Identification of the Extracellular Matrix (ECM) Binding Motifs of Tissue Inhibitor of Metalloproteinases (TIMP)-3 and Effective Transfer to TIMP-1*

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Tissue inhibitor of metalloproteinases (TIMPs) are the endogenous inhibitors of the zinc-dependent endopeptidases of the matrix metalloproteinase families. There are four mammalian TIMPs (TIMP-1 to -4) but only TIMP-3 is sequestered to the extracellular matrix (ECM). The molecular basis for the TIMP-3:ECM association has never been fully investigated until now. In this report, we identify the unique amino acid configuration that constitutes the basis of the ECM binding motif in TIMP-3. By systematically exchanging the subdomains of the TIMPs and exhaustive mutation of TIMP-3, we have identified the surface residues directly responsible for ECM association. Contrary to the accepted view, we have found that TIMP-3 interacts with the ECM via both its N- and C-terminal domains. The amino acids involved in ECM binding are all basic in nature: Lys-26, Lys-27, Lys-30, Lys-76 of the N-terminal domain and Arg-163, Lys-165 of the C-terminal domain. Replacement of these residues with glutamate (E) and glutamine (Q) (K26/27/30/76E + R163/K165Q) resulted in a soluble TIMP-3 devoid of ECM-adhering ability. Using the ECM binding motif derived from TIMP-3, we have also created a TIMP-1 mutant (K26/27/30 + K76 transplant) capable of ECM association. This is the first instance of TIMPs being intentionally rendered soluble or ECM-bound. The ability to prepare TIMPs in soluble or ECM-bound forms also opens new avenues for future TIMP research.

Tissue inhibitor of metalloproteinases (TIMPs) are the endogenous regulators of the zinc-dependent endopeptidases of the MMPs (matrix metalloproteinases, matrixins) and their close associates, the ADAM (a disintegrin and metalloproteinase) and the ADAMTS (ADAM with thrombospondin type II repeats) families. There are four members of mammalian TIMPs (TIMP-1, -2, -3, -4) and each TIMP has its own specific profile of inhibition against the metalloproteinases (MMPs). TIMP-1, for instance, inhibits the majority of the MMPs with the exception of MMP-19 and the membrane-type MT-MMPs (1–3). As well as this, TIMP-1 is also a known inhibitor of ADAM-10 but its activity against ADAM-17 (TNF-α-converting enzyme, TACE) has been shown to be minimal (4, 5). TIMP-3, on the contrary, inhibits all known interstitial and membrane-bound MMPs as well as several key ADAMs and ADAMTSs, the most notable being ADAM-10, ADAM-17, and the aggrecanases ADAMTS-4 and ADAMTS-5 (6). Collectively, TIMPs regulate the enzymatic activities of all MMPs and many of the known ADAM and ADAMTS proteins.

TIMPs are relatively small proteins of ca. 21–28-kDa molecular mass. They exert their inhibitory functions on the MPs by forming 1:1 non-covalent stoichiometric complexes with the catalytic domains of the enzyme targets (7, 8). The four TIMPs share a relatively high degree (40–50%) of sequence homology (9) but to date, only the structures of TIMP-1 and -2 have been resolved by protein crystallography or nuclear magnetic resonance (NMR) (PDB 1UEA and PDB 1BR2) (7, 10). The overall conformation of the two TIMPs is remarkably comparable: both are multidomain proteins with very similar, near-superimposable N- and C-terminal configurations. The N-terminal domain of the TIMPs encompasses the first two-thirds of the polypeptides. Structurally, the domain resembles a five antiparallel β-stranded barrel typical of the oligosaccharide/oligonucleotide binding (OB) moiety. The C-terminal subdomain is made up of the remaining 50–60 amino acids, and its structure is generally less well defined. There are six disulfide bonds in the TIMPs, three within each domain. Although the structures of TIMP-3 and -4 are yet to be determined, mutagenesis and kinetic studies carried out on the two TIMPs suggest an overall similarity in tertiary configuration with those of TIMP-1 and -2 (11, 12).

TIMP-3 is unique, because it is the sole TIMP regulator of one of the most versatile ADAM sheddases, TACE (ADAM-17) (4). Furthermore, TIMP-3 is also renowned for its ability to induce apoptosis in mammalian cells by stabilization of the three death receptors TNF-RI, FAS, and TRAIL-RI (13, 14). Another extraordinary quality that distinguishes TIMP-3 from the others is its ability to adhere to the extracellular matrix (ECM). Whereas TIMP-1, -2, and -4 are all soluble proteins, TIMP-3 is largely sequestered to the ECM in both its glycosylated (27 kDa) and unglycosylated (24 kDa) forms. Indeed,
TIMP-3 was first discovered as a novel ECM-adhering molecule from chicken embryo fibroblasts undergoing early stages of oncogenic transformation (15). To date, there has been no systematic research to investigate whether this ECM-adhering attribute has any bearing upon the biological activities of the inhibitor. We are interested in the development of TIMP-3 to counter TACE-related inflammatory diseases such as rheumatoid arthritis, psoriasis, and Crohn’s disease (16, 17). To succeed in making a therapeutic TIMP of high specificity, it will be essential to understand the molecular rationale that underpins TIMP-3:ECM association to formulate an effective strategy of delivery.

