Identification and Enzymatic Characterization of Two Diverging Murine Counterparts of Human Interstitial Collagenase (MMP-1) Expressed at Sites of Embryo Implantation

Remodeling of fibrillar collagen in mouse tissues has been widely attributed to the activity of collagenase-3 (matrix metalloproteinase-13 (MMP-13)), the main collagenase identified in this species. This proposal has been largely based on the repeatedly unproductive attempts to detect the presence in murine tissues of interstitial collagenase (MMP-1), a major collagenase in many species, including humans. In this work, we have performed an extensive screening of murine genomic and cDNA libraries using as probe the full-length cDNA for human MMP-1. We report the identification of two novel members of the MMP gene family which are contained within the cluster of MMP genes located at murine chromosome 9. The isolated cDNAs contain open reading frames of 464 and 463 amino acids and are 82% identical, displaying all structural features characteristic of archetypal MMPs. Comparison for sequence similarities revealed that the highest percentage of identities was found with human interstitial collagenase (MMP-1). The new proteins were tentatively called Mcol-A and Mcol-B (Murine collagenase-like A and B). Analysis of the enzymatic activity of the recombinant proteins revealed that both are catalytically autoactivable but only Mcol-A is able to degrade synthetic peptides and type I and II fibrillar collagen. Both Mcol-A and Mcol-B genes are located in the A1–A2 region of mouse chromosome 9, Mcol-A occupying a position syntenic to the human MMP-1 locus at 11q22. Analysis of the expression of these novel MMPs in murine tissues revealed their predominant presence during mouse embryo development, particularly in mouse trophoblast giant cells. According to their structural and functional characteristics, we propose that at least one of these novel members of the MMP family, Mcol-A, may play roles as interstitial collagenase in murine tissues and could represent a true orthologue of human MMP-1.

Controlled degradation of the extracellular matrix is an essential event in a variety of physiological conditions involving connective tissue remodeling such as embryonic growth and development, uterine involution, ovulation, bone growth and resorption, and wound healing (1, 2). In addition, excessive breakdown of connective tissue plays an important role in a number of pathological processes such as rheumatoid arthritis, atherosclerosis, pulmonary emphysema, and tumor invasion and metastasis (1, 2). Among the diverse proteolytic enzymes potentially involved in these physiological and pathological processes, many studies have focused on matrix metalloproteinases (MMPs),1 a family of structurally related endopeptidases collectively capable of degrading the major protein components of the extracellular matrix and basement membranes. At present, 20 different human MMPs have been characterized at the amino acid sequence level (3). According to structural and functional characteristics, these human MMPs can be classified into at least six different subfamilies of closely related members: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs (MT-MMPs), and other MMPs.

The collagenase subfamily of human MMPs consists of three distinct members: fibroblast collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13). An additional collagenase called collagenase-4 has been identified in Xenopus laevis (4), but to date the putative orthologues of this enzyme in other vertebrate species have not been described. Biochemical characterization of all these collagenases has revealed that they share the ability to cleave fibrillar collagens at a specific peptide bond, resulting in the generation of fragments of about three-fourths and one-fourth the size of the intact molecule. Then, the resulting fragments denature spontaneously to gelatin in physiological temperature and become susceptible to degradation by other MMPs (5–8). Interestingly, kinetic studies have revealed that each human collagenase shows distinct substrate preferences toward the diverse fibrillar collagens. Thus, MMP-1 degrades preferentially type III collagen (6), MMP-8 prefers type I collagen (7), and MMP-13 degrades type II collagen 6-fold more effectively than type I and type III collagens (8). It is also remarkable that MMP-13 displays about 40-fold stronger gelatinolytic activity than MMP-1 and MMP-8 (8). On the basis of these data, we have previously

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proposed that the different human collagenases have evolved as specialized enzymes to participate in the remodeling of tissues with different collagen composition (8). The observation that the three human collagenases exhibit distinct tissue distribution and are subjected to different regulatory mechanisms (9, 10) is also consistent with the idea that they may play different functional roles in both physiological and pathological processes. To provide further experimental support to this proposal, it is essential that animal models be available in which the activity of the different enzymes can be selectively manipulated. However, these studies have been seriously hampered by the inability to detect the murine orthologue of MMP-1. In fact, to date only murine MMP-8 and MMP-13 have been identified and characterized at the amino acid sequence level (11–13), whereas all attempts from many different groups to isolate murine MMP-1 have been repeatedly unsuccessful. These data have suggested that MMP-1 may be functionally substituted in murine tissues by other enzymes with collagenolytic activity such as MMP-8 and MMP-13. Nevertheless, the possibility that additional as yet unidentified murine enzymes could be structurally or functionally related to human MMP-1 cannot be definitively ruled out. To evaluate this possibility, we have performed an extensive screening of murine genomic and cDNA libraries using as probe the full-length cDNA for human MMP-1. As a direct result of this work, we report herein the identification of two novel members of the MMP gene family originally selected by their positive hybridization with the human MMP-1 probe, and contained within the cluster of MMP...
genes located at murine chromosome 9. We also describe the expression of the genes in *Escherichia coli* and perform an analysis of the enzymatic activity of the recombinant proteins. Finally, we analyze the expression of these novel MMPs in murine tissues with the finding of their predominant presence at sites of embryo implantation.

