

Molecular Cloning and Characterization of Human Tissue Inhibitor of Metalloproteinase 4*

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John Greene[‡], Mingsheng Wang[§], Yiliang E. Liu[§], Lisa A. Raymond[‡], Craig Rosen[‡], and Yuenian E. Shi^{§1}

From [‡]Human Genome Sciences, Inc., Rockville, Maryland 20850-3338 and [§]Department of Pediatrics, Long Island Jewish Medical Center, The Long Island Campus for the Albert Einstein College of Medicine, New Hyde Park, New York 11042

The tissue inhibitors of metalloproteinases (TIMPs) constitute a family of proteins, of which three members have so far been described. Using the expressed sequence tag sequencing approach, we have identified a novel TIMP-related cDNA fragment and subsequently cloned a fourth human TIMP (TIMP-4) from a human heart cDNA library. The open reading frame encodes a 224-amino acid precursor including a 29-residue secretion signal. The predicted structure of the new protein shares 37% sequence identity with TIMP-1 and 51% identity with TIMP-2 and -3. The protein has a predicted isoelectric point of 7.34. The open reading frame-directed expression of TIMP-4 protein in MDA-MB-435 human breast cancer cells showed metalloproteinase inhibitory activity on reverse zymography. By Northern analysis, only the adult heart showed abundant TIMP-4 transcripts with a 1.4-kilobase predominant transcript band; very low levels of the transcripts were detected in the kidney, placenta, colon, and testes, and no transcripts were detected in the liver, brain, lung, thymus, and spleen. This unique expression pattern suggests that TIMP-4 may function in a tissue-specific fashion in extracellular matrix homeostasis.

Matrix metalloproteinases (MMPs)¹ play a critical role in ECM homeostasis. Controlled remodeling of the ECM is an essential aspect in the process of normal development, and deregulated remodeling has been indicated to have a role in the etiology of diseases such as arthritis, periodontal disease, and cancer metastasis (1–5). The overproduction and unrestrained activity of MMPs has been linked to malignant conversion of tumor cells (4–12). The down-regulation of MMPs may occur at the levels of transcriptional regulation of the genes and activation of secreted proenzymes and through interaction with specific inhibitor proteins such as TIMPs. TIMPs are secreted multifunctional proteins that play pivotal roles in the regulation of ECM metabolism. Their most widely recognized action is as inhibitors of matrix MMPs. Thus, the net MMP activity in the ECM is the result of the balance between activated enzyme levels and TIMPs levels. Augmented MMP activity is associ-

ated with the metastatic phenotype of carcinomas, especially breast cancer (7–9, 13–16); the decreased production of TIMP could also result in greater effective enzyme activity and invasive potentials (17–19). These results suggest that an increase in the amount of TIMPs relative to MMPs could function to block tumor cell invasion and metastasis. In fact, tumor cell invasion and metastasis can be inhibited by up-regulation of TIMP expression or by an exogenous supply of TIMPs (17, 40–44).

Three mammalian TIMPs have been characterized at the sequence level: TIMP-1 (20), TIMP-2 (21), and TIMP-3 (22–25, 36). The proteins are classified based on structural similarity to each other as well as their ability to inhibit matrix metalloproteinases. There have been other reports of inhibitors of metalloproteinases (IMPs) with characteristics different from these known TIMPs. In some cases these activities result from alternate forms of the known TIMPs. For instance, a report describes one IMP present in the conditioned media of human bladder carcinoma to be a partially glycosylated form of TIMP-1 and another to be a partially processed and degraded form of TIMP-2 (25). There are additional reports that describe sources and characteristics of IMP activity, but the gene products associated with these activities have not been delineated (26).

Individual TIMP family members may have specific physiological roles. This notion is supported by several lines of evidence. First, although TIMPs are essentially interchangeable in their capabilities as inhibitors of MMPs, they are distinguished by the formation of specific complexes with different pro-MMPs (27–29). Secreted MMP-2-TIMP-2 and MMP-9-TIMP-1 complexes may represent an additional function for TIMPs in controlling activation of specific latent MMPs. Unlike TIMP-1 and -2, TIMP-3 has a unique association with the ECM (30). Second, the expression of TIMP genes is quite different. The TIMP-1 gene is highly inducible at the transcriptional level in response to many cytokines and hormones (31–34). Likewise, TIMP-3 expression is not only induced in response to mitogenic stimulation but also is subject to cell cycle regulation (22), suggesting that TIMP-3 expression may represent an invaluable tool for the analysis of cell cycle progression, terminal differentiation, and replicative senescence. In contrast, TIMP-2 expression, like that of MMP-2 with which it interacts, is largely constitutive (21, 37).

