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Molecular Cloning and Expression of Collagenase-3, a Novel Human Matrix Metalloproteinase Produced by Breast Carcinomas*

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A cDNA coding for a new human matrix metalloproteinase (MMP) has been cloned from a cDNA library derived from a breast tumor. The isolated cDNA contains an open reading frame coding for a polypeptide of 471 amino acids. The predicted protein sequence displays extensive similarity to the previously known MMPs and presents all the structural features characteristic of the members of this protein family, including the well conserved PRCGXPD motif, involved in the latency of the enzyme and the zinc-binding domain (HEXGHXXXXXHS). In addition, this novel human MMP contains in its amino acid sequence several residues specific to the collagenase subfamily (Tyr-214, Asp-235, and Gly-237) and lacks the 9-residue insertion present in the stromelysins. According to these structural characteristics, the MMP described herein has been tentatively called collagenase-3, since it represents the third member of this subfamily, composed at present of fibroblast and neutrophil collagenases. The collagenase-3 cDNA was expressed in a vaccinia virus system, and the recombinant protein was able to degrade fibrillar collagens, providing support to the hypothesis that the isolated cDNA codes for an authentic collagenase. Northern blot analysis of RNA from normal and pathological tissues demonstrated the existence in breast tumors of three different mRNA species, which seem to be the result of the utilization of different polyadenylation sites present in the 3′-noncoding region of the gene. By contrast, no collagenase-3 mRNA was detected either by Northern blot or RNA polymerase chain reaction analysis with RNA from other human tissues, including normal breast, mammary fibroadenomas, liver, placenta, ovary, uterus, prostate, and parotid gland. On the basis of the increased expression of collagenase-3 in breast carcinomas and the absence of detectable expression in normal tissues, a possible role for this metalloproteinase in the tumoral process is proposed.

A distinctive characteristic of malignant tumors is their ability to invade normal tissues and spread to distant sites giving rise to metastasis. These processes involve degradation of the different components of the extracellular matrix and appear to require the action of proteolytic enzymes produced either by the tumor cells themselves or by the surrounding stromal cells (Liotta *et al.*, 1991; Mignatti and Rifkin, 1993). Among the variety of proteinases with potential involvement in facilitating invasion and metastasis, a large number of studies have focused on matrix metalloproteinases (MMPs),[1] a group of highly related enzymes that are involved in the remodeling of the connective tissue during many normal or pathological conditions (Woessner, 1991; Matrisian, 1992; Murphy and Docherty, 1992).

Based on structural and functional considerations, the MMPs constitute a single evolutionary protein superfamily that can be classified into at least three different families of closely related members: collagenases, gelatinases, and stromelysins. All of them are similar in that they are secreted in a latent form, contain a zinc-binding site, and can be inhibited by chelating agents and tissue-specific inhibitors. However, they differ with respect to their substrate specificity (Nagase *et al.*, 1991). Thus, collagenases cleave the native helix of types I, II, and III fibrillar collagens at a single peptide bond, generating fragments approximately 3/4 and 1/4 the size of the original molecule (Welgus *et al.*, 1981; Hasty *et al.*, 1987). Gelatinases degrade types IV, V, VII, and X collagens and elastin and may act synergistically with interstitial collagenases in the degradation of fibrillar collagens (Fessler *et al.*, 1984; Collier *et al.*, 1988; Wilhelm *et al.*, 1989; Senior *et al.*, 1991). Finally, stromelysins have a broad substrate specificity and are able to degrade many extracellular proteins, including proteoglycans, laminin, and fibronectin (Chin *et al.*, 1985; Wilhelm *et al.*, 1987; Quantin *et al.*, 1989; Murphy *et al.*, 1991).

At present nine human MMPs have been isolated and characterized, including the recently described elastolytic metalloproteinase produced by human alveolar macrophages (Shapiro *et al.*, 1993). Two of these human MMPs belong to the collagenase subgroup and have been named fibroblast and neutrophil collagenases (Goldberg *et al.*, 1986; Hasty *et al.*, 1990). The gelatinase subclass is composed of two members, 72- and 92-kDa type IV collagenases (Collier *et al.*, 1988; Wilhelm *et al.*, 1989), whereas the remaining four human MMPs, stromelysins-1, -2, and -3 and matrilysin (Whitham *et al.*, 1986; Muller *et al.*, 1988; Basset *et al.*, 1990), have been initially included in the stromelysin class. However, the substrate specificity of stromelysin-3 has not yet been clearly defined and according to structural comparisons and chromosomal location of the gene (Levy *et al.*, 1992), this enzyme does not appear to belong to any

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X75308.

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[1] The abbreviations used are: MMP, matrix metalloproteinase; APMA, 4-aminophenylmercuric acetate; bp, base pair(s); kb, kilobase pair(s); TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

of the previously described subclasses and may represent the first member of a new MMP subfamily (Murphy *et al.*, 1991; Basset *et al.*, 1993).

A comparison of the amino acid sequence of the different members of the MMP family reveals that these proteinases share several domains with apparent specific functions: a pre-domain encoding the leader sequence that targets these proteinases for secretion, a pro-domain of about 80 amino acids involved in the maintenance of the latency of the enzymes, a catalytic domain of approximately 170 residues which contains the zinc-binding region, and finally, a COOH-terminal fragment of about 200 amino acids, which is absent in matrilysin and that shows sequence similarity to hemopexin, a heme-binding serum protein. In these domains, there are two short sequence motifs that are particularly well conserved in all MMPs characterized to date (Van Wart and Birkedal-Hansen, 1990). Thus, the propeptide region contains in all cases the sequence PRCG(V/N)PD, in which the cysteine residue seems to be essential for maintaining enzyme latency. On the other hand, the catalytic domain of all these proteinases contains a short sequence HE*X*GH*XXXXX*HS that is thought to be involved in the coordination of the zinc atom at the active site (Sánchez-López *et al.*, 1988; Springman *et al.*, 1990). The occurrence of these highly conserved sequences opens the possibility to identify putative additional members of the MMP gene family by PCR-based homology cloning, using degenerate oligonucleotides encoding these two structural motifs.

