Tissue Inhibitor of Metalloproteinases-3 (TIMP-3) Is an Extracellular Matrix-associated Protein with a Distinctive Pattern of Expression in Mouse Cells and Tissues*

Kevin J. Leco‡‡, Rama Khokha‡‡, Nadine Pavloff**‡‡, Susan P. Hawkes**, and Dylan R. Edwards‡‡‡

From the ‡‡Department of Pharmacology and Therapeutics, University of Calgary, Calgary, Alberta, T2N 4N1, Canada, the **Department of Oncology, London Regional Cancer Center, London, Ontario, N6A 4L6, Canada, and the ‡‡‡Department of Pharmacy, University of California, San Francisco, California 94143-0446

We have isolated cDNA clones corresponding to a novel mouse metalloproteinase inhibitor. Five overlapping cDNA clones contain most of the information for a prominent 4.5-kilobase transcript that was detected in RNA from mouse fibroblasts and adult tissues. Sequence analysis revealed an open reading frame (ORF) for a protein of 212 amino acids that is 80% identical to chicken inhibitor of metalloproteinases-3 (ChIMP-3). The 3'-untranslated sequence also showed remarkable conservation with the chicken gene. The ORF directed the expression of a 24-kDa protein in COS-1 cells that localized to the extracellular matrix (ECM). On the basis of these similarities we propose to identify the new gene as murine tissue inhibitor of metalloproteinases-3 (TIMP-3). Mouse C3H 10T1/2 fibroblasts produced a 24-kDa metalloproteinase inhibitor that also localized to the ECM and was recognized by a polyclonal antibody to ChIMP-3. Like TIMP-1, TIMP-3 was highly inducible in mouse C3H 10T1/2 fibroblasts by phorbol ester (PMA), epidermal growth factor (EGF), and transforming growth factor-β1, but nuclear run-on assays showed that the on/off transcription kinetics were faster for TIMP-3 than TIMP-1. A major difference in vitro was the stimulation of expression of TIMP-3 by dexamethasone which inhibits EGF- and PMA-induced TIMP-1 transcription. Also, TIMP-3 showed a distinctive pattern of expression in adult tissues with abundant transcripts detected in kidney, lung, and brain but only low levels detected in bone, a prominent location of TIMP-1 transcripts. We propose that TIMP-3 functions in a tissue-specific fashion as part of an acute response to remodeling stimuli.

Tissue inhibitors of metalloproteinases (TIMPs)* are secreted multifunctional proteins that play pivotal roles in the regulation of extracellular matrix (ECM) metabolism (Woessner, 1991; Denhardt et al., 1989). Their most widely recognized action is as inhibitors of matrix metalloproteinases (MMPs), a family of zinc-dependent enzymes that includes collagenases, stromelysins, and gelatinases which in combination are able to degrade all of the proteinaceous components of ECMs (Alexander and Werb, 1991; Matsubara, 1992; Birkedal-Hansen et al., 1993). Two mammalian TIMPs have been described: though sharing only approximately 42% amino acid identity TIMP-1 and TIMP-2 are essentially interchangeable in their capabilities as inhibitors of active MMPs, but they are distinguished by the formation of specific complexes with different gelatinases (Goldberg et al., 1989; Wilhelm et al., 1989; Kleiner et al., 1992; Murphy et al., 1992). These gelatinase A-TIMP-2 and gelatinase B-TIMP-1 complexes may reflect an additional function for TIMPs in controlling the activation of specific latent MMPs. However, an important additional aspect of the biology of TIMPs is that they possess growth promoting properties: both TIMP-1 and TIMP-2 act as erythroid potentiating activities that stimulate the growth of erythroid precursors in vitro (Gasson et al., 1985; Stetler-Stevenson et al., 1992) and in vivo (Niskanen et al., 1988), and recent evidence suggests that TIMP-1, in particular, is a serum mitogen for a wide array of cultured cells (Bertaux et al., 1991; Hayakawa et al., 1992).

A third member of the TIMP family was recently identified in chickens (Staskus et al., 1991; Pavloff et al., 1992). Chicken inhibitor of metalloproteinases-3 (ChIMP-3) shows slightly more structural similarity to TIMP-2 than TIMP-1 proteins (42% amino acid identity versus 28%), but its localization to the ECM sets it apart from the mammalian TIMPs described to date. The 21-kDa ChIMP-3 was originally isolated as a transformation-induced protein in chicken fibroblasts (Blenis and Hawkes, 1983, 1984). Yang and Hawkes (1992) reported that ChIMP-3 promotes detachment from the ECM of cells that are in the process of acquiring the transformed phenotype and stimulates the growth of nontransformed cells in low serum conditions.