At present, there is no consensus among researchers as to the exact location of the ECM binding motif in TIMP-3. On the one hand, it appears that the C-terminal domain is critical for ECM association since N-TIMP-3 (TIMP-3 devoid of a C-terminal domain) displays no propensity for ECM association while T2:T3 chimera (i.e. N-TIMP-2 fused with C-TIMP-3) is capable of adhering to the ECM, albeit with lesser affinity (18). On the other hand, N-TIMP-3 has also been shown to bind to heparin with affinity similar to that of full-length TIMP-3, suggesting that the binding motif is situated at the N-terminal domain (19). To clarify the confusion, we embarked on a series of site-directed and domain-exchanged mutagenesis leading to the final uncovering of the ECM binding motif. In this report, we examined earlier findings by Langton et al. (18) and showed that both the N and C termini of TIMP-3 were implicated in ECM association. To find out the residues involved, a strategy was devised to split the molecule into two independent entities to simplify the process of identification. This brought about successful delineation of the ECM binding modules on the N- and C-terminal domains and a soluble TIMP-3 devoid of ECM binding ability. The final part of the results demonstrates how our understanding of the motif has been adapted to engineer a TIMP-1 variant equally capable of ECM association.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals and reagents in this study were purchased from Sigma Chemicals unless otherwise stated. Vent and Kod DNA polymerases for mutagenesis were purchased from New England Biolabs and Merck Biosciences, respectively. All restriction enzymes for subcloning are also from New England Biolabs. DNA primers for mutagenesis were synthesized by MWG-Biotech AG. Anti-human TIMP-3 monoclonal antibody, 136-13H4, was a kind gift from Daiichi Chemical Industries Ltd, Takaoka, Japan.

**Construction of TIMP Chimeras and Site-directed Mutagenesis of TIMP-3**—All TIMP-1, -2, -3, and -4 cDNAs used in this study are of human origin. TIMP chimeras (TX:TX) were typically produced in two stages. The first stage of the mutagenesis process involved independent amplification of the N- and the C-terminal domains of the desired TIMPs by PCR before ligation to create an uncorrected TX:TX template in pcDNA 3.1 mammalian expression vector (Invitrogen, Paisley, UK). To make the interdomain ligation possible, an NdeI restriction site was incorporated into the 3'- and 5'-ends of the PCR fragments encoding the two domains. The second stage of the process entailed elimination and replacement of the NdeI site by a pair of primers encoding the correct amino acid sequence. This was achieved by QuikChange® site-directed mutagenesis (Stratagene) following the recommended protocols. Mutants of simpler designs were constructed by direct amplification of the cDNAs with long distance Kod DNA polymerase. All constructs in this study have been sequenced to confirm that no unwanted mutations had been introduced during the mutagenesis process.

**Transient TIMP Transfection**—A total of four adherent cell lines were used in this study: immortalized HeLa cells, MCF-7 human breast adenocarcinoma cells, HT1080 human fibrosarcoma cells, and transformed African green monkey kidney fibroblast COS-7 cells. The procedure for transfection is a slight modification of the protocol recommended in the product leaflet accompanying the FuGene® 6 transfection reagent (Roche Applied Science). As a general rule, the cells were typically seeded to 30–40% confluency the day before transfection in 6-well tissue culture plates in 1.5 ml 10% fetal calf serum-enriched Dulbecco’s modified Eagle’s medium-Glutamax® media (Invitrogen). 1 μg of plasmid DNA was used for transfection throughout this work. Plasmid DNA was added to 3 μl of FuGene premixed with 97 μl serum-free media for 20 min before dropwise addition to the cells. 24 h post-transfection, the media were replaced by fresh DMEM-Glutamax® but with reduced fetal calf serum (2.5%) content. Incubation was allowed for a further 3 days at 37 °C in 5% CO2 incubator before the ECM, media, or cell lysates were harvested for analysis.

**ECM Harvesting**—Confluent cell layers were detached from the culture plates by preincubation in 5 ml of phosphate-buffered saline (PBS) supplemented with 5 mM EDTA and 5 mM EGTA, pH 7.4 at 37 °C for 1 h. Cell layers were lifted off the plates by twice gentle washing of the dishes using PBS with added EGTA/EDTA (5 mM each). The cell pellets were stored at −20 °C until required for Western blot analysis. The plates were rinsed for the final time with PBS and examined under a low power microscope to ensure that no cell debris remained on the dishes. Whole ECM was harvested into 100 μl of non-reducing SDS-PAGE loading buffer (2.4 ml of 1 mM Tris-HCl pH 6.8, 3 ml of 20% SDS, 3 ml of glycerol, and 6 mg of bromphenol blue made up to 10 ml with sterile water) with the help of cell scrapers. Samples were stored at −20 °C until required. We typically used up to 5 μl of ECM samples and 4 μl of conditioned media for reverse zymography analysis.