In this work, and as part of our studies directed to investigate the involvement of proteolytic enzymes in breast cancer (Sánchez *et al.*, 1992a, 1993; Díez-Itza *et al.*, 1993), we have used this cloning strategy to search putative novel MMPs produced by breast carcinomas. We describe the cloning of a gene coding for a novel member of the MMP gene family, here designated collagenase-3. We also report the expression of the gene by a vaccinia virus recombinant that produced an active collagenase-3. Finally, we show that this gene is expressed by human mammary carcinomas but not by the normal resting mammary gland or by a number of different human tissues. According to these results, the expression of this proteolytic enzyme may be of importance in the malignant transformation of the mammary tissue.

EXPERIMENTAL PROCEDURES

Materials—Specimens of human breast tumors were obtained from women who had undergone surgery for primary breast carcinoma; human placenta was obtained immediately after delivery; other tissue specimens were from autopsies performed within 15 h after death. Tissue samples were frozen in liquid nitrogen and stored at −70 °C until used. The RNA-PCR kit used for the reverse transcription of total RNA and cDNA amplification was from Perkin-Elmer. Oligonucleotides were synthesized by the phosphoramidite method in an Applied Biosystems DNA synthesizer (model 381A) and purified by polyacrylamide gel electrophoresis according to standard procedures (Maniatis *et al.*, 1982) or used directly after synthesis. The poly(A)$^+$ RNA purification and cDNA synthesis kits were from Pharmacia Biotech Inc. (Uppsala, Sweden). Restriction endonucleases and other reagents used for molecular cloning were purchased from Boehringer (Mannheim, Germany). Double-stranded DNA probes were radiolabeled with [^{32}P]dCTP (3000 Ci/mmol) using a commercial random-priming kit from Pharmacia Biotech Inc. Reagents for amino acid sequencing were from Applied Biosystems. Synthetic peptide (Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH) for enzyme activity assays was from Bachem (Bubendorf, Switzerland), whereas radioactively labeled Type I collagen was kindly provided by Dr. M. A. Lizarbe (Universidad Complutense, Madrid, Spain).

PCR Amplification of Breast Carcinoma RNA—Total RNA from a breast carcinoma was isolated by guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). cDNA synthesis was carried out with the RNA-PCR Kit from Perkin-Elmer. The reverse transcription was performed for 1 h at 42 °C with 1 μg of total RNA and random hexamers as primer. The whole mixture was used for PCR with two degenerate oligonucleotides corresponding to the highly conserved cysteine switch and zinc-binding domains of MMPs (5′-CCN(AC)GNTG(CT)GGNGTNCC and 5′-TGNCC(AG)AA(TC)TC(AG)TGNGC, respectively) (100 pmol/reaction). PCR reaction was carried out in a Techne PHC-3 Thermal Cycler for 40 cycles of denaturation (94 °C, 1 min), annealing (45 °C, 1 min), and extension (72 °C, 2 min). The PCR products were phosphorylated with T4 polynucleotide kinase, and the DNA band of the expected size (approx 0.4 kb) was gel-purified and ligated in the *Sma*I site of pEMBL19. DNA from 30 independent clones was isolated and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using the Sequenase Version 2.0 kit (U. S. Biochemical Corp.). All nucleotides were identified in both strands. Sequence ambiguities were solved by substituting dITP for dGTP in the sequencing reactions. Computer analysis of DNA and protein sequences was performed with the GCG software package of the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

Construction and Analysis of a Breast Tumor cDNA Library—Breast carcinoma poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography using a commercial kit from Pharmacia. Double-stranded cDNA was synthesized with the You-Prime cDNA synthesis kit (Pharmacia) using oligo(dT) as primer and ligated into the *Eco*RI site of λgt11. About 3×10^5 plaque-forming units of the resulting library (without previous amplification) were plated using *Escherichia coli* Y1088 as host and analyzed according to the method of Benton and Davis (1977) using the partial MMP cDNA cloned by RNA-PCR as probe. Hybridization to the radiolabelled probe was carried out for 18 h in 6 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 5 × Denhardt's (1 × Denhardt's = 0.02% bovine serum albumin, 0.02% polyvinylpirrolidone, 0.02% Ficoll), 0.1% SDS, and 100 μg/ml denatured herring sperm DNA. Subsequently, the filters were washed twice for 1 h at 60 °C in 1 × SSC, 0.1% SDS and subjected to autoradiography. Following plaque purification, the cloned insert was excised by *Not*I digestion, repaired with Klenow, subcloned into the *Sma*I site of pEMBL19, and sequenced as previously described.

Northern Blot Analysis—Samples of about 40 μg of total RNA were separated by electrophoresis in 1.4% agarose-formaldehyde gels. After assessing RNA integrity and equal loading by observing the appearance of the ribosomal RNAs, blotting onto Hybond N nylon filters was carried out. Filters were prehybridized at 42 °C for 3 h in 50% formamide, 5 × SSPE (1 × SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 2 × Denhardt's, 0.1% SDS, and 100 μg/ml denatured herring sperm DNA and then hybridized for 48 h under the same conditions. Filters were washed with 0.2 × SSC, 0.5% SDS for 2 h at 65 °C and exposed to autoradiography.

Expression in E. coli—Plasmid pNot3a, which contains the full-length cDNA for human collagenase-3, was digested consecutively with *Not*I, *Hin*dIII, and nuclease S1, and the 1.5-kb blunt-ended fragment containing the entire coding sequence was purified and ligated to the expression vector pET3c (Rosenberg *et al.*, 1987), previously treated with *Nde*I and nuclease S1. The resulting plasmid, called pETI9, was transformed into *E. coli* strain BL21(DE3). BL21(DE3) cells transformed with the expression plasmid pETI9 or with pET3c without insert were grown in LB broth containing 200 μg/ml ampicillin at 37 °C for about 16 h, diluted 1/100 with the same medium, and grown to a A_{600} of 1.0. Then, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM and the incubation was continued for another 3 h. Cells were collected by centrifugation, resuspended in 0.05 volume of TBS (50 mM Tris/HCl, pH 8, 150 mM NaCl), lysed by using a French press, and centrifuged at 20,000 × *g* for 20 min at 4 °C. The insoluble fraction of the extract was washed with the same volume of 2 M urea in TBS and finally solubilized with 1 volume of 8 M urea in TBS and centrifuged as before.

