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Langton, K.P., Barker, M.D. and McKie, N. (1998) Localization of the Functional Domains of Human Tissue Inhibitor of Metalloproteinases-3 and the Effects of a Sorsby's Fundus Dystrophy Mutation. *Journal of Biological Chemistry*, 273 (27). pp. 16778-16781. ISSN 0021-9258

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# Localization of the Functional Domains of Human Tissue Inhibitor of Metalloproteinases-3 and the Effects of a Sorsby's Fundus Dystrophy Mutation\*

(Received for publication, March 9, 1998)

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A transient COS-7 cell expression system was used to investigate the functional domain arrangement of tissue inhibitor of metalloproteinases-3 (TIMP-3), specifically to assess the contribution of the amino- and carboxyl-terminal domains of the molecule to its matrix metalloproteinase (MMP) inhibitory and extracellular matrix (ECM) binding properties. Wild type TIMP-3 was entirely localized to the ECM in both its glycosylated (27 kDa) and unglycosylated (24 kDa) forms. A COOH-terminally truncated TIMP-3 molecule was found to be a non-ECM bound MMP inhibitor, whereas a chimeric TIMP molecule, consisting of the NH<sub>2</sub>-terminal domain of TIMP-2 fused to the COOH-terminal domain of TIMP-3, displayed ECM binding, albeit with a lower affinity than the wild type TIMP-3 molecule. Thus the functional domain arrangement of TIMP-3 is analogous to that seen in TIMP-1 and -2, namely that the NH<sub>2</sub>-terminal domain is responsible for MMP inhibition whereas the COOH-terminal domain is most important in mediating the specific functions of the molecule. A mutant TIMP-3 in which serine 181 was changed to a cysteine, found in Sorsby's fundus dystrophy, a hereditary macular degenerative disease, was also expressed in COS-7 cells. This gave rise to an additional 48-kDa species (possibly a TIMP-3 dimer) that retained its ability to inhibit MMPs and localize to the ECM. These data favor the hypothesis that the TIMP-3 mutations seen in Sorsby's fundus dystrophy contribute to disease progression by accumulation of mutant protein rather than by the loss of functional TIMP-3.

four members (TIMP-1–TIMP-4) (1–4). The balance between MMPs and TIMPs regulates the integrity of the proteinaceous extracellular matrix (ECM) and thus plays a key role in a wide range of physiological processes that include embryonic development, connective tissue remodeling, wound healing, glandular morphogenesis, and angiogenesis. An imbalance in MMP/TIMP expression has been implicated in various diseases such as erosive joint disease, cardiovascular disease, and cancer (reviewed in Refs. 5–7).

The TIMPs form high affinity 1:1 complexes with the active forms of most MMPs (reviewed in Ref. 8) but show varying specificity for different pro-MMPs allowing TIMPs to control the activation of specific MMPs (9–12). Activities have also been ascribed to the TIMPs that are independent of their ability to inhibit MMPs; for example, anti-angiogenic and erythroid-potentiating activities have been described for TIMP-1 that are independent of MMP inhibition (13, 14). Likewise TIMP-2 shows MMP independent inhibition of endothelial tube formation (15). These differences in TIMP specificities, together with the finding that TIMPs are regulated differently at the transcriptional level (reviewed in Refs. 16 and 17), have led to the hypothesis that the individual family members have specific physiological roles rather than being interchangeable protease inhibitors.

