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Strategies for Cloning New MMPs and TIMPs

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1. Introduction

Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) play important roles in the remodeling of connective tissues associated with normal mammalian development and growth, and in the degradative processes accompanying diseases such as rheumatoid arthritis, pulmonary emphysema or tumor cell invasion and metastasis ***(1)***. Because of the importance of these proteins in both normal and pathological conditions, over the last years many groups have tried to clone the diverse MMPs mediating these matrix remodeling events as well as the different TIMPs able to balance their proteolytic activities. The first evidence for the occurrence of MMPs was reported about 35 yr ago by Gross and Lapiere who described the presence of diffusible collagenolytic factors in tissue cultures of bullfrog tadpoles ***(2)***. Some years after this finding, several groups independently reported the existence of naturally occurring metalloproteinase inhibitors known as TIMPs and active against most members of the MMP family ***(3)***. The utilization of standard biochemical methods allowed the isolation of the first MMP and TIMP family members and their subsequent physico-chemical characterization. However, these studies were seriously hampered by the small amount of proteases and inhibitors usually found in normal conditions. The observation that these proteins were much more abundant in a series of pathological conditions such as inflammatory or tumor processes or during extracellular matrix remodeling events, facilitated the identification of additional members of both families and the molecular cloning of the first MMPs and TIMPs. More recently, the advent of more powerful molecular biology techniques and improved cloning strategies has made it possible to identify a large number of novel MMP and

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TIMP family members. To date, 18 distinct MMPs have been identified, cloned, and characterized in vertebrates *(4)*. In addition, MMPs have been also cloned from embryonic sea urchin *(5)*, green alga *(6)* and soybean leaves *(7)*. The complexity of the TIMP family has also expanded during the last years and a total of 4 distinct inhibitors with ability to control the proteolytic activity of MMPs have been cloned and characterized at the molecular level *(8)*. These new additions to the growing list of MMPs and TIMPs have provided much more complexity to the field but have also opened new views on the role of these proteins in normal and pathological processes. Thus, evidence is accumulating that MMPs are not exclusively involved in the proteolytic degradation of extracellular matrix components, playing also direct roles in essential cellular processes such as differentiation, proliferation, angiogenesis and apoptosis *(9)*. Similarly, TIMPs appear to have additional roles other than their direct inhibition of MMP proteolytic activity, and a number of reports have described their involvement in cell growth *(10)*. The delineation of expanding roles for these proteins in a wide variety of biological processes has also reinforced previous observations indicating that misregulation of these proteases and inhibitors can have important pathological consequences. Nevertheless, it seems clear that most of this progress has been only possible by the cloning of an unexpected large number of these proteins. This chapter will give an overview of the different strategies used for cloning MMPs and TIMPs and their application to the identification and characterization of putative yet unknown members of these protein families that play essential roles in both normal and pathological conditions.

2. General Methods for Cloning MMPs and TIMPs

A survey of the literature on MMPs and TIMPs indicates that the strategies used for cloning the distinct members of these families can be summarized in four different groups (**Table 1**). However, it should be noticed that in some cases, a combination of several of these different approaches has been required to clone some specific family members. Furthermore, it is remarkable that some MMPs have been cloned by other procedures not involving an oriented search specifically aimed at cloning MMPs or TIMPs. This is the case for rat stromelysin-1 (MMP-3) first cloned as an oncogene-transformation induced gene in rat embryo fibroblasts *(11)*, or for human stromelysin-3 (MMP-11) cloned from a subtractive breast cancer cDNA library as a gene specifically expressed in stromal cells surrounding epithelial tumor cells *(12)*.

2.1. Methods Based on Purification and Biochemical Characterization of MMPs and TIMPs

The first MMP and TIMP family members were cloned by following approaches initially based on the isolation of the corresponding proteins from