**EXPERIMENTAL PROCEDURES**

**Materials**—A high density gridded mouse P1 artificial chromosome (PAC) genomic library was supplied by the Human Genome Mapping Resource Center (Cambridgeshire, UK). Restriction endonucleases and other reagents used for molecular cloning were from Roche Molecular Biochemicals (Mannheim, Germany). Oligonucleotides were synthesized in an Applied Biosystems (Foster City, CA) model 392A DNA synthesizer. Double-stranded DNA probes were radiolabeled with \( \alpha^{32}\text{P}\)dCTP (3000 Ci/mmol) from Amersham Pharmacia Biotech (Buckinghamshire, UK) using a commercial random-priming kit purchased from the same company.

**Screening of a Mouse Genomic Library**—The mouse PAC genomic library was hybridized with a \( \alpha^{32}\text{P}\)dCTP-labeled cDNA probe corresponding to full-length human MMP-1 probe (ATCC number 57684). Hybridization and washes were performed at 60°C. After autoradiographic exposure of the filters, 24 positive clones were detected, and 7 of them were further analyzed by extensive Southern blotting and DNA sequencing of isolated fragments.

**cDNA Cloning of Mouse Mcol-A and Mcol-B**—Oligonucleotides derived from the coding exons of the previously isolated genomic DNA sequences were used as primers for RT-PCR amplification of RNA from mouse embryos using the RNA-PCR kit from PerkinElmer Life Sciences. All PCR assays were carried out in a GeneAmp 2400 or 9700 PCR system from PerkinElmer Life Sciences. Full-length cDNA of Mcol-A and Mcol-B was obtained by RT-PCR amplification and further assembly of two overlapping fragments of each gene, covering from the ATG sequence to the stop codon of the previously identified genomic fragments.

**Nucleotide Sequence Analysis**—DNA fragments of interest were sequenced by the dideoxy chain termination method, using the Sequenase Version 2.0 kit (U.S. Biochemicals, Cleveland, OH), and the ABI-Prism DNA sequencer (Applied Biosystems). Computer analysis of DNA and protein sequences was performed with the GCG software package of the University of Wisconsin Genetics Computer Group. A phylogenetic tree was constructed to examine the evolutionary relationships between the homologous mouse MMPs clustered in human chromosome 11 and mouse chromosome 9 was constructed on-line at the United Kingdom Human Genome Mapping Project Resource Center, using PIE, which provides a [www interface to programs included in the PHYLIP software package.](http://www.phylip.com/)

**Fluorescent in Situ Hybridization on Mouse Chromosomes**—Labeling of the probes was performed by using 2 \( \mu\)g of PAC or BAC DNA in a nick translation reaction with biotin-16-dUTP. Biotinylated probes were hybridized to mouse male metaphase chromosomes and detected using two avidin-fluorescein layers. Chromosomes were diamine-2-phenylinodole dihydrochloride-banded, and images were captured in a Zeiss axiophot fluorescence microscope equipped with a charge-coupled device camera (Photometrics). The specific probe for mouse chromosome 9 was the telomeric probe BAC 55J6 corresponding to the marker D9Mit152 (14).

**Northern Blot Analysis**—Nylon filters containing 20 \( \mu\)g of RNA of murine tissues were prehybridized at 42°C for 3 h in 50% formamide, 5X SSPE (1X = 150 mM NaCl, 10 mM NaH2PO4, 1 mM EDTA, pH 7.4), 10X Denhardt’s solution, 2% SDS, and 100 \( \mu\)g/ml denatured herring sperm DNA, and then hybridized for 20 h under the same conditions. Filters were washed with 0.1X SSC, 0.1% SDS for 2 h at 50°C and exposed to autoradiography. RNA integrity and equal loading was assessed by hybridization with an actin probe.

**In Situ RNA Hybridization**—Digoxigenin-11-UTP-labeled single-stranded RNA probes were prepared with digoxigenin RNA-labeling mix and the corresponding T3 or T7 RNA polymerase (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Mcol-A probe was a 770-bp BamHI fragment, Mcol-B probe was a 700-bp BamHI/HindIII fragment, and MMP-9 probe was a 1353-bp BamHI fragment, and all of them were subcloned in pBluescript (Stratagene) vector. In situ hybridization was performed on paraformaldehyde-fixed tissue sections from 9.5-day postcoitum (dpc) mouse embryos and 10.5-dpc rat embryos, essentially as described (15).