Since the introduction of the expressed sequence tag (EST) sequencing approach, many novel human genes have been discovered and isolated (38). With the rapidly growing repertoire of human ESTs, we took advantage of automated EST sequence analysis to identify novel TIMP-related genes. We describe here the full-length sequence of a novel member of the TIMP family, and we examined the expression of this new

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¹ To whom correspondence should be addressed: Pediatric Research Center, Schneider Children Hospital, Long Island Jewish Medical Center, New Hyde Park, NY 11042. Tel.: 718-470-3086; Fax: 718-470-6744; E-mail: Shi@aecom.yu.edu.

¹ The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; IMP, inhibitor of metalloproteinases; ECM, extracellular matrix; EST, expressed sequence tag; bp, base pair; kb, kilobase.

member, TIMP-4, in a variety of tissues. We have also demonstrated an MMP inhibitory activity of the expressed TIMP-4 protein.

MATERIALS AND METHODS

Reagents—Restriction enzymes, T7 polymerase, a random primer DNA-labeling kit, and digoxigenin-labeled nucleotides were obtained from Boehringer Mannheim. [³²P]dATP was purchased from Amersham Corp.

Molecular Cloning of TIMP-4 Full-length cDNA Sequence—We have used EST analysis to search for a new TIMP. A data base containing approximately 500,000 human partial cDNA sequences (expressed sequence tags) has been established in a collaborative effort between the Institute for Genomic Research and Human Genome Science, Inc., using high throughput automated DNA sequence analysis of randomly selected human cDNA clones (38). Sequences of TIMP-related genes were searched for using the blastn and tblastn algorithms (39). An EST from a human brain library, which demonstrated homology to TIMPs, was completely sequenced and found to be a partial clone lacking the sequence at the 5'-end. The coding region and 3'-untranslated region of this clone were excised from the Bluescript vector by digestion with the restriction endonucleases *EcoRI* and *XhoI* and used to generate a radiolabeled probe. This probe was used to screen a Northern blot of total RNAs from several human tissues. The highest level of expression of the putative novel TIMP was noted in RNA from adult heart. We next generated a cDNA library from human heart. Poly(A) mRNA from heart tissue was obtained using Oligotex beads. Five micrograms of this mRNA were used to construct a directional cDNA library in the Stratagene Unizap vector using the Stratagene cDNA library kit. One million clones of the primary library were amplified, and an aliquot was excised to yield Bluescript SK plasmid clones. These clones were screened with the probe generated by *EcoRI* and *XhoI* digestion of the positive clone from a human brain library as described above. Positive clones were rescreened, both by hybridization and polymerase chain reaction analysis, using a Bluescript reverse primer and an antisense primer (5'-GACTGTCCACTTGGCACTTCT-3') specific for the putative TIMP-related gene in the 3'-untranslated region. The full-length cDNA was completely sequenced using ABI 373a automated fluorescent sequencer protocols.

Northern Analysis—Total RNA was extracted from tissues according to the method of Chomczynski and Sacchi (45). The RNA from human breast cancer cells was prepared using the RNeasy B RNA isolation kit (Tel-Test, Inc.) based on the manufacturer's instructions. Equal aliquots of RNA were electrophoresed in a 1.2% agarose gel containing formaldehyde and transferred to a nylon membrane (Boehringer Mannheim). The membrane was prehybridized with ExpressHyb hybridization solution (Clontech, Inc.) at 68 °C for 30 min. The hybridization was carried out in the same solution with a ³²P-labeled TIMP-4 probe (1.5 × 10⁶ cpm/ml) for 1 h at 68 °C. The membrane was then rinsed in 2 × SSC (1 × SSC contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) containing 0.05% SDS three times for 30 min at room temperature, followed by two washes with 0.1 × SSC containing 0.1% SDS for 40 min at 50 °C. The full-length TIMP-4 cDNA was isolated from the Bluescript vector, following *EcoRI* and *XhoI* digestion, and used as a template for preparation of a random-labeled cDNA probe. The riboprobe is a 390-base segment extending from nucleotides 800–1,189 (the end of the 3'-end of the cDNA). This riboprobe, which covers 85% of the 3'-untranslated region, was generated by *PstI* digestion of the Bluescript vector, followed by RNA synthesis with T7 polymerase.

