 22342–22347
18. Henriet, P., Rousseau, G. G., and Eeckhout, Y. (1992) *FEBS Lett.* **310,** 175–178
19. Bergman, I., and Loxley, R. (1963) *Anal. Chem.* **35,** 1961–1965
20. Goldring M. B., and Krane, S. M. (1987) *J. Biol. Chem.* **262,** 16724–16729
21. Dayer, J.-M., Stephenson, M. L., Schmidt, E., Karge, W., and Krane, S. M. (1981) *FEBS Lett.* **124,** 253–256
22. Delaissé, J. M., Eeckhout, Y., Neff, L., François-Gillet, C., Henriet, P., Su, Y., Vaes, G., and Baron, R. (1993) *J. Cell Sci.* **106,** 1071–1082
23. Birkedal-Hansen, H. (1987) *Methods Enzymol.* **144,** 140–171
24. Nagase, H., Jackson, R. C., Brinckerhoff, C. E., Vater, C. A., and Harris, E. D., Jr. (1981) *J. Biol. Chem.* **256,** 11951–11954
25. Matsudaira, P. (1990) *Methods Enzymol.* **182,** 602–613
26. Ausubel, F. M. (eds) (1995) in *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, New York
28. Whitham, S. E., Murphy, G., Angel, P., Rahmsdorf, H.-J., Smith, B. J., Lyons, A., Harris, T. J. R., Reynolds, J. J., Herrlich, P., and Docherty, A. P. J. (1986) *Biochem. J.* **240,** 913–916
29. Ye, Q.-Z., Johnson, L. L., Hupe, D. J., and Baragi, V. (1992) *Biochemistry* **31,** 11231–11235
30. Bailey, A. J., Sims, T. J., Avery, N. C., and Halligan, E. P. (1995) *Biochem. J.* **305,** 385–390
31. Hirose, T., Patterson, C., Pourmotabbed, T., Mainardi, C. L., and Hasty, K. A. (1993) *Proc. Natl. Acad. Sci U. S. A.* **90,** 2569–2573
32. Fields, G. B., Van Wart, H. E., and Birkedal-Hansen, H. (1987) *J. Biol. Chem.* **262,** 6221–6226
33. Sottrup-Jensen, L., and Birkedal-Hansen, H. (1989) *J. Biol. Chem.* **264,** 393–401
34. Mattot, V., Raes, M. B., Henriet, P., Eeckhout, Y., Stehelin, D., Vandenbunder, B., and Desbiens, X. (1995) *J. Cell Sci.* **108,** 529–535
35. Gack, S., Vallon, R., Schmidt, J., Grigoriadis, A., Tuckermann, J., Schenkel, J., Weiher, H., Wagner, E. F., and Angel, P. (1995) *Cell Growth Differ.* **6,** 759–767
36. Light, N. D., and Bailey, A. J. (1980) *Biochem. J.* **189,** 111–124