Table 1
Strategies for Cloning MMPs and TIMPs

Name	Source	References
	Purification and biochemical characterization of MMPs or TIMPs	
Collagenase-1 (MMP-1)	Human skin fibroblasts	***13***
Collagenase-2 (MMP-8)	Human neutrophils	***17,19***
Stromelysin-1 (MMP-3)	Human skin fibroblasts	***20***
Gelatinase A (MMP-2)	Human ras-transformed bronchial cells	***21***
Gelatinase B (MMP-9)	Human SV40-transformed fibroblasts	***22***
Envelysin	*Paracentrotus lividus* hatched embryos	***5***
Green alga MMP	*Chlamydomonas reinhardtii* vegetative cells	***6***
Soybean MMP	Soybean leaves	***7***
TIMP-1	Human fetal lung fibroblasts	***24***
TIMP-2	Human fetal heart cells	***28***
	Low-stringency screening with MMP or TIMP probes	
Stromelysin-2 (MMP-10)	Rat genomic library screened with rat MMP-3	***29***
Matrilysin (MMP-7)	Human tumors cDNA library screened with h-MMP-3	***30***
Collagenase-4 (MMP-18)	Xenopus tadpole cDNA library screened with h-MMP-3	***32***
	RT-PCR with degenerate oligonucleotides	
Collagenase-3 (MMP-13)	Human breast carcinoma	***35***
MT1-MMP (MMP-14)	Human placenta	***38***
MT2-MMP (MMP-15)	Human kidney carcinoma	***39***
MT3-MMP (MMP-16)	Human oral melanoma	***40***
MT4-MMP (MMP-17)	Human breast carcinoma	***41***
Metalloelastase (MMP-12)	Murine macrophages	***42***
Enamelysin (MMP-20)	Porcine enamel organ	***43***
X-MMP (MMP-21)	Xenopus embryos	***44***
TIMP-3	Human breast carcinoma	***45***
	Screening of Expressed Sequence Tag-databases	
MMP-19	Human liver	***54***
TIMP-4	Human heart	***8***

different sources using standard biochemical techniques. After purification of the proteins of interest, partial amino acid sequences can be obtained by automatic Edman degradation of the intact protein or of peptides derived by enzy-

matic or chemical cleavage of the purified proteins. The obtained amino acid sequences can then be used for designing oligonucleotide probes encoding these protein fragments. Finally, the oligonucleotide probes are used as probes to screen cDNA libraries in which the specific MMPs or TIMPs are presumed to be expressed at high levels.

This approach was first successfully used for cloning human fibroblast collagenase (MMP-1 or collagenase-1) from skin fibroblasts ***(13)***. To this purpose, procollagenase-1 was purified from conditioned medium of cultured adult skin fibroblasts (WUN 80547 cell strain) using cation-exchange and gel filtration chromatography. Since the N-terminal residue of the intact protein was blocked, a preparation of purified procollagenase-1 was subjected to cyanogen bromide cleavage and the resulting peptides were purified by reverse-phase high performance liquid chromatography (HPLC) and sequenced. Then, a mixture of 32 oligonucleotides, 17-bases long, encoding part of the amino acid sequence determined for a cyanogen bromide peptide was synthesized. The selected protein sequence (His-Phe-Asp-Glu-Asp-Glu) contained amino acids that had the lowest degree of degeneracy in the corresponding codons, thus diminishing the probe complexity. This oligomer was 5' end-labeled with polynucleotide kinase and hybridized to a cDNA library constructed from collagenase-producing human skin fibroblasts mRNA. Nucleotide sequencing of isolated clones hybridizing with the probe revealed the presence of an ORF encoding a protein of 469 amino acids with a predicted molecular weight of 51,929, which was called fibroblast collagenase. The sequence of this first cloned human MMP showed homology to an oncogene-induced rat protein of unknown function at that time and that we now know corresponds to rat stromelysin-1 ***(11)***. Shortly after, other groups reported the cloning of collagenase-1 from different sources following a similar approach ***(14,15)***. Since then, collagenase-1 has been the subject of a wide variety of biochemical, enzymatic, genetic, and clinical studies that have extended the knowledge of multiple normal and pathological aspects of this first human representative of the MMP family (reviewed in **ref.** ***16***).

A similar strategy was subsequently used for the cloning of neutrophil collagenase (MMP-8 or collagenase-2) ***(17)***. Partial amino acid sequences derived from the purified proenzyme were used to deduce 50 bases long synthetic oligonucleotides. In this case, a computer program was utilized to design "guessmers" based on codon usage, dinucleotide frequency, and potential probe self-complementarity ***(18)***. The probes were used to screen a granulocyte cDNA library derived from mRNA of a patient with chronic granulocytic leukemia. Collagenase-2 was also cloned by Hasty et al. ***(19)*** from RNA of a granulocytic leukemia patient although they used as probe a 24-mer oligonucleotide derived from the Zn-binding region of collagenase-1. In both cases, the

isolated cDNA clones encoded a 467-residue protein with about 58% identity to human collagenase-1 and displaying the same domain structure, including a signal peptide, an 80-residue propeptide, a catalytic domain with a Zn-binding site and a C-terminal hemopexin-like domain. Further studies revealed that recombinant collagenase-2 degraded type I collagen into the 3/4 and 1/4 fragments characteristic of mammalian interstitial collagenase activity. Thus, definitive evidence was provided that neutrophil collagenase is a member of the MMP family distinct from fibroblast collagenase, in both structural and enzymatic properties.