The third member of the TIMP gene family, TIMP-3, is unique because it is associated with the insoluble ECM (18). TIMP-3 was originally identified as a molecule up-regulated in viral transformation of chicken fibroblasts, a process in which it acted as a growth factor in low serum conditions and tempered the attachment of cells (19). Transcription of TIMP-3 has been shown to be regulated by a wide variety of cytokines and growth factors (18) and to be up-regulated during early G<sub>1</sub> phase of cell cycle progression (20). Limited characterization of human TIMP-3 has shown it to be an effective MMP inhibitor in line with the other family members and to be produced in both glycosylated and unglycosylated forms (21). Like TIMP-1 and -2, TIMP-3 has been shown to have anti-angiogenic activity (22). It has been reported that TIMP-1 and -2 consist of 2 functional domains, their NH<sub>2</sub>-terminal domains being responsible for MMP inhibition and their COOH-terminal domains performing their specific functions (23, 24). Structural studies on these molecules have shown that their NH<sub>2</sub>-terminal domains have very similar tertiary structures, both being members of the oligonucleotide/oligosaccharide binding fold family (25, 26). Amino acid sequence comparisons suggest that TIMP-3 will have a similar functional domain arrangement (27) and a similar tertiary structure to TIMP-1 and -2, enforced by six disulfide bonds that have been shown to form in TIMP-1 (26, 28) and are assumed to form in the other TIMP family members.

The matrix metalloproteinases (MMPs)<sup>1</sup> are a family of zinc-dependent endopeptidases that exist in both secreted and membrane bound forms. The enzymes are initially expressed as inactive pro-enzymes becoming activated by proteolytic cleavage of their amino termini. The activity of MMPs is tightly regulated by the tissue inhibitors of metalloproteinases (TIMPs), a family of secreted proteins currently comprising

\* This work was supported by the Arthritis and Rheumatism Council, the British Heart Foundation, and Yorkshire Cancer Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: MMP, matrix metalloproteinase; AMD, age-related macular degeneration; ECM, extracellular matrix; SFD, Sorsby's fundus dystrophy; TIMP, tissue inhibitor of metalloproteinases; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

Mutations to TIMP-3, all of which introduce an extra cysteine residue into exon 5 (which forms part of the COOH-terminal domain of the molecule), have been linked to the macular degenerative disease Sorsby's fundus dystrophy (SFD) (17, 29) in which patients typically lose central vision in middle age (fourth or fifth decade) (30). SFD is an autosomal dominant disorder characterized by abnormal deposits and thickening in Bruch's membrane, atrophy of the choriocapillaris, retinal pigment epithelium and photoreceptors, and subretinal neovascularization. The effects of the SFD mutations on TIMP-3 function are unknown. The aim of this study was to determine whether TIMP-3 exhibits a similar functional domain structure to that of TIMP-1 and -2 and to begin to investigate the effects of SFD mutations on TIMP-3 function

#### EXPERIMENTAL PROCEDURES

**Expression Plasmid Construction**—Synthetic oligonucleotide primers were used to create expression constructs by standard polymerase chain reaction-based techniques, in each case 5' *Eco*RI and 3' *Sal*I sites were introduced to allow cloning into the pCi.neo expression vector (Promega UK). All polymerase chain reaction reactions were performed using *Pwo* DNA polymerase (Boehringer Mannheim UK). Wild type TIMP-3, including the proregion, was amplified from vector pCDNATIMP-3 (a kind gift from Dr. M. Wick, University of Marburg, Germany) by polymerase chain reaction. TIMP-3 in which Asn<sup>184</sup> was changed to Glu (N184Q) or Ser<sup>181</sup> was changed to Cys (S181C) were created by introducing the mutations into the 3' primer. Truncated TIMP-2 (full-length TIMP-2 clone was a kind gift from Dr. D. Rowan, University of Newcastle upon Tyne, UK), and TIMP-3 constructs ( $\Delta$ TIMP-2 and  $\Delta$ TIMP-3) were amplified using 3' primers that converted residues Cys<sup>127</sup> and Cys<sup>122</sup>, respectively, into stop codons. The TIMP-2-TIMP-3 chimera was created by overlap extension polymerase chain reaction (31), such that the coding sequence for the proregion of TIMP-2 and residues 1–126 of the mature protein were fused to residues 121–189 of TIMP-3. All constructs were cloned into pCi.neo expression vector by digestion with *Eco*RI and *Sal*I restriction endonucleases (Boehringer Mannheim UK) and ligation with T4 DNA ligase (Promega UK). Plasmids for transfection were prepared with plasmid midi or maxi kits (QIAGEN Ltd, UK). DNA sequences were confirmed by sequencing on an Applied Biosystems automated DNA sequencer.