The notion that individual TIMP family members may have specific physiological roles is supported by gene expression studies. The TIMP-1 gene is highly inducible at the transcriptional level in response to many cytokines (Edwards et al., 1993). The TIMP-3 gene is highly inducible at the transcriptional level in response to many cytokines (Edwards et al., 1993). These cytokines include interferons, interleukins, and tumor necrosis factor-α.
Mouse TIMP-3: Cloning and Expression Analysis

1987, 1992; Lotz and Guerne, 1991), hormones (Mann et al., 1991; Sato et al., 1991) and the tumor promoter phorbol 12-myristate 13-acetate (PMA; Murphy et al., 1985; Edwards et al., 1986), and it is also sensitive to transformation by the ras oncogene (Tuck et al., 1991; Leco et al., 1992; Su et al., 1993). Likewise ChIMP-3 is sensitive to transformation by the src oncogene (Blenis and Hawkes, 1983) and production of the inhibitor by nontransformed chicken cells is stimulated by PMA (Blenis and Hawkes, 1984). In contrast, TIMP-2 expression, like that of progelatinase A with which it interacts, is largely constitutive (Stetler-Stevenson et al., 1990; Leco et al., 1992). Where responses of TIMP-2 to stimuli have been observed, the outcomes are opposite to those seen for TIMP-1 (Stetler-Stevenson et al., 1990; Denhardt et al., 1993). Tissue localization data show that high levels of TIMP-1 transcripts are found at sites of active remodeling, such as developing bone in the early mouse embryo where its expression overlaps significantly with that of members of the transforming growth factor-β family (TGF-β1; Nomura et al., 1989; Flenniken and Williams, 1990).

In this paper we report the isolation of a novel member of the mammalian TIMP family. On the basis of its high degree of similarity to ChIMP-3, its ECM localization, and its recognition by anti-ChIMP-3 antisera, we propose that this gene is the murine homolog of ChIMP-3 and that it be designated as mTIMP-3. Our data indicate that mTIMP-3 shares many features of the inducible expression of mTIMP-1 in response to epidermal growth factor (EGF), TGF-β1 and PMA, although it responds with faster transcription kinetics. A significant difference is that mTIMP-3 expression is induced by dexamethasone, which inhibits EGF-induced TIMP-1 mRNA production in mouse C3H 10T1/2 fibroblasts. Moreover, mTIMP-3 shows a pattern of expression in adult mouse tissues that differs markedly from that of either TIMP-1 or TIMP-2. Our data establish mTIMP-3 as a new member of the mammalian TIMP family that has distinctive biochemical characteristics and a unique pattern of expression that argues for a specialized function in vivo.

MATERIALS AND METHODS
cDNA Library Screening—A λ ZAP II cDNA library derived from mouse PCC4 teratocarcinoma cell mRNA (Stratagene) was screened using the ChIMP-3 clone D cDNA insert (Pavloff et al., 1992). Approximately 6 × 10⁸ phage were plated on 12.5 cm bacterial dishes, and phage plaques were transferred to 132-mm Hybond N nylon membranes (Amersham) using standard protocols (Sambrook et al., 1989). The ChIMP-3 cDNA insert was radiolabeled with (α-3²⁵)PdCTP by nick translation (Sambrook et al., 1989) to a specific activity of >10⁸ cpm/μg. Filters were prehybridized in a buffer containing 5% formamide, 5 × Denhardt's solution, 5 × SSC, 1% SDS, and 0.1 mg/ml single-stranded salmon sperm DNA for 2 h. Filters were then transferred for prehybridization buffer containing 8 × 10⁶ cpm/ml labeled ChIMP-3 probe and incubated at 42 °C overnight. Filters were washed once in 1 × SSC, 0.2% SDS at room temperature for 10 min, once in 1 × SSC, 0.2% SDS at 42 °C for 30 min, and twice in 0.2 × SSC, 0.2% SDS at 42 °C for 10 min. Filters were then air-dried and exposed to XAR5 x-ray film (Eastman Kodak) for 48 h. Five positive phage plaques were identified and then recovered from the bacterial dishes and replated (Sambrook et al., 1989). Successive rounds of plating and hybridization were performed until all phage plaques on a single dish were identified as positive. Single positive phage plaque for each clone was used for an in vivo excision reaction (according to Stratagene) which yielded the cDNA insert within the EcoRI site of the phBluescript II SK+ plasmid. The five plasmid clones were subjected to restriction digestion with the enzymes EcoRI, BamHI, HindIII, HincII, and Pst I, electrophoresed on agarose gels, transferred to Hybond N nylon membranes, and probed with radiolabeled cDNA insert. A restriction map of the five overlapping cDNA clones was generated (Fig. 1A), and individual hybridizing restriction fragments were purified by agarose gel electrophoresis onto DEAE paper (Sambrook et al., 1989). Fragments were ligated into appropriately restriction-digested phBluescript II KS+ plasmids.