The cloning of human stromelysin-1 was also based on the same approach described above for cloning human collagenases-1 and -2 ***(20)***. This enzyme was purified from human skin fibroblast (WUN 80547) conditioned medium by successive chromatographies in Zn-chelate-Sepharose, DEAE-Sepharose, and reactive red-agarose, and then subjected to Edman degradation. The N-terminal sequence of the 45-kDa active enzyme (Thr-Phe-Pro-Gly-Ile-Pro) was used to synthesize a 17-base long oligonucleotide probe. This probe contained 3 deoxyinosine residues at codon nucleotide positions of fourfold degeneracy in order to limit the number of oligonucleotides in each probe mixture. Then, a cDNA library from human fibroblasts (WUN 80547) was screened with this probe and the positive clones characterized by nucleotide sequencing. Structural analysis indicated that stromelysin-1 has 477 residues and the same overall organization as collagenases, confirming their evolutionary relationship. The sequence of stromelysin-1 from human skin fibroblasts was in agreement with that of a putative stromelysin cDNA clone isolated from a human gingival fibroblast cDNA library by cross-hybridization to a partial rabbit stromelysin-1 cDNA clone ***(14)***.

The two members of the gelatinase subfamily of MMPs, 72K and 92K type IV collagenases or gelatinases A and B, were also originally cloned following the approach used for cloning of collagenases and stromelysins ***(21,22)***. Gelatinase A was purified from H-*ras*-transformed bronchial epithelial cells by Zn chelate-Sepharose, reactive green-agarose and AcA-44 size-exclusion chromatography. The purified protein was digested with trypsin and the resulting fragments purified by reverse-phase HPLC and subjected to amino acid sequencing. The sequence of one of these peptides was used to construct a 21-mer oligonucleotide with 2 inosine residues at positions of fourfold codon degeneracy. The probe was used to screen a cDNA library of human skin fibroblast mRNA leading to the isolation of a number of positive clones. Structural analysis of these clones revealed that gelatinase A had a domain organization similar to that of collagenases and stromelysins. However, an additional domain was found consisting of 175 residues with homology to the type II motif of the collagen-binding region of fibronectin. Similarly, gelatinase B was

purified from conditioned medium of SV40-transformed fibroblasts or TPA-differentiated monocytic leukemia U937 cells by successive chromatographies in reactive red-agarose, gelatin-Sepharose, Aca-44, and phenyl-Sepharose. The N-terminal sequence of the proenzyme was reverse-translated to generate a 48-nucleotide long probe containing 3 inosine residues. This probe was then used to screen a cDNA library from TPA-treated HT1080 fibrosarcoma cells with the finding of a series of positive clones encoding a 707 amino acid-long proenzyme of predicted Mr 78,426. Gelatinase B consists of a series of domains shared by most MMPs including the signal sequence, the propeptide, the catalytic region, and the C-terminal hemopexin-like domain. In addition, it contains the fibronectin-like domain also present in gelatinase A and a unique 54-amino acid long proline-rich domain homologous to the α2 chain of type V collagen ***(21,22)***. The cloning of both human gelatinases has facilitated the subsequent cloning of the homologous enzymes in numerous species including mouse, rat, chicken, Xenopus, and newt. In addition, it has opened the possibility to perform a variety of biochemical studies which have demonstrated that gelatinases share broad overlapping substrate specificities, but differ considerably in terms of their transcriptional regulation, glycosylation patterns, and activation mechanisms. Finally, clinical studies have shown that gelatinases are usually overexpressed in a wide diversity of pathological conditions, suggesting that they may be general targets for future therapeutic intervention.

Furthermore, it is of interest that all MMPs from nonvertebrate origin characterized to date have been cloned through this general approach based on the previous purification of the corresponding enzymes. Thus, the sea-urchin MMP (also called hatching enzyme or envelysin) was cloned by immunoscreening of a blastula stage cDNA library from *Paracentrotus lividus* embryos with a polyclonal antibody raised against the enzyme purified from culture supernatants of hatched embryos ***(5)***. Similarly, a green alga MMP involved in cell wall degradation to facilitate gamete fusion was cloned by screening of a cDNA library from *Chlamydomonas reinhardtii* vegetative cells with a 57-mer probe derived from the N-terminal sequence of the purified gamete lytic enzyme ***(6)***. Finally, the first MMP identified in higher plants has been recently cloned by using degenerate oligonucleotides derived from the sequence of the protein purified from soybean leaves ***(7)***. Although the biological significance of the presence of a MMP in plants remains unclear, it has been proposed that it may be involved in the tissue remodeling events which must occur during leaf expansion. Alternatively, an attractive albeit speculative hypothesis, is that the enzyme may play a defensive role against insects in plant leaves, through digestion of the collagen-like proteins within the midgut lining of insect pests, thus disrupting the normal digestive physiology ***(7)***.

In addition to these efforts directed to cloning MMPs from different sources, several groups have tried to clone and characterize their naturally occurring inhibitors, known as TIMPs, and playing a critical role in controlling the matrix remodeling that takes place during normal development and in diseases such as cancer and arthritis. Based on structural and functional comparisons, these inhibitors constitute a protein family that in humans is composed at least by four different members which have been cloned by different methods. The first two members of this family, TIMP-1 and TIMP-2, have been cloned by following the classical approach involving the previous purification of the corresponding proteins. TIMP-1 is an ubiquitous glycoprotein that binds tightly to the active form of multiple MMPs and is also found associated specifically with the latent form of gelatinase B ***(23,24)***. TIMP-1 was purified from human amniotic fluid and from the culture media of human fetal lung fibroblasts and subjected to N-terminal amino acid sequencing. Based on this information, a single 69-base oligonucleotide probe capable of encoding the 23 N-terminal amino acids of TIMP-1 was synthesized. To avoid codon degeneracy, the codons used for probe designing were those reported to appear most frequently in human genes. The 69-mer probe was used to identify positive colonies from a human fetal lung fibroblast cDNA library ***(24)***. The isolated clones encoded a protein of 207 amino acids whose sequence was identical to a protein with erythroid-potentiating activity purified from medium of T-lymphoblast cells infected by HTLV-II virus ***(25)***. This finding opened the possibility, subsequently confirmed, that TIMPs may have functions in cell growth distinct than those derived from their ability to inhibit MMPs ***(3,10)***. This growth promoting activity of TIMP-1 is shared with TIMP-2, a 21 kDa nonglycosylated protein described in 1989 by several independent groups ***(26,27)***. Human TIMP-2 was cloned from a fetal human heart library probed with a bovine TIMP-2 probe ***(28)***. The bovine inhibitor had been previously cloned by using degenerate oligonucleotides derived from the N-terminal sequence of the protein purified from aortic endothelial cells. The isolated clones for TIMP-2 code for a mature protein of 194 residues and a signal peptide of 26 amino acids. This sequence is virtually identical to that of TIMP-2 cloned from a melanoma cell cDNA library screened with a 45-mer probe derived from the amino acid sequence of the inhibitor purified from cultured medium of melanoma cells. TIMP-2 shares about 40% identity with TIMP-1, including 12 conserved cysteine residues, and displays similar inhibitory properties against active MMPs. In addition, it binds with high affinity to the latent form of gelatinase A. However, both inhibitors differ in the regulation of their expression since TIMP-1 is responsive to a variety of external stimuli, such as phorbol esters, growth factors, cytokines, and serum, whereas TIMP-2 expression is for the most part constitutive (reviewed in **ref. *3***).

2.2. Methods Based on Low-Stringency Hybridization of Genomic or cDNA Libraries with MMP or TIMP Probes

The availability of MMP and TIMP cDNAs cloned by the RT-PCR strategy opened the possibility to clone new family members by using methods based on low-stringency hybridization of genomic or cDNA libraries with probes derived from the isolated MMP or TIMP cDNAs. This strategy was first successfully used by Breathnach et al. to clone rat stromelysin-2 through low-stringency screening of a rat genomic library with a cDNA probe for rat stromelysin-1 *(29)*. Subsequent cDNA cloning and structural analysis revealed that this second representative of the stromelysin subfamily of MMPs is 71% identical in sequence to stromelysin-1. However, their regulatory mechanisms are markedly different since stromelysin-1 is induced by a variety of cytokines, growth factors, and tumor promoters in fibroblast cells, whereas stromelysin-2 gene is not usually a target of any of these factors *(29)*. Shortly after, the same group used this approach to search for stromelysin-related genes that could be involved in tumor invasion and metastasis *(30)*. After hybridization of a cDNA library prepared from RNAs extracted from a pool of primary and metastatic tumors with a stromelysin-1 probe, a clone encoding human stromelysin-2 was isolated. Furthermore, this experiment led to the cloning of a novel member of the family originally named pump-1 and subsequently known as matrilysin (MMP-7). Structural analysis has revealed that matrilysin is synthesized without a hemopexin-like domain and thus it is comparably small in size. It can cleave a wide array of extracellular matrix substrates and can activate procollagenase-1, progelatinase A, and progelatinase B. The homologous rat enzyme has been cloned and its presence at very high levels in involuting rat uterus has been described, suggesting an essential participation of matrilysin in this reproductive process *(31)*.