**Reverse Zymography**—Reverse zymography was performed in 12.5% SDS-PAGE gel containing 0.5 mg/ml gelatin and 0.33 ml/5 ml BHK-conditioned media expressing gelatinase-A enzyme (20). Following electrophoresis, gels were washed twice, 30 min each, first with 2.5% Triton X-100 in water and the second time with 2.5% Triton X-100 in 50 mM Tris-HCl, pH 7.5. Gels were briefly rinsed with water before overnight incubation at 37 °C in 5 mM CaCl2, 50 mM Tris-HCl, pH 7.5. The following day, gels were stained for 30 min in 0.25% Coomassie Blue G-250 followed by destaining in a solution containing 30% methanol and 1% acetic acid. The presence of TIMPs was identified as dark bands on a light background.

**Western Blotting**—Protein contents from the whole cell lysates (from cell pellets dissolved in 200 μl of reducing SDS-PAGE sample buffer; 8 μl used in each lane), ECM (10 μl), and
conditioned media (6 μl) were separated by SDS-PAGE electrophoresis before transfer to nitrocellulose membrane. Following incubation in anti-TIMP-3 antibody (8 μg/ml) for 1 h, the membrane was briefly washed in PBS and probed with sheep anti-mouse secondary antibody (Jackson IR Laboratories) for another hour. TIMP-3 was detected with ECL® Western blotting Reagent (Amersham Biosciences) by 30 s to 1 min exposure to film.

RESULTS

Scanning TIMP-3 for ECM Binding Motif: Unsuccessful Attempts to Create a Soluble TIMP-3—The ECM binding profiles of wild-type human TIMPs (TIMP-1 to -4) using a HeLa cell expression mode are shown in Fig. 1A (left panel). Among the four TIMPs, only TIMP-3 was sequestered to the ECM, whereas TIMP-1, -2, and -4 were all highly water soluble. Two forms of TIMP-3 were detected in the ECM: an unglycosylated form (24 kDa) and a glycosylated form (27 kDa) (Fig. 1A, upper panel, highlighted by arrows). Although both forms of TIMP-3 were ECM-sequestered, there were some recombinant proteins in the conditioned media possibly because of leakage from an oversaturated ECM. At the outset of this study, we repeated the same experiments by Langton et al. (18) but with a C-terminally tagged version of TIMP-3 included. The C-terminal hexa-histidine tag was added because we intend to purify TIMP-3 in the near future and hence the interest in knowing its effects on the ECM binding characteristic of the molecule. The solubility and ECM binding profiles of untagged TIMP-3, histidine-tagged TIMP-3 and N-TIMP-3 in transiently transfected HeLa cells are shown in the right panel of Fig. 1A. Indeed, both wild-type TIMP-3 (24 kDa) and its histidine-tagged variant (25 kDa) were clearly ECM-associated. N-TIMP-3, in contrast, was exclusively water-soluble, and no trace of the protein could be discerned in the ECM.

The conclusion appeared straightforward: the ECM binding motif of TIMP-3 must reside within the C-terminal domain as N-TIMP-3 was totally incapable of adhering to the ECM. To find out if the primary sequence of TIMP-3 contains any recognized heparin binding motifs, we BLAST-searched TIMP-3 with proteins in the Brookhaven Protein Data Bank (PDB) but failed to retrieve any significant hits with known heparin binding sequences (such as the “XBBXBX” and “XBBBXXBX” motifs whereby “B” denotes basic residues and “X” represents other amino acids) (21, 22). Neither can a canonical integrin binding motif (e.g. RGD motif) be detected from homology comparison (Fig. 1B). Undefined exploration, on the other hand, would certainly entail mutations of a very large repertoire of residues with no guarantee of success. Because of the apparent lack of an identifiable motif, we used molecular modeling to find alternative explanations. Fig. 1C is a model of TIMP-3 created on the backbones of TIMP-1 and -2. Eleven lysine and arginine residues are highlighted in the figure, six on the N-terminal domain (Lys-26, Lys-27, Lys-30, Lys-71, Arg-100 and Arg-100) and five on the C-terminal domain (Lys-123, Lys-125, Lys-137, Arg-163, and Lys-165). These residues were singled out because they are all basic, non-conserved, in close proximity to each other and most importantly, their side chains are exposed to the surface in considerable high density, suggesting a possible role in heparin association. With this rationale, we proceeded to try to identify an ECM binding motif located on the C-terminal domain of TIMP-3.

The outcomes of the study are shown in Fig. 2. As explained previously, five surface-exposed basic residues were deemed appropriate for alanine scanning mutagenesis on C-TIMP-3: Lys-123, Lys-125, Lys-137, Arg-163, and Lys-165. Fifteen single, double, triple, and multiple lysine/arginine-to-alanine mutants were created in the study. With the exception of three compounded mutants that were not detectable by reverse zymography (i.e. K123/125/163A, K123/125/163/165A and K123/125/137/163/165A), all the rest were still clearly ECM-sequestered. None of the mutants was exclusively soluble as had been hoped.