Sequence Analysis—Sequence analysis was performed using the Sequenase version 2.0 DNA sequencing kit (U. S. Biochemical Corp.) and by the University Core DNA Sequencing Service (Calgary, Alberta, Canada). Protein was sequenced by the Genedata ScienceSequencing Package from the Molecular Biology Resource (MBIR), Baylor College of Medicine, Department of Cell Biology. Comparison of the deduced amino acid sequence of ChIMP-3 with other sequences in the data bank was performed using the pattern-induced multisequence alignment (PIMA) algorithm of Smith and Smith (1990), which employs secondary structure-dependent gap penalties for comparative protein modeling.

Cell Culture and Preparation of RNA, Conditioned Media, and ECM—For preparation of RNA, mouse C3H 10T1/2 cells were grown to confluence in 75 cm² culture flasks in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12) media supplemented with 10% fetal calf serum (FCS; Life Technologies, Inc.). The cells were then rendered quiescent by incubation in serum-free DMEM/F-12 media for 18–24 h. Stimulations were performed by addition of dexamethasone, EGF, PMA, and/or TGF-β1 to final concentrations of 10⁻⁸ m, 50 ng/ml, 10⁻⁷ m, and 2 ng/ml, respectively, for times indicated in the figures. Stimulations were terminated by addition of a guanidinium isothiocyanate/1% formaldehyde, followed by transfer to Hybond N nylon membranes (Sambrook et al., 1989). Filters were hybridized with nick-translated cDNA probes as described under "cDNA Library Screening." After exposure to x-ray film, filters were stripped by incubation in prehybridization buffer at 70 °C for 10 min, then rehybridized with the probes indicated in the figures. Samples of RNA (20 μg) from mouse tissues were electrophoresed as described above and transferred to GeneScreen plus (DuPont NEN). The filters were probed with [3²⁵]P-labeled cDNA inserts for mTIMP-1, mTIMP-3, and glyceraldehyde-3-phosphate dehydrogenase, each at 1.5 × 10⁶ cpm/ml. The cDNA probes were radiolabeled by random priming, and hybridization was performed according to Church and Gilbert (1984). Nuclear run-on analyses were performed as described previously by Edwards and Mahadevan (1992).

Expression of TIMPs in COS-1 Cells—An EcoRI-EcoRI fragment from the mTIMP-3 clone C4 (Fig. 1A) containing the entire coding region plus approximately 225 bp of 5'-untranslated and 900 bp of 3'-untranslated sequence was ligated into the EcoRI site of the eukaryotic expression vector pXMT2, to yield pXMT2 mTIMP-3. Correct orientation of the inserted cDNA was confirmed by DNA sequence analysis. Conditioned media and ECM from COS-1 cells transfected with pXMT2-1 and mouse TIMP-2 vectors has been described previously (Leco et al., 1992). The monkey kidney cell line, COS-1, was used to transiently express the TIMP-3 cDNA to assay for a functional protein product. The COS-1 cells were seeded at a density of 1 × 10⁶ cells/100-mm dish in DMEM/F-12 plus 10% FCS. The following day cells were transiently transfected with 10 μg of indicated plasmid DNA using the calcium phosphate precipitation method of Chen and Okayama (1987). After 24 h, the medium was replaced with fresh DMEM/F-12 plus 10% FCS. After 18 h recovery, the...
Mouse TIMP-3: Cloning and Expression Analysis

The data shown in Fig. 4 demonstrate that transcription of mTIMP-3 expression plasmid; there was no corresponding augmentation of activity in conditioned media from the same transfection, suggesting that the expressed protein preferentially localizes to the ECM. In contrast, transfection with either human TIMP-1 or mouse TIMP-2 expression vectors led to increased activities at the expected sizes of 28 and 21 kDa, but only in samples of conditioned media. The background 24-kDa inhibitor activity seen in all of the ECM samples probably represents endogenous COS-1 cell TIMP-3.

