Murine tissue inhibitor of metalloproteinases-4 (*Timp*-4): cDNA isolation and expression in adult mouse tissues

Kevin J. Leco[1,a], Suneel S. Apte[b], Gary T. Taniguchi[c], Susan P. Hawkes[c], Rama Khokha[d], Gilbert A. Schultz[a], Dylan R. Edwards[a,*]

[a]*Department of Medical Biochemistry, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta T2N 4N1, Canada*
[b]*Cleveland Clinic Foundation, Department of Biomedical Engineering, 9500 Euclid Avenue, Cleveland, OH 44195, USA*
[c]*Departments of Biopharmaceutical Sciences and Pharmaceutical Chemistry, University of California, San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143-0446, USA*
[d]*Ontario Cancer Institute, Princess Margaret Hospital, 610 University Avenue, Toronto, Ontario M5G 2M5, Canada*

Abstract We have isolated cDNA clones corresponding to a new member of the murine tissue inhibitor of metalloproteinase (TIMP) family, designated *Timp*-4. The nucleotide sequence predicts a protein of 22 609 Da that contains the characteristic 12 cysteine TIMP signature. TIMP-4 is more closely related to TIMP-2 and TIMP-3 than to TIMP-1 (48%, 45% and 38% identity, respectively). Analysis of *Timp*-4 mRNA expression in adult mouse tissues indicated a 1.2 kb transcript in brain, heart, ovary and skeletal muscle. This pattern of expression distinguishes *Timp*-4 from other *Timp*s, suggesting that the TIMP-4 protein may be an important tissue-specific regulator of extracellular matrix remodelling.

Key words: Tissue inhibitor of metalloproteinases; Extracellular matrix; cDNA cloning; Gene expression

1. Introduction

Tissue inhibitors of metalloproteinases (TIMP) are a family of secreted proteins whose primary recognized function is the ability to inhibit the active forms of matrix metalloproteinases (MMP) [1,2]. Since MMPs are recognized as the principal class of enzymes involved in extracellular matrix (ECM) degradation, TIMPs provide critical control of the rate of ECM metabolism during normal tissue remodelling [3,4]. Thus processes such as wound healing [5], ovulation [6,7], embryo implantation [8–11] and mammary gland involution [12,13] are characterized by orchestrated changes in the production of MMPs and TIMPs, supporting the notion that the local balance between the activities of MMPs and TIMPs is pivotal in determining the rate of ECM turnover. Disruption of this balance has been implicated in the pathological tissue damage seen in a number of disease states including tumour invasion and metastasis [14,15], cardiovascular disease [16] and multiple sclerosis [17].

*Corresponding author. Fax: (1) (403) 283-8727.
E-mail: dedwards@acs.ucalgary.ca

[1]*Present address:* Ontario Cancer Institute, Princess Margaret Hospital, 610 University Avenue, Toronto, Ontario, M5G 2M5, Canada.

Abbreviations: TIMP-4 designates protein; *Timp*-4 and TIMP4 are symbols for the mouse and human genes, respectively.

The sequences of the mouse *Timp*-4 and human TIMP-4 genes described here have been submitted to GenBank.

Given the importance of the TIMP proteins during ECM remodelling, we undertook a degenerate oligonucleotide cloning strategy to search for novel members of the *Timp* gene family. Isolated RNA from several murine tissues, including embryo, decidua, placenta, and heart were analysed by reverse transcription-polymerase chain reaction (RT-PCR) for the presence of new *Timp*-like cDNAs. We present here the identification of a new member of the murine *Timp* family, which has been designated *Timp*-4.

2. Materials and methods

2.1. RNA isolation

Tissues to be used for RNA extraction were collected from 12-week-old female CD1 mice (for testes RNA one 9-month-old male CD1 mouse was used), frozen on dry ice, and stored at −70°C until processed. Frozen tissue was homogenized and RNA prepared as previously described [18].