**COS-7 Cell Transfection**—COS-7 cells were routinely cultured in Dulbecco's modified Eagle's medium + 10% fetal calf serum in a 37 °C, 5% CO<sub>2</sub> humidified incubator. Transfections were performed by DEAE-dextran/dimethyl sulfoxide shock (32). 48 h after transfection the cells were transferred to serum-free Dulbecco's modified Eagle's medium and allowed to grow for a further 48 h. Cells, conditioned media and ECM were then harvested according to Ref. 18. The majority of the conditioned medium was then concentrated approximately 15-fold. The protein concentrations of the fractions were then determined using BCA Protein Assay Reagent (Pierce & Warriner UK Ltd.), and equal protein amounts were analyzed by protease/substrate SDS-polyacrylamide gel electrophoresis.

**Protease/Substrate SDS-Polyacrylamide Gel Electrophoresis (Reverse Zymography)**—Samples were analyzed on 12 or 15% acrylamide protease/substrate gels according to Ref. 33, except that phorbol myristate acetate stimulated U937 cell-conditioned medium at a final concentration of 10% (v/v) was used as a source of gelatinase activity (34), and gelatin was incorporated into the gel at a final concentration of 2.5 mg/ml. Following staining with Coomassie Brilliant Blue (Sigma Chemical Co., UK), inhibitory species were visualized as dark blue bands against a clear background.

**Immunoblotting**—Reduced or nonreduced (heated for 10 min at 95 °C with or without 2% 2-mercaptoethanol, respectively) samples were separated on 10% SDS-polyacrylamide gel electrophoresis gels with biotinylated molecular mass markers (Amersham International plc UK) prior to blotting onto a polyvinylidene difluoride membrane (Pierce & Warriner (UK) Ltd.) in 10 mM CAPS + 10% methanol (pH 11.0) at 60 mA overnight. Membranes were blocked by incubation with Tris-buffered saline, pH 7.5 + 3% bovine serum albumin + 0.2% Tween 20 for 2 h. Rabbit anti-TIMP-3 antibody (Insight Biotechnology Ltd UK) was diluted (1:500) in Tris-buffered saline + 3% bovine serum albumin + 0.05% Tween 20 (antibody dilution buffer) and incubated with the blots for 2 h. Membranes were washed extensively in Tris-buffered saline and Tris-buffered saline + 0.1% Tween 20, prior to incubation with horseradish peroxidase-labeled secondary antibody (Sigma UK) (1:10,000

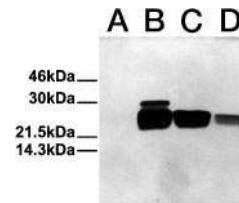


FIG. 1. Reverse zymogram of ECM from transfected COS-7 cells. Lane A, mock transfected; lane B, wild type TIMP-3 transfected; lane C, N184Q-TIMP-3 transfected; lane D, TIMP-2-TIMP-3 chimera transfected. Positions of molecular mass markers are indicated on the left.

and streptavidin-horseradish peroxidase conjugate (1:1, 500) (Amersham International plc UK) in antibody dilution buffer for 30 min. Membranes were then washed as before and developed with ECL reagent (Amersham International plc UK).

#### RESULTS

**Mock Transfected COS-7 Cells**—No inhibitory species were detected in reverse zymograms of ECM or unconcentrated conditioned medium from the pCi.neo mock transfected COS-7 cells (Fig. 1, lane A, and Fig. 2, lane A). However 15-fold concentration of the conditioned medium revealed the presence of three inhibitors (Fig. 3, lane A): two major bands at approximately 28 and 22 kDa (most likely TIMP-1 and TIMP-2, respectively) and a minor, approximately 24-kDa species (possibly partially degraded or deglycosylated TIMP-1 or trace amounts of TIMP-3). No cross-reactivity was observed between these fractions and the anti-TIMP-3 antibody in Western blotting, whereas the anti-TIMP-3 antibody cross-reacted with an approximately 22-kDa protein in the 15-fold concentrated conditioned medium (data not shown).