Amino Acid Sequencing—Direct sequencing of recombinant collagenase-3 was carried out by the method of Matsudaira (1987). Proteins present in the 8 M urea extract were separated by SDS-polyacrylamide gel electrophoresis, blotted onto an Immobilon transfer membrane (Millipore), and stained with Coomassie Blue, and the membrane carrying the recombinant protein was placed directly into the reaction chamber of a model 477A Sequencer (Applied Biosystems). Edman degradation was performed according to the Blott program indicated by the manufacturer. The anilinothiazolinones were converted to phenylthiohydantoin derivatives in the automatic conversion flask of the Sequencer and quantified with an on-line phenylthiohydantoin analyzer (model 120A; Applied Biosystems).

Antiserum Production and Western Blotting—1 ml of 8 M urea extract was electrophoresed through a 12% polyacrylamide gel, and the portion of the gel containing the recombinant protein was excised, ground, and incubated with 2 ml of deionized water at 37 °C for about 20 h with

sporadic vortexing. 1 ml of SDS-PAGE-purified protein was used to immunize a New Zealand White rabbit according to the method described by Vaitukaitis (1981). The rabbit was bled 6 weeks after the injection, and IgGs were purified by chromatography through a DEAE-cellulose column (Whatman DE52) equilibrated and eluted with 20 mM phosphate buffer, pH 7.2. Finally, the obtained antibodies (diluted 1/1000) were used for Western blot analysis as previously described (Sánchez *et al.*, 1992a, 1992b).

Generation of Vaccinia Virus Recombinants—Vaccinia virus expressing human collagenase-3 was obtained using a plaque selection system.[2] Plasmid pRB-col3 was obtained by inserting an *Eco*RI/*Hin*dIII fragment containing the gene downstream of a vaccinia virus synthetic early/late promoter, in plasmid pBR21. Confluent monolayers of CV-1 cells in T25 flasks were infected with 1 plaque forming unit/cell vaccinia virus vRB12 clone a4 and transfected with 10 μg of plasmid pRB-col3. At 2 days post-infection, the progeny virus was harvested. The recombinant, termed VV-col3, was selected by two consecutive rounds of plaque purification on BSC-1 cell monolayers. For production of the recombinant protein, preconfluent BSC-1 cells in 900-cm^2 roller bottles were infected with wild-type vaccinia virus (strain WR) or VV-col3 at a multiplicity of infection of about 5 plaque forming units/cell. Extracellular medium and cell extracts were harvested at 24 h post-infection.

Enzyme Activity Measurements—10-μl aliquots of medium harvested from cells infected with collagenase-3 recombinant virus or with wild type virus were incubated in the presence of 1 mM APMA. After 4 h at room temperature, samples were diluted with 90 μl of assay buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij 35 (v/v), 0.02% sodium azide) and incubated 24 h either with 0.1 mM synthetic peptide (Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH) or with reconstituted insoluble fibrils of ^{14}C-radiolabeled type I collagen. Hydrolysis of the peptide was evaluated by measuring the absorbance at 365 nm after extraction with ethyl acetate as described (Masui *et al.*, 1977). Degradation of fibrillar collagen was determined by liquid scintillation counting of the solubilized material.

Immunohistochemical Staining—Immunohistochemical assays were performed on 6-μm formalin-fixed paraffin-embedded tissue sections using the avidin-biotin procedure (Hsu *et al.*, 1981). Endogenous peroxidase and nonspecific binding were blocked by sequential incubation of the sections in 10% hydrogen peroxide solution and in normal serum. Incubation with antiserum against recombinant collagenase-3 (diluted 1:500 in 20 mM phosphate buffer, pH 7.2) was performed at 4 °C for 16 h. Then, the slides were incubated with the second biotinylated antibody obtained from Dako (Dako, Denmark) and the avidin-biotin complex reagent (Vector Laboratories, Burlingame, CA). After 30 min at room temperature, the reaction was developed with 0.06% diaminobenzidine and 0.01% hydrogen peroxide. Finally, the sections were counterstained with a modification of the formaldehyde-thionine method (Tolivia and Tolivia, 1985), dehydrated, cleared in eucalyptol, and mounted with Eukitt. Specificity of staining was determined by using controls that involved incubation of tissue sections alone or with an equal amount of IgG from nonimmunized rabbits.

RESULTS

Identification and Cloning of a cDNA for Human Collagenase-3—To identify new members of the human MMP family produced by breast tumors, two degenerate oligonucleotides were designed from two domains highly conserved among the different members of this proteinase family. After RNA-PCR of total RNA isolated from a mammary carcinoma (I-9), a band of the expected size (about 0.4 kb) was obtained and cloned in the plasmid vector pEMBL19. DNA from 30 independent clones (I9-1 to I9-30) was isolated and sequenced. Analysis of the nucleotide sequence of these clones revealed that 12 of them corresponded to a cDNA with a high degree of similarity to MMPs present in the data bases but distinct from all previously characterized proteinases belonging to this gene family.

In order to obtain a full-length cDNA for this putative novel MMP, a cDNA library was prepared using, as starting material, poly(A)$^+$ RNA from the same breast carcinoma used for the RNA-PCR experiment described above. Upon screening of approximately 3×10^5 plaque-forming units using the PCR generated cDNA as probe, three positive clones were identified.