2.2. Design of degenerate oligonucleotides for PCR

Degenerate primers were designed based on the VIRAK and (H/E)CLW(T/M)D motifs found in TIMP-1, -2 and -3 from all animals for which the corresponding genes have been cloned [19]. The oligonucleotides used were:

5′-primer: 5′-**ACTGGGATCC**GT[C,G]AT[A,C][A,C]G[A,G]GC-[C,T]AAA
3′-primer: 5′- **GCATAAGCTT**C[C,G][A,G]TCCA[C,G]A[A,G]-[A,G]CA[C,G]T[C,G]

Degenerate positions are indicated by square brackets. In addition to flanking nucleotides the 5′- and 3′-primers contained sequences for *Bam*HI and *Hin*dIII restriction enzyme sites, respectively (shown in bold) to facilitate cloning. These oligonucleotides were predicted to generate *Timp*-specific cDNA fragments of approximately 450 bp.

2.3. Isolation of murine Timp-4 cDNAs

For RT-PCR, 1 µg of RNA from either day-6.5 embryo with decidua, day-8.5 embryo with decidua, day-11.5 embryo, day-11.5 placenta, day-13 embryo, day-13 placenta, or adult heart was reverse transcribed using Superscript RT (Gibco-BRL) according to manufacturer's instructions. One-tenth of the RT product was used for PCR with the degenerate TIMP oligonucleotide primers. Forty cycles of PCR, each consisting of 1 min at 94°C, 2 min at 45°C, and 2 min at 72°C were performed using Taq polymerase (Pharmacia) in a Techne PHC-2 thermal cycler. PCR products of approximately 450 bp were digested with *Bam*HI and *Hin*dIII, isolated from an agarose gel, and subcloned into pBluescript KS− (Stratagene) using standard procedures [20]. Following transformation, bacteria were grow on LB agar containing 50 µg/ml ampicillin and colonies were transferred to nylon filters (Hybond N, Amersham). Filters were hybridized with a mixture of [32P]dCTP-labelled *Timp*-1, *Timp*-2, and *Timp*-3 cDNA probes [19,21,22] and washed as previously described [19]. Dried filters were exposed to XAR5 X-ray film (Eastman Kodak). Filters were

then hybridized with [³²P]dCTP-labelled first-strand cDNA (prepared as described above), washed and exposed to X-ray film. Colonies hybridizing only to the cDNA and not to *Timp*-1, -2, and -3 were grown in liquid culture and plasmid DNA isolated [19]. One clone (subsequently designated pBSmTIMP-4) chosen for automated sequence analysis (University Core DNA Sequencing Service, University of Calgary) contained a cDNA insert related to, but distinct from, *Timp*-1,-2 and -3. This cDNA was labelled with [³²P]dCTP and used to screen a lambda ZAP murine heart cDNA library (Stratagene) according to manufacturer's instructions. Two identical clones contained 5′-sequence information not included in the original PCR fragment. A mouse brain cDNA library in lambda gt11 (generated from 18-day-old mice at the Molecular Biology core of the Mental Retardation Research Center at the University of California, Los Angeles, and provided by Dr. Wendy Macklin, Cleveland Clinic Foundation) was used for isolation of the 3′-end of the mouse *Timp*-4 cDNA. An aliquot of the library was boiled in distilled water and used as a template for PCR using a forward primer derived from the sequence of pBSmTIMP-4 (5′-AGCCTGAATCATCACTACC) together with lambda gt11 flanking primers. An 850 bp cDNA was cloned into pGEM-T (Promega) by T/A cloning and automated sequence analysis carried out (Molecular Biotechnology Core Facility of the Cleveland Clinic Foundation). Both strands of all cDNAs were sequenced, and DNA or protein sequences were generated using GCG software (Genetics Computer Group).