The expression of mTIMP-3, in comparison with that of mTIMP-1 and mTIMP-2, in fibroblasts and adult mouse tissues is presented in Figs. 4–8. Northern blot analysis revealed the presence of a single major transcript class at around 4.5 kb; there is apparently size heterogeneity, because the mTIMP-2 transcript (3.5 kb) runs on the same blots as a much tighter band (Figs. 4 and 7). Minor RNAs of lower molecular mass are also present but it is unclear whether these are functional mRNAs or breakdown products of the larger form. No transcripts have been observed in the 0.9–1.0 kb range that are characteristic of other murine TIMPs.

The data shown in Fig. 4 demonstrate that transcription of mTIMP-3 in mouse fibroblasts is highly inducible by stimuli that affect ECM remodeling. Expression of mTIMP-3 was induced in confluent serum-deprived mouse C3H/10T1/2 fibroblasts by exposure to PMA and TGF-β1, and these two agents in combination led to a superinduction of mRNA at early times of identity occurring throughout the predicted coding region (data not shown). Remarkably, sequences downstream of the ORF of the mouse gene were very similar to the 3′-untranslated region of ChIMP-3. One A+T-rich stretch of 47 bp is missing only one nucleotide from the corresponding region of ChIMP-3 (Fig. 1D). In contrast, the 5′-untranslated sequences of the two genes are poorly conserved. Third, the extensive tracts of 5′- and 3′-untranslated regions are the longest so far described for a member of the TIMP family. Assuming that the cDNA clones we have isolated have not suffered sequence rearrangements, the start of translation of the ORF is 1.1 kb from the 5′ terminus of the longest cDNA (clone 7.1), and we estimate at least 2.7 kb of 3′-untranslated sequence. These features differ markedly from the organization of the mature 0.9-kb TIMP-1 transcript in mouse and human cells.

The expression of TIMP-3 sequences with sequences of TIMP-related proteins from human, mouse, pig, cow, rabbit, and chicken in the EMBL/GenBank is shown in Fig. 2. Analysis of these data indicates that 46 amino acid residues are conserved among all the proteins listed. This represents 21% sequence identity. There is 27 and 40% identity between the mouse TIMP-3 and consensus sequences for the TIMP-1 and TIMP-2 proteins, respectively. These calculations do not take into account the significance of any gaps in the alignments.

The mTIMP-3 cDNA in pXMT2 was transfected into COS-1 cells and conditioned media, and ECM fractions were analyzed for metalloproteinase inhibitor activity by reverse zymography. Fig. 3 shows that the ECM fraction contained a prominent activity at 24 kDa that was increased approximately 3-fold in the sample prepared from cells transfected with the mTIMP-3 expression plasmid; there was no corresponding augmentation of activity in conditioned media from the same transfection, suggesting that the expressed protein preferentially localizes to the ECM. In contrast, transfection with either human TIMP-1 or mouse TIMP-2 expression vectors led to increased activities at the expected sizes of 28 and 21 kDa, but only in samples of conditioned media. The background 24-kDa inhibitor activity seen in all of the ECM samples probably represents endogenous COS-1 cell TIMP-3.

The expression of mTIMP-3, in comparison with that of mTIMP-1 and mTIMP-2, in fibroblasts and adult mouse tissues is presented in Figs. 4–8. Northern blot analysis revealed the presence of a single major transcript class at around 4.5 kb; there is apparently size heterogeneity, because the mTIMP-2 transcript (3.5 kb) runs on the same blots as a much tighter band (Figs. 4 and 7). Minor RNAs of lower molecular mass are also present but it is unclear whether these are functional mRNAs or breakdown products of the larger form. No transcripts have been observed in the 0.9–1.0 kb range that are characteristic of other murine TIMPs.

The data shown in Fig. 4 demonstrate that transcription of mTIMP-3 in mouse fibroblasts is highly inducible by stimuli that affect ECM remodeling. Expression of mTIMP-3 was induced in confluent serum-deprived mouse C3H/10T1/2 fibroblasts by exposure to PMA and TGF-β1, and these two agents in combination led to a superinduction of mRNA at early times.

* N. S. Kishnani, P. W. Staskus, T.-T. Yang, F. R. Masiarz, and S. P. Hawkes, submitted for publication.
Mouse TIMP-3: Cloning and Expression Analysis

Fig. 1. Organization and sequence analysis of mouse TIMP-3 (mTIMP-3) cDNA clones. A, restriction mapping and positional alignments of cDNA clones 1, 4, 7.1, 7.2, and 11. Also shown is the region corresponding to the ChIMP-3 cDNA (Pavloff et al., 1992). B, DNA sequence and translation of the ORF of mouse TIMP-3. Arrows show schematically the direction and length of sequencing runs that were used to compile the data, along with the identity of the cDNA clone that was sequenced. C, one-letter amino acid code comparison of the sequences of the putative 3′-untranslated region of mTIMP-3 and the corresponding region of ChIMP-3, commencing in each case at the nucleotide immediately downstream from the stop codon.