Expression of TIMP-4 in Human Breast Cancer Cells—The human TIMP-4 full-length sequence was subcloned into the pCI-neo mammalian expression vector (Promega) downstream of the human cytomegalovirus promoter to generate the pCITIMP4 expression vector. Forty micrograms of pCITIMP4 or the control pCI-neo plasmids were transfected into MDA-MB-435 human breast cancer cells by the calcium phosphate-mediated method as described previously (46). Thirty G418-resistant individual clones were selected in the selection medium containing 800 µg/ml G418, subcloned, and characterized by *in situ* hybridization and Northern blot analysis. TIMP-4-producing clones were grown in serum-free defined medium. The conditioned media were collected at 40 h after culturing cells in serum-free Dulbecco's modified Eagle's medium, concentrated approximately 10-fold using an Amicon hollow fiber concentrator with a *M_r* 10,000 cutoff. The inhibitory activity was subsequently analyzed on reverse zymography SDS-polyacrylamide gel electrophoresis.

Electrophoretic Analysis by Reverse Zymography—Samples of conditioned media from TIMP-4-producing clones and control clones were

adjusted to the same protein concentration and electrophoresed on a 0.1% SDS, 12% polyacrylamide protease substrate gel (47). The gel was incubated in collagenase buffer (50 mM Tris, pH 7.4, 0.2 M NaCl, 5 mM CaCl₂, 1% Triton X-100, and 0.7 µg/ml of recombinant MMP2) at 37 °C overnight to allow digestion of gelatin in the gel. The MMP inhibitory activities of samples were visualized by Coomassie Blue R-250 (Sigma) staining and destaining as described previously (48).

RESULTS

Molecular Cloning of TIMP4 cDNA—We have searched a data base of human genes identified by the EST method. The automated screening revealed an EST from a human brain library with a 45% sequence homology to the TIMP-2 protein. The clone was completely sequenced. A putative stop codon was located; however, a start codon (ATG) could not be located at the 5'-end. The length of the open reading frame was also shorter than expected for a 22–28-kDa protein in the TIMP family. Therefore, it was concluded that this cDNA clone did not encode the entire putative TIMP protein, and that a segment at the 5'-end containing the start codon was missing. To obtain the full-length sequence of the putative new TIMP gene, the identified cDNA clone was prepared as a probe and was used to investigate the expression of this new putative TIMP gene in a variety of human tissues by Northern blot analysis. Because the highest expression of this new putative TIMP gene was identified in human heart, we next generated a cDNA phage library from a human adult heart and screened 1 million clones for an additional 5'-sequence. As a result, a number of clones were identified, and the longest of these was sequenced and found to contain the full-length cDNA sequence of the putative new TIMP gene.

The nucleotide sequence determined from this clone and the predicted corresponding amino acid sequence are shown in Fig. 1. The full-length cDNA sequence contains 1,189 bp with a 672-bp open reading frame: 59 bp in the 5'-untranslated region and 458 bp of a 3'-untranslated sequence. The open reading frame extends from the initiation A60TG codon to TAG732 stop. The open reading frame encodes a protein of 224 amino acids. A hydrophobic leader sequence at the amino terminus conforms to a consensus signal peptide with a predicted cleavage site following an alanine residue located at position 29 in the precursor (Fig. 1). Removal of the signal sequence results in a mature protein of 195 amino acids, having a calculated molecular mass of 22 kDa, which is in close agreement with the molecular mass range of the TIMP family. The deduced amino acid sequence predicts a protein with an isoelectric point of 7.34. Comparison of the predicted amino acid sequence with the sequences of human TIMP-related proteins is shown in Fig. 2. After optimal alignment, the putative protein shows 37% sequence identity and 57% similarity to TIMP-1 and 51% identity and 70% similarity to TIMP-2 and -3. These calculations do not take into account the significance of any gaps in the alignments. The predicted protein structure of the putative new protein shares several essential features that are characteristic of the TIMP family, including 12 completely conserved cysteine residues in the corresponding positions that form intrachain disulfide bonds that fold the protein into a two domain structure (49). The presence of a consensus sequence, VIRAK, which has been proposed to serve a hallmark of the TIMP family (36, 50), was also observed in the most conserved first 22 amino acids located at the amino-terminal region.