2.4. Isolation of human TIMP-4

The cDNA insert from pBSmTIMP-4 was used to screen a lambda gt11 human placenta cDNA library (Clontech) and one positive clone was obtained. The insert of this phage could not be excised with *Eco*RI, therefore PCR with lambda gt11 flanking primers was used to amplify the insert. The PCR product of approximately 1.2 kbp was cloned into pGEM-T. Automated sequence analysis with vector and internal primers was carried out (Molecular Biotechnology Core Facility of the Cleveland Clinic Foundation).

2.5. Protein sequence analysis

Protein sequence analysis was performed by use of the Wisconsin Sequence Analysis Package (Version 8, Genetics Computer Group). Comparison of the deduced amino acid sequence of mouse TIMP-4 with other TIMP sequences was performed using the Pattern-Induced Multisequence Alignment (PIMA) algorithm [23,24] which employs secondary structure-dependent gap penalties for comparative protein modelling.

2.6. Northern blot analysis

RNA was collected as described above. For ovaries and uterus, tissue from three animals was pooled to minimize effects of female cycling. Ten micrograms of total RNA from each tissue was electrophoresed on 1.1% agarose gels containing 2.2 M formaldehyde and transferred to nylon membranes (Amersham) as previously described [19]. Replicate filters were hybridized with ³²P-labelled *Timp*-1, -2, -3 and -4 cDNA probes followed by 18S ribosomal RNA cDNA to ensure equal loading [19].

3. Results

3.1. Cloning and characterization of mouse Timp-4 cDNAs

A degenerate oligonucleotide-PCR strategy was used to identify a novel murine *Timp* cDNA. Degenerate primers were devised based on two short sequence motifs that are well-conserved in all of the known TIMPs from diverse species, namely the VIRAK sequence close to the N-terminus of mature TIMP proteins (5′-primer) and the consensus (H/E)CLW(T/M)D amino acid sequence (3′-primer) that lies in the C-terminal domain [19]. The primers were used for RT-PCR with RNA isolated from murine embryos, decidua, placenta and adult heart. Only heart RNA yielded a cDNA that was similar to, but distinct from, *Timp*-1, -2 and -3. Overlapping clones isolated from mouse heart and brain cDNA libraries plus the original PCR product were subjected to se-

Fig. 1. A: DNA and predicted amino acid sequences of murine TIMP-4. The schematic underneath the sequence shows the origins of the three cDNA clones used to compile the composite *Timp*-4 sequence. B: Comparison of mouse and human TIMP-4 sequences.

quence analysis and a composite cDNA and predicted amino acid sequence of mouse *Timp*-4 is presented in Fig. 1A. Within the 940 bp cDNA, sequence from nucleotides 1 through 310 was obtained from the heart library cDNA clone, sequence from nucleotides 141 through 539 was obtained from the original PCR product, and sequence from nuclotides 441–940 was obtained from the brain library cDNA clone. The open reading frame within the cDNA sequence encodes a predicted protein of 225 amino acids which contains hallmarks of TIMP proteins including 12 precisely conserved cysteine residues, and the VIRAK and ECLWTD features. The sequence of the mouse TIMP-4 protein was compared with the predicted amino acid sequence of a partial human TIMP-4 cDNA clone that correponds to the region from serine-44 to the C-termi-

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                                        ↓
TIMP-1    1 .MMAPFASLASGI.LLLLSLIAS....SKACSCAPPHPQTAFCNSDLVIR  44
TIMP-2    1 .MGAAARSLRLALGLLLLASLVRP...ADACSCSPVHPQQAFCNADVVIR  46
TIMP-3    1 ......MTPWLGL.VVLLSCWSLGHWGAEACTCSPSHPQDAFCNSDIVIR  43
TIMP-4    1 MPWSPLAALSWALVLRLLALLWPPG.RGEACSCAPAHPQQHFCHSALVIR  49