(i.e. 2.0-4.5 h) after exposure. This pattern of responsiveness is similar to that displayed by mTIMP-1 and distinct from that of mTIMP-2 which we have previously shown to be expressed in an essentially constitutive fashion in mouse fibroblasts (Leco et al., 1992). Like TIMP-1, the PMA- and cytokine-mediated induction of mTIMP-3 depends upon ongoing protein synthesis (data not shown). Maximal induction of mTIMP-3 mRNAs occurs from 3.0-4.5 h after stimulation, preceding the peak of mTIMP-1 transcript accumulation between 4.5 and 9.0 h. Nuclear run-on assays that were carried out using nuclei isolated from cells exposed to the same treatment combinations (Fig. 5) showed that increased gene transcription activity preceded the inductions seen in Fig. 4. Transcription of mTIMP-3 had more rapid onset kinetics (peaking at 3.0 h and declining by 4.5 h) than that of mTIMP-1, which increased continuously through to 4.5 h after stimulation. The superinduction of both mTIMP-1 and mTIMP-3 by the combination of PMA and TGF-β1 was also clearly demonstrated in the run-on transcription assays.

The data presented above are reflected in an increased mTIMP-3 activity found localized in the ECM of serum-deprived C3H 10T1/2 cells treated with PMA and TGF-β1. Fig. 6, A and B, show reverse zymographic analysis of conditioned media and ECM, respectively, in which mTIMP-3 was detected as an inhibitor with an apparent size of 23-24 kDa, found only in ECM samples. Confirmation that this protein is mTIMP-3 is provided by recognition with antibodies to ChIMP-3 on a Western blot (C). In comparison with the control, TGF-β1 stimulated the production of mTIMP-3, and to a lesser extent, so did the combination of PMA and TGF-β1. Two other inhibitors with apparent sizes of 29 and 30 kDa were also present in the ECM samples. Both were stimulated by TGF-β1. Levels of TIMP-2 found in conditioned media are unaffected by any of the treatments, whereas levels of TIMP-1 are stimulated slightly by TGF-β1 and to a greater extent by PMA and the combination of the two.

Another important distinction between the regulation of mTIMP-1 and mTIMP-3 expression was observed when mouse fibroblasts were treated with the synthetic anti-inflammatory glucocorticoid dexamethasone, either alone or in combination with EGF or PMA (Fig. 7). Dexamethasone inhibited TIMP-1 mRNA induction by both EGF and PMA, as has been observed
FIG. 2. Comparison of the deduced amino acid sequences of mTIMP-3 and other TIMPs. Species of origin for the various TIMPs include: bovine (b), porcine (p), human (h), murine (m), and chicken (c). Boldface letters indicate amino acids conserved among all TIMPs; lowercase letters indicate amino acids conserved between mTIMP-3 and TIMP-1 proteins; underlined letters indicate amino acids conserved between mTIMP-3 and TIMP-2 proteins. The bracket indicates the beginning of the mature proteins.

Mouse TIMP-3: Cloning and Expression Analysis

Previously, for its effects on interleukin-6-induced expression in rat hepatocytes (Roeb et al., 1993). It should be noted that in C3H 10T1/2 fibroblasts used in the present study, EGF does not activate protein kinase C (Edwards and Mahadevan, 1992), causing a more marginal reduction of the PMA-induced level, essentially abolishing EGF-induced TIMP-1 expression while sensitive to steroid inhibition. In sharp contrast to the data for mTIMP-1, mTIMP-3 expression was stimulated by dexamethasone treatment alone and co-administration with either EGF or PMA. The observation that mTIMP-3 expression is induced equally well in males and females, and the repair and regeneration of damaged tissues. The balance between MMPs and TIMPs underlies this control:

**DISCUSSION**

Controlled remodeling of ECMs is an essential aspect of normal animal development, pregnancy-induced changes in females, and the repair and regeneration of damaged tissues.
Mouse TIMP-3: Cloning and Expression Analysis