**Construction of Expression Vectors for Mcol-A and Mcol-B**—Enzyme Assays—Enzymatic activity of purified recombinant Mcol-A and Mcol-B against fibrillar collagens was followed by SDS-PAGE. All assays were performed in 50 mM Tris/HCl, 5 mM CaCl2, 150 mM NaCl, and 0.05% (w/v) Brij-35, pH 7.6, for 16 h at 37°C (17). The enzyme/substrate ratio (w/w) used in these experiments was 1/10. Enzymatic activity was also analyzed using the synthetic fluorescent substrates Mca-Pro-Leu-Glu-Leu-Dpa-Ala-Arg-NH2 (QF-24), Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH2 (QF-35), and Mca-Pro-Cha-Gly-Nva-His-Ala...
Diverging Murine Orthologues of Human MMP-1

Identification, Cloning, and Sequence Analysis of Two Novel MMPs—To identify putative murine MMPs structurally related to human interstitial collagenase (MMP-1), we screened a mouse PAC genomic library using as a probe a full-length cDNA coding for this human protease. After hybridization under low stringency conditions, several PAC clones were selected on the basis of positive hybridization to the probe. The inserts contained in these clones were characterized by endonuclease restriction analysis and selected fragments showing hybridization with the MMP-1 cDNA probe were cloned and subjected to nucleotide sequencing. This analysis allowed the identification of two DNA fragments, derived from PAC 528 C11, whose nucleotide sequences were similar to those previously determined for other murine MMPs. Further sequence analysis of these fragments and comparison with the exon-intron distribution of other MMP genes led us to identify several putative exons of a presumably novel MMP gene. To try to determine the complete structure of this MMP, studies were undertaken to isolate a full-length cDNA encoding this enzyme. To do that, two primers covering the start and stop codons identified in the putative first and last exons of the cloned MMP gene were synthesized and used for RT-PCR amplification of total RNA obtained from mouse embryos. The PCR-amplified product was cloned, and its identity was confirmed by nucleotide sequencing. Computer analysis of the obtained sequence (Fig. 1A) revealed an open reading frame coding for a protein of 464 amino acids with a predicted molecular mass of 53.5 kDa, which was tentatively called Mcol-A (Murine collagenase-like A).

Further analysis of additional clones obtained by RT-PCR amplification of murine embryos RNA with oligonucleotides derived from the sequence determined for Mcol-A, revealed the presence of sequences highly related to but distinct from that determined for this novel MMP. A full-length cDNA for this apparently distinct MMP was isolated following the same strategy as above and then characterized by nucleotide sequencing. Analysis of the resulting sequence (Fig. 1B) allowed the finding of an open reading frame encoding a protein of 463 residues, with a calculated molecular mass of 53.5 kDa, and tentatively called Mcol-B. Genomic clones for this second MMP gene were also identified from DNA fragments obtained from PAC 519 F1 and allowed to confirm the sequence determined by analysis of the cDNA amplified by RT-PCR of murine embryos RNA. A comparison of the deduced amino acid sequences determined for Mcol-A and Mcol-B showed that they were closely related, exhibiting about 82% identities between them. Pairwise comparisons for sequence similarities between the identified amino acid sequences (Fig. 1C) and those determined for other murine MMPs showed that the maximum percentage of identities was found with mouse neutrophil collagenase (MMP-8) (48% and 45% with Mcol-A and Mcol-B, respectively). Interestingly, a higher percentage of identities (58% in amino acids and 74% in nucleotides) was found with human interstitial collagenase (MMP-1). This comparative sequence analysis also revealed that both Mcol-A and Mcol-B display all structural features characteristic of archetypal MMPs, including signal sequences, prodomain regions with the conserved Cys motifs, and a catalytic domain, as present in 2TCL. The figures were modeled with MolMol and rendered with Megapov and POV-Ray (from the POV-Ray site on the Web).