TIMP-1   45 AKFMGSPEINE.......TTLYQRYKIKMTKMLKGFKAVGNAADIRYAYT  87
TIMP-2   47 AKAVSEKEVDSGNDIYGNPIKRIQYEIKQIKMFKGPD.....KDIEFIYT  91
TIMP-3   44 AKVVGKKLVKEGP......FGTLVYTIKQMKMYRGFSK...MPHVQYIHT  84
TIMP-4   50 AKISSEKVVPASKD.PADTQKLIRYEIKQIKMFKGFEKA...KDIQYVYT  95

TIMP-1   88 PVMESLCGYAHKSQNRSEEFLITGRLR.NGNLHISACSFLVPWRTLSPAQ 136
TIMP-2   92 APSSAVCGVSLDVGG.KKEYLIAGKAEGDGKMHITLCDFIVPWDTLSITQ 140
TIMP-3   85 EASESLCGLKLEVN..KYQYLLTGRVY.EGKMYTGLCNFVERWDHLTLSQ 131
TIMP-4   96 PFDSSLCGVKLETNS.HKQYLLTGQILSDGKVFIHLCNYIEPWEDLSLVQ 144

TIMP-1  137 QRAFSKTYSAGCGVCTVFPCLSIPCKLESDTHCLWTDQVL.VGSEDYQSR 185
TIMP-2  141 KKSLNHRYQMGC.ECKITRCPMIPCYISSPDECLWMDWVTEKSINGHQAK 189
TIMP-3  132 RKGLNYRYHLGC.NCKIKSCYYLPCFVTSKNECLWTDMLSNFGYPGYQSK 180
TIMP-4  145 RESLNHHYHQNC.GCQITTCYAVPCTISAPNECLWTDWLLERKLYGYQAQ 193

TIMP-1  186 HFACLPRNPGLCTWRSLGAR.......... 205
TIMP-2  190 FFACIKRSDGSCAWYRGAAPPKQEFLDIEDP 220
TIMP-3  181 HYACIRQKGGYCSWYRGWAPPDKSISNATDP 211
TIMP-4  194 HYVCMKHVDGICSWYRGHLHLRKEYVDIIQP 224
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Fig. 2. Comparison of predicted amino acid sequences of mouse TIMPs. The sequences used for comparison include TIMP-1 [21,28] (these sequences are identical and differ at nine positions from another mouse TIMP-1 sequence in [29]); TIMP-2 [30], chosen in preference to [22] as there are two differences (H and E at positions 12 and 195) in which the chosen mouse sequence shows a better match with human TIMP-2; TIMP-3 [19]. The shaded areas represent 75% identity. The arrow indicates the N-terminus of the mature protein after cleavage of the signal peptide.

nus of the mouse protein, revealing 91% identity and 96% similarity (Fig. 1B).

3.2. Protein sequence comparison

The deduced amino acid sequence of mouse TIMP-4 includes a 29-residue signal peptide and a mature protein of 195 amino acids. Assuming no post-translational modifications, the latter is predicted to be 22 609 Da with a pI of 7.73. There are no N-glycosylation motifs in the sequence. A comparison of the mouse TIMP-4 sequence with sequences for other members of the TIMP family within this species is presented in Fig. 2, where numbering of the amino acid residues begins with the first residue of the signal peptide. TIMP-4 is more closely related to TIMP-2 and TIMP-3 than TIMP-1, exhibiting 48%, 45% and 38% identity, and 66%, 63% and 54% similarity, respectively. In this respect, in the region of residues 59–64 in TIMP-2 (where there is a NDIYGN insert) TIMP-4 also has an insert of KDPAD (residues 62–66) and like both TIMP-2 and TIMP-3, TIMP-4 has an extended 'tail' at the C-terminus. The signal peptide of mouse TIMP-4 is the longest of the four TIMPs (29 amino acids compared to 24, 26 and 23 for TIMP-1, -2 and -3, respectively). The mature protein contains a particularly large number of histidines (12 compared to 4–6 for the other three mouse TIMPs). It has a significantly higher isoleucine content than TIMP-1 and TIMP-3 (14 residues versus 7) and like TIMP-3 a relatively high tyrosine content.