The extensive similarity of the predicted amino acid sequence with TIMPs suggests that the putative new protein is a novel member of the human TIMP family and should be designated human TIMP-4.

Tissue Expression—Tissue-specific transcription of TIMP-4 was examined by Northern blotting on 20 µg of total RNAs from various human adult tissues (Fig. 3). As expected, the

	CCTGCTGGGGCCGTCCAGTCCCCACAGCTCACAGGCTCAGTCGCGGATCTGCAGTGTC	
1	-+-----+-+-----+-+-----+-+-----+	60
	TGCCTGGGAGCCCTCGGCCCGGCCAAGCTGGGTGCTGTTGCTGCGGCTGCTGGCGTTGC	
61	-+-----+-+-----+-+-----+-+-----+	120
	P G S P R P A P S W V L L L R L L A L L	
	TGCGGCCCCCGGGGCTGGGTGAGGCATGCAGCTGCGCCCCGGCGCACCTCAGCAGACA	
121	-+-----+-+-----+-+-----+-+-----+	180
	R P P G L G E A C S C A P A H P Q Q H I	
	TCTGCCACTCGGCATTGTGATTCGGGCCAAAAATCTCCAGTGAGAAGGTAGTTCCCGCCA	
181	-+-----+-+-----+-+-----+-+-----+	240
	C H S A L V I R A K I S S E K V V P A S	
	GTGCAGACCCCTGCTGACACTGAAAAATGCTCCGGTATGAATAAACAGATAAAAGATGT	
241	-+-----+-+-----+-+-----+-+-----+	300
	A D P A D T E K M L R Y E I K Q I K M F	
	TCAAAGGGTTTGAGAAAAGTCAAGGATGTTTCAGTATATCATATACGCCCTTTTGACTCTTCCC	
301	-+-----+-+-----+-+-----+-+-----+	360
	K G F E K V K D V Q Y I Y T P F D S S L	
	TCTGTGGTGTGAAACTAGAAGCCAACAGCCAGAAGCAGTATCTCTTGA CTGGTCAGGTCC	
361	-+-----+-+-----+-+-----+-+-----+	420
	C G V K L E A N S Q K Q Y L L T G Q V L	
	TCAGTGATGGAAAAAGTCTTCATCCATCTGTGCAACTACATCGAGCCCTGGGAGGACCTGT	
421	-+-----+-+-----+-+-----+-+-----+	480
	S D G K V F I H L C N Y I E P W E D L S	
	CCTTGGTGCAGAGGGAAAGTCTGAATCATCACTACCATCTGAACGTGGCTGCCAAATCA	
481	-+-----+-+-----+-+-----+-+-----+	540
	L V Q R E S L N H H Y H L N C G C Q I T	
	CCACTGCTACACAGTACCCTGTACCATCTCGGGCCCTTAACGAGTGCCCTCGGAACAGI	
541	-+-----+-+-----+-+-----+-+-----+	600
	T C Y T V P C T I S A P N E C L W T D W	
	GGCTGTTGGAACGAAAGCTCTATGTTTACCAGGCTCAGCATTATGTCTGTATGAAGATG	
601	-+-----+-+-----+-+-----+-+-----+	660
	L L E R K L Y G Y Q A Q H Y V C M K H V	
	TTGACGGCACCTGCAGCTGGTACCGGGGCCACCTGCCTCTCAGGAAGGAGTTTGTGTACA	
661	-+-----+-+-----+-+-----+-+-----+	720
	D G T C S W Y R G H L P L R K E F V D I	
	TCGTTACAGCCCTAGTAGGACCAGTGACCATCACATCCCTTCAAGAGTCTTGAAGATCAA	
721	-+-----+-+-----+-+-----+-+-----+	780
	V Q P *	
	GCCAGTTCTCCTTCCCTGCAGAGCTTTGGCCATTACCACCTGACCTCTTGTGCCAGCTA	
781	-+-----+-+-----+-+-----+-+-----+	840
	ATAAGAAGTGCCAAGTGAGCAGTCTGGCCACTGTCAAGCGAGGGAAGGGGCCATGACTTT	
841	-+-----+-+-----+-+-----+-+-----+	900
	TCTGCCCTTGCCCTCAGCCTGTTGCCCTGCCCTCCCAAAACCCATTAGTCTAGCCTTGTAGC	
901	-+-----+-+-----+-+-----+-+-----+	960
	TGTTACTGCAAGTGTCTTCTTGCTTAGTCTGTTTTCTAAAGCCAGGAGTATTCCCTTT	
961	-+-----+-+-----+-+-----+-+-----+	1020
	CCTCCCCAGGAATATGTGTTTTCTTTTGTCTTAATCGATCTGGTAGGGGAGAAATGGCGA	
1021	-+-----+-+-----+-+-----+-+-----+	1080
	ATGTCATACACATGAGATGGTATATCCTTGCGATGTACAGAAATCAGAAGGTGGTTTGACA	
1081	-+-----+-+-----+-+-----+-+-----+	1140
	GCATCATAAACAGGCTGACTGGCAGGAATGAAAAAAAAAAAAAAAAAAAA	
1141	-+-----+-+-----+-+-----+-+-----+	1189