An additional example of the usefulness of this method is represented by the recent cloning of *Xenopus* collagenase-4, the last identified member of the collagenase subfamily of MMPs *(32)*. Thus, this novel MMP has been cloned by screening a tadpole intestinal cDNA library with a human stromelysin-1 probe, under reduced stringency conditions. The finding of collagenase-4, a MMP whose expression is activated during amphibian metamorphosis, is a representative example of the presence of these enzymes during the extensive remodeling that occurs as larval tissues degenerate and adult organogenesis takes place, and emphasizes again the use of this model system as an excellent source for the cloning of novel family members.

In addition to its demonstrated validity for the cloning of novel MMPs, the strategy based on low stringency hybridization with previously cloned family members has been widely used for cloning orthologue enzymes from MMPs or

TIMPs originally isolated in other species. A representative example of this approach is the cloning of human macrophage metalloelastase (MMP-12) by using the murine cDNA as the probe to screen a human genomic DNA library. An isolated genomic fragment including two exons of the human gene was subsequently used as probe to isolate full-length cDNA clones from a human alveolar macrophage cDNA library ***(33)***. Similarly, the human homologue of porcine enamelysin (MMP-20) has been recently cloned in our laboratory by screening of a human genomic library with a porcine enamelysin cDNA. The information deduced from isolated genomic clones was further used to PCR-amplify a full-length cDNA for human enamelysin ***(34)***. Recombinant human enamelysin is able to degrade amelogenin, the major protein component of the enamel matrix. This fact together with its highly restricted expression to dental tissues strongly suggests that human enamelysin plays a central role in the process of tooth enamel formation ***(34)***.

2.3. Methods Based on RT-PCR Amplification with Degenerate Oligonucleotides

Once several members of both MMP and TIMP families had been cloned, reverse-transcriptase polymerase chain reaction (RT-PCR) strategies using degenerate oligonucleotides encoding conserved sequences within the distinct proteins have provided an excellent choice to identify novel family members. The cloning of collagenase-3, MT-MMPs and TIMP-3 from human tissues are illustrative examples of the suitability of this procedure, but the technique has been widely applied by different groups to reveal additional members of these protein families in different species.

The cloning of human collagenase-3 was the result of studies directed to look for the presence of new proteases in tumor tissues ***(35)***. Tumor specimens are an appropriate starting material to identify novel members of the MMP family because malignant processes require the combined action of over-expressed proteases that degrade the connective tissue to facilitate tumor growth and spread to distant sites. To clone collagenase-3, we utilized degenerate oligonucleotide primers spanning two highly conserved amino acid sequences in known MMPs and reverse-transcribed RNA from human breast carcinomas. The conserved structural motifs corresponded to the activation locus (Pro-Arg-Cys-Gly-Val-Pro-Asp) containing the Cys residue essential for the maintenance of the latency of these enzymes, and the Zn-binding site (Val-Ala-Ala-His-Glu-Phe-Gly-His) present in the catalytic domain of all MMPs. After synthesizing two degenerate oligonucleotides encoding these conserved motifs and performing RT of total RNA from a mammary carcinoma, a band of the expected size (about 400 bp) was obtained and cloned. Analysis of the nucleotide sequence of different clones revealed that some of them had a

sequence similar to previously characterized human MMPs. Screening of a breast cancer cDNA library prepared from the same tumor employed for the RT experiment, and using the PCR generated fragment as probe, led to the identification of a positive clone. The isolated cDNA encoded a polypeptide of 471 amino acids with all structural features characteristic of MMPs. In addition, this novel human MMP contained in its amino acid sequence several residues specific to the collagenase subfamily of MMPs which led to the name of collagenase-3, since it represented the third member of this subfamily composed at that time of fibroblast and neutrophil collagenases. Further studies performed with recombinant collagenase-3 produced in different eukaryotic expression systems confirmed that it is a *bona fide* collagenase active against fibrillar collagens as anticipated from its amino acid sequence. Finally, it is of interest that collagenase-3 has also been cloned from IL-1 stimulated human chondrocytes by using this RT-PCR approach with degenerate oligonucleotides ***(36)***. Further studies have extended these findings and revealed that collagenase-3 may play a critical role in the destruction of articular cartilage during arthritic processes, thus providing an additional dimension to the study of this MMP ***(37)***.

The relevance of the PCR-based approach for MMP cloning is also demonstrated by the cloning of the different members of the MT subfamily of MMPs, whose discovery has represented a significant step to elucidate the mechanisms that underlie the pericellular activity of proteolytic enzymes ***(38)***. In fact, all four distinct human MT-MMPs characterized to date have been cloned from different sources by RT-PCR with degenerate oligonucleotides ***(38–41)***. Using primers corresponding to the above mentioned Cys-switch and Zn-binding sites, Sato et al. cloned in 1994 the first of these integral plasma membrane enzymes from a human placenta cDNA library. Since then, 3 additional MT-MMPs have been cloned using primers derived from the same regions and RNAs from different tumor tissues as templates: a kidney carcinoma for MT2-MMP, an oral melanoma for MT3-MMP, and a breast carcinoma for MT4-MMP. Full-length sequences for these enzymes were subsequently obtained by screening appropriate cDNA libraries. Structural comparisons between the four MT-MMPs have revealed that in addition to all typical domains shared with other MMPs, these four enzymes have a 10–12 residue insert with a furin-like recognition motif, and a final 75–105 residue insert containing a transmembrane sequence of about 24 residues and a short cytoplasmic C-terminal tail. Despite this similar structural organization, Northern blot analysis has shown that each MT-MMP shows a unique pattern of expression. Thus, MT1-MMP is expressed in numerous normal tissues, but is undetectable in brain and leukocytes. MT2-MMP is also undetectable in brain and leukocytes but is much more abundant than MT1-MMP in liver, heart, and skel-

etal muscle. MT3-MMP is mainly present in brain, placenta, lung, and heart, whereas MT4-MMP expression is very strong in leukocytes and brain. Functional analysis of MT-MMPs have revealed that these enzymes induce specific activation of pro-gelatinase A at the cell surface, thus being prime candidates to act as upstream activators in the gelatinase A proteolytic cascade involved in the tissue destruction accompanying tumor processes ***(38)***.

Further examples of the application of the PCR-based homology cloning strategy with the universal MMP degenerate primers from Cys-switch and Zn-binding sites, include the identification of murine macrophage metalloelastase (MMP-12), porcine enamelysin (MMP-20) and amphibian X-MMP (MMP-21). The first of these enzymes was cloned by screening a murine macrophage cDNA library with a specific probe generated by two rounds of RT-PCR amplification ***(42)***. The first round was carried out with the Cys-switch and Zn-binding derived primers and total RNA from P88D1 murine macrophages. The second round PCR was performed on an aliquot of the first round reaction mixture using the same Zn-reverse primer and a nested degenerate oligonucleotide based on the N-terminal sequence of the purified 22 kDa active enzyme. The deduced amino acid sequence of mouse MMP-12 predicts a 462 amino acid protein that is a distinct member of the MMP family. Enamelysin was cloned using RNA from porcine enamel organ as the template for RT-PCR ***(43)***. The amplified 401 bp length fragment was then used for screening a cDNA library from porcine enamel, resulting in the isolation of a novel cDNA encoding a 483 amino acid residues protein similar in size to collagenases and stromelysin. However, enamelysin lacks distinctive residues of these two MMP subfamilies and thus cannot be classified into these MMP groupings. Similarly, X-MMP does not belong to any of the established MMP subfamilies. X-MMP is the most recently identified member of this protein family and has been cloned by RT-PCR using RNA from Xenopus embryos in early developmental stages and the two universal MMP-primers ***(44)***. X-MMP is a 604 residue protein distantly related to other members of the family and unlike other MMPs, it has a vitronectin-like insert in its propeptide domain and lacks a proline-rich hinge region between its catalytic and C-terminal domains. A putative human homologue of X-MMP has not yet been reported.

The PCR-based homology cloning strategy has also been applied to search for novel members of the TIMP family. Thus, based on the fact that TIMPs are usually overexpressed in tumor tissues as part of a defensive response, we cloned TIMP-3 from human breast carcinomas using degenerate oligonucleotides encoding conserved sequences present in TIMP-1 and TIMP-2 ***(45)***. The forward primer covered the central part of a conserved motif located in the N-terminal region of these inhibitors (Ile-His-Pro-Gln-Asp-Ala), whereas the sequence of the reverse primer was derived from a highly conserved region

surrounding a Trp residue of the C-terminal of TIMPs (Glu-Cys-Leu-Trp-Thr-Asp). After RT-PCR amplification of total RNA isolated from a breast carcinoma, a band of the expected size (about 0.4 kb) was obtained and cloned. Nucleotide sequence analysis of isolated clones revealed that three of them were similar to the two human TIMPs previously isolated. To obtain a full-length cDNA for this putative novel TIMP, a cDNA library was prepared using as starting material polyA$^+$ RNA from the same breast carcinoma used for the original RT-PCR experiment. Upon screening of this library using the PCR generated cDNA as probe, two positive clones were obtained. Nucleotide sequence analysis of the largest one revealed an ORF coding for a protein of 211 amino acids, and a predicted molecular mass of about 24 kDa. This novel member of the family was called TIMP-3 and shares 40% identity with TIMP-1 and 45% with TIMP-2, including the 12 cysteine residues which are conserved among all these proteins ***(45)***. A similar strategy was also used by Silbiger et al. to clone human TIMP-3, although they used fetal kidney as the source of RNA and the forward oligonucleotide was derived from a distinct region (Val-Ile-Arg-Ala) and contained a restriction site to facilitate the subsequent cloning of the amplified products ***(46)***. The sequences deduced for TIMP-3 cloned by following this approach are virtually identical to that reported by Apte et al. who cloned a cDNA for TIMP-3 by screening of a human placenta cDNA library with a probe specific for the murine inhibitor ***(47)***. The cloning and sequencing of human TIMP-3 has also revealed that it is the homologue of a chicken inhibitor of metalloproteases called ChIMP-3, which was first isolated as a transiently expressed 21 kDa protein in the extracellular matrix of transforming chick fibroblasts ***(48)***. Further studies have provided a biochemical characterization of this third member of the TIMP family with the finding that it is an effective inhibitor of MMPs, as well as of endothelial cell migration and angiogenesis. However, unlike the other TIMPs which are soluble, TIMP-3 is unique in being a component of extracellular matrix. TIMP-3 is produced by many epithelia and at high levels in cartilage and muscle, and its expression is induced in response to mitogenic stimulation, being subjected to a strict cell cycle regulation ***(49)***. Finally, it is worthwhile mentioning that mutations in the human TIMP-3 gene cause Sorsby's fundus dystrophy, a dominantly inherited form of blindness, thus being the first member of the MMP and TIMP families in which genetic defects have been found ***(50)***.

In summary, this approach has been of extraordinary value to clone novel members of the MMP and TIMP families. Several situations rather specific for MMPs and TIMPs have facilitated the success of this approach and may even favor its future application for the cloning of as yet unknown members of these families. Thus, both protein families have highly conserved sequences of enough length (6–7 amino acids) to design specific oligonucleotides. In addition,

these regions are relatively devoid of amino acids such as Ser, Leu, or Arg displaying a high grade of degeneracy in their respective codons. The use of inosine at positions of fourfold degeneracy diminishes the complexity of the oligonucleotide mixture but in our experience good results can be equally obtained without the introduction of this base. Some groups have used oligonucleotides containing restriction sites at their ends to facilitate cloning of the PCR-amplified fragments, but this introduces additional mismatches into the primers which in some cases may lead to a low efficiency in the amplification process. It is also remarkable that the distance separating the diverse forward and reverse primers designed for cloning MMPs or TIMPs is about 300–400 bp, which is optimal because is easy to amplify and allows complete sequencing of the amplified fragments in a single sequencing run. Finally, an additional advantage of this strategy is that can be applied to small amounts of RNA extracted from a number of normal and pathological tissues in which these proteins may be overexpressed.

2.4. Methods Based on Screening of Expressed Sequence Tag-Databases

Expressed sequence tags (ESTs) are short nucleotide sequences derived by partial sequencing of the inserts of randomly selected cDNA clones ***(51)***. More than 900,000 human ESTs generated from a large variety of tissue sources have now been deposited in a public database named dbEST. In addition, ESTs from model organisms are also being introduced in separate data bases, thus expanding the usefulness of this approach. Because the majority of the source cDNA libraries are normalized in vitro to reduce the high variation in abundance among the clones that represent individual mRNA species, it is likely that the dbEST already contains a significant percentage of all genes present in the human genome ***(52)***. Consequently, the dbEST represents an invaluable and easily accessible tool to look for novel genes through automated computer search of ESTs with sequence similarity to genes of interest ***(53)***. To this purpose, one of the most powerful methods is to conduct a homology search with the Basic Local Alignment Search Tool (BLAST), a program capable of rapidly detecting even very distant sequence similarities in a statistically rigorous manner. Where similarities are detected it is possible to make functional inferences concerning the encoded protein based on what is known about the function of the matched sequence. Recently, this vast source of data has also been used for cloning novel MMPs and TIMPs, MMP-19 and TIMP-4 being the first examples of the successful application of the EST-based approach to the identification and cloning of members of these protein families.

To clone human MMP-19, we first performed an extensive analysis of the dbEST with the BLAST algorithm, looking for sequences with homology to human MMPs. This search led to the identification of a short DNA sequence

(R55624, Merck EST project) which, when translated, showed a significant similarity to a region of the propeptide domain found in all previously characterized MMPs. A cDNA containing part of this EST was obtained by PCR amplification of total DNA prepared from a human liver cDNA library. Then, the amplified fragment was used as a probe to screen the same liver cDNA library, with the finding of several positive clones encoding a putative novel MMP which was tentatively called MMP-19. This protein exhibits the domain structure typical of MMPs but lacks specific features that would otherwise distinguish it as a collagenase, stromelysin, gelatinase or MT-MMP, suggesting that MMP-19 may represent the first member of a new MMP subfamily. Furthermore, in contrast to most MMPs, MMP-19 is expressed in a variety of normal human tissues, including placenta, lung, pancreas, ovary, spleen, and intestine suggesting that it may play a specialized role in these tissues ***(54)***.

The same approach has also been used for cloning human TIMP-4, the most recently identified member of this family of proteinase inhibitors ***(8)***. In this case, the automated search of the dbEST revealed an EST from a human brain cDNA library with high sequence similarity (about 45% identities) to TIMP-2. This EST was then used as a probe to examine the expression in a variety of human tissues by Northern blot analysis. Because the highest expression was found in heart, a cDNA library from human heart was screened with the EST probe. A full-length cDNA was obtained, coding for a protein of 224 amino acids, including a 29-residue signal sequence. After optimal alignment, TIMP-4 shows about 50% identity to TIMP-2 and TIMP-3, and 37% to TIMP-1. The predicted protein shares several essential features that are characteristic of the TIMP family including the consensus sequence Val-Ile-Arg-Ala-Lys present in the N-terminal region and proposed to serve as a hallmark of this family of inhibitors. The sequence deduced for TIMP-4 also displays the 12 conserved cysteine residues involved in the formation of the six disulfide bonds that fold these proteins in a two domain structure. In addition to these structural similarities, recombinant TIMP-4 produced in eukaryotic cell possesses an inhibitory activity against MMPs and is secreted extracellularly thus confirming that the cloned TIMP-4 is a new member of the TIMP family. In addition, recombinant TIMP-4 inhibits tumor cell invasion across reconstituted basement membranes. This finding together with the observation that this novel TIMP is expressed in stromal cells surrounding breast carcinomas suggests that TIMP-4 may reflect a host response to try to balance the local tissue degradation associated to tumor cell invasion. These studies open the possibility of TIMP-4 mediated antitumor and antimetastatic activities for controlling cancer progression ***(55)***.

3. Conclusions and Perspectives

Over the last few years there has been a dramatic increase in the complexity of MMP and TIMP families. New proteases and inhibitors with unexpected novel functions have been cloned from human tissues as well as from a number of vertebrate species. Furthermore, the first MMPs from sea urchins, green algae and plants have been recently cloned and functionally characterized. The methods used for cloning these genes have clearly shifted from biochemical-based procedures, to molecular biology-based approaches, and more recently to computer search-based strategies. The classical approach to identifying MMPs and TIMPs started with the purification and biochemical characterization of a certain protein with an interesting biological activity as a protease or as a protease inhibitor. Subsequently, the gene encoding this protein of interest was cloned by using oligonucleotide probes derived from structural information of the purified protein, or less frequently, by immunoscreening of cDNA libraries with antibodies raised against the purified protein. After generalization of PCR-based techniques, this classical approach to cloning MMPs and their specific inhibitors has been largely replaced by RT-PCR methods with oligonucleotides derived from conserved sequences in these protein families. The successful application of this strategy by different research groups has considerably expanded the number of MMPs and at least eight new enzymes have been identified following this procedure. To our view, the RT-PCR approach still represents an alternative for the cloning of new members of these families, specially if it is applied to RNA from fetal tissues or other conditions involving extensive tissue remodeling in which MMPs or TIMPs are usually more abundant. Nevertheless, it is tempting to speculate that computer search of dbEST will be the method of choice for cloning novel MMPs and TIMPs. To date, only one member of each of these families has been cloned through this approach. However, it should be noted that most ESTs currently deposited on data bases are derived from genes expressed in normal tissues under physiological conditions in which MMPs or TIMPs may be largely under represented. The extension and generalization of EST projects to specific pathologic conditions, such as arthritic, inflammatory, or tumor processes in which these genes may be over expressed will provide an invaluable tool for the identification of new MMPs or TIMPs. Likewise, the conclusion of the large scale genome sequencing projects will provide a definitive view of the number and identity of MMPs and TIMPs present in human tissues as well as in model organisms. Their cloning and functional characterization will shed light on the contribution of these proteins to both normal and pathological processes. Finally, the targeted modulation of MMP and TIMP levels in vivo through genetic manipulation of the cloned genes will help to elucidate the biological significance of the apparent functional redundancy of these continuously growing protein families.

During editing of this chapter, three novel human MMPs: MMP-23, MT5-MMP, and MT6-MMP have been cloned ***(56–59)***. The cloning strategies have involved the screening of EST-databases or the use of information derived from human genome sequencing projects.

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