3.3. RNA expression analysis

Expression profiles for Timp-1, -2, -3 and -4 mRNAs in adult mouse tissues were compared by Northern blot analysis which is presented in Fig. 3. As has been observed previously [25,26], Timp-1 mRNA was found predominantly in ovary and bone. Timp-2 mRNA was detected in many tissues examined, with high levels observed in lung, ovaries, brain, testes

and heart. Abundant expression of Timp-3 mRNA was observed in kidney, lung, heart, brain and ovary [19,27]. In contrast to the patterns seen with Timp-1, -2 and -3, expression of Timp-4 mRNA was restricted to brain, heart, ovaries, skeletal muscle and skin. In other studies, we failed to detect expression of Timp-4 by Northern blot analysis in normal or Ha-ras-transformed C3H10T1/2 mouse fibroblasts, though the other three Timp genes showed characteristic cell line-specific and stimulus-responsive patterns of expression [19,21,22].

4. Discussion

Due to its essential nature in the maintenance of tissue structure, the integrity of the ECM must be preserved in the adult animal. However, in many normal physiological processes remodelling of the ECM is required. During ovulation, endometrial cycling, mammary gland development, wound healing, and embryo implantation and development, the ECM is locally degraded to effect the changes required. The MMP family of enzymes, by virtue of their ability to degrade all of the major protein components of the ECM [1,3,4], are key factors in the modulation of the local matrix environment. As such, control over the activity of these enzymes is exerted at several levels, including gene expression, proenzyme activation, and by inhibition of active enzyme by TIMPs [1–4]. Until now the family of TIMP proteins contained three members, TIMP-1, -2 and -3. In this report, we describe the isolation of a cDNA encoding a new member of the TIMP family. The predicted amino acid seqence of this cDNA encodes a protein containing the 12 cysteine residues and the VIRAK sequence conserved in all TIMP proteins. By these criteria we designate the new protein murine TIMP-4.

The amino acid sequence of mouse TIMP-4 has regions of similarity with all of the other known TIMP proteins. Moreover, a high degree of evolutionary conservation is evident
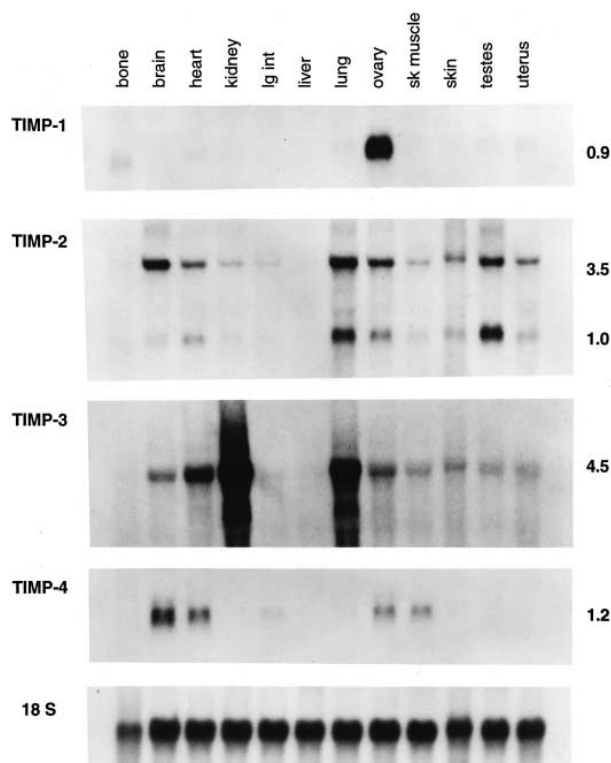
Fig. 3. Northern blot analysis of adult murine tissues with *Timp*-1, -2, -3 and -4 probes. Hybridization to 18S ribosomal RNA demonstrates equivalent loading between samples. Transcript sizes were determined relative to the migration of 28S and 18S ribosomal RNAs. lg int, large intestine; sk muscle, skeletal muscle.

