ADAMTS1 Interacts with, Cleave, and Modifies the Extracellular Location of the Matrix Inhibitor Tissue Factor Pathway Inhibitor-2*

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ADAMTS1 is an extracellular metalloproteinase known to participate in a variety of biological processes that includes inflammation, angiogenesis, and development of the urogenital system. Many of its functions rely on its catalytic activity, which thus far has been limited to the cleavage of the matrix proteoglycans aggrecan and versican. However, it is likely that other substrates exist. Using a yeast two-hybrid screen, we identified the Kunitz-type inhibitor, tissue factor pathway inhibitor-2 (TFPI-2), as a binding partner of ADAMTS1. The interaction was confirmed by several biochemical and cell-based assays. In addition, our studies revealed alterations in the pattern of TFPI-2-secreted isoforms and in its extracellular location caused by the specific action of ADAMTS1. Interestingly, we found that TFPI-2 is a novel substrate of ADAMTS1. The cleavage removes a protease-sensitive C-terminal region in TFPI-2, altering its binding properties. The proposed role of TFPI-2 as a maintenance factor of extracellular remodeling suggests the indirect function of ADAMTS1 as an additional homeostatic player by its ability to alter the extracellular location of TFPI-2 and, therefore, to disrupt the remodeling machinery, a phenomenon directly associated to pathologies such as atherosclerosis and tumor progression.

The extracellular milieu has been recognized as a dynamic scenario that directly influences proliferation, survival, migration, and biosynthetic activities of cells. The constituents of this extracellular environment include growth factors, chemokines, cell surface proteins, and an extensive list of matrix components with structural and signaling properties. Matrix proteases and their respective inhibitors are also important components of the extracellular milieu with the added value that these molecules modify, degrade, or inflict functional alterations to most of the components previously listed (1). In fact, modification of extracellular components by processing and/or proteolysis is a frequent event during morphogenesis and tissue repair and, in an altered manner, during the progression of various pathological conditions (2).

The ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family of proteases includes a group of 19 secreted enzymes with a more restricted spectrum of catalytic activities than the well known matrix metalloproteinases (MMP) (3). ADAMTS1, the first member described (4), has been shown to display anti-angiogenic properties (5). Systemic deletion of adams1 in mice resulted in multiple defects in the urogenital system (6, 7). The enzyme is known to have catalytic activity toward matrix proteoglycans, particularly versican and aggrecan (8, 9).

Mechanistically, it is not evident that the outcomes of the gene-deletion studies can be linked with the catalytic profile known for this enzyme. Thus, it is possible that either other domains contribute to the spectrum of biological functions and/or that additional biologically relevant substrates are yet to be identified. The modular structure of ADAMTS1 allows for a multiplicity of protein-protein interactions that are likely to affect its biological actions. Here, we have exploited the yeast two-hybrid system to identify potential interacting proteins. The screen resulted in the identification of tissue factor pathway inhibitor-2 (TFPI-2) as a binding partner for ADAMTS1.

TFPI-2 was first identified as a serine protease inhibitor containing Kunitz domains (10, 11). Its inhibitory abilities have been demonstrated toward tissue factor (TF)-Factor VIIa complex, trypsin, plasmin, and kallikrein (12). Consequently, this inhibitor affects the plasmin- and trypsin-mediated activation of matrix metalloproteinases proMMP-1 and proMMP-3 (13). In addition, direct inhibition of MMP1, -2, -9, and -13 activities have been reported (14). TFPI-2 is secreted by several cell types, including endothelial cells (15), smooth muscle cells, fibroblasts (16), keratinocytes (17), and syncytiotrophoblasts (18). Its preferential association to the extracellular matrix (ECM) has been documented (19), and several reports suggest a pivotal role of TFPI-2 in the maintenance and regulation of ECM remodeling. In fact, altered levels of TFPI-2 have been associated with pathological conditions, such as atherosclerosis (14) and tumor growth, although its action in this second case appears paradoxical and dependent on the tumor model studied. For example, its inhibited role was observed in models of glioma invasion (20) and fibrosarcoma growth and metastasis (21). In contrast, TFPI-2 exerts a pro-invasive effect on hepatocarcinoma (22). In aggressive uveal melanoma, this molecule was found to be up-regulated when compared with poorly aggressive melanoma (23). Interestingly, in this latter study, TFPI-2 was implicated in processes of vasculogenic mimicry, an alternative pathway for tumor perfusion that is independent of angiogenesis (for review, see Ref. 24).

In this study we confirmed the interaction between ADAMTS1 and

1. To whom correspondence should be addressed: Laboratori de Angiogènesi, Institut de Recerca Hospital Universitari Vall d’Hebron, Psg. Vall d’Hebron 119–129, Barcelona 08035, Spain. Tel: 34-93-489-4167; Fax: 34-93-274-6026; E-mail: jcrodrig@ir.vhebron.net.

2. The abbreviations used are: MMP, matrix metalloproteinase; ADAM, a disintegrin and metalloproteinase domain; ADAMTS, a disintegrin-like and metalloproteinase with thrombospondin type I motifs; ECM, extracellular matrix; PMA, phorbol 12-myristate 13-acetate; TFPI-2, tissue factor (TF) pathway inhibitor 2; TSR, thrombospondin type I repeat; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; pAb, polyclonal antibody; mAb, monoclonal antibody; RIPA, radioimmune precipitation assay.