RESULTS

Identification, Cloning, and Sequence Analysis of Two Novel MMPs—To identify putative murine MMPs structurally related to human interstitial collagenase (MMP-1), we screened a mouse PAC genomic library using as a probe a full-length cDNA coding for this human protease. After hybridization under low stringency conditions, several PAC clones were selected on the basis of positive hybridization to the probe. The inserts contained in these clones were characterized by endonuclease restriction analysis and selected fragments showing hybridization with the MMP-1 cDNA probe were cloned and subjected to nucleotide sequencing. This analysis allowed the identification of two DNA fragments, derived from PAC 528 C11, whose nucleotide sequences were similar to those previously determined for other murine MMPs. Further sequence analysis of these fragments and comparison with the exon-intron distribution of other MMP genes led us to identify several putative exons of a presumably novel MMP gene. To try to determine the complete structure of this MMP, studies were undertaken to isolate a full-length cDNA encoding this enzyme. To do that, two primers covering the start and stop codons identified in the putative first and last exons of the cloned MMP gene were synthesized and used for RT-PCR amplification of total RNA obtained from mouse embryos. The PCR-amplified product was cloned, and its identity was confirmed by nucleotide sequencing. Computer analysis of the obtained sequence (Fig. 1A) revealed an open reading frame coding for a protein of 464 amino acids with a predicted molecular mass of 53.5 kDa, which was tentatively called Mcol-A (Murine collagenase-like A).

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To further explore the structural relationship between human MMP-1 and murine Mcol-A and Mcol-B, we next performed a more detailed sequence analysis with special emphasis aimed at comparing a series of residues conserved in all collagenases described to date and proposed as essential determinants of collagenase specificity. These residues include Tyr-210, Asp-231, and Gly-233 according to human MMP-1 numbering (5, 21). The equivalent residues at these three positions in Mcol-A are Phe-208, Asp-229, and Gly-231, whereas in Mcol-B these residues are Phe-208, Asp-229, and Glu-231, respectively (Fig. 1C). Therefore, it seems that Mcol-A is more...
related to collagenases than Mcol-B at least in terms of occurrence of residues important for this activity. This structural analysis also revealed that both Mcol-A and Mcol-B contain an RGD (Arg-Gly-Asp) motif in the catalytic domain. This motif is present at equivalent position in the MMP-1 sequence from all species in which this protein has been characterized, but not in other MMPs, providing additional evidence on the structural relationship between MMP-1 and the newly identified family members Mcol-A and Mcol-B. By contrast, both enzymes lack the nine-residue insertion present in the hinge region of all stromelysins. They also lack the fibronectin-like domain present in gelatinases, the C-terminal extension rich in hydrophobic residues characteristic of MT-MMPs, and the furin activation motif (RXR/KR) mediating the intracellular activation of MT-MMPs and stromelysin-3 (22, 23). In summary, and taking collectively all these structural comparisons, most data point to the inclusion of Mcol-A and Mcol-B as members of the collagenase subfamily, although they cannot be unequivocally classified within this group on the exclusive basis of their amino acid sequence characteristics.

Physical Mapping of Mcol-A and Mcol-B Genes—To determine the chromosomal location of murine genes encoding Mcol-A and Mcol-B, metaphase spreads from a male mouse were hybridized with the biotinylated PACs 528 C11 and 519 F1 enclosing these genes and with the telomeric marker of chromosome 9, BAC 55J6. After single- and double-fluorescent in situ hybridization experiments with both probes, fluorescent signal corresponding to in situ hybridization experiments with both probes, fluorescent signal corresponding to

<table>
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<tr>
<th>PAC</th>
<th>519 F1</th>
<th>528 C11</th>
<th>365 N22</th>
<th>360 L22</th>
<th>636 I22</th>
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</thead>
<tbody>
<tr>
<td>Mcol-A</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mcol-B</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>MMP-8</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>MMP-3</td>
<td>−</td>
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<td>+</td>
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</tr>
<tr>
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<td>−</td>
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<td>MMP-13</td>
<td>−</td>
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<td>−</td>
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According to the above I bands of 80 kbp. Taken collectively, these data show that

![Fig. 3. Degradation of fibrillar type I and II collagen by Mcol-A and Mcol-B. Lane 1, Mcol-B was incubated with type I collagen for 24 h at 25 °C; lane 2, Mcol-B was incubated with type I collagen for 24 h at 25 °C; lane 3, type I collagen buffer control; lane 4, Mcol-B was incubated with type II collagen for 24 h at 25 °C; lane 5, Mcol-A was incubated with type II collagen for 24 h at 25 °C; lane 6, type II collagen buffer control. The reaction products were analyzed by SDS-PAGE and Coomasie Blue staining. The positions of the respective three-fourths and one-fourth fragments are indicated by arrows on the left and right.](http://www.jbc.org/)

<table>
<thead>
<tr>
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<th>Human MMP-1</th>
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<tbody>
<tr>
<td>Gelatin</td>
<td>1.44</td>
<td>0</td>
<td>120</td>
</tr>
<tr>
<td>Casein</td>
<td>0.07</td>
<td>0</td>
<td>Not determined</td>
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TABLE II Determination of $k_{on}/K_M$ for three quenched fluorescent peptide substrates for Mcol-A and Mcol-B

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Data were taken from Knäuper et al. (8).

mentioned that after submission of this manuscript two short genomic sequences (AZ349151 and AZ443051) containing partial information for Mcol-A and Mcol-B have been released to the Genome Survey Sequence data bank. According to our gene structure analysis, AZ349151 would contain the exon 2 of Mcol-A, whereas AZ443051 would contain information for exon 7 of Mcol-B.