because the protein sequence predicted from a partial human TIMP-4 cDNA clone that spans more than 80% of the protein coding region showed 91% amino acid identity with the murine TIMP-4. TIMP-4 is most closely related to TIMP-2 and like this protein it has no N-glycosylation motifs in its primary sequence. At 22 609 Da the predicted mature TIMP-4 product is the largest core protein of all the mouse TIMPs (compared to 20 243 Da, 21 729 Da and 21 676 Da for TIMP-1, -2 and -3, respectively). However, a striking feature of mouse TIMP-4 is that at five positions, previously considered to be conserved for all known TIMPs, the TIMP-4 sequence deviates. These five residues are invariant in 17 TIMP-1, -2 and -3 sequences from various species in available databanks (data not shown). Most notable among these substitutions are two in the highly conserved N-terminus of the protein within the sequence AFCN(S,A)(D,E)(L,V,I)VIRAK where A and N (underlined) are substituted by H at positions 40 and 43 in TIMP-4 (Fig. 2). Other substitutions include G to N, S to A and A to V at positions 155, 172 and 196, respectively, in the mouse TIMP-4 sequence. These latter three differences are also found in the partial human TIMP-4 clone indicating that they may have functional significance. It will be interesting to determine whether the histidines at positions 40 and 43 are also present in TIMP-4 from different species.

Another interesting substitution that may have implications for the mechanism of inhibition of MMPs by TIMPs occurs at position 45 in mouse and human TIMP-4, where A replaces D/E in all other TIMP sequences. The negatively charged amino acid at this position, which precedes several hydropho-

bic residues, was previously proposed to be suitably placed to interact with the zinc within MMPs and render them inactive upon binding of the hydrophobic amino acids to the S1′–S2′–S3′ region of the enzymes [1]. We have not yet expressed the TIMP-4 protein and therefore we do not know whether this sequence will yield an active metalloproteinase inhibitor. If it does, a different mechanism of inhibition of MMPs by TIMP-4, at least, would need to be invoked. There is further support for a review of the proposed mechanism since a recent site-directed mutagenesis study established that a D to Y change at this position in mouse TIMP-1 did not impair the ability of the protein to inhibit MMPs [31].

The expression pattern of murine *Timp*-4 mRNA in adult mouse tissues is distinct from that of the other *Timp*s. Abundant *Timp*-4 mRNA was detected in adult brain, heart, ovary and skeletal muscle, possibly reflecting the highly structured nature of these tissues. Expression of all four *Timp* mRNAs was detected in ovary, a finding which implies the need for exquisite control of matrix remodelling in this tissue. Roles for TIMP-1 in the regression of the corpus luteum (CL) in the cycling mouse and for TIMP-3 in the maintenance of structural integrity of the CL in the pregnant mouse have been suggested [7]. In a rat pseudopregnant model, TIMP-3 again appears to be involved in the maintenance of the CL [6]. It will be interesting to define any role that the TIMP-4 protein may play in these systems or other hormonally regulated events such as the development and function of the mammary gland and the normal cycling of the uterus. Preliminary evidence indicates that the *Timp*-4 mRNA is regulated during post-natal mouse mammary development (R.K., unpublished observation). A function for TIMP-4 in the physiology and/or pathology of the brain, heart and skeletal muscle must await further experimentation. It is important to note that by in situ hybridization and Northern blot analysis, *Timp*-4 mRNA could not be detected during mouse embryogenesis (K.J.L. and S.S.A., unpublished observation), perhaps explaining why the cDNA was not isolated from mouse embryo or placental RNA.

In summary, we have isolated a cDNA encoding a new member of the TIMP family of proteins, murine TIMP-4. Although TIMP-4 shares some common features with the other TIMPs it is clearly distinct in both its amino acid sequence and expression profile in the mouse.

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