The classic approach to identifying novel proteins begins with the discovery of an interesting biological activity. This protein is then purified and biochemically characterized, and subsequently, the gene is cloned. Since the introduction of the EST sequencing approach and the availability of tens of thousands of ESTs, researchers can now shift their attention to high throughput cDNA cloning in conjunction with structural similarity analysis as an accelerated method of protein discovery. In this regard, the nucleic acid sequences of randomly picked

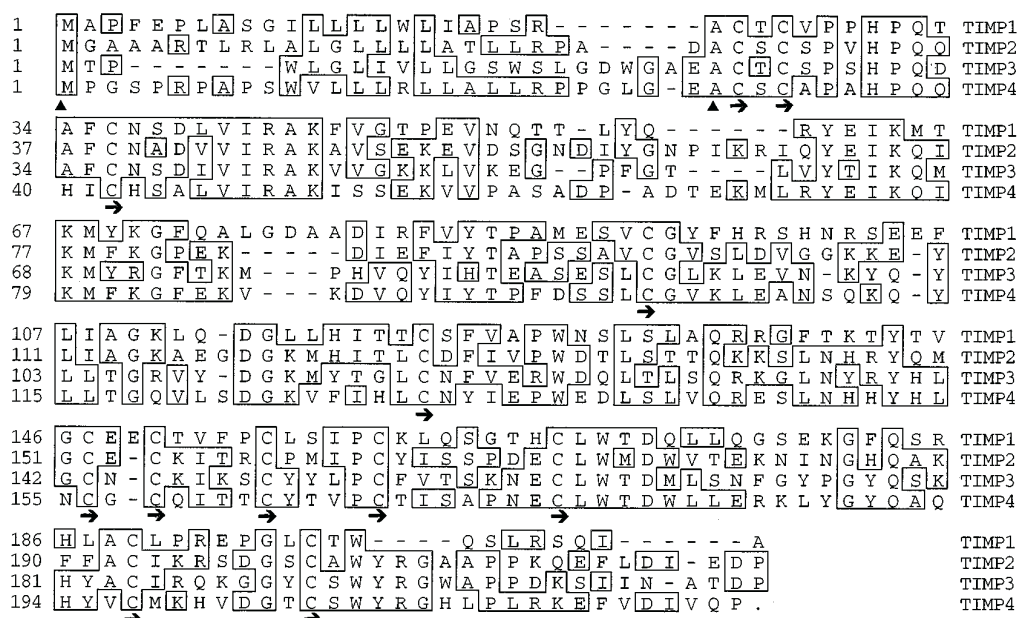


FIG. 2. Comparison of the predicted amino acid sequence of human TIMP-4 with human TIMP-1–TIMP-3. The available amino acid sequence of TIMP-1 (accession number P01033), TIMP-2 (accession number P16035), and TIMP-3 (accession number P35625) were obtained from the SwissProt data base and aligned with the TIMP-4 deduced sequence using the clustal method of the MegAlign program from the DNASTAR software package. Conserved bases are boxed; the 29-amino acid putative signal sequence is shown between two triangles (Δ), and the 12 conserved cysteine residues are labeled with arrows.