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**1** The abbreviations used are: MMP, matrix metalloproteinase; ADAM, a disintegrin and metalloproteinase domain; ADAMTS, a disintegrin-like and metalloproteinase with thrombospondin type I motifs; ECM, extracellular matrix; PMA, phorbol 12-myristate 13-acetate; TFPI-2, tissue factor (TF) pathway inhibitor 2; TSR, thrombospondin type I repeat; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; pAb, polyclonal antibody; mAb, monoclonal antibody; RIPA, radioimmune precipitation assay.
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TFPI-2 by several independent approaches. Moreover, our results indicate that the interaction regulates the extracellular distribution of TFPI-2 through the proteolytic processing at its C-terminal end. These effects were observed in both cell culture and tumor xenograft assays. Taken together, these findings suggest that ADAMTS1 is a regulator of TFPI-2 function by modulating its distribution within the extracellular microenvironment, implicating a role for ADAMTS1 in pathological processes that includes, but is not restricted to, tumor progression and atherosclerosis.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—The MATCHMAKER Two-hybrid System 2 (Clontech Laboratories Inc.) was used according to the manufacturer. A cDNA fragment encoding amino acid residues 540–666 of human ADAMTS1 was amplified by PCR and inserted into the pSA2–1 vector that was previously digested with NotI and Sall. The resulting GAL-4BD-ADAMTS1540–666 chimera protein was used as bait to screen a human placenta MATCHMAKER cDNA library (Clontech). Both bait and library vectors were simultaneously introduced into Saccharomyces cerevisiae CG-1945, and double transformants were selected on synthetic minimal medium with dextrose but lacked tryptophan, leucine, and histidine. A total of ~7 × 105 clones was screened. Putative positive colonies were re-streaked and tested for β-galactosidase activity using a filter assay. DNA fragments from positive colonies were amplified by PCR using the MATCHMAKER 5′- and 3′-AD LD-Insert Screening Amplimers and then sequenced.

Expression Vectors—Bicistronic TFPI-2-green fluorescent protein expression vector was obtained as follows. pZem 229 plasmid (10) containing the TFPI-2-cDNA was digested with EcoRI and subsequently cloned into the EcoRI pIRE2-EGFP (BD Biosciences Clontech). To generate the TFPI-2-Myc expression vector (scheme in Fig. 1B), pZem229 was used as a template to amplify and create new restriction sites with the following oligonucleotides: TFPI-2 forward 5′-CTT GGT CTA CATT CCC ATG GAC CCC GCT GCG-3′, TFPI-2 reverse 5′-CTT TGG TAC CTG CTT TCT CCG AAT TTG C-3′ (EcoRI and KpnI restriction sites are in bold, and the starting ATG is underlined). The resulting PCR product was then cloned into pCR2.1 (Invitrogen) using the TOPO-TA cloning kit, and the TFPI-2-cDNA was obtained by subsequent digestion with EcoRI and KpnI. This fragment was inserted into KpnI/EcoRI pcDNA 3.1/Myc-His(−) B expression vector (Invitrogen). This construct contains a functional Myc-His epitope at the C-terminal end. The C-terminal-truncated TFPI-2 (ΔCTFPI-2-Myc) expression vector (scheme in Fig. 1B) was generated by amplification of pZem229 with the same forward primer noted above and the reverse primer 5′-CTT GGT ACC CTT TTT CAA AGC TTT TGG-3′. The same procedure described above was used to subclone this truncated form into KpnI/EcoRI pcDNA 3.1/Myc-His(−) B expression vector (Invitrogen).

Full-length human ADAMTS1, zinc-binding site mutant E385A-ADAMTS1, and ΔTSRs-ADAMTS1 (Met-Dis: 1–556) constructs have previously been described (9, 25). Recombinant human TFPI-2 and p87-ADAMTS1 were purified as described (10, 26).

Cell Culture, Transfection, and Treatments—Mouse lung endothelial (LE) and 293T cells were cultured at 37 °C with 5% CO2 under saturated conditions (PBS, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1 mM EDTA).

N-Glycosidase F Treatment—Conditioned medium was methanol-precipitated and re-suspended in 50 μl of reaction buffer (100 mM sodium phosphate, pH 7.0, 2% SDS, 0.1 mM EDTA). Samples were incubated for 3 min at 100 °C, and then 0.1% Triton X-100 was added. Enzymatic incubation was performed at 37 °C for 20 h in the presence of 1 unit of N-glycosidase F (Roche Applied Science).

In Vitro Digestion of TFPI-2—Conditioned medium from stable TFPI-2-Myc overexpressing 293T cells was purified under denaturing conditions (PBS, pH 8.0, 8 M urea) with nickel nitrilotriacetic acid-agarose (Qiagen). Bound protein was washed with PBS and equilibrated with reaction buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 10 mM CaCl2, 10 μM ZnCl2, 5 μg/ml heparin). Digestions were performed at 37 °C for 16 h. Then samples were concentrated with StrataClean resin (Stratagene, Cedar Creek, TX), resolved by SDS-PAGE, and analyzed by Western blot with Myc and TFPI-2 antibodies.

Co-immunoprecipitation Studies—Cells lysates or conditioned media were preabsorbed with protein G-Sepharose 4 Fast Flow (Amersham Biosciences) for 30 min at 4 °C in a rotation wheel. This material was then incubated with the appropriate antibody overnight at 4 °C. Protein G-Sepharose was added for 1 h in the same condition. Finally, immunoprecipitates were pelleted, rinsed 3 times with RIPA/PBS (1:3), resuspended in loading buffer, and analyzed by SDS-PAGE. The antibodies used were monoclonal anti-ADAMTS1 clone 3E4C6B4 (9) and monoclonal anti-Myc (a generous gift from Dr. Arribas, Vall d’Hebron University Hospital Research Institute, Barcelona, Spain).