If TIMP-3 could not be rendered soluble by such extensive mutations at the C-terminal domain, we next wondered if the goal could be achieved by mutation of the N-terminal domain. Given the strength of the affinity of N-TIMP-3 for heparin in vitro, it is possible that the motif for ECM association is indeed located on the N-terminal domain as suggested by Yu et al. (19). A series of N-terminal mutants were created to examine this possibility. Of the basic residues on N-TIMP-3, six were of particular interest: Lys-26, Lys-27, Lys-30, Lys-71, Lys-76, and Arg-100 (K26, K27, K30, K71, K76, and R100; Fig. 1B, C). Instead of mutating these residues individually and collectively to generate a large pool of single and multiple mutants, a different approach was taken. Centering on Lys-26, we systematically increased the number of residues incorporated in our mutation targets until all six lysines/arginine were included in the final mutant. In line with this new strategy, only six lysine/arginine-to-glutamate (K/R to E) mutants were made, each carrying an extra mutation than its predecessor: (i) K26E (ii) K26/30E (iii) K26/27/71E, K26/27/30/76E (iv) K26/27/30/76E (v) K26/27/30/76E + K71E, and lastly (vi) K26/27/30/76E + R100E. As shown in Fig. 3A, mutants carrying K71E and R100E mutations were noticeably absent in the ECM and conditioned media of HeLa cells transfected with the cDNAs (hence the mutations are highlighted in bold). Subsequent Western blotting on the +K71E mutant with an anti-TIMP-3 antibody confirmed the absence of these mutants because of incorrect post-translational processing (Fig. 3B). Probably as a result of the increased negativity in surface polarity, mutant K26/27/76E and K26/27/30/76E migrated somewhat faster than their wild-type counterpart. Under reducing conditions, the pace of migration was restored to that of the wild-type TIMP-3, as confirmed by the Western blot of the K26/27/30/76E mutant in Fig. 3B. Despite the arguable increased solubility of the triple and quadruple mutants K26/27/76E and K26/27/30/76E, all the TIMP-3s demonstrated the property of ECM association.

Splitting of the TIMPs: Creation of T3:TX and TX:T3 Chimeras—The unsuccessful attempt to produce a soluble TIMP-3 after exhaustive mutations at its N- and C-terminal domains necessitated a re-evaluation of our previous investigation strategy. Using a different approach, we separated the N- and C-domains into two independent entities in the expectation that this would simplify the identification process. TIMP-1, -2, and -4 are all highly water soluble. By fusing the N- and C-terminal domains of TIMP-3 to these soluble TIMPs
Unmasking the ECM Binding Motif of TIMP-3

Despite the fact that the vast majority of the T1:T3 and T2:T3 chimeras were partitioned to the conditioned media, there were still a small proportion of the proteins that were sequestered to the ECM.

Taken together, our data indicated that the ECM binding motif of TIMP-3 was made up of two independent modules, one on each domain of the molecule. Resolving the issue henceforth involved independent delineation of the modules from both termini of the TIMP.

Delineating the ECM Binding Module on the N-terminal Domain of TIMP-3—All T3:TX chimeras and their site-directed mutants could be expressed and secreted and the yields of the recombinant proteins were consistently higher than that of wild-type TIMP-3. Fig. 5 summarizes the ECM binding profiles of T3:T1, T3:T2, and T3:T4 and their various site-directed mutants. The mutations introduced to identify the ECM binding module on N-TIMP-3 were essentially the same as those mentioned in the first section of the “Results”: (i) K26E single mutant (ii) K26/30E double mutant (iii) K26/27/76E triple mutant, and (iv) K26/27/30/76E quadruple mutant. Among the three, T3:T1 was the most vulnerable to mutations. A single substitution (K26E) was sufficient to dissociate most of the recombinant protein from the ECM. Replacement of a further lysine (K26/30E) resulted in a mutant that was entirely soluble. T3:T2 followed, instead of two mutations, three lysine-to-glutamate substitutions (K26/27/76E) were required to render the chimer non-ECM adherent. The chimera most tightly bound to the ECM was T3:T4. Despite four mutations (K26/27/30/76E) at its N-terminal, there was still a minute trace of the recombinant protein sequestered to the ECM (Fig. 5C).

It is of interest to note that the ECM binding affinities of the T3:TX chimeras are not strictly related to the number of C-terminal lysine/arginine residues of the particular TIMP with which N-TIMP-3 is fused. With five lysines and three arginines, C-TIMP-2 boasts the highest number of basic residues (seven in C-TIMP-4; five in C-TIMP-1; Fig. 1B) and yet, the most independently, we could effectively narrow down the ECM binding motif to a specific part of the molecule. To investigate the possible roles of the N-terminal domain, fusion proteins were therefore made between N-TIMP-3 and the C-terminal domains of TIMP-1, -2, and -4. The new series of TIMP chimeras are henceforth named T3:T1, T3:T2, and T3:T4, respectively (or T3:TX in abbreviation). To study the effect of the C-terminal domain, likewise, C-TIMP-3 was made to fuse with the N-terminal domains of TIMP-1, -2, and -4 to create T1:T3, T2:T3, and T4:T3 chimeras (TX:T3 in short). In total, six chimeras were made (three T3:TX and three TX:T3) and transfected into HeLa cells.