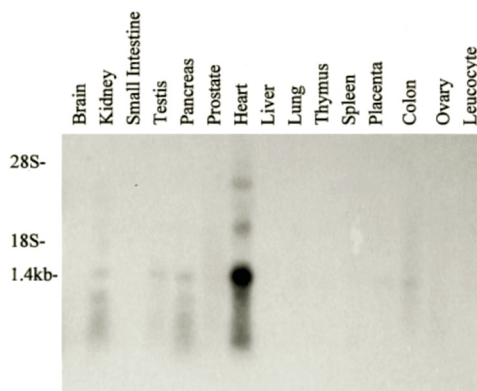


FIG. 3. Expression of the TIMP-4 gene in a variety of normal adult human tissues. Twenty micrograms of total RNA were analyzed in Northern blotting. A strong hybridizing band of 1.4 kilobases was recognized in the lane corresponding to RNA from adult heart. Additional bands with much lower intensities corresponding to mRNA species of about 4.1, 2.1, 1.2, and 0.97 kb were also detected. The integrity of the RNA samples was ascertained by direct visualization of the ribosomal RNAs in the stained gel.

cDNAs from established EST data bases are searched and analyzed by the BLAST program for sequence similarity to the protein of interest. 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 sequences. Using this approach, we identified an EST with high sequence homology to TIMP-2 and, subsequently, the novel TIMP-4 gene was cloned using this EST as a probe.

The predicted protein structure of TIMP-4 shows several interesting features. First, as expected, essential features of other TIMPs are conserved, including the location of 12 Cys residues, as well as their relative spacing and the presence of a 29-amino acid leader sequence, which presumably is cleaved to produce the mature protein (13). Second, the mature protein has an expected size of 22 kDa, which is similar to the sizes of TIMP proteins. Expressed rTIMP-4 protein migrates as a 24-kDa protein by reverse zymography SDS-polyacrylamide gel

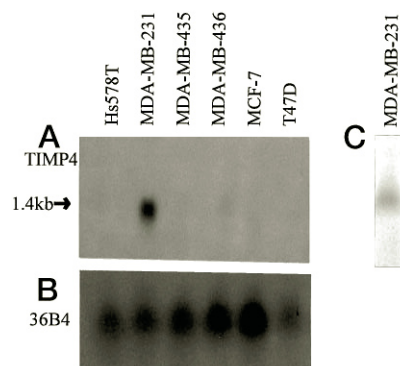


FIG. 4. Northern analysis of TIMP-4 expression in human breast cancer cells. RNAs were isolated and subjected to Northern analysis by hybridization with either a full-length cDNA probe (A) or a 390-base riboprobe, which represents a specific nucleotide sequence of the 3'-untranslated TIMP-4 (C). The integrity of the RNA was ascertained by hybridization with a housekeeping gene, 36B4 (B). Each lane contained 20 μ g of total RNA. Northern analysis failed to detect the TIMP-4 transcript in most breast cancer cell lines, except MDA-MB-231 cells, which showed a strong 1.4-kb TIMP-4 transcript; a very weak hybridization signal was also detected in MDA-MB-436 cells.

electrophoresis at nonreducing conditions, which is consistent with that obtained for other TIMPs (25). Third, the deduced amino acid sequence of TIMP-4 predicts a protein with an isoelectric point of 7.34, the most neutral human TIMP protein at the physiological condition (pH 7.4) comparing with values of 8.00, 6.45, and 9.04 for human TIMP-1, TIMP-2, and TIMP-3, respectively (24). Fourth, as expected, TIMP-4 has a highly conserved amino-terminal domain similar to other TIMPs. The amino-terminal 126 amino acid residues of mature TIMP-1 (51) and the amino-terminal 127 residues of mature TIMP-2 (52, 35) have been shown to be adequate for the inhibition of MMPs, suggesting that this part of the proteins is functionally critical for inhibition of MMPs. In this region, the first 22 amino acids of the mature proteins is the most conserved among the TIMPs; 16 of the first 22 amino acids (73%) are identical among human TIMP-1–TIMP-3. However, the first 22 amino acids of mature