Production of Recombinant Mcol-A and Mcol-B in E. coli and Analysis of Their Enzyme Activity—According to the above

2 M. Balbín and C. López-Otin, unpublished results.
described data, Mcol-A and Mcol-B have some structural features characteristic of members of the collagenase subfamily of MMPs, and we therefore expressed them in E. coli, refolded, and purified them as described under “Experimental Procedures.” Our protocol was originally established to successfully refold procollagenase-3 and a transmembrane deletion mutant of MT1-MMP and allows the correct folding of collagenolytic MMPs (16). Therefore, we used this strategy to carry out a preliminary analysis of the ability of both refolded Mcol-A and Mcol-B to cleave triple-helical collagens. In addition, it’s remarkable that SDS-PAGE analysis of the refolded proteins under reducing and nonreducing conditions revealed that they migrated faster under nonreducing conditions, indicating correct folding of the C-terminal domain, a prerequisite for our analysis of collagenolysis. Mcol-A was autoproteolytically converted to the active enzyme form (M, 44,000) during the dialysis step employed to remove the imidazole from the enzyme preparation after purification using nickel-nitrilotriacetic acid-agarose (not shown). In contrast, a large proportion of the Mcol-B preparation was still in the proenzyme form (M, 56,000 and M, 54,000) following purification and dialysis, with minor bands at 46,000 and 43,000. It was, however, noted that Mcol-B underwent autoproteolytic conversion to these two lower molecular species (M, 46,000 and M, 43,000) when incubated for 24 h at 25 °C, a process that was inhibited by EDTA (not shown). When both enzyme preparations were analyzed for enzymatic activity, only Mcol-A was able to hydrolyze triple-helical type I and type II collagen into three-fourths and one-fourth fragments (Fig. 3), whereas Mcol-B remained inactive. These data indicate that, although we were unable to show enzymatic activity for Mcol-B versus macromolecular or quenched fluorescent peptide substrates, the propeptide domain was autoproteolytically removed, strongly suggesting that the enzyme is active. Nevertheless, the possibility that Mcol-B is incompletely folded cannot be ruled out. Activation trials for Mcol-B with either trypsin alone or in combination with stromelysin revealed no change in the ability of the enzyme to degrade macromolecular substrates. Thus Mcol-B might have a very restricted substrate specificity, which will need further investigation in the future.

The enzymatic activity of Mcol-A was analyzed in more detail. The enzyme was shown to hydrolyze three quenched fluorescent peptide substrates with distinct kcat/Km values, summarized in Table II. The Mcol-A activity versus these quenched fluorescent substrates was inhibited by TIMP-1, thereby indicating that this enzyme is a typical MMP (not shown). Comparison of the kcat/Km values of Mcol-A with human MMP-1 revealed that they were considerably reduced, indicating that, although the active site may be different, it still allows collagenolysis. Active site titrations were performed using the quenched fluorescent substrates and a standard TIMP-1 solution of known concentration that allowed us to determine the specific activity of Mcol-A against 14C-labeled rat type I collagen, 14C-labeled rat gelatin, and 14C-labeled β-casein. The results obtained are shown in Table III, which also shows data for human MMP-1. Our data revealed that, although 14C-labeled rat type I collagen represents a substrate for Mcol-A, the enzyme was unable to hydrolyze gelatin. This is a very surprising result, because all known human collagenases hydrolyze gelatin and thus Mcol-A may represent a more specific collagenase, needing the triple-helical conformation for activity. Additionally, 14C-labeled β-casein was also cleaved by Mcol-A, but the specific activity determined was extremely low (Table III).