**Wild Type TIMP-3 Transfected COS-7 Cells**—Reverse zymograms of ECM from cells transfected with plasmid pCITIMP-3 revealed the presence of two inhibitory species: a major one at approximately 24 kDa and a minor one at approximately 27 kDa (Fig. 1, lane B). These correspond to the previously reported molecular masses of unglycosylated and glycosylated TIMP-3, respectively (21), and are in agreement with the results of mouse TIMP-3 expression in COS-1 cells (18). The same cells grown in the presence of 5  $\mu$ g/ml tunicamycin expressed only the 24-Da band (data not shown), indicating that the 27-kDa band is indeed an *N*-glycosylated variant of TIMP-3. Western blotting with anti-TIMP-3 antibody only identified a 24-kDa protein in the ECM from these cells (data not shown), and this may reflect a reduced affinity of this antibody for the glycosylated form of TIMP-3. The conditioned medium from these cells displayed the same pattern and levels of inhibitors as the mock transfected cells. Thus recombinant human TIMP-3 produced from COS-7 cells is localized solely to the ECM.

**N184Q-TIMP-3 Transfected COS-7 Cells**—TIMP-3 has been shown to be *N*-glycosylated (21). To determine whether glycosylation affects ECM localization, COS-7 cells were transfected with a TIMP-3 mutant in which the only potential *N*-glycosylation site, Asn<sup>184</sup>, was changed to a Gln. These transfected cells produced a single strong inhibitory species of approximately 24 kDa in their ECM (Fig. 1, lane C), corresponding to the molecular mass of unglycosylated TIMP-3. This was confirmed by Western blotting with anti-TIMP-3 antibody. No additional inhibitory species were detected in conditioned medium from these cells.

**$\Delta$ TIMP-3 Transfected COS-7 Cells**—No additional inhibitory species (relative to mock transfected cells) could be detected in the ECM or unconcentrated conditioned medium of COS-7 cells transfected with  $\Delta$ TIMP-3, which lacks the COOH-terminal domain (data not shown). 15-fold concentration of the condi-

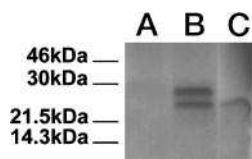


FIG. 2. Reverse zymogram of conditioned media from transfected COS-7 cells. Lane A, mock transfected; lane B, TIMP-2-TIMP-3 chimera transfected; lane C, TIMP-2-TIMP-3 chimera transfected and tunicamycin-treated. Positions of molecular mass markers are indicated on the left.

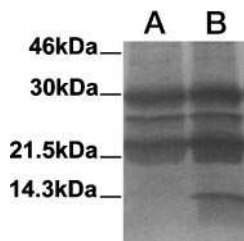


FIG. 3. Reverse zymogram of concentrated conditioned media from transfected COS-7 cells. Lane A, mock transfected; lane B,  $\Delta$ TIMP-3 transfected. Positions of molecular mass markers are indicated on the left.

tioned medium, however, revealed the presence of a unique low molecular mass inhibitory species of approximately 13 kDa (Fig. 3, lane B), correlating well with the predicted size of  $\Delta$ TIMP-3 of 13.9 kDa. The amino-terminal domain of TIMP-3 is therefore a soluble molecule and is sufficient for MMP inhibition.