As shown in Fig. 4A, all the T3:TX chimeras were partially sequestered to the ECM regardless of the nature of their C-terminal domains. Furthermore, it appears that T3:T1 and T3:T4 chimeras were produced in two different forms of slightly varied molecular mass (highlighted by an asterisk in Fig. 4A). The conclusion from the T3:TX panel was unambiguous: the ECM binding motif must be found on the N-terminal domain of TIMP-3 as all T3:TX were capable of ECM association. However, when the C-TIMP-3 chimeras T1:T3 and T2:T3 were subjected to similar analysis, both chimeras also exhibited clear, though weaker signs of ECM association (highlighted by an asterisk in Fig. 4B).

FIGURE 1. A, left panel, reverse zymography of ECM and conditioned media of HeLa cells transiently transfected with wild-type TIMP-1, -2, -3, and -4. Whereas TIMP-1, -2, and -4 were all highly water soluble, TIMP-3 was ECM-sequestered. The positions of the endogenous TIMP-1 (28 kDa), TIMP-2 (21 kDa), TIMP-3 (24 and 27 kDa, corresponding to unglycosylated and glycosylated forms of the inhibitor), TIMP-4 (22 kDa) in the ECM and conditioned media are labeled for easy recognition. The 24-kDa smeared band in TIMP-1-transfected sample (lane 2) that co-migrated with TIMP-3 is probably an incompletely glycosylated form of TIMP-1. Right panel, wild-type TIMP-3, TIMP-3 with a C-terminal hexahistidine tag and N-TIMP-3 transiently expressed in HeLa cells. TIMP-3 both with and without histidine tag displayed clear signs of ECM association whereas N-TIMP-3 was exclusively soluble. N-TIMP-3 was never found to be associated to the ECM. The presence of TIMP-3 in the conditioned media of cells overexpressing the protein was a common occurrence, possibly because of leakage of the inhibitor from an oversaturated ECM. Apart from the 24 kDa (unglycosylated) and 27 kDa (glycosylated) forms of TIMP-3, there are several minor bands of lower molecular mass (<24 kDa, lanes 2 and 3) that we believe, are either partially degraded products of TIMP-3 or an assortment of TIMP-3s with differently oriented N- and C-terminal subdomains. These minor TIMP-3 bands are also visible in pCDNA-transfected sample (lane 1). Asterisks are used to highlight the recombinant TIMPs in the ECM and the media. Bar, sequence alignment of human TIMP-1, -2, -3, and -4. The eleven amino acids selected for preliminary mutagenesis (N-terminal: K26, K27, K30, K71, K76, R100; C-terminal: K123, K125, K137, R163, and K165) are labeled and highlighted by shadowed boxes. C, model of TIMP-3 depicting the relative positions of the eleven residues. Whereas the N-terminal six residues are arranged in a neat linear band, the C-terminal residues form a concentric circle at the top of the domain.

FIGURE 2. Reverse zymography of TIMP-3 with single, double, triple, and multiple mutations at the C-terminal domain: (A) ECM and (B) conditioned media. Alanine scanning mutagenesis was carried out to screen five lysine and arginine residues (K123, K125, K137, R163, and K165) at the C-terminal domain of TIMP-3. Note the dissimilar levels of expression among the mutants. Compounded mutants such as K123/125/137/163/165A were unyielding to correct processing; the recombinant proteins could not be detected in either the ECM or the conditioned media with reverse zymography. The presence of endogenous TIMP-1 (28 kDa), TIMP-2 (21 kDa), and TIMP-3 (24 kDa) in the conditioned media are also labeled for clarity.

FIGURE 3. Reverse zymography of TIMP-3 with single, double, triple, and multiple mutations at the N-terminal domain. Alanine scanning mutagenesis was carried out to screen five lysine and arginine residues (K123, K125, K137, R163, and K165) at the N-terminal domain of TIMP-3. Note the dissimilar levels of expression among the mutants. Compounded mutants such as K123/125/137/163/165A were unyielding to correct processing; the recombinant proteins could not be detected in either the ECM or the conditioned media with reverse zymography. The presence of endogenous TIMP-1 (28 kDa), TIMP-2 (21 kDa), and TIMP-3 (24 kDa) in the conditioned media are also labeled for clarity.