Tumor Xenograft Assays—293T clones were trypsinized, washed twice, and re-suspended in serum-free Dulbecco’s modified Eagle’s medium. Nu/Nu Balb/c mice were subcutaneously injected in the right back with 5 × 106 cells/200 μl. Mice weight and tumor size were assessed every 3 days after cell injection. When the tumors reached 1.5 cm2, mice were sacrificed, and the tumors were removed for further analysis. Tumor volumes were calculated by the equation D × d2 × π/6, where D is the tumor diameter at its widest, and d is at its smallest (27).

Heparin Purification—The affinity of ADAMTS1 and TFPI-2 proteins to heparin has been previously reported (19, 26). Samples were incubated with pre-equilibrated heparin beads (Amersham Biosciences) overnight at 4 °C under constant agitation. Heparin beads were then washed twice with 100 mM NaCl and twice more with PBS.
Finally, samples were pelleted, resuspended in loading buffer, and analyzed by SDS-PAGE.

**Immunoblot Analysis**—Cell layers and tumor samples were homogenized and lysed with RIPA buffer containing 1 mM of phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin. Cell debris was discarded by centrifugation (20 min at 16,000 × g), and protein suspension was quantified using the Bio-Rad DC protein assay (Bio-Rad). When needed cell lysates were precipitated with 3 volumes of methanol at −20 °C overnight. The precipitate was washed twice with acetone and left to dry at room temperature. Conditioned medium was concentrated with StrataClean resin. Samples were subjected to SDS-PAGE or Tricine-SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked with 5% nonfat milk and incubated with the antibodies polyclonal rabbit anti-TFPI-2 (28) and anti-TFPI (Schuell). Membranes were incubated with the antibodies polyclonal rabbit anti-TFPI-2 (28) and anti-TFPI (Schuell) and monoclonal anti-actin (clone AC-40, Sigma). After incubation with the appropriate secondary antibodies conjugated to peroxidase, signal was detected with the SuperSignal chemiluminescence kit (Pierce). Membranes were stripped as necessary using the Bio-Rad DC protein assay (Bio-Rad). When needed cell lysates were subjected to immunoprecipitation with Myc and ADAMTS1 monoclonal antibodies (Fig. 2A). Both α and β TFPI-2 isoforms were pulled down with the Myc antibody; the γ TFPI-2 isoform, however, was not adequately resolved. In addition, p87-ADAMTS1 was observed to co-immunoprecipitate with the Myc antibody in the presence of TFPI-2, suggesting a specific interaction between these two proteins. To avoid antibody cross-reactivity, Western blot analyses were done with rabbit and guinea pig polyclonal antibodies to detect TFPI-2 and ADAMTS1 proteins, respectively. When the ADAMTS1 monoclonal antibody that recognizes all the forms of the protease was used in immunoprecipitation experiments, we observed the co-precipitation of both α- and β-TFPI-2 isoforms; again, the γ-TFPI-2 isoform was not identified (Fig. 2A). Also, in this experiment, the antibodies used for the final detection were different from the ones used to pull down the complexes.

**RESULTS**

**Identification of TFPI-2 as an Interacting Protein of ADAMTS1**—A yeast two-hybrid screen of a human placenta cDNA library was performed to identify ADAMTS1 interacting proteins. Given the multidomain structure of ADAMTS1 and the high probability of false positive clones, we decided to perform the screen using discrete modular domains of the coding region. A fragment of human ADAMTS1 containing the first thrombospondin type I repeat (TSR) and part of the cysteine-rich region (encoding amino acid residues 540–666) was used as bait (Fig. 1A). Among several positive clones identified, the sequencing of the clone F2.3.3 included an insert that corresponded to the complete 3′-end (from nucleotide 258) of the human tissue factor pathway inhibitor-2 cDNA (accession number NM_006528). This sequence encoded for the entire C-terminal TFPI-2 protein (from amino acid residue 62) that included the last two Kunitz inhibitory domains (Fig. 1A).

**Interaction of TFPI-2 and ADAMTS1 in a Cell Culture Model**—To confirm the ADAMTS1/TFPI-2 interaction, we performed co-immunoprecipitation studies in a mammalian cell-based system. 293T cells that constitutively overexpressed ADAMTS1 were transiently transfected with a TFPI-2-Myc chimera construct (scheme in Fig. 1B) or a control vector. Conditioned medium from these cells was independently subjected to immunoprecipitation with Myc and ADAMTS1 monoclonal antibodies (Fig. 2A). Both α and β TFPI-2 isoforms were pulled down with the Myc antibody; the γ TFPI-2 isoform, however, was not adequately resolved. In addition, p87-ADAMTS1 was observed to co-immunoprecipitate with the Myc antibody in the presence of TFPI-2, suggesting a specific interaction between these two proteins. To avoid antibody cross-reactivity, Western blot analyses were done with rabbit and guinea pig polyclonal antibodies to detect TFPI-2 and ADAMTS1 proteins, respectively. When the ADAMTS1 monoclonal antibody that recognizes all the forms of the protease was used in immunoprecipitation experiments, we observed the co-precipitation of both α- and β-TFPI-2 isoforms; again, the γ-TFPI-2 isoform was not identified (Fig. 2A). Also, in this experiment, the antibodies used for the final detection were different from the ones used to pull down the complexes.