**TIMP-2-TIMP-3 Chimera Transfected COS-7 Cells**—This molecule was constructed to determine whether the COOH-terminal domain of TIMP-3 is sufficient for ECM localization. The unconcentrated conditioned medium from these cells contained two strongly stained inhibitory species with approximate molecular masses of 24 and 27 kDa (Fig. 2, lane B). These correspond well to the predicted molecular masses of the unglycosylated and glycosylated chimeras, assuming that the glycosylation of the chimera is analogous to that of wild type TIMP-3 (no *N*-glycosylation sites are present in the amino-terminal domain of TIMP-2). Culturing the same cells with 5  $\mu$ g/ml tunicamycin led to the loss of the higher molecular mass band (Fig. 2, lane C), confirming that this molecule is an *N*-glycosylated MMP inhibitor. The ECM produced by these cells contained increased levels of an approximately 24-kDa inhibitory species, but the 27-kDa band was absent (Fig. 1, lane D). Cells transfected with  $\Delta$ TIMP-2, a TIMP-2 mutant truncated at Glu<sup>126</sup> but lacking the TIMP-3 COOH-terminal domain, produced no additional inhibitory species in their ECM (relative to mock transfected cells) but had an additional inhibitory species of approximately 15 kDa in their conditioned medium (data not shown), corresponding closely to the calculated molecular mass for  $\Delta$ TIMP-2 of 14.1 kDa.

**S181C-TIMP-3 Transfected Cells (SFD Mutation)**—Unconcentrated and concentrated conditioned media from COS-7 cells transfected with the SFD mutant TIMP-3 in which Ser<sup>181</sup> is changed to Cys contained no additional inhibitory species relative to the mock transfected cells (data not shown). However, analysis of the ECM from these cells revealed the presence of small amounts of an inhibitory species at approximately 24 kDa and also a novel inhibitory species at approximately 48 kDa (Fig. 4). These molecular masses correspond to those of unglycosylated TIMP-3 monomer and dimer, respectively. Western blotting with the anti-TIMP-3 antibody confirmed that both bands contained TIMP-3 (Fig. 5).

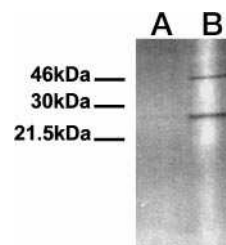


FIG. 4. Reverse zymogram of ECM from S181C-TIMP-3 transfected COS-7 cells. Lane A, mock transfected; lane B, S181C-TIMP-3 transfected. Positions of molecular mass markers are indicated on the left.

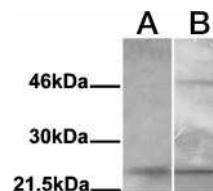


FIG. 5. Western blots of ECM from S181C-TIMP-3 transfected COS-7 cells probed with anti-TIMP-3 antibody. Lane A, reduced; lane B, nonreduced. Positions of molecular mass markers are indicated on the left.

#### DISCUSSION

The data presented in this report confirm that TIMP-3 shows an analogous functional domain structure to TIMP-1 and -2 in which the NH<sub>2</sub>-terminal domain is responsible for MMP inhibition and the COOH terminus confers the specific biochemical properties of the molecule. Specifically we have shown that the three NH<sub>2</sub>-terminal domains (comprising residues 1–121) are sufficient for MMP inhibition, whereas the three COOH-terminal domains (comprising residues 121–189) confer most of the unique ability of the molecule to localize to the ECM. Wild type TIMP-3 was solely localized to the ECM and was undetectable in the conditioned medium, whereas the reverse was true for  $\Delta$ TIMP-3, lacking the three COOH-terminal domains. However, the TIMP-2-TIMP-3 chimera was found in both the ECM and the conditioned medium. Replacement of the NH<sub>2</sub>-terminal domains of TIMP-3 with those of TIMP-2 may partially inhibit the ability of the COOH-terminal domain to bind to the ECM in the normal way, or this may be indicative of a contribution of the amino terminus of TIMP-3 to ECM localization. The latter explanation is supported by the hypothesis that TIMP-3 binds to the ECM component hyaluronate via regions of basic amino acids (19), because both domains of TIMP-3 contain large numbers of positive charges that could contribute to such an interaction. Interestingly although both unglycosylated and glycosylated forms of wild type TIMP-3 were only detectable in the ECM, the glycosylated form of the TIMP-2-TIMP-3 chimera was only seen in the conditioned medium, whereas the non-glycosylated form was detected in both ECM and conditioned medium. This indicates that the decreased affinity of the chimera for the ECM is further reduced by glycosylation. This would support the idea that basicity contributes to the ECM localization of TIMP-3 as glycosylation might be expected to counter such an electrostatic interaction. Tunicamycin treatment of wild type TIMP-3 together with mutation of the potential *N*-glycosylation site, Asn<sup>184</sup>, confirm that glycosylation does occur at this residue but that this is not essential for either MMP inhibition or ECM localization.

The finding that mutations in the TIMP-3 gene are linked to the hereditary macular degenerative disease SFD (29) implied that TIMP-3 could play a unique role in the eye. The symptoms of SFD could either be because of insufficiency of normal TIMP-3 or a negative effect of the mutant molecule itself. By

expressing the S181C mutant form of TIMP-3 (the most frequently observed SFD mutation) in COS-7 cells, we hoped to begin to address this problem. Our data show for the first time that although expressed at lower concentrations than wild type TIMP-3, the SFD mutant is functional with respect to both ECM localization and MMP inhibition. Additionally the mutation gives rise to a novel molecule approximately twice the size of the wild type TIMP-3, which is lost upon reduction with 2-mercaptoethanol. The most likely explanation for this is that the free cysteine residue present in this mutant (and also in all other known SFD mutations) causes TIMP-3 to dimerize or associate with another molecule of identical size.

These data tend to support the hypothesis that the SFD phenotype is caused by the mutant TIMP-3 molecule rather than haploinsufficiency. The haploinsufficiency model requires that expression from the normal allele (as SFD is a dominant disease) is incapable of compensating for the mutated one. The facts that TIMP-3 has been shown to inhibit angiogenesis and that neovascularization is one of the symptoms of the disease have been used to argue in favor of this model (22). However, subretinal neovascularization is not always observed in SFD (35). Additionally it has been shown that night blindness in SFD is significantly responsive to treatment with high doses of vitamin A, suggesting that the disease may be at least partially a result of nutrient starvation of the retinal pigment epithelium (36). Such starvation may well be caused by thickening of Bruch's membrane or atrophy of the choriocapillaris, both of which are observed in SFD and both of which may give rise to neovascularization as a secondary event.

Although SFD is very rare, its clinical and histopathological features are almost identical to those of the most severe or "wet" form of age-related macular degeneration (AMD). AMD has a later age of onset than SFD and is the most common cause of blindness in the elderly of developed countries. Although recent work (37) has shown that some of the milder or "dry" forms of AMD are associated with mutations in the *ATP-binding cassette transporter retina* (ABCR) gene, other gene defects have yet to be identified. Interestingly, although AMD is not associated with heritable mutations in TIMP-3, all drusen (lipid-rich deposits) associated with Bruch's membrane in AMD eyes stain strongly with anti-TIMP-3 antibodies (17). If the two diseases are indeed closely related this would tend to suggest that it is an excess rather than insufficiency of TIMP-3 that gives rise to the SFD phenotype. One possibility is that the high molecular mass TIMP-3 complex we have observed is less prone to degradation than the wild type molecule and so gradually accumulates in the eye. Because the molecule remains functional this would imply that it is inhibition of ECM remodeling that gives rise to the SFD phenotype. It is tempting to speculate that similar TIMP-3 complexes may give rise to the symptoms of AMD. This could result from abnormal disulfide bond formation or somatic mutation giving rise to a more gradual TIMP-3 accumulation than in SFD. Interestingly a high molecular mass TIMP-3 immunoreactive complex has been previously reported in tissue taken from a 69-year-old human eye (38). TIMP-3 accumulation could therefore be a common phenomenon in old age that is exacerbated in SFD and AMD.

*Acknowledgments*—We are grateful for the encouragement of Prof. R. G. Russell and to the Krebs Institute Biomolecular Synthesis Service for providing oligonucleotides and automated DNA sequencing.

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