FIGURE 4A. All the T3:TX chimeras were partially sequestered to the ECM regardless of the nature of their C-terminal domains. Furthermore, it appears that T3:T1 and T3:T4 chimeras were produced in two different forms of slightly varied molecular mass (highlighted by an asterisk in Fig. 4A). The conclusion from the T3:TX panel was unambiguous: the ECM binding motif must be found on the N-terminal domain of TIMP-3 as all T3:TX were capable of ECM association. However, when the C-TIMP-3 chimeras T1:T3 and T2:T3 were subjected to similar analysis, both chimeras also exhibited clear, though weaker signs of ECM association (highlighted by an asterisk in Fig. 4B).
tightly binding of the three is T3:T4. The impact exerted by the C-terminal domain, therefore, is very much dependent on the pattern of distribution as well as the spacing between the individual residues involved.

**Delineating the ECM Binding Module on the C-terminal Domain of TIMP-3**—Because T4:T3 was undetectable by reverse zymography, only the chimeras of T1:T3 and T2:T3 could be used to identify the ECM binding module on C-TIMP-3. Following the same strategy outlined in section 1, the five most conspicuous C-terminal lysines and arginine were targeted for mutation: Lys-123, Lys-125, Lys-137, Arg-163, and Lys-165. Instead of alanine, the residues were mutated to glutamine (Q) as we found, during the course of this study, that TIMP-3-bearing glutamine substitutions (i.e. X-to-Q) were generally more easily expressed than those with alanine substitutions (data not shown). A clear and consistent profile emerged from the two panels of chimeras: while mutants with Lys-123 (K123Q), Lys-125 (K125Q), and Lys-137 (K137Q) mutations retained their ECM binding characteristics, those with Arg-613 (R163Q) and Lys-165 (K165Q) mutations had little, if any, affinity for the ECM. Furthermore, the profiles were identical in both panels of T1:T3 and T2:T3 chimeras (Fig. 6). Thus, the conclusion from this study was that Arg-163 and Lys-165 were essential parts of the ECM binding module on the C-terminal domain of TIMP-3.

**Reunion of the Two Domains: the Creation of a Soluble TIMP-3**—We had so far treated the ECM binding motif as two independent N- and C-terminal modules. Could a soluble TIMP-3 be made by the reunion of the two domains? To this end, we combined the four N-terminal mutations (K26E, K27E, K30E, and K76E, identified in section 3) with the two C-terminal mutations (R163Q and K165Q, identified in section 4) to generate a compounded TIMP-3 mutant: K26/27/30/76E/H11001. The ECM binding profiles of the mutant in HeLa, MCF-7, HT1080, and COS-7 are shown in Fig. 7. Indeed, the mutant was exclusively soluble and no trace of the protein was detected in the ECM. As with previous Lys-to-Glu mutants, the soluble TIMP-3 also migrated slightly faster than its wild-type TIMP-3 counterpart. Transfection and overexpression of the mutant in MCF-7, HT1080, and COS-7 cell lines all yielded recombinant proteins that were soluble and non-ECM-adhering (Fig. 7, B–D).

**From TIMP-3 to TIMP-1: Transplantation of the ECM Binding Motif**—If a soluble TIMP-3 could be engineered by reshaping the basicity of its surface landscape, could TIMP-1, -2, and -4 be rendered ECM-sequestered by the transplantation of the motif from TIMP-3? Of the three soluble TIMPs, TIMP-1 was considered the most appropriate as we had previous experience in converting the TIMP into a potent TACE inhibitor with a Ki value (0.14 nM) comparable to that of TIMP-3 (0.22 nM) (23).
recreate a fully functional ECM binding motif on TIMP-1, a minimum of six mutations was expected to be necessary to make up the ECM binding ensemble: Lys-26, Lys-27, Lys-30, Lys-76, of the N-terminal module and Arg-163 and Lys-165 of the C-terminal module. For the ease of DNA manipulation, however, the six lysines/arginine were subdivided into three “epitopes” according to their locations. The three epitopes were: (i) Lys-26, Lys-27, and Lys-30 (in short, K26/27/30) collectively as the first epitope (ii) Lys-76 (i.e. K76) singly as the second epitope, and finally (iii) the C-terminal duo Arg-163 and Lys-165 (i.e. R163/K165) as the third epitope. Sections of TIMP-1 corresponding to the three epitopes, namely (i) Thr26-Pro27-Glu28-Val29-Asn30 (ii) Ser80-Glu81, and (iii) Pro168-Arg169-Glu170 were subsequently excised and replaced by those of TIMP-3. The blueprint of the new ECM-aimed TIMP-1 is best explained by its sequence alignment with that of wild-type TIMP-1 (Fig. 8A). The reason residue Glu-28, Glu-81, and Arg-169 were also mutated to those of TIMP-3 was to avoid undesirable inter-residue clashes between the newly transplanted ECM motif and the adjacent amino acids. The hypothetical surface landscapes of TIMP-1 before and after the mutagenesis are illustrated in Fig. 8, B and C. Incidentally the first epitope K26/27/30 directly deleted one of the known N-linked glycosylation sites of TIMP-1 (i.e. Asn-30) while the second epitope, Lys-76, was only two amino acids from the second glycosylation site (Asn-78) of TIMP-1 identified by Caterina et al. (24) in 1998 (Fig. 8A, highlighted by CHO arrows). Hence, we expected the mutant TIMP-1s to lose either one or both of its glycosylations as a result of the mutations.