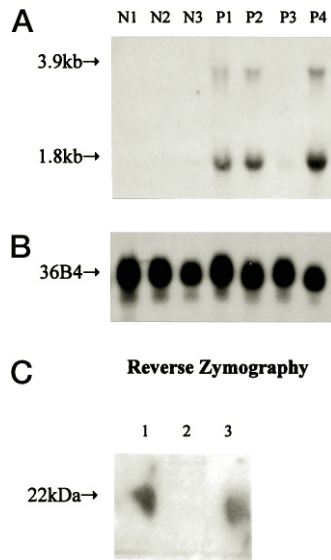


FIG. 5. Metalloproteinase inhibitory activities produced by transforming human breast cancer cells. The human breast cancer cell line MDA-MB-435 was transfected with either the pCITIMP4 plasmid containing the full-length TIMP-4 cDNA or the control pCI-neo plasmid, and the TIMP-4 positive clones were selected as described under "Materials and Methods." **A**, Northern blot of RNAs from both control and TIMP-4 transfected clones. Total RNAs were isolated from three control pCI-neo transfected clones (N1–N3) and four TIMP-4-transfected clones (P1–P4) and then subjected to Northern blot analysis with a random-labeled, full-length TIMP-4 probe. Strong TIMP-4 transcripts were detected in three of four transfected clones; clone P3 shows low level TIMP-4 expression. In contrast, no endogenous TIMP-4 transcripts were detected in any of the control clones. The integrity of the RNAs and loading control were ascertained by hybridization with a housekeeping gene, 36B4 (**B**). **C**, analysis of MMP inhibitory activity by reverse zymography. Conditioned media were prepared from one control clone (N1) and two TIMP-4-producing clones (P1 and P4), concentrated, and analyzed by protease substrate gel electrophoresis as described under "Materials and Methods." Lane 1, clone P1; lane 2, clone N1; lane 3, clone P4. Arrow, molecular mass of the expressed TIMP-4 protein.

TIMP-4 show a decreased sequence identity with other TIMPs: 63% identical to TIMP-1 and TIMP-2 and 59% identical to TIMP-3. The consensus sequence CXCXPXHPQXAFCNXDX-VIRAK (single amino acid code; X, any amino acid) has been proposed to serve a diagnostic hallmark of the TIMPs being present in TIMP-1–TIMP-3 (36). Because TIMP-4 has a less conserved sequence in this region, with only 12 of 22 amino acids identical in all four TIMPs, we suggest the use of consensus sequence VIRAK (positions 47–51; Fig. 2) as a diagnostic hallmark of the TIMP family. We have shown that TIMP-4 is more homologous to TIMP-2 and TIMP-3 than to TIMP-1.

Tissue expression of TIMP-4 appears limited. Although large amounts of transcript were detected in the heart, much lower levels of expression were detected in the kidney, pancreas, colon, and testes; no TIMP-4 transcripts were detected in other tissues, such as liver, brain, lung, thymus, small intestine, and spleen. TIMP-4 may function in a tissue-specific fashion as part of an acute response to tissue remodeling. It is interesting to note that the highest level of TIMP-4 expression is seen in the heart, in which human cancer metastasis rarely occurs. The possibility that the high expression of TIMP-4 in the heart may contribute the inability of malignant cells to invade needs further consideration.

We have expressed TIMP-4 in MDA-MB-435 human breast cancer cells in an effort to investigate the biological significance of this new TIMP in tumor growth and metastasis. Since TIMPs block the activities of MMPs, the net inhibitory activity of TIMPs might be important in preventing malignant progres-

sion from the benign to the metastatic phenotype. In fact, tumor cell invasion and metastasis can be blocked by up-regulation of TIMP expression or an exogenous supply of TIMPs (17, 40–44). Alternatively, down-regulation of TIMP-1 and -2 has been reported to contribute significantly to the invasive potential of human glioblastoma (19). We have analyzed the MMP inhibitory activities of the expressed rTIMP-4 from the conditioned media of transfected clones. As expected, rTIMP-4 proteins expressed from human breast cancer cells possess an inhibitory activity against MMP and are secreted extracellularly, thus confirming that the novel protein is a new member of the TIMP family.

In summary, we have cloned and sequenced a novel human TIMP gene designated TIMP-4, the expression of which is tissue-specific. We have also presented evidence indicating the MMP inhibitory activity of the expressed TIMP-4 protein.

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