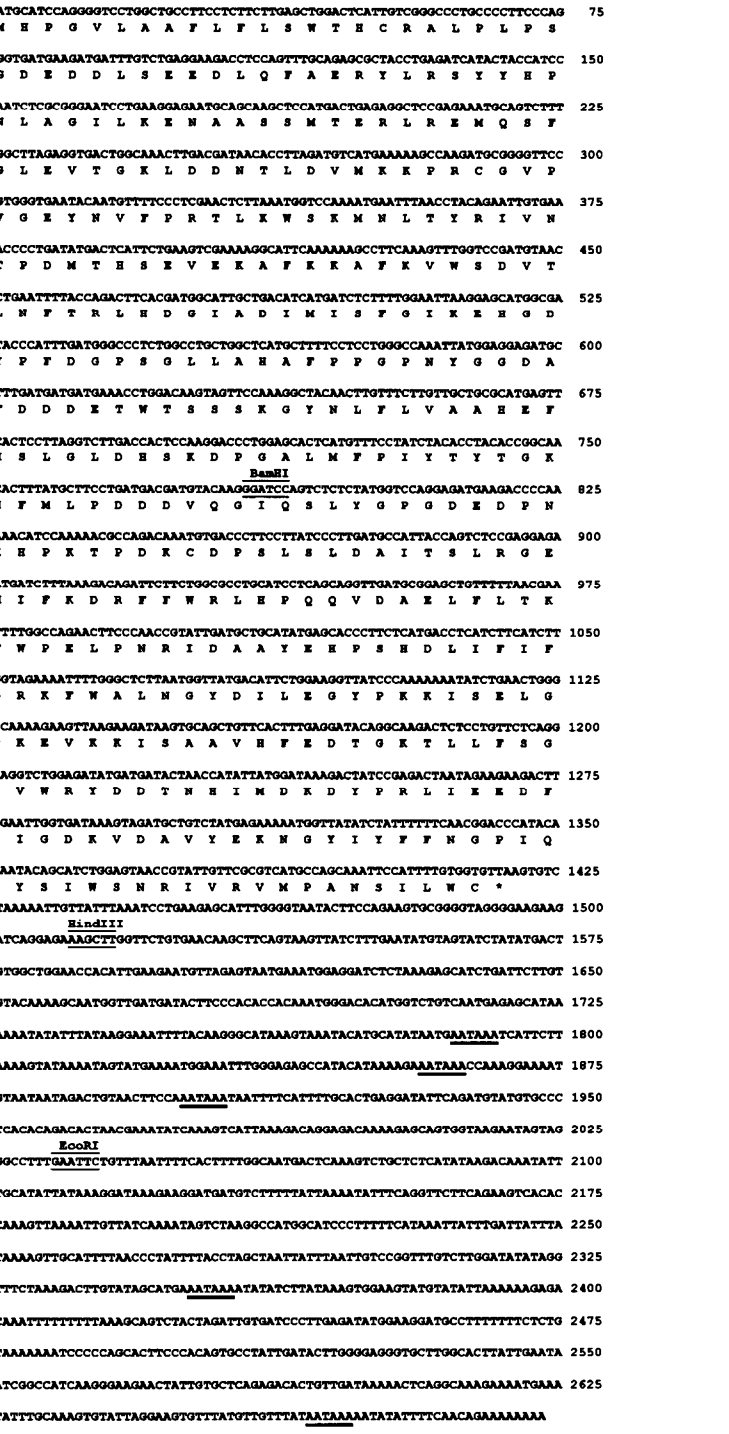

FIG. 1. **Nucleotide sequence of collagenase-3 cDNA.** The deduced amino acid sequence is shown *below* the nucleotide sequence. Restriction sites relevant for subcloning in M13 and in the expression plasmid are indicated. Putative polyadenylation sites are *underlined*.

One of them, named I9c9, had an insert of 2.7 kb, which could be large enough to contain the complete coding information for a MMP. The nucleotide sequence of the cloned cDNA (Fig. 1) revealed an open reading frame 1413 bp long, starting with an ATG codon at position 5 and ending with a TAA codon at position 1418. This open reading frame codes for a protein of 471 amino acids containing all the characteristic features of MMPs and with a predicted molecular weight of 53,759 that is very similar to those corresponding to other MMPs belonging to the collagenase and stromelysin subgroups (Murphy and Docherty,

[2] R. Blasco and B. Moss, manuscript in preparation.

A

	268	284
FIBROBLAST	RSQNPVQ----------	PIGPQTPKAC
FIBROBL BOV	PSQNPTQ----------	PVGPQTPEVC
FIBROBL PIG	PSENPVQ----------	PSGPQTPQVC
FIBROBL RAB	PSQNPSQ----------	PVGPQTPKVC
OSTEOBL RAT	PGDEDPN----------	PKHPKTPEKC
OSTEOBL MOU	PGDEDPN----------	PKHPKTPEKC
NEUTROPHIL	LSSNPIQ----------	PTGPSTPKPC
COLLAGENASE-3	**PGDEDPN---------**	**PKHPKTPDKC**
STROMELYSIN-1	PPPDSPETPLVPTEPVPPEPGTPANC	
STROMELYSIN-2	PPPASTEEPLVPTKSVPSGSEMPAKC	
STR-1 RAT	PPTESPDVLVVPTKSNSLDPETLPMC	
STR-2 RAT	ARP-SSDATVVPVPSVSPKPETPVKC	

B

	213	238
FIBROBLAST	EYNLHRVAAHELGHSLGLSHSTDIGA	
FIBROBL BOV	DYNLYRVAAHEFGHSLGLAHSTDIGA	
FIBROBL PIG	DYNLYRVAAHELGHSLGLSHSTDIGA	
FIBROBL RAB	NYNLYRVAAHELGHSLGLSHSTDIGA	
OSTEOBL RAT	GYNLFIVAAHELGHSLGLDHSKDPGA	
OSTEOBL MOU	GYNLFIVAAHELGHSLGLDHSKDPGA	
NEUTROPHIL	NYNLFLVAAHEFGHSLGLAHSSDPGA	
COLLAGENASE-3	**GYNLFLVAAHEFGHSLGLDHSKDPGA**	
	*	* *
STROMELYSIN-1	GTNLFLVAAHEIGHSLGLFHSANTEA	
STROMELYSIN-2	GTNLFLVAAHELGHSLGLFHSANTEA	
STR-1 RAT	GTNLFLVAAHELGHSLGLFHSANAEA	
STR-2 RAT	GTNLFLVAAHELGHSLGLFHSNNKES	

FIG. 2. **Comparison of protein sequences from collagenases and stromelysins around the proposed critical region for substrate specificity.** The available amino acid sequences of collagenases and stromelysins were extracted from the SwissProt data base and used for multiple alignment. Numbering corresponds to collagenase-3. *A*, amino acid sequences around the 9-residue insertion characteristic of stromelysins; *B*, region containing distinctive residues between collagenases and stromelysins. Residues that are conserved in all collagenases and which are distinct in stromelysins are indicated with an *asterisk*.