**Further validation of the interaction was achieved on 293T parental cells previously treated with phorbol esters (PMA). This stimulus is known to induce the expression of TFPI-2 (30), and here we demonstrate that it also induces ADAMTS1 expression. For these assays conditioned medium from 293T cells that were either treated or not treated with PMA for 48 h was harvested and immunoprecipitated with the ADAMTS1 monoclonal antibody. As expected, the analysis of these complexes revealed the presence of both ADAMTS1 and TFPI-2 only when the cells were stimulated with PMA (Fig. 2B).**

**TFPI-2 Is a Substrate of ADAMTS1**—TFPI-2 is a broad-spectrum protease inhibitor that targets both serine proteases and matrix metal-

![FIGURE 1. A, schematic representation of the ADAMTS1 and TFPI-2 proteins. The inset shows the ADAMTS1 domain used as bait and the TFPI-2 interacting region as revealed by the yeast two-hybrid screen. SP, signal peptide; Cys-Rich, cysteine-rich domain; GAL4, yeast transcription activator; BD, binding domain; AD, activating domain, Disint, disintegrin. B, scheme of the TFPI-2 constructs used. Numbers indicate the amino acid residues of the human TFPI-2 protein.]
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FIGURE 2. Interaction of ADAMTS1 and TFPI-2 in a cell-based system. A, 293T cells that overexpress ADAMTS1 were transiently transfected with the TFPI-2-Myc or mock expression vector. 3 ml of conditioned medium were collected, preabsorbed, and subjected to co-immunoprecipitation (IP) with either a Myc or ADAMTS1 monoclonal antibody as described under "Experimental Procedures." Total extracts are the result of concentrating 0.5 ml of the same conditioned medium subjected to immunoprecipitation. B, after parental 293T cells were treated with PMA for 48 h, 8 ml of conditioned medium were preabsorbed and subjected to co-immunoprecipitation with an ADAMTS1 monoclonal antibody as described under "Experimental Procedures." To visualize the total amounts of both proteins, 2 ml of the same conditioned medium were concentrated. Western blots were performed with polyclonal antibodies for ADAMTS1 and TFPI-2. α, β, and γ represent different glycosylated TFPI-2 forms; p110, p87, and p65 represent different ADAMTS1 forms.

![Image](252x390 to 564x733)

loproteases. Thus, we first speculated that TFPI-2 could function as an endogenous inhibitor of ADAMTS1. To test this hypothesis, we evaluated the effect of TFPI-2 on aggrecan and syndecan-4 proteolytic assays, because these proteoglycans are known substrates for ADAMTS1 (9). However, the presence of TFPI-2 did not alter the proteolytic activity of ADAMTS1 on these substrates (data not shown). We then considered the potential effect of ADAMTS1 on TFPI-2. Cells overexpressing the inhibitor were transfected with either wild-type ADAMTS1 or the catalytically inactive form (zinc-binding site mutant E385A-ADAMTS1 (9)). As seen in Fig. 3A, three last three lanes, the presence of ADAMTS1 resulted in increased levels of TFPI-2 in the conditioned media together with an apparent shift in the pattern of secreted isoforms. In contrast, this effect was not observed on cells transfected with the catalytically inactive ADAMTS1. To better understand this process, we used two types of arrows to indicate the different states of TFPI-2 isoforms; the black arrow indicates the primary TFPI-2 products, and the white arrow corresponds to the forms originated in the presence of ADAMTS1. Stars next to the bands also denote the new forms. We evaluated TFPI-2 levels in the presence of heparin, known to release ECM-bound and cell surface-anchored TFPI-2 (19). Although the overall quantities of soluble TFPI-2 were equivalent under these conditions, the mentioned changes in the pattern of TFPI-2-secreted isoforms persisted in the presence of ADAMTS1 (Fig. 3A). When the cell layer compartment was analyzed, the α-isofrom of TFPI-2 was found to be predominant (Fig. 3A). A slight decrease of this form was observed in the presence of ADAMTS1, but no evidence of additional products appeared, which is indicative of the extracellular nature of this event. According to these data, we further explored the possibility that these changes in TFPI-2 are due to proteolysis by ADAMTS1.

It has been previously reported that differences between TFPI-2 isoforms are due to post-translational glycosylation events (31). To facilitate our evaluation, the glycosylation inhibitor tunicamycin was included in this analysis. Tunicamycin treatment resulted in the appearance of two main bands of TFPI-2 (Fig. 3B); that is, the highly glycosylated α isoform that appears more abundantly in normal conditions and the under-glycosylated γ TFPI-2 isoform. Treatment with tunicamycin did not fully prevent the synthesis of the α isoform but increased the level of the γ isoform. Interestingly, the differences in the pattern of secreted isoforms imposed by ADAMTS1 persisted, and the treatment with tunicamycin made the shift more apparent (Fig. 3B). In addition, treatment with the metalloprotease inhibitor BB94 caused the normalization of the TFPI-2 pattern in the presence of ADAMTS1. This effect was clearly noted in the presence of tunicamycin (Fig. 3B). ADAMTS1 was also evaluated to verify that tunicamycin and BB94 treatments did not alter its relative levels in the conditioned medium.