Indeed, the operation transformed an otherwise soluble TIMP-1 into an ECM-bound protein. Interestingly, only the first two epitopes (i.e. K26/27/30 + K76) were required for the
transformation to take place. As anticipated, TIMP-1 transplanted with the two N-terminal epitopes had no glycosylation potential. The molecular mass of the K26/27/30/H11001 K76 mutant was estimated to be 21 kDa, a significant difference from the 28 kDa as noticed in wild-type TIMP-1 (Fig. 9A). Mutants carrying the third epitope (R163/K165) failed to be expressed, be it singly or in combination with the N-terminal epitopes. Despite this, it is quite likely that the adhesive role of the duo had been replaced by an arginine (−Pro−Arg169−Glu−) situated right in middle of the C-TIMP-1 segment intended for replacement (Fig. 8A). To rule out the possibility that the novel ECM binding property was an inherent quality of an unglycosylated TIMP-1, single and double knock-out mutants (N30Q and N30/78Q) were also created to confirm that unglycosylated TIMP-1 had no ECM association capability (Fig. 9A, right panel).

Further expression and examination of the mutants in MCF-7, HT1080, and COS-7 cells confirmed the ability of the TIMP-1 to associate to the ECM (Fig. 9, B–D). It also appeared that the ECM components secreted by HeLa had a noticeably higher affinity for TIMP-1 bearing only the first module (26/27/30K). The protein adhered much more readily to the ECM of HeLa than those of other cell lines.

DISCUSSION

Fig. 10 compares, hypothetically, the surface features of wild-type TIMP-3 with that of the soluble mutant K26/27/30/76E/H11001 R163/K165Q created in this work. The four residues that collectively constitute the N-terminal ECM binding module, namely Lys-26, Lys-27, Lys-30, and Lys-76, are highlighted for special attention in the figure. The Arg-163 and Lys-165 duo that make up the C-terminal module, on the other hand, are situated at the far end of the molecule (Arg-163 shielded from view). The most pressing issue we need to address as a direct result of this study is to find the reason these two apparently far-flung modules could co-operatively form the basis of a potent ECM binding motif. The clue to the answer, we believe, lies in the TIMP-1 mutant that had just been rendered ECM-
bound in this work (Fig. 8). A shared feature among the two TIMPs, apart from the newly transplanted ECM binding module, is a cluster of four highly compact but essentially conserved lysine residues (namely Lys-22, Lys-41, Lys-44, and Lys-118, labeled in gray) that, in cooperation with the newly transplanted ECM binding module, converts TIMP-1 into a potent ECM-adhering molecule just like TIMP-3.

FIGURE 8. A, blueprint of an ECM-adhering TIMP-1: sequence alignment with wild-type TIMP-1. TIMP-1 is glycosylated at Asn-30 and Asn-78 (arrowed CHO). The three sections of TIMP-1 excised and replaced by the ECM binding motif of TIMP-3 are in shadow for easy recognition: (i) first: 26TPEVN30 replaced by KKLVK (ii) second: 80SE81 replaced by KY and (iii) third: 168PRE170 replaced by ROK. Structures of (B) wild type TIMP-1 (PDB 1UEA) and (C) a model of its ECM binding equivalent, K26/27/30 + K76. Transplantation of the first and second epitopes renders the molecular surface of TIMP-1 much more basic in disposition. Note the cohort of conserved lysine residues (Lys-22, Lys-41, Lys-44, and Lys-118, labeled in gray) that, in cooperation with the newly transplanted ECM binding module, converts TIMP-1 into a potent ECM-adhering molecule just like TIMP-3.
likely to be proteoglycan components such as heparan sulfate, chondroitin sulfate or one of the equivalents (25). We attempted to model a heparin oligomer onto TIMP-3 but because of the rigidity of the polysaccharide caused by rotational restrictions about the anomeric linkages, a good quality TIMP-3:heparin co-model could not be created.

Another critical question that required answering is the identity of the common element on the C termini of TIMP-1, -2, and -4 that stabilizes the interactions between N-TIMP-3 and the ECM binding partner(s). Sequence alignment of the four TIMPs reveals that the two Arg-163 and Lys-165 residues that form the basis of the C-terminal module are in fact not unique to TIMP-3. All the TIMPs have within the narrow section one or two lysines/arginine (Arg-169 for TIMP-1; Lys-169 and Arg-170 for TIMP-2; Lys-170 for TIMP-4; Fig. 1B) that could reasonably replace the duo from TIMP-3. Insofar as we are concerned, this is the most probable explanation as to the reason all T3:TX chimeras were able to associate to the ECM. However, there is also the possibility that, without the structural cooperation of the C-terminal domain, a proper N-terminal ECM binding module simply cannot be sustained because of perturbation in the overall tertiary configuration.