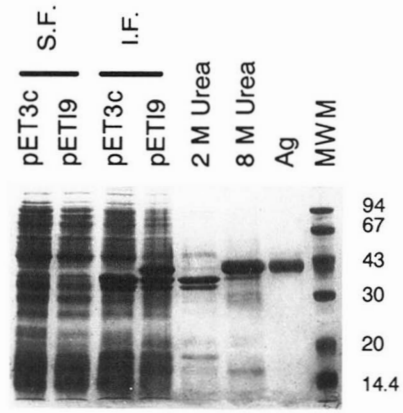

FIG. 3. **Production of collagenase-3 in *E. coli* BL21(DE3).** 5-μl aliquots of soluble (*S.F.*) and insoluble (*I.F.*) fractions of the bacterial extracts, as well as of proteins solubilized with the indicated concentrations of urea, and 1 μl of purified collagenase-3 (*Ag*) were analyzed by SDS-PAGE. The size in kilodaltons of the molecular size markers (*MWM*) is shown at *right* of figure.

1993). Amino acid sequence comparison with the remaining human MMPs showed that the similarity ranges from 50.5% (neutrophil collagenase) to 36% (stromelysin-3). However, when the comparison was performed with all sequences contained in the data bank, the highest amino acid sequence similarity (86%) was found with rat and mouse collagenases produced by uterine smooth muscle cells, osteosarcoma cells, and osteoblasts and whose primary structure has been recently elucidated (Quinn *et al.*, 1990; Henriet *et al.*, 1992). Over the last years, it has been widely assumed that these proteinases are the murine counterparts of human fibroblast interstitial collagenase and consequently, they have been designated as MMP-1 (Clohisy *et al.*, 1992; Scott *et al.*, 1992). However, the finding that the human MMP described herein is more closely related to these enzymes than fibroblast collagenase strongly suggests that these murine collagenases are enzymes distinct from fibroblast collagenase.

A more detailed amino acid sequence comparison of this novel human MMP with the remaining MMPs characterized to date prompted us to include it in the subclass of collagenases. Thus, according to recent structure-function relationship studies, the specific action of interstitial collagenases on triple helical collagen is determined by the presence of a 16-amino acid sequence in their COOH-terminal domain (Hirose *et al.*, 1993). In stromelysins, this region contains an insertion of 9 amino acids, whose introduction in the corresponding sequence of neutrophil collagenase results in complete loss of the collagenolytic activity of this chimeric enzyme (Hirose *et al.*, 1993). A comparative examination of this domain in the identified open reading frame shows the presence of this critical region for substrate specificity against fibrillar collagens and the absence of the 9 residues characteristic of stromelysins (Fig. 2*A*). In addition, this novel human MMP also contains in its predicted amino acid sequence the 3 residues (Tyr-214, Asp-235, and Gly-237) that are conserved in all collagenases characterized to date and which are never present in stromelysins (Fig. 2*B*). According to these structural comparisons with other MMPs, the putative new family member identified in this work has been tentatively called collagenase-3, since it represents the third member of this subclass composed at present of fibroblast and neutrophil collagenases. Furthermore, this proposed name attempts to reflect the parallelism of this enzyme with stromelysin-3, a recently described MMP that has been also found associated with breast carcinomas (Basset *et al.*, 1990, 1993). On the other hand, and following the nomenclature system proposed by Okada *et al.* (1986), we would assign number 13 to the novel human MMP here described, number 12 being the murine and human metalloelastases recently isolated by Shapiro *et al.* (1992, 1993).

Expression of the Collagenase-3 cDNA in E. coli and in Vaccinia Virus—As a previous step to examine the functional relevance of collagenase-3 and to obtain specific antibodies that could aid in the identification of the native protein in human tissues, studies were undertaken to express collagenase-3 cDNA in *E. coli*. For this purpose, a 1.5-kb fragment containing the entire open reading frame was subcloned in the expression vector pET3c (Rosenberg *et al.*, 1987). The resulting plasmid, called pETI9, was transformed into *E. coli* BL21(DE3), and the transformed bacteria were induced to produce the recombinant protein. Extracts were prepared from the induced bacteria and analyzed by SDS-PAGE (Fig. 3). The insoluble fraction of the extract showed a major band, corresponding to a polypeptide of about 40 kDa, which was not present in the bacteria carrying the control plasmid. After washing with 2 M urea, the recombinant protein was solubilized with 8 M urea, electrophoresed, blotted onto a polyvinylidine difluoride membrane, and subjected to direct amino acid sequencing. The obtained partial sequence (MNLTYRIVNYTPDMT) matches to residues 116–130 of the deduced amino acid sequence for collagenase-3, suggesting the occurrence of a proteolytic processing event. By analogy with other MMPs, which undergo autocatalytic fragmentation around the cysteine-switch sequence of the pro-domain, recombinant collagenase-3 could be self-processed during cell culture and purification originating the 40-kDa form detected in the present work. However, the participation of some bacterial proteolytic activity with a trypsin-like specificity required to cleave the Lys-Met peptide bond found to be cleaved in recombinant procollagenase-3, cannot be excluded. This