To gain information as to the approximate location of the cleavage site, we used the TFPI-2 construct that possessed a Myc-tag in the C-terminal region (TFPI-2-Myc) (scheme in Fig. 1B). Analysis of conditioned medium of cells transfected with this construct revealed that the TFPI-2 forms that appeared shifted in the presence of ADAMTS1 (white arrows and stars in Fig. 4A) were not recognized by the Myc antibody, although the amounts of protein were substantial. This observation indicated that the processing of TFPI-2-Myc occurred at the C-terminal fragment that provokes the release of the

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We also performed deglycosylation treatments with N-glycosidase F of this conditioned medium containing secreted TFPI-2-Myc in the absence and presence of ADAMTS1. Although not fully efficient, such treatment provoked an enrichment of under-glycosylated forms, and the mentioned changes in motility persisted, supporting the existence of a catalytic process. Again, re-probing of this membrane with the Myc antibody confirmed the deletion of the C-terminal fragment in all forms by the action of ADAMTS1 (Fig. 3).

**FIGURE 3. Identification of TFPI-2 as a substrate of ADAMTS1.** A, 293T cells that overexpress TFPI-2 were transiently transfected with the full-length ADAMTS1 (ATS1), the catalytic inactive mutant E385A-ADAMTS1 (Inact. ATS1), or a mock expression vector (Control). 24h after transfection cells were serum-deprived and, when indicated, treated with heparin. 0.5 ml of conditioned media and 100 µg of cell layer were concentrated and subjected to analysis by Western blot with the TFPI-2 polyclonal antibody. B, 293T cells that overexpress TFPI-2 were transiently transfected with full-length ADAMTS1 or a mock expression vector. 24h after transfection cells were serum-deprived and treated with BB94 and tunicamycin as indicated. Conditioned medium was harvested 48h after treatment, and the presence of TFPI-2 and ADAMTS1 was determined by Western blot analysis with the detailed antibodies. Black arrows indicate the primary TFPI-2 isoforms, and white arrows indicate the modified isoforms in the presence of ADAMTS1. Stars point to the modified isoforms in the presence of the protease.

**FIGURE 4. ADAMTS1 cleaves TFPI-2 at its C-terminal domain.** A, 293T cells that overexpress TFPI-2-Myc were transiently transfected with full-length ADAMTS1 or a mock expression vector. 24h after transfection cells were serum-deprived. After 48h, 0.5 ml of conditioned media were concentrated and subjected to analysis by Western blot with a TFPI-2 polyclonal antibody. The same Western was stripped and re-probed with a Myc monoclonal antibody. B, from cells in A, 0.5 ml of conditioned media were methanol-precipitated and incubated in the presence of N-glycosidase F. Samples were finally analyzed by Western blot with TFPI-2 and Myc antibodies. C, from cells in A, 8 ml of conditioned media were concentrated, resolved in 18% Tricine gels, and analyzed by Western blot with a Myc monoclonal antibody. D, purified TFPI-2-Myc protein was incubated with reaction buffer and 293T-conditioned media in the absence and presence of purified (pATS1) and non-purified ADAMTS1 (npATS1). These samples and the starting material were resolved and analyzed by Western blot. Black arrows indicate the primary TFPI-2 isoforms, and white arrows indicate the modified isoforms in the presence of ADAMTS1. Stars point to the modified isoforms in the presence of the protease.
Importantly, we were able to visualize the released C-terminal fragment with an apparent molecular mass of ~6 kDa by concentration of high amounts of conditioned media and further resolution in Tricine gels (Fig. 4B).

To prove that this cleavage is performed by ADAMTS1, we took advantage of the His tag present in the TFPI-2-Myc chimera. We purified this recombinant form under denaturing conditions using nickel nitritotriacetic acid-agarose and treated it at 37 °C for 16 h with purified recombinant ADAMTS1 (pATS1 in Fig. 4D) and compared that with conditioned media containing non-purified ADAMTS1 (npATS1 in Fig. 4D). This digestion raised the same pattern of bands, and the deletion of the C-terminal end of TFPI-2-Myc was confirmed by Western blot analysis with the Myc antibody (Fig. 4D).

**Protease Sensitivity of TFPI-2 C-terminal Region**—A close analysis of this sequence reveals an enrichment of basic residues (Arg and Lys) that suggested its sensitivity to plasmin and thrombin proteases. In addition, the equivalent region in TFPI is sensitive to the action of such enzymes (32, 33). Accordingly, the addition of plasmin and thrombin to 293T cells overexpressing TFPI-2-Myc construct caused a similar cleavage pattern observed with ADAMTS1 (Fig. 5A). The release of the C-terminal fragment was demonstrated by analysis with a Myc antibody. To confirm the specificity of these catalytic events and the independence of the ADAMTS1 action, we used specific serine protease inhibitors for plasmin and thrombin such as aprotinin and leupeptin, respectively. As expected, the presence of these inhibitors blocked the action of plasmin and thrombin, but it did not have any effect on ADAMTS1 activity.
The observed shifts in the sizes of TFPI-2 isoforms indicated that the cleavage sequence for ADAMTS1 and serine proteases is located in the final 2–2.5-kDa portion of TFPI-2. To test this further, we generated a truncated TFPI-2 form (ΔCTFPI-2-Myc) that lacked the cleaved region (from amino acid 215) but still retained the last Kunitz-type domain and that ended at amino acid 208. A Myc tag was also added at the C-terminal end of this truncated version (scheme in Fig. 1B). Transfection experiments with this TFPI-2 form in the absence and presence of ADAMTS1 showed no changes in the pattern of secreted isoforms in contrast with the full-length TFPI-2-Myc construct (Fig. 5B). Re-probing the same membrane with a Myc antibody showed an identical profile to that given by the TFPI-2 antibody. These results demonstrated that the cleavage site is located at the deleted region, and it does not affect the Kunitz inhibitory domains.

