Thrombospondin Type 1 Repeats Interact with Matrix Metalloproteinase 2

REGULATION OF METALLOPROTEINASE ACTIVITY*

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Thrombospondins are thought to function as inhibitors of angiogenesis. However, the mechanism(s) of this activity is not well understood. In this study, we have used the yeast two-hybrid system to identify proteins that interact with the thrombospondins 1 (TSP1) and 2 (TSP2) propeptide-like type 1 repeats (TSR). One of the proteins identified that interacted with both TSR was matrix metalloproteinase 2 (MMP2). The isolated MMP2 cDNA clone encoded amino acid residues 237–655, which include the fibronectin-like gelatin binding region flanking the catalytic center and the carboxyl hemopexin-like region. Further testing of this clone demonstrated that the TSR interacted with the NH₂-terminal region of the MMP2 that contains the catalytic domain. The protein interaction observed in yeast was further demonstrated by immunoprecipitation and Western blotting using purified intact TSP1, TSP2, MMP2, and MMP9. Although MMP2 interacted with TSP1 and TSP2 via its gelatin-binding domain or a closely mapping site, neither TSP1 nor TSP2 was degraded by MMP2 in vitro. Tissue culture and in vitro assays demonstrated that the presence of purified TSR and intact TSP1 resulted in inhibition of MMP activity. The ability of TSP1 to inhibit MMP3-dependent activation of pro-MMP9 and thrombin-induced activation of pro-MMP2 suggests that the TSR may inhibit MMP activity by preventing activation of the MMP2 and MMP9 zymogens.

The thrombospondin (TSP) family of proteins includes at least five related extracellular matrix glycoproteins encoded by separate genes (1). Each thrombospondin contains NH₂- and COOH-terminal globular domains flanking different structural modules (Ref. 2; Fig. 1A). The modules common to all TSP proteins include epidermal growth factor-like type 2 and Ca²⁺-binding type 3 repeats. In addition to these common features, TSP1 and TSP2 share two additional modules: a procollagen homology region and three propeptide-like type 1 repeats (TSR).

The NH₂ and COOH termini contain regions that are low in cysteine content, do not possess internal repeating motifs, and do not show homology with other proteins (3). The amino-terminal domain is largely responsible for the heparin binding properties of thrombospondins (4, 5). The procollagen region contains a region homologous to a cysteine-rich domain in the NH₂-terminal propeptide of the α₁-chain of type 1 procollagen (6). The type 1 repeats are homologous to sequences in a variety of proteins found in protozoans (7–9), invertebrates (10), and mammals (11–16).

Thrombospondin 1 has been the most extensively studied TSP protein to date. TSP1 contains a growing number of sites that have been implicated in interactions with more than 30 cell surface and matrix proteins, including structural proteins (e.g. collagen and fibronectin), cell surface receptors (e.g. integrins, syndecans, and CD36), enzymes (e.g. elastase and plasmin), and cytokines (e.g. transforming growth factor-β1) (17). Because of its ability to interact with such a wide variety of proteins, TSP1 has been implicated in a number of biological processes including coagulation, cell adhesion, cell growth, modulation of cell-cell and cell-matrix interactions, control of tumor growth and metastases, and angiogenesis (18–21). However, the relative importance and physiological roles of these interactions are poorly understood.

TSP1 was initially recognized as an inhibitor of angiogenesis with the isolation from the medium of the hamster cell line BHK21/c113 of a 140-kDa protein capable of inhibiting neovascularization induced by angiogenic factors (22). Subsequent structure-function studies (23) localized the anti-angiogenic region of TSP1 to the procollagen-like region and the TSR. Intact TSP2 has also been shown to inhibit angiogenesis in vitro and in vivo (24, 25). Recently, examinations of mice with a disrupted TSP2 gene (26) demonstrated increased tissue vascularity, consistent with the proposed anti-angiogenic activity of TSP2. Since TSP2 shows poor sequence homology to TSP1 in the procollagen region but good matches in the TSR region, it has been suggested that the anti-angiogenic activity of TSP2 maps to the TSR (27). Previous studies showed that the sequence CSVTCG within the TSR of TSP1 interacts with the surface receptor glycoprotein CD36 (28) and mediates the in vitro and in vivo inhibitory effects of TSP1 on endothelial cells (29, 30). However, although peptides containing the sequence VTCG were the most effective in their anti-angiogenic properties, corresponding peptides lacking VTCG and different peptides derived from the procollagen region were also active. Furthermore, recent studies showed that the TSP1 TSR inhibited epithelial cell proliferation and migration in a CD36-independent manner (31). These observations led to the conclusion that more than one structural domain and possible mechanism are involved in the inhibition of neovascularization by TSP1.

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† The abbreviation used are: TSP, thrombospondin; TSR, TSP1 and -2 propeptide-like type 1 repeat(s); BAEV, bovine aortic endothelial cells; PACE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; APA, p-aminohippuric acid; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyraoside; hTSP1, human platelet TSP1; hMMP2, human MMP2; PBS, phosphate-buffered saline.

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The pAS2-TSP2-type 1 construct was used for screening the cDNA that contained approximately 55 amino acids each, as a bait for screening an activation domain-cDNA translational fusion library using the yeast two-hybrid system. The obtained clones were then screened with a TSP1 type 1 repeat construct to identify proteins interacting with both TSP2 and TSP1 or only TSP2. One such protein, interacting with both thrombospondins, is MMP2. The binding is mediated by residues 237–445 of MMP2, which include the fibronectin-like gelatin binding domain and catalytic center domain, and results in inhibition of MMP catalytic activity.

**MATERIALS AND METHODS**

**Cells and Media**—The Saccharomyces cerevisiae strain Y190 (CLONTECH) was used for cDNA library screening and for direct testing of protein-protein interactions. The cells were grown at 30 °C either in rich YPD medium or minimal media with appropriate amino acid supplements. The minimal media were SD–Trp, in which tryptophan was omitted; SD–Leu, in which leucine was omitted; SD–Trp,Leu, in which tryptophan and leucine were omitted; and SD–Trp,Leu,His, in which tryptophan, leucine, and histidine were omitted.

The cell line ECV304 was obtained from ATCC (Manassas, VA). Bovine aortic endothelial cells (BAEC) were isolated from aorta obtained from a local slaughterhouse. Cells were maintained in M199 (ECV304) or Dulbecco's modified Eagle's medium (BAEC) supplemented with 10% heat-inactivated fetal bovine serum, nonessential amino acids, 2 mM glutamine, 100 μM β-mercaptoethanol, and 1 mM 3-azoni-1,2,4-triazole and tested at least twice for color reactivity in the β-galactosidase colony filter assay. The present study was undertaken to systematically identify proteins interacting with both TSP2 and TSP1 or only TSP2. One such protein, interacting with both thrombospondins, is MMP2. The binding is mediated by residues 237–445 of MMP2, which include the fibronectin-like gelatin binding domain and catalytic center domain, and results in inhibition of MMP catalytic activity.

**Plasmid Constructs**—Constructs for use in the yeast two-hybrid system were based on the MATCHMAKER GALA two-hybrid system 2, which allows using vectors pAS1 and pACT2 (CLONTECH). The pAS2–1 vector carries the nutritional marker TRP1 and allows the design of constructs, referred to as the bait, for the expression of fusion proteins carboxy to the GALA(1–147) DNA binding domain. The pACT2 vector carries the nutritional marker LEU2 and allows the design of constructs, referred to as the prey, for expression of fusion proteins carboxy to the GALA(484–851) activation domain. To construct a bait with a second reading frame, the vector pAS2 (CLONTECH) was transformed into Y190 cells and the cells were harvested, resuspended in an electrophoresis sample buffer for SDS-PAGE analysis.

**Immunoprecipitation and Western Blotting**—A tube containing purified human platelet TSP1 (0.3 μg; Calbiochem) or in vitro translated murine TSP2 and purified MMP2 or MMP9 (0.1 μg; Roche Molecular Biochemicals) was incubated at 4 °C in 1× PBS for 4 h. The solution was precleared by adding 20 μl of protein G-Plus/protein A-agarose beads (Calbiochem) or protein A-agarose beads (Sigma) for 1 h at 4 °C. The supernatant was incubated at 4 °C for 16 h in the presence of anti-MMP2 or anti-MMP9 antibody (1:200; Trippe Point Biologies) and protein G-Plus/protein A or protein A-agarose beads. The beads were washed, precipitated, washed repeatedly with PBS, and resuspended in electrophoresis sample buffer for SDS-PAGE analysis.

**