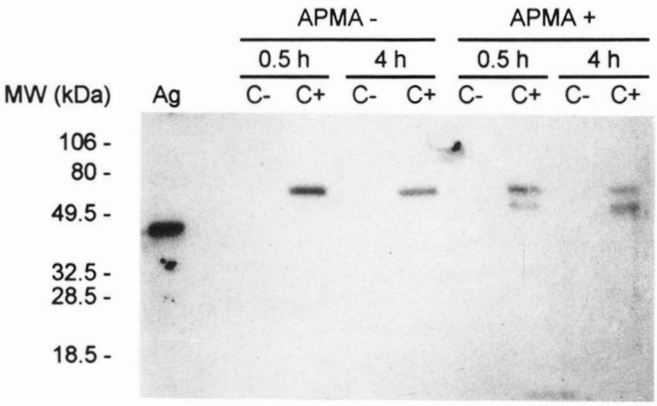

FIG. 4. **Production of collagenase-3 in vaccinia virus.** Samples of medium harvested from cells infected with the collagenase-3 recombinant virus (*lanes C+*) or with wild type virus (*lanes C−*) were incubated at room temperature in the presence or absence of 1 mM APMA. After the indicated times, 5-μl aliquots were removed and analyzed by Western blot with antibodies against purified collagenase-3 produced in *E. coli*. *Lane Ag* is recombinant collagenase-3 from *E. coli*.

truncated collagenase-3 was then used for obtaining polyclonal antibodies, as well as for performing functional studies. However, all attempts to detect any proteolytic activity of the recombinant protein on collagen, casein, or gelatin substrates were unsuccessful (data not shown). In relation to this, it is remarkable that similar negative results have been recently described for recombinant stromelysin-3 produced in *E. coli* by using the same T7 phage RNA polymerase system employed in this work (Murphy *et al.*, 1993).

Since the above results suggested that the bacterially produced protein was incorrectly folded, studies were undertaken to produce human collagenase-3 in an eukaryotic expression system. To do that, the complete cDNA coding for human collagenase-3 was cloned in a plasmid designated pRB21, which contains a strong synthetic vaccinia virus early/late promoter and the complete coding region for vp37, the major protein in the external envelope of extracellular infectious vaccinia virions that is essential in the process of plaque formation in cell monolayers (Blasco and Moss, 1991). The resulted plasmid pRB-col3 was used to transfect CV-1 cells infected with vRB12, a vaccinia virus lacking the vp37 gene and therefore unable to form plaques. After selection of the recombinant viruses by successive rounds of plaque purification on BSC-1 cell monolayers, a vaccinia virus recombinant designated VV-col3 was obtained and used to examine the production of human collagenase-3. In this way, proteins present in medium harvested from cells infected with VV-col3 or with wild type vaccinia virus (strain WR), were separated by SDS-PAGE and analyzed by Western blot with antibodies against purified collagenase-3 produced in *E. coli*. As shown in Fig. 4, a single band of about 65 kDa was detected in the extracellular medium from cells infected with the recombinant virus but not in that obtained from cells infected with wild type vaccinia virus. Furthermore, when the medium was treated with 4-aminophenylmercuric acetate (APMA), an organomercurial agent known to specifically activate MMPs, an additional immunoreactive band of about 55 kDa was detected (Fig. 4). Since the activation of MMPs involves the proteolytic removal of the corresponding profragment, the above results strongly suggested that the vaccinia virus expression system was able to produce and secrete to the medium a recombinant collagenase-3 that could be useful to perform the required functional studies. In addition, the fact that the observed immunoreactive bands were of a higher size than those calculated from the amino acid sequence seemed to indicate that the vaccinia virus system was also able to effectively glycosylate collagenase-3 in some or all of the three potential *N*-glycosylation sites contained within the presumed active form of the molecule (Fig. 1).

In order to examine the enzymatic activity of this recombinant protein, both collagenase-3-containing medium and extracellular medium harvested from cells infected with wild type vaccinia virus were treated with APMA and incubated with different substrates for MMPs. As can be seen in Fig. 5, collagenase-3-containing medium degraded Type I collagen, as well as the synthetic peptide (Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH) commonly used as a substrate for assaying vertebrate collagenases (Masui *et al.*, 1977; Kleine *et al.*, 1993). In addition, this proteolytic activity was fully abolished by EDTA, an inhibitor of metalloproteinases (Fig. 5). By contrast, we failed to observe any degrading activity on gelatin or casein zymograms. Taken together, these results indicate that collagenase-3 is a *bona fide* matrix metalloproteinase with a degrading activity on fibrillar collagen clearly in accordance with that anticipated from its amino acid sequence (Fig. 2).

Expression Analysis of Collagenase-3 in Normal and Tumoral Human Tissues—To study the expression of the collagenase-3 gene in both normal and pathological human tissues, samples from several tissues (uterus, placenta, liver, prostate, parotid gland, breast, fibroadenomas, and mammary carcinomas) were collected. Total RNA was isolated from the samples and analyzed by Northern blot, using the full-length collagenase-3 cDNA as probe. Three hybridizing bands were recognized by the probe in the lane corresponding to RNA from a breast carcinoma (Fig. 6*A*), while none of them was present in any of the examined tissue specimens. These bands correspond to mRNA species of approximately 2, 2.5, and 3 kb, respectively. Since several putative polyadenylation signals can be recognized in the 3′-noncoding sequence of the cloned collagenase-3 cDNA (Fig. 1), the three RNA bands could be the result of utilization of different polyadenylation sites. To examine this possibility, the same filter was stripped and rehybridized using as probe a *Dra*I-*Eco*RI fragment 232 bp long, corresponding to the 3′-end of the cDNA (Fig. 6*A*). This probe only recognized the largest mRNA species, demonstrating that the three mRNA bands detected with the full-length probe differ in their 3′-untranslated regions.