In addition, to confirm that the tested serine proteases acted at the same region as ADAMTS1, we performed similar assays with the truncated ΔCTFPI-2-Myc construct resistant to ADAMTS1 activity. Neither plasmin nor thrombin exerted any catalytic action on this TFPI-2 construct that lacked the C-terminal region (Fig. 5C), confirming the sensitivity of this region to proteases of diverse nature.

Requirements for the Cleavage of TFPI-2—Because ADAMTS1 and TFPI-2 are secreted molecules that associate with the ECM and the cell surface, we explored the requirements of proximity and/or co-location between these two factors for the cleavage of TFPI-2 to occur. First, in addition to the co-transfection assays that illustrated a potential cis cleavage and autocrine mode of action, we performed co-culture experiments with two cell populations that overexpressed ADAMTS1 and TFPI-2 independently. Under both experimental conditions we observed the same shift in TFPI-2 pattern in the presence of ADAMTS1 (Fig. 6A). These results demonstrated that ADAMTS1 activity on TFPI-2 could take place in a juxtacrine, paracrine, and/or autocrine manner.

As shown above, the interaction between ADAMTS1 and TFPI-2 is mediated by the ADAMTS1 domain containing the TSRs. However, such experiments did not determine whether the interaction is required for the cleavage of TFPI-2. Now, 293T cells overexpressing TFPI-2 were transiently transfected with either full-length ADAMTS1 or a truncated ADAMTS1 construct that lacked all TSRs but still contained an intact catalytic domain (25). Although high levels of the truncated ADAMTS1 form were observed by Western blot analysis with a Myc antibody (Fig. 6B, right panels), cleaved forms of the TFPI-2 molecule were not detected (Fig. 6B, left panels). In contrast, the full-length protease released the expected isoforms of TFPI-2.

Effects of ADAMTS1 in the Extracellular Location of TFPI-2 Protein in Vivo—To test the significance of the described cleavage of TFPI-2 at its C-terminal domain, we performed several experiments with potential functional consequences. As a first approach, we used an endothelial cell model as a recognized cellular target for the action of both molecules. We added exogenous ADAMTS1 and TFPI-2 purified proteins on endothelial cell monolayers. In this assay we confirmed the affinity of TFPI-2 for the ECM compartment (Fig. 7A). In addition, we evaluated the levels of TFPI-2 anchored to the cellular compartment in the presence of p87-ADAMTS1 purified protein. TFPI-2 levels in the cell layer were significantly diminished when p87 was added (Fig. 7A). This is likely the result of the direct cleavage of the C-terminal domain of TFPI-2 that provides affinity to matrix components. Although this assay
confirmed the displacement of TFPI-2 by ADAMTS1, the experiment cannot rule out the possibility that ADAMTS1 targets additional proteins in the cell layer that are directly responsible for TFPI-2 anchorage. As a second approach, we used total conditioned media from TFPI-2-Myc overexpressing 293T cells that were previously transfected with ADAMTS1 or control vector. The presence of TFPI-2 in these conditioned media was demonstrated earlier (Fig. 4A). After endothelial cultures were treated with these conditioned media for 24 h, we analyzed both cell layer and conditioned media. According to our previous observations, most TFPI-2 remained soluble, and the isoforms that anchored to the cellular compartment appeared to be highly glycosylated and uncleaved (Fig. 7B). A slight but consistent decrease of TFPI-2 bound to the cell layer was observed in the presence of ADAMTS1. This observation is consistent with the cleavage of TFPI-2. Conversely, analysis of the conditioned media showed an increase of cleaved TFPI-2 that lacked the Myc-containing C-terminal end in the presence of ADAMTS1. We also observed important levels of the cleaved TFPI-2 form even in the absence of ADAMTS1 (third lane, Fig. 7B); this is probably due to the activity of additional proteases, in agreement with our previous results.

To assess the magnitude of a functional in vivo interaction between ADAMTS1 and TFPI-2, we performed tumor xenograft assays in mice with cell lines overexpressing these molecules. Given the results presented above showing that ADAMTS1 could act in a juxtacrine and/or paracrine manner, we approached these assays by co-injecting equal amounts of ADAMTS1 and TFPI-2 overexpressing cells alone (with control cells) or in combination. The presence of ADAMTS1 was confirmed in those tumors extracts that originated from ADAMTS1-expressing cells. No changes in ADAMTS1 levels were noted by the presence or absence of TFPI-2-expressing cells. However, we did observe markedly reduced protein levels of TFPI-2 in those tumors originated by the co-injection of TFPI-2 and ADAMTS1-expressing cells (Fig. 8A). These results, although more dramatic, are in agreement with our previous observations in a cell-based system where ADAMTS1 promoted TFPI-2 removal/solubilization from the ECM and cell surface to the culture medium. To assess if changes in TFPI-2 protein levels might be originated by alterations in mRNA expression in the xenograft or by the predominant growth of ADAMTS1-expressing cell population versus the co-injected TFPI-2-expressing cells, we extracted total RNA from tumor samples and performed reverse transcription followed by primere-specific PCR. As shown in Fig. 8B, TFPI-2 mRNA levels were consistently similar in the absence or presence of ADAMTS1. Once we ruled out the possibility of changes in RNA expression, we attempted to evaluate TFPI-2 levels in the plasma. Unfortunately, our experimental approach was not sensitive enough to detect TFPI-2 levels (data not shown). We concluded that TFPI-2 protein levels were decreased in tumor xenografts in a manner that is dependent on the presence of ADAMTS1 and is likely the result of TFPI-2 processing by ADAMTS1. Regarding the final destination of the released TFPI-2, it is not clear if it was rapidly degraded or was redirected to an unknown location. To ascertain that this effect is specific for TFPI-2, we analyzed the endogenous presence of the closely related TFPI protein. We first verified the basal levels of this protein as a single 31-kDa band. Further determinations of TFPI in the different tumor extracts did not show any evidence of mobilization or proteolytic cleavage in the presence or absence of ADAMTS1 (Fig. 8C), confirming the specificity of the findings for TFPI-2.

**DISCUSSION**

ADAMTSs represent a family of extracellular Zn$^{2+}$-dependent proteases characterized by the presence of a disintegrin domain and a varied number of TSRs (3). Similar to MMP and ADAM proteases, its multidomain structure supports the idea that their functionality depends on interactions with cell surface and ECM components. Although previously questioned, recent reports have demonstrated the benefits of the yeast two-hybrid system to characterize interactions between extracellular proteins of mammalian origin (34, 35). Here, we provide additional evidence that supports the contribution of this methodology. We observed the interaction of the protease ADAMTS1 with the Kunitz-type inhibitor TFPI-2. Such interaction was confirmed in a mammalian model by co-immunoprecipitation assays. Furthermore, additional studies demonstrated the ability of ADAMTS1 to cleave TFPI-2 at the C-terminal end.

With the rationale that a cleavage event would be preceded by a physical interaction, Overall et al. (36) developed a yeast two-hybrid approach that consisted of the use of an inactive catalytic domain of a protease. According to our data, the use of domains proximal to the catalytic region appears to be an additional approach to discover their potential substrates. The relevance of such noncatalytic regions for the final action of metalloproteinases has already been outlined for hemopexin-containing MMPs (37). In the case of ADAMTS proteases, the C-terminal region containing the TSRs and the spacer region appears to mediate binding of the protease to its substrate, acting as an anchor to support the final catalytic activity and providing substrate specificity. Here, we demonstrated that the truncated ADAMTS1 form that lacked all TSRs was unable to induce the cleavage of TFPI-2. This confirms the relevant role of this domain in mediating substrate interaction. Similar characteristics have been shown for ADAMTS4 that, significant to its in vivo regulation, is naturally processed at its C-terminal end (38–40). Regarding ADAMTS1, it has been reported that the C-terminal region supports anchorage to the cell surface and promotes

**FIGURE 7. ADAMTS1 affects the location of TFPI-2 protein in vivo.** A, lung endothelial cells were treated with purified TFPI-2 (3.2 μg) and p87-ADAMTS1 (4 μg). After 36 h of treatment, the cell layer and conditioned (Cond.) media were collected and analyzed by Western blot with a TFPI-2 antibody. B, lung endothelial cells cultures were treated with conditioned media from TFPI-2-Myc overexpressing 293T cells transiently transfected with ADAMTS1 or control vector. After 24 h of treatment, cell layer and conditioned media were collected and analyzed by Western blot with TFPI-2 and Myc antibodies. Black arrows indicate the primary TFPI-2 isoforms, and white arrows indicate the modified isoforms in the presence of ADAMTS1.

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a higher anti-angiogenic activity than a C-terminal-truncated form (26). Here, a two-hybrid screen revealed that the ADAMTS1 fragment containing the first TSR and part of the cysteine-rich region is responsible for its interaction with TFPI-2. However, our in vivo analysis showed exclusively the p87-ADAMTS1 species but not the p65. This truncated ADAMTS1 form includes the region used in the two-hybrid approach, but it is possible that its affinity is weaker and transient, so we were not able to detect it in the co-precipitation assays. In addition, the strongest binding of p87-ADAMTS1 could be enhanced by the presence of two additional TSRs, domains that have been extensively implicated in protein-protein interaction (41). Furthermore, this TSR sequence has been found to bind MMPs (42), and interestingly, the full glycoprotein TSP1 was found to interact with the inhibitor TFPI, which is highly similar to TFPI-2. These authors, however, did not provide a detailed characterization of the domains implicated in the interaction (43).

From a functional point of view, ADAMTS1 was first identified from a cachexigenic adenocarcinoma cell line associated to inflammatory processes (4), and later the human protein was shown to display anti-angiogenic activity (5). adams1 knock-out mice displayed defects during growth and in the urogenital system (6, 7). Studies on an ADAMTS1 transgenic/apoe-deficient mouse model suggested a role of this protease as an atherosclerosis-promoter agent (44). To date only the matrix proteoglycans aggregan and versican have been reported to be substrates of ADAMTS1 (8, 9). Our work adds a new substrate to this growing list of candidates and also provides the finding that TFPI-2 extracellular location is significantly altered by ADAMTS1. Still, the relevance of these substrates and their relative contribution to the described ADAMTS1-related phenotypes needs further studies.

The preferential association of TFPI-2 to the cell surface and ECM has already been stated, and several reports suggest its pivotal role in the maintenance and regulation of ECM remodeling. Initial studies on the structure of TFPI-2, which is highly similar to TFPI, attributed its homeostatic role to its serine protease inhibitory activity. This revealed important implications to tumor cell growth and invasion (21, 45). Another relevant role of TFPI-2 as an indirect inhibitor of MMP activity was supported by its capacity to regulate the activation of proMMP1 and proMMP3 by serine proteases such as plasmin and trypsin (13). Surprisingly, a more recent report suggested the direct inhibition of MMPs by TFPI-2 with relevance in atherosclerotic processes, probably preceded by the respective protease-inhibitor interaction (14). In accordance to its recognition as a wide spectrum metalloproteinase inhibitor, we first speculated that TFPI-2 could function as a potential inhibitor of ADAMTS1; however, our experiments did not confirm such a prediction. Although our approaches included both in vitro and in vivo systems, it is conceivable that we are missing some requirements for the inhibition to occur.

In connection with the relevance of TFPI-2 as a vascular homeostatic factor that prevents atherosclerotic complications, ADAMTS1 has been found to be up-regulated in atherosclerotic plaques and to exert an opposite function to that of TFPI-2 (44). The mobilization of TFPI-2 in our in vivo studies supported the hypothesis that up-regulation of ADAMTS1 could act to mobilize TFPI-2 from specific cellular microenvironments, as observed during the progression of atherosclerosis (14). This event consequently triggers the predominant action of MMPs and plaque instability. The xenograft approach used in this study corroborated findings from the cell-based analysis that demonstrated the capacity of ADAMTS1 to mobilize TFPI-2 in a manner that does not require their synthesis by the same cell. The tumor xenograft model reproduces a multicellular scenario in which different cell subtypes provide distinct extracellular factors. Although the mRNA levels of TFPI-2 do not change in the presence or absence of the protease, we consistently observed a dramatic down-regulation of protein levels in the tumor extract when ADAMTS1 was overexpressed. Interestingly, we did not detect TFPI-2 in the circulation; thus, we predict that the protein might be rapidly degraded or redirected to a site of unknown location. Although an inhibition of tumor growth by TFPI-2 has been reported in a xenograft model with HT1080 fibrosarcoma cells (21), our tumor studies with 293T-modified cells were inconclusive, and we did not observe major changes related with the overexpression of TFPI-2 in the presence or absence of the protease ADAMTS1. Most likely, the overall requirements of the cell line used here in the xenograft model are distinctive in comparison to HT1080. A thorough analysis of these tumors that includes the examination of MMP and serine proteases whose activity has been found to be modulated by TFPI-2 is currently in progress.

Functional mobilization of extracellular proteins has been previously shown in a tumor context. Vascular endothelial growth factor, for example, is constitutively present in the tumor, but the association to its specific receptor is activated by the action of proteases, mainly MMPs; this event triggers the angiogenic switch during carcinogenesis (46). In

**FIGURE 8.** ADAMTS1 affects the location of TFPI-2 in tumor xenografts. A, a tumor lysate (500 μg of total protein) from different xenografts in the absence or presence of ADAMTS1 and TFPI-2 were heparin-purified and analyzed by Western blot for the presence of these molecules with the respective polyclonal antibodies. The determination of loading levels.

B. ADAMTS1: TFPI2: A, +, +, +, +, - p110 p87 ADAMTS1 pAb ADAMTS1 pAb TFPI2 pAb TFPI2 pAb actin actin Ab

C. ADAMTS1: TFPI2: A, +, +, +, +, - p110 p87 ADAMTS1 pAb ADAMTS1 pAb TFPI2 pAb TFPI2 pAb actin actin Ab

D. ADAMTS1: TFPI2: A, +, +, +, +, - p110 p87 ADAMTS1 pAb ADAMTS1 pAb TFPI2 pAb TFPI2 pAb actin actin Ab

**A.** ADAMTS1: TFPI2: A, +, +, +, +, - p110 p87 ADAMTS1 pAb ADAMTS1 pAb TFPI2 pAb TFPI2 pAb actin actin Ab

**B.** ADAMTS1: TFPI2: A, +, +, +, +, - p110 p87 ADAMTS1 pAb ADAMTS1 pAb TFPI2 pAb TFPI2 pAb actin actin Ab

**C.** ADAMTS1: TFPI2: A, +, +, +, +, - p110 p87 ADAMTS1 pAb ADAMTS1 pAb TFPI2 pAb TFPI2 pAb actin actin Ab
our case, the presence of the inhibitor TFPI-2 appears constant and, in theory, responsible to maintain an adequate proteolytic balance. A subsequent up-regulation of ADAMTS1 causes TFPI-2 re-location and a consequent alteration of the proteolytic balance. As mentioned, TFPI-2 is secreted by various cell types and is mainly deposited in the ECM. Expression of this inhibitor appears to be required for appropriate adhesion and maintenance of endothelial cell cultures (28) and also participates in the process of vascular mimicry observed on aggressive melanoma (23). Interestingly, ADAMTS1 was found to display antiangiogenic activity targeting endothelial cells (5). Our data confirm the participation in the inhibitory properties of the protease, not just in a angiogenic activity targeting endothelial cells (5). Regarding other metalloproteinase inhibitors (52), such as TIMPs (time inhibitor of matrix metalloproteinases), the related ADAMTS1 Cleaves and Alters TFPI-2 Extracellular Location

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