The mTIMP-3 ORF encodes a protein that has TIMP activity and displays ECM association. Expression vectors containing either human TIMP-1, mouse TIMP-2, or mouse TIMP-3 cDNAs and the parent vector pXMT2 were transfected into COS-1 cells as described under "Materials and Methods." After culture in medium lacking serum, samples of conditioned media and ECM were analyzed by reverse zymography SDS-PAGE (top) and by silver staining after SDS-PAGE (bottom). The TIMP-1 and TIMP-2 constructs directed the expression of proteins of the expected sizes (28 and 22 kDa, respectively) that were detected by staining and MMP inhibitory activity only in conditioned media. In contrast, the TIMP-3 vector gave rise to a 24-kDa inhibitor activity that was present in ECM but not conditioned medium. A minor 27-kDa inhibitor was also detected by reverse zymography.

as examples, tumor cell invasion and metastasis can be blocked by up-regulation of TIMP expression or an exogenous supply of TIMPs (Khokha et al., 1989, 1992), and TIMPs can block mammary gland involution after lactation (Talhouk et al., 1992). The work described here introduces a new member of the mamalian TIMP family, on which we confer the title mouse TIMP-3 because of its high degree of sequence identity with ChIMP-3 (82% at the level of mature protein), its recognition by anti-ChIMP-3 antibodies and its property of ECM association. Furthermore, we show that mTIMP-3 is expressed in a stimulus- and tissue-specific fashion, indicating that it likely plays an important role in regulating tissue architecture and remodeling in particular physiological contexts.

The predicted protein structure of mTIMP-3 shows several interesting features. First, as expected, essential features of other TIMPs are conserved, including the location of 12 Cys residues that form intrachain disulfide bonds that fold the protein into a two-domain structure (Williamson et al., 1990; Murphy et al., 1991) and the presence of a 23-amino acid leader sequence, which presumably is cleaved to produce the mature protein. Second, the mature protein has an expected size of 21,676 Da, which is smaller than the predicted size of ChIMP-3 (21,825 Da; Pavloff et al., 1992). However, on reverse zymograms the mouse protein migrates as a species which is approximately 2000 Da larger than ChIMP-3. Similar observations have been made for human TIMP-3, which co-migrates with its mouse counterpart on reverse zymograms. Despite conservation of a potential site of N-linked glycosylation (NAT) at the C terminus of the protein, we have evidence that this difference in size is not due to N-linked glycosylation, and further characterization of the mammalian proteins will be necessary to explain this observation. Third, the deduced amino acid sequence of mTIMP-3 predicts a very basic protein

Fig. 3. The mTIMP-3 ORF encodes a protein that has TIMP activity and displays ECM association. Expression vectors containing either human TIMP-1, mouse TIMP-2, or mouse TIMP-3 cDNAs and the parent vector pXMT2 were transfected into COS-1 cells as described under "Materials and Methods." After culture in medium lacking serum, samples of conditioned media and ECM were analyzed by reverse zymography SDS-PAGE (top) and by silver staining after SDS-PAGE (bottom). The TIMP-1 and TIMP-2 constructs directed the expression of proteins of the expected sizes (28 and 22 kDa, respectively) that were detected by staining and MMP inhibitory activity only in conditioned media. In contrast, the TIMP-3 vector gave rise to a 24-kDa inhibitor activity that was present in ECM but not conditioned medium. A minor 27-kDa inhibitor was also detected by reverse zymography.

Fig. 4. Accumulation of mTIMP-3 transcripts is induced by PMA and TGF-β1 in mouse fibroblasts. Northern blot analysis of TIMP gene expression in total cellular RNA isolated from confluent serum-deprived mouse C3H 10T1/2 fibroblasts and cells treated for the indicated times with PMA (10⁻⁸ M), TGF-β1 (2 ng/ml, 8 x 10⁻¹ⁱ M), or combinations of the two agents. Blots were hybridized sequentially with nick-translated cDNA probes corresponding to mTIMP-1, mTIMP-3, mTIMP-2, and last, mouse 18S rRNA. Note that there is some residual signal from mTIMP-3 on the mTIMP-2 panel shown, which appears just above the 3.5-kb mTIMP-2 transcript. Mouse TIMP-3 transcripts accumulate in response to PMA and TGF-β1 and are superinduced by these two agents in combination. This characteristic is similar to mTIMP-1, although mRNA levels and transcription rates peak earlier for mTIMP-3 than for mTIMP-1. In contrast, mTIMP-2 expression shows only slight increases in stimulated cells.