Homology Modeling of Mcol-A and Mcol-B—The homology models deduced for the catalytic domains of Mcol-A and Mcol-B show a clear superimposable pattern with the catalytic domain of human MMP-1, consistent with the significant sequence similarity between them (Fig. 4, A–C). Likewise, the molecular surfaces of these domains are also very similar (Fig. 4D). Analysis of specificity determinants further strengthens the close relationship between MMP-1, Mcol-A, and Mcol-B. An essential factor for MMP specificity is the size of the S1′ pocket (30–32). The depth of this hydrophobic pocket is largely determined by the side chain of the residue present at position 214 (MMP-1 numbering) (Fig. 4C). Most MMPs have a Leu residue at this position, and consequently their S1′ sites are very deep and form a channel across the protein, allowing the digestion of substrates with large P1′ side chains. Interestingly, MMP-1 as well as the novel murine enzymes have large residues at this position (Arg and Tyr, respectively), occluding the S1′ channel and leaving a cavity that can only accept middle-sized substrates (Fig. 4C) (33, 34). The character of the S1′ subsite depends mainly on residue 180, which is hydrophilic in MMP-1, Mcol-A, and Mcol-B (Asn, Lys, and His, respectively) and hydrophobic in the other MMPs. Taken together, these structural data support that the novel murine enzymes are more closely related to MMP-1 than to other MMPs. Furthermore, analysis of the molecular models depicted for Mcol-A and Mcol-B provides some clues to explain the observed differences in the catalytic activity of both enzymes. Thus, residue 181 is Leu in most MMPs, including Mcol-A and MMP-1, but Mcol-B is unique by possessing a Phe residue at this position. This bulky residue could hamper the access of the substrate to the active site cleft (Fig. 4, A and B). In addition, there is a series of residues in Mcol-B that could be important in terms of catalytic differences with M-colla. These include the Gly residue at position 233 (MMP-1 numbering) that is absolutely conserved in all collagenases but in Mcol-B is replaced by Glu, as well as the acidic residues 194 and 201 involved in calcium binding in MMP-1, which are changed to amide residues (Asn and Gln, respectively) in Mcol-B (Fig. 1C).

Analysis of Mcol-A and Mcol-B Expression during Murine Embryogenesis—To study Mcol-A and Mcol-B expression in murine tissues, we first performed RT-PCR amplification using specific oligonucleotides and RNA prepared from a variety of tissues (uterus, kidney, ovary, lung, and placenta) and embryos at different stages of development. These analyses revealed that Mcol-A and Mcol-B were expressed during fetal development. Mcol-A and Mcol-B expression during murine embryogenesis was also confirmed by Northern blot analysis (Fig. 5). Specific hybridization with Mcol-A probe was detected in the yolk sac and uterine tissue adjacent to mouse embryos at 9.5 and 10.5 dpc but not in tissue from embryos of 13.5, 15.5, 17.5, and 19.5 dpc. This specific hybridization signal was also observed in the rat. In this case, bands were observed in the yolk sac and uterine tissue adjacent to rat embryos of 11.5 and 12.5 dpc. These days of gestation correspond to the beginning of development of the chorionallantoic placenta. Hybridization was also detected in RNA obtained from placenta at 13.5, 15.5, and 17.5 dpc. To examine the identity of the cells responsible for the production of Mcol-A and Mcol-B in the murine tissue during embryogenesis, we carried out an in situ hybridization on tissue sections of rat and mouse embryos and adjacent tissue from 8.5 to 16.5 dpc. A clear expression for both Mcol-A and Mcol-B was found in a low number of extra-embryonic cells located at the maternal interface (Fig. 6). By contrast, no transcripts were detected in either the embryo or the maternal decidua. Hybridizations with the two probes in adjacent serial sections demonstrated that both enzymes were expressed in the same cells, although staining for Mcol-B was much weaker. Expression of both genes was restricted to a network of cells at the periphery of the embryo in contact with the adjacent decidual cells. Pos-
positive cells were morphologically identified as trophoblast giant cells. To further identify these positive cells, adjacent serial sections were hybridized with antisense probes for MMP-9, which is considered as a typical marker for terminally differentiated trophoblast giant cells (35). Cells positive for Mcol-A and Mcol-B showed high expression levels of MMP-9, although cells expressing MMP-9 but not Mcol-A and Mcol-B were also found. Detection of Mcol-A and Mcol-B transcripts was restricted to sections of 9.5 and 10.5 dpc in mice and 10.5 and 11.5 dpc in rat embryos. In both cases the expression level significantly decreased with age of embryos and virtually no expression was found in mouse embryos older than 10.5 dpc.

DISCUSSION

This work describes the identification of two murine metalloproteases Mcol-A and Mcol-B, cloned as a result of their positive hybridization with a probe for human MMP-1. According to their structural and functional characteristics, we propose that at least one of these novel members of the MMP family, Mcol-A, may play roles as interstitial collagenase in murine tissues and could represent a true orthologue of human MMP-1.

Over the last years, the number and identity of collagenolytic enzymes produced by murine tissues has been a debated question within the MMP field. Studies performed by several groups have demonstrated the existence in mouse and rat cells and tissues of the corresponding orthologues of neutrophil collagenase (MMP-8) (11) and collagenase-3 (12, 13). However, and somewhat surprisingly, to date no evidence of occurrence of murine interstitial collagenase (MMP-1) has been reported. This is specially puzzling if we consider that MMP-1 was likely responsible of at least part of the collagenolytic activity discovered by Gross and Lapière in 1962 from the tail of the metamorphosing tadpole (36), as well as the first human MMP cloned and characterized at the amino acid sequence level (21). Furthermore, MMP-1 orthologues have been identified and

![Fig. 4. Homology models of the catalytic domains of Mcol-A and Mcol-B.](http://www.jbc.org/)

- **A**, ribbon representations of the models of Mcol-A and Mcol-B superimposed to the catalytic domain of human MMP-1. The RO 31-4724 substrate analogue of MMP-1 and the histidine side chains that coordinate the catalytic Zn are also shown.  
- **B**, detailed view of the interaction between RO 31-4724 and residues Leu-181 of Mcol-A, and Phe-181 of Mcol-B.  
- **C**, cross-section of the catalytic domains of MMP-1, MMP-8, and modeled Mcol-A showing the different shapes of the S1’ pocket. The residues in position 214, which determine the size of this pocket, are also shown. MMP-1 and MMP-8 substrate analogs are shown in blue and green, respectively.  
- **D**, molecular surface of the catalytic domains of MMP-1, Mcol-A, and Mcol-B. Standard view showing the active site of the molecules. Electrostatic potentials lower than –1.8 V are in red, higher than 1.8 V are in blue, and neutral is in white. Intermediate values are interpolated.

![Fig. 5. Expression of Mcol-A and Mcol-B in mouse tissues.](http://www.jbc.org/)

- **A**, RT-PCR was performed on 1 μg of RNA from whole embryos or placenta at the indicated days of embryonic development, with specific oligonucleotides for Mcol-A and Mcol-B as primers. 20 μl of the final product were separated on a 2% agarose gel. Bl lane shows RT-PCR performed without added template. The standard lane is Marker V from Roche Molecular Biochemicals.  
- **B**, samples of 20 μg of total RNA from whole embryo (W.E.), yolk sac (Y.S.), or placenta (P.) at the indicated days of development were separated by agarose gel electrophoresis under denaturing conditions, blotted onto nylon filters, and analyzed by hybridization with full-length cDNA for Mcol-A. Filters were exposed to autoradiography at –70 °C for 7 days with Kodak BIOMAX M8 films and screens. The positions of the 28 S and 18 S RNA are indicated.

entiated trophoblast giant cells (35). Cells positive for Mcol-A and Mcol-B showed high expression levels of MMP-9, although cells expressing MMP-9 but not Mcol-A and Mcol-B were also found. Detection of Mcol-A and Mcol-B transcripts was restricted to sections of 9.5 and 10.5 dpc in mice and 10.5 and 11.5 dpc in rat embryos. In both cases the expression level significantly decreased with age of embryos and virtually no expression was found in mouse embryos older than 10.5 dpc.
cloned in a number of species including *Homo sapiens*, *Bos taurus*, *Sus scrofa*, *Oryctolagus cuniculus*, and *Rana catesbiana* (21, 37–39). A partial cDNA sequence presumably encoding a C-terminal fragment of the guinea pig MMP-1 has been also reported, although it has been proposed that the guinea pig is more closely allied with lagomorphs than with rodents (40). Our approach to the identification of a putative murine orthologue of MMP-1 involved a combined strategy based on screening of a mouse PAC genomic library with a human MMP-1 cDNA probe, followed by RT-PCR amplification of mouse embryo RNA with oligonucleotides derived from the sequence of genomic clones hybridizing with the MMP-1 probe. This strategy led us to identify two murine cDNAs coding for proteins with a series of structural features present in MMPs, and more specifically in members of the collagenase subfamily. Both Mcol-A and Mcol-B were most similar to human interstitial collagenase (MMP-1). The overall identities (74% in nucleotides and 58% in amino acids) are maintained throughout the different domains of these proteins and are similar to those found in the comparison of mouse and human MMP-12 (76% in nucleotides and 61% in amino acids) but are lower than those shared by mouse and human orthologues of most MMPs. Nevertheless, both Mcol-A and Mcol-B contain a characteristic RGD motif present in MMP-1 from all species in which this collagenase has been characterized but not in other MMPs. Conversely, both enzymes are devoid of any structural features defining members of other MMP subfamilies such as stromelysins, gelatinases, and MT-MMPs. An analysis of a phylogenetic tree constructed to evaluate the evolutionary relationships of mouse Mcol-A and Mcol-B to other MMPs also revealed that human MMP-1 was the most closely related to these novel proteases (Fig. 7). Finally, molecular modeling experiments based on the crystal structures known for diverse MMPs have confirmed that the overall fold of the catalytic domains of Mcol-A and Mcol-B is topologically very similar to that of MMP-1, including the small size of the S1′ specificity pocket. Nevertheless, there are considerable differences at the amino acid sequence level between Mcol-A and Mcol-B, which might be involved in structural features that determine different enzymatic activities.
In addition to the above features that suggest that both murine enzymes are structurally related to MMP-1, we have also provided functional evidence that at least Mcol-A exhibits the ability to act as a collagenolytic enzyme. In fact, recombinant Mcol-A displays proteolytic activity against type I and type II fibrillar collagens, although its specific activity versus fibrillar type I collagen is much lower than that described for human MMP-1 or MMP-13. Mcol-B is apparently devoid of collagenolytic activity, although it can autoactivate when incubated for 24 h at 25 °C or stored for prolonged periods of time at 4 °C. On the other hand, genomic studies have indicated that both Mcol-A and Mcol-B exhibit the same exon-intron distribution as human MMP-1, and their proximal promoter region is significantly similar to that of human MMP-1. Furthermore, fine chromosomal mapping of the region containing these murine genes has revealed that Mcol-A seems to be located at a position syntenic to the MMP-1 locus in the human genome. On these bases, together with the above enzymatic analysis, we can conclude that Mcol-A is closer to MMP-1 than Mcol-B in both structural and functional terms. Thus, Mcol-A could be a homologue of MMP-1 in murine tissues, Mcol-B being the result of a specific gene duplication event that has retained a number of MMP-1 features but that has also accumulated some changes resulting in an impairment of its ability to act as a collagenolytic enzyme. As mentioned above, sequence comparisons and molecular modeling of the catalytic domains of these enzymes have suggested some specific features of Mcol-B that could contribute to the observed catalytic differences with Mcol-A and human MMP-1. Nevertheless, further studies involving site-directed mutagenesis experiments will be required to elucidate the molecular basis for the differential activities among all these closely related members of the MMP family.

To investigate the functional role of these novel MMPs, we have also examined the tissue distribution of both Mcol-A and Mcol-B in murine tissues. According to RT-PCR and Northern blot analysis, these enzymes are mainly produced in yolk sac and uterine tissue adjacent to mouse embryos at early times during implantation. In situ hybridization demonstrated that expression of both genes is restricted to trophoblast giant cells present at the embryo/maternal interface, although in all cases, the expression level of Mcol-A is higher than that of Mcol-B. According to these expression analyses, it is tempting to speculate that these novel murine proteases may participate in embryo implantation. The implantation of the mammalian embryo into the uterine stroma is a highly controlled process of tissue invasion that involves extensive remodeling of extracellular matrix components to accommodate the growing embryo as well as to establish the vascular structures necessary for transplacental exchange (41, 42). This process is initiated by the attachment of the blastocyst to the uterine epithelium on day 4.5 of mouse development and ceases on day 10.5, with placental function beginning on day 11. The observation that both Mcol-A and Mcol-B are produced by trophoblast cells on days 9.5–10.5 suggests that these enzymes may contribute to the final stages of the invasive process. Similarly, the observation that gelatinase B, whose expression peaks at 7.5 days, colocalizes with Mcol-A and Mcol-B could be indicative of the occurrence of a putative proteolytic cascade involving these proteases. Nevertheless, the possibility that Mcol-A and Mcol-B are involved in other processes distinct from blastocyst invasion, including angiogenesis regulation or collagen turnover accompanying decidualization, cannot be ruled out. It is also noteworthy in the context of the putative relationships between these novel murine MMPs and human MMP-1, that this interstitial collagenase has been also found to be produced by trophoblastic cells during human pregnancy (43, 44). These data suggest that the parallelisms between human MMP-1, and murine Mcol-A and Mcol-B, could be extended to their respective expression patterns. However, it is still unclear if the murine enzymes will share its wide distribution in processes such as wound healing or tumor progression, in which the presence of human MMP-1 has been repeatedly described (45, 46).

In conclusion, our structural and functional analysis suggest that Mcol-A and Mcol-B can be considered as putative murine counterparts of MMP-1, although it seems that they have diverged much more rapidly than other mouse and human MMP orthologues. Furthermore, on the basis of differences between both enzymes, it is tempting to speculate that Mcol-A is functionally closer to MMP-1 than Mcol-B. Further studies will be required to provide definitive evidence on the proposal that the newly identified murine MMPs, and more specifically, Mcol-A represent structural and functional counterparts of MMP-1. It will be also of interest to evaluate if the observed structural divergence between them may underlie diverging functional roles for these proteolytic enzymes.

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REFERENCES


FIG. 7. Schematic illustration of evolutionary relationships between human and mouse MMPs. The phylogenetic tree includes the diverse MMPs clustered in human chromosome 11 and mouse chromosome 9 and was constructed on-line at the United Kingdom Human Genome Mapping Project Resource Center, using PIE, which provides a web interface to programs included in the PHYLIP software package.
Identification and Enzymatic Characterization of Two Diverging Murine Counterparts of Human Interstitial Collagenase (MMP-1) Expressed at Sites of Embryo Implantation

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