In an attempt to increase the sensitivity of detection of putative collagenase-3 expression in both normal and pathological human tissues, we performed PCR-RNA analysis with RNAs obtained from a wide variety of samples including breast carcinomas, breast fibroadenomas and normal tissues, as well as with RNAs obtained from breast cancer cell lines. Oligonucleotides 5′-TCATGACCTCATCTTC and 5′-GAACAGCTGCACTTAT were used as the primer pair in a RNA-PCR experiment to amplify a 134-bp segment corresponding to nucleotides 1,030–1,163 of the collagenase-3 cDNA. RNA-PCR omitting the reverse transcriptase step was used as a control of RNA-dependent amplification. The quality of the studied RNAs was checked by PCR amplification of the reversed transcribed RNAs using a pair of primers (5′-CGGCGAGTACAACAAAGCCA and 5′-CACAGCGTAGATCTGGAAAG) that directed the amplification of a 219-bp segment corresponding to the cDNA sequence of the human cystatin C gene, a housekeeping gene that is expressed in all the tissues studied so far (Abrahamson *et al.*, 1990; Freije *et al.*, 1991). The results obtained indicated that amplification of a cDNA segment from collagenase-3 RNA was detected in RNA from eight different breast carcinomas but not in RNA from normal resting mammary gland, three different breast fibroadenomas, liver, placenta, ovary, uterus, prostate, or parotid gland (Fig. 6*B* and data not shown), supporting the results obtained by Northern blot analysis. In addition, no collagenase-3 expression could be detected by PCR in RNA from

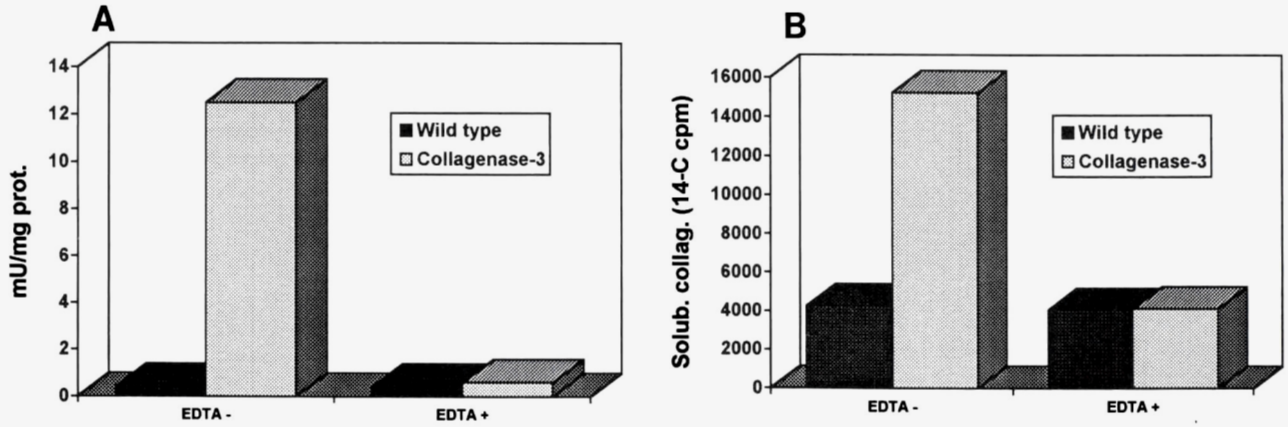

FIG. 5. **Enzymatic activity of recombinant collagenase-3.** Hydrolysis of metalloproteinase substrates incubated with medium harvested from cells infected either with recombinant collagenase-3 vaccinia virus or with wild type virus. Assays were performed in the presence or absence of 50 mM EDTA. *A*, hydrolysis of Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH. One unit of activity corresponds to 1 μmol of hydrolyzed peptide × min^{-1}. *B*, hydrolysis of insoluble type I [^{14}C]collagen. Activity is measured as ^{14}C-solubilized hydrolyzed material.

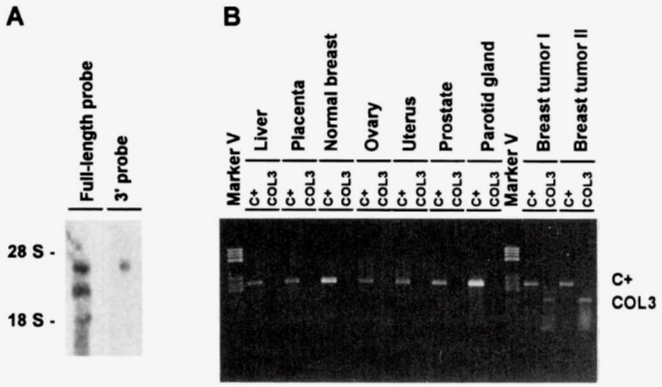

FIG. 6. **Expression analysis of collagenase-3 in normal and pathological human tissues.** *A*, about 40 μg of total RNA from a breast carcinoma were separated by agarose gel electrophoresis, blotted onto nylon filters, and analyzed by hybridization with the full-length cDNA for collagenase-3 or with a *Dra*I-*Eco*RI fragment corresponding to the 3′-end of the cDNA. The integrity of the RNA was ascertained by direct visualization of the stained gel and the nylon membrane under UV light. The positions of 28 and 18 S rRNA are shown. *B*, RNA-PCR was performed on 1 μg of RNA from the samples indicated in a volume of 100 μl. *COL3 lanes* indicate RNA-PCR amplification of a segment of collagenase-3 cDNA. *C+ lanes* indicate RNA-PCR amplification of a segment of cystatin C cDNA. 5 μl (*C+ lanes*) or 20 μl (*COL3 lanes*) of the final product were separated on a 2% agarose gel run in Tris/borate/EDTA. pBR322 digested with *Hae*III (Marker V, Boehringer Mannheim) was used as a size marker.

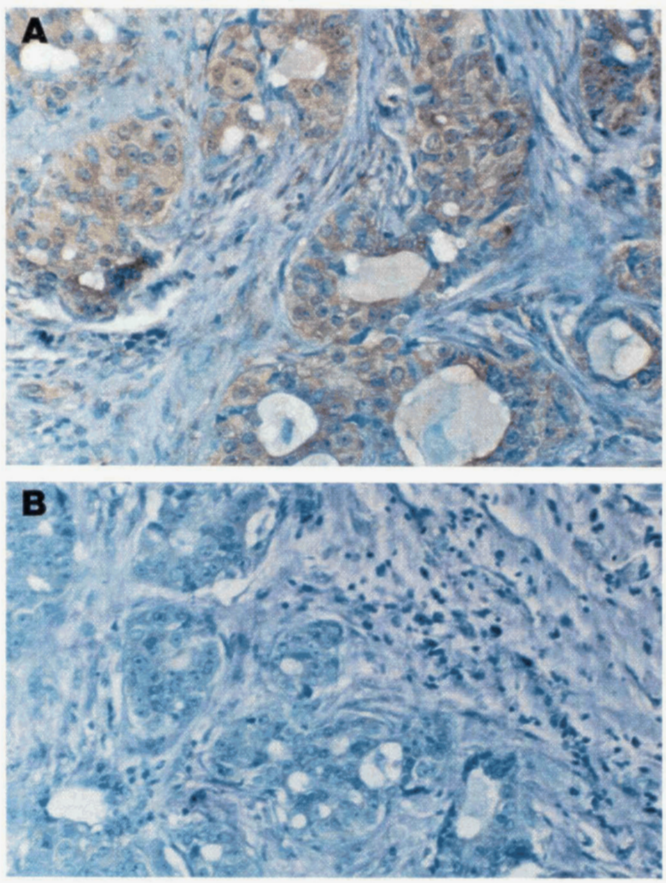

FIG. 7. **Immunohistochemical staining of collagenase-3 in human breast cancer.** Tissue sections were incubated with anti-collagenase-3 diluted 1:500 in phosphate buffer (*A*) or with phosphate buffer alone (*B*). Sections were counterstained with formaldehyde-thionine. Original magnification, × 270.

three different breast cancer cell lines: T47-D, MCF-7, and ZR75–1.

The production of collagenase-3 by breast carcinomas was also observed at the protein level by immunohistochemical analysis of tissue sections from breast carcinomas. Representative examples of these immunohistochemical studies are presented in Fig. 7. As can be seen, a strong collagenase-3 immunoreactivity was detected in the cytoplasm of breast cancer cells, although in some cases, a slight immunoreactivity could also be observed in the surrounding stromal cells.

DISCUSSION

In this work we describe the molecular cloning, functional studies, and expression analysis of human collagenase-3, a novel MMP produced by breast carcinomas but not by either the normal resting mammary gland or a number of examined normal human tissues. The identification and cloning of the cDNA for this new tumor proteinase was performed by screening of a breast carcinoma cDNA library with a probe obtained by using a PCR-based homology cloning strategy with primers deduced from conserved sequences among MMPs. The deduced amino acid sequence for collagenase-3 displays significant sequence similarity to the previously known members of this proteinase family, including the three domains that are conserved among all of them: the pre-domain encoding a hydrophobic leader sequence, the pro-domain containing the well conserved PRCG*X*PD motif involved in maintaining the latency of these enzymes, and the catalytic domain with the HE*X*GH*XXXXX*HS motif containing the His and Glu residues

considered to be the critical catalytic zinc-binding sites. The identified open reading frame also contains the hemopexin-like domain found in the COOH-terminal region in all family members with the exception of matrilysin.

The inclusion of this novel MMP in the collagenase subclass of MMPs was initially based on structural comparisons, since collagenase-3 contains in its amino acid sequence a number of features characteristic of this specific subfamily of MMPs. Thus, collagenase-3 lacks the 9-amino acid insertion present in all stromelysins and the fibronectin-like domain characteristic of the gelatinases but contains several residues specific to the collagenase subfamily (Tyr-214, Asp-235, and Gly-237) (Fig. 2). Since these residues have been proposed as fundamental determinants of collagenase specificity (Sánchez-López *et al.*, 1993), its presence in collagenase-3 strongly suggested that this enzyme belonged to the collagenase subfamily of MMPs. Functional analysis of collagenase-3 produced in an eukaryotic expression system provided definitive support for our proposal that this enzyme is an authentic collagenase. Thus, by using a vaccinia virus expression system, we were able to produce a recombinant collagenase-3 which was active against Type I collagen as well as against a synthetic peptide used for assaying interstitial collagenases. According to these data, the vaccinia virus expression system herein used appears to be appropriate for production of active human MMPs, thus opening the possibility to extend its use in the functional expression of other members of this protein family.

In this work, we have also performed an analysis of collagenase-3 expression in a number of normal human tissues, benign and malignant breast tissue specimens, and breast cancer cell lines. According to the obtained results, collagenase-3 expression was detected by PCR analysis in all examined breast carcinomas but not in other tissues including normal breast, mammary fibroadenomas, liver, placenta, ovary, uterus, prostate, and parotid gland, nor in T47-D, MCF-7, and ZR75–1 breast cancer lines. This expression analysis also revealed the occurrence in breast carcinomas of three different mRNA species, which seem to be the result of the utilization of different polyadenylation sites present in the 3′-flanking region of the collagenase-3 gene. Alternative utilization of three polyadenylation sites has also been reported for cathepsin B, a lysosomal cysteine proteinase that is overproduced in malignant tumors including breast carcinomas (Qian *et al.*, 1989; Sloane, 1990). Interestingly, the presence of multiple transcripts is detected in tumor cells but not in normal tissues, suggesting that the post-transcriptional processing pathway of the cathepsin B gene may be modified in malignant tumors (Qian *et al.*, 1991). A similar situation could also occur in the case of collagenase-3, although the lack of detection of significant collagenase-3 expression in any examined normal tissues precludes at present further studies directed to precisely establish the possible association of multiple collagenase-3 transcripts with malignancy.

A final question regarding the occurrence of human collagenase-3 in breast carcinomas makes reference to its possible participation in the tumorigenic process. In relation to this, the finding here described of increased collagenase-3 expression in breast tumors compared to normal tissues or benign tumors is consistent with the hypothesis that this enzyme may be involved in the lytic processes associated with invasive breast cancer lesions. In this regard, our recent finding in breast carcinomas of a novel tissue inhibitor of metalloproteinases designated TIMP-3 (Uría *et al.*, 1994) raises interesting questions about the possibilities of collagenase-3 inhibition in this specific type of tumor. Studies are in progress to elucidate this question as well as to establish the functional significance of collagenase-3 and its precise role among the increasing number of proteinases with potential involvement in the malignant transformation of human mammary tissue.

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