3 S. P. Hawkes, P. W. Staskus, and N. S. Kishnani, unpublished data.
4 S. P. Hawkes and P. W. Staskus, unpublished data.
with a pI of 9.16. Furthermore, like ChIMP-3 it is tyrosine-rich, with 19/188 residues compared with 6/181 for mTIMP-1 and 7/194 for mTIMP-2. However, only the positions of Tyr^{62} and Tyr^{139} are precisely conserved in all other TIMPs. We do not yet know the significance of the high pI and Tyr content: one possibility is that they are related to the unique ECM localization of mTIMP-3 and ChIMP-3. Fourth, two very interesting discrepancies between mTIMP-3 and all other TIMPs, including ChIMP-3, are the presence of a His at residue 83 instead of a Tyr and a Tyr at residue 137 replacing either His or Lys in every other TIMP. These residues are located in loops 1 and 3 of the large N-terminal domain that has been demonstrated to possess MMP inhibitory activity (Murphy et al., 1991). Their exact interconversion in mTIMP-3 is suggestive of some functional relevance, perhaps implying that in the three-dimensional structure of the protein they are in close proximity and are required for interaction with the active site of MPMs. Finally, it should be noted that with the exception of two triplet motifs containing Trp residues (WLG and WSL), the leader sequences of mTIMP-3 and ChIMP-3 show poor sequence conservation. A possible role for the leader sequence in targeting a secreted protein to the ECM is suggested by the work of Rathjen et al. (1990) who demonstrated that a matrix-associated form of leukemia inhibitory factor arose from alternate exon usage that generated a MRCRIV motif at the N terminus of the protein. We see no similar structure in either mTIMP-3 or ChIMP-3, indicating that if the propeptide sequences are important for correct trafficking of these proteins an alternative mechanism must apply.

We were surprised to find a high degree of sequence match in the 5'-untranslated regions of mTIMP-3 and ChIMP-3. Although it is possible that the identity of 47 out of 48 bp positioned close to the end of translation of both proteins is fortuitous, we think it more likely that this reflects a regulatory function. It is possible that this sequence motif may play a role in stabilization of mTIMP-3 transcripts, because in PMA-simulated C3H 10T1/2 fibroblasts the t_{1/2} of mTIMP-3 RNA is approximately 9 versus 6 h for mTIMP-1. This is despite the presence in the mTIMP-3 5'-untranslated region of at least three AUUUA elements that have been linked to the rapid turnover of immediate-early growth factor-responsive genes such as c-fos (Shaw and Kamen, 1986; You et al., 1992). Alternatively, they may reflect an additional level of control over mRNA translation, such as that provided by the iron-responsive element in the 5'-region of ferritin mRNAs (Rouault et al., 1988) or a translation control element in the 3'-noncoding sequences of 81 interferon mRNAs (Grafi et al., 1993). Further experimentation is necessary to define the function of this interesting motif.

We observed dramatic changes in the transcriptional activity of mTIMP-3 in response to cytokines, tumor promoters, and anti-inflammatory agents. In cultured mouse fibroblasts, PMA, EGF, TGF-β1, and dexamethasone induced mTIMP-3 mRNA accumulation; for TGF-β1 and PMA we demonstrated corresponding increases in gene transcription by nuclear run-on assays. Moreover, TGF-β1 and dexamethasone augmented and prolonged induction when added in combination with EGF or PMA, as compared with the effects of the latter two agents used on their own. The response to TGF-β1 mirrored that of mTIMP-1, although with faster kinetics: mTIMP-3 transcripts peak between 3 and 4.5 h after stimulation, compared with a 4.5-9-h maximum for mTIMP-1.5 As observed previously, mTIMP-2 expression is only modestly responsive to exogenous stimuli (Stetler-Stevenson et al., 1990; Leco et al., 1992). Dexamethasone treatment elicited strikingly different effects on the expression of mTIMP-1 and mTIMP-3, with EGF- and PMA-induced mTIMP-1 expression being inhibited by dexamethasone, although to different extents.

\[ D. \text{R. Edwards, unpublished data.}\]
Dexamethasone exerts opposite effects on mTIMP-1 and mTIMP-3 expression. Northern blot analysis of RNA isolated from unstimulated C3H 10T1/2 mouse fibroblasts and cells exposed for either 3 or 6 h to dexamethasone (Dex, 10^{-6} M), EGF (50 ng/ml), PMA (10^{-7} M), or combinations of EGF plus dexamethasone or PMA plus dexamethasone. Replicate blots were hybridized with the nick-transcribed mTIMP-1, mTIMP-2, mTIMP-3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes. Dexamethasone alone induced mTIMP-3 expression and further stimulated the induction seen with EGF and PMA. In contrast, dexamethasone inhibited EGF- and PMA-induced mTIMP-1 expression, although to different extents.

We conclude from our in vitro expression data that there are probably some cis-acting transcription regulatory elements that are common to the mTIMP-1 and mTIMP-3 genes. A binding site for transcription factor AP1 within the promoter region of mTIMP-1 has been implicated in activation of its expression by serum growth factors and PMA (Campbell et al., 1991; Edwards et al., 1992; Ponton et al., 1992), and a recent mutational analysis that we have carried out indicates that this site is necessary for basal and inducible expression. We predict that the mTIMP-3 gene will also contain an essential promoter-proximal AP1 site. However, it is clear that mTIMP-3 must also possess regulatory elements distinct from the mTIMP-1 machinery because of (i) the differential effects of EGF and PMA on induction and (ii) the opposing effects of dexamethasone on expression of the two genes.

Glucocorticoids inhibit AP1-mediated activation of many genes, including the MMPs collagenase and stromelysin-1 (Shapiro et al., 1991), potentially via direct interaction of the glucocorticoid receptor with AP1 in the absence of DNA binding (Jonat et al., 1990; Yang-Yen et al., 1990; Korppola et al., 1993). Other genes such as ovalbumin and prolactin have composite AP1 nuclear hormone receptor response elements leading to more complex cross-talk between steroid- and cytokine-mediated signaling pathways (Diamond et al., 1990; Gaub et al., 1990). We predict, therefore, that the mTIMP-3 gene should contain a functional glucocorticoid response element present either as a composite AP1-glucocorticoid response element motif or as an autonomous binding site that interacts with other elements involved in inducible expression. Our data demonstrating suppression of mTIMP-1 expression by dexamethasone agree with previous studies of human alveolar macrophages (Shapiro et al., 1991) and rat hepatocytes (Roeb et al., 1993). However the induction of mTIMP-3 by dexamethasone adds an important new aspect to the anti-inflammatory action of glucocorticoids that may be quantitatively as significant as the suppression of MMP production.

The unique attributes of the mTIMP-3 gene control apparatus are further revealed in the differential expression of TIMPs in adult mouse tissues. Although some tissues such as lung, ovary, and uterus have appreciable levels of RNAs for both mTIMP-1 and mTIMP-3, in others expression was essentially mutually exclusive. This is the case in bone, a major site of mTIMP-1 expression, where we noted that mTIMP-3 transcripts were rare. The reverse is the case for kidney and brain, which express only TIMP-3 at appreciable levels. We do not yet understand the significance of these observations, which should become clearer when the results of detailed comparative in situ hybridization studies are available. Moreover, to understand the functional significance of tissue-specific expression we have to know what activities of the TIMPs are important in a particular context: are they present solely as MMP inhibitors, or are their growth promoting or ECM interaction properties of paramount importance?

In summary, we have isolated a novel murine TIMP gene, designated mTIMP-3, whose expression is tissue-specific and regulated in vitro by an array of stimuli that influence ECM remodeling, including a tumor promoter, cytokines and an anti-inflammatory agent. The mTIMP-3 protein is predominantly matrix-associated, distinguishing it from TIMP-1 and TIMP-2.

These observations indicate that mTIMP-3 has a specialized role and that it is probably not interchangeable with other members of the family.

Acknowledgments—We thank Narendra Kishnani (University of California San Francisco) for probing the Western blot, Leslie Taylor (University of California San Francisco computer graphics lab) for help with the computer analysis, and Pamela Leco for assistance with cell culture.

REFERENCES

Alexander, C. M., and Werb, Z. (1991) in Cell Biology of Extracellular Matrix (Hay, E. D., ed) pp. 255-302, Second Ed., Plenum Press, New York
Bertaux, B., Honebeck, W., Eisen, A. Z., and Dubertret, L. (1991) J. Invest. Derma tol. 97, 679-85
Birkedal-Hansen, H., Moore, W. G. I., Bodden, N. K., Windsor, L. J., Birkedal- Hansen, B., Decarlo, A., and Ingler, A. (1993) Crit. Rev. Oral Biol. Med. 4, 197-250
Blenis, J., and Hawkes, S. P. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 770-774
Blenis, J., and Hawkes, S. P. (1984) J. Biol. Chem. 259, 11563-11570
Boone, T. C., Johnson, M. J., De Clerck, Y. A., and Langley, K. E. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2890-2894
Campbell, C. E., Flenniken, A. M., Skup, D., and Williams, B. R. G. (1991) J. Biol. Chem. 266, 7199-7206

* D. R. Edwards, unpublished data.