The impact of the present study on future TIMP research is profound. Not only can we now evaluate, for the first time, the effect of ECM association on the cellular activity of TIMP-3, the breakthrough in transplantation also allows us to engineer a new breed of ECM-adhering TIMP-1 with potential for cell targeting and delivery. We have previously engineered a TIMP-1 mutant that is highly TACE-active (V4S/TIMP-3-AB-loop/V69L/T98L, $K_i$ value 0.14 nM) (23). It is our future intention to combine the TACE-inhibiting function with the ECM binding characteristic into a novel, therapeutic TIMP-1 suitable for use against TACE. An ECM-adhering TIMP-1 can also be employed as bait to pull-down some or possibly even all of the ECM component(s) that interact with TIMP-3 by means of affinity chromatography.

The findings from this study can also explain the reason that mutated TIMP-3s from Sorsby Fundus Dystrophy (SFD) have similar affinity for ECM with that of wild-type TIMP-3. SFD is a retinal degenerative disorder caused by accumulation of mutated TIMP-3 in the Bruch’s membrane that will eventually lead to complete loss of vision. Majid et al. (26) recently published an article comparing the ECM binding
characteristics of wild-type TIMP-3, and four of its SFD mutants with over eight individual ECM components ranging from collagens to heparin (26). Although the SFD mutations all reside on the C-terminal domain (i.e., Ser-156, Gly-167, Tyr-168, and Ser-181), none coincides with the ECM binding residues identified in this work (i.e., Arg-163 and Lys-165), hence the reason for their indistinguishable affinity for the ECM. Indeed, subsequent binding/elution analysis carried out by the same authors confirmed that the SFD mutations have no effect on the extent to which TIMP-3 associates to the ECM components.

A major constraint that has rendered this project significantly more challenging than expected is the inability of many mutants to be expressed in transfected mammalian cells. Replacements of lysine and arginine on the C terminus of TIMP-3, irrespective of the locus, by amino acids of acidic nature such as glutamate result in constructs that cannot be properly processed.3 Alanine and glutamine appear to be better tolerated, although the level of expression is still below that of wild-type TIMP-3. The same is true for certain loci on the N terminus, a good example being Lys-71 introduced in Fig. 3. Suspected to be part of the ensemble involved in ECM association, Lys-71 was once considered a prime target until exhaustive attempts to express its glutamate and glutamine replacements (K71E, K71Q) under a variety of conditions proved fruitless. Throughout the course of this study, we have had to make do with a very limited range of targets, so too are the choices of residues chosen to replace them.

A distinctive difference between the present study and those before is the approach we adopted to tackle the problem. Bearing in mind the complexity of the ECM components, its constituents include a diverse range of proteins, glycoproteins, proteoglycans, and hyaluronic acid, our foremost concern was to ensure that the ECM media chosen for binding analysis were an accurate reflection of the physiological milieu. Hence, only ECM secreted by mammalian cells was used as the binding medium. Apart from HeLa cells, ECM from MCF-7, HT1080, and COS-7 cells were also employed to ensure the reproducibility of the binding results. No binding/elution assay was performed with immobilized forms of ECM components such as heparin-agarose or multi-adhesive ECM array plates as these agents were considered biased and non-physiological. The second obvious difference was our decision to include all four TIMPs in the construction of the chimeras. Instead of focusing on TIMP-3 alone, we employed TIMP-1, -2, and -4 as fusion partners whenever and wherever appropriate. From the panel of TX:TX chimeras, a great deal has been learned about the domain interactions of TIMP-3 without which the ECM binding motif would never have been revealed.

In conclusion, we have resolved the molecular rationale that underpins the ability of TIMP-3 to associate with the ECM. We have also conclusively demonstrated that ECM binding by TIMP-3 is a direct consequence of its basicity. This unique feature can be transferred to the other TIMP(s) if the right selection of residues is transplanted. The work presented here represents the completion of the first phase of our enterprise to delineate the molecular mechanism that governs TIMP-3:ECM association. The next phase of this study will be geared toward uncovering the ECM binding partner(s) and to examine the relative inhibitory capability of soluble and ECM-associated TIMPs.

3 G. Murphy, unpublished data.

**Unmasking the ECM Binding Motif of TIMP-3**

*FIGURE 10. Hypothetical models of wild-type TIMP-3 and its soluble mutant, K26/27/30/76E + R163/K165Q. A, transparent and full surface views of the molecule showing the side chains and surface charges responsible for its ECM-adhering property. The N-(K26/27/30/76) and C-terminal (R163/K165) ECM binding modules are highlighted and labeled for attention (with the exception of K165, which is hidden from view). Between the N- and the C-terminal modules is a cohort of highly conserved basic residues (Lys-22, Lys-42, Lys-45, and Lys-110, labeled in gray) that could act as a bridge to interconnect the N- and C-terminal modules into a powerful ECM binding motif (B) TIMP-3 upon mutation to its soluble form, K26/27/30/76E + R163/K165Q. The surface charges are evidently more negative after mutations.*
Unmasking the ECM Binding Motif of TIMP-3

Acknowledgment—We thank Philippa Dodds for her invaluable input during the early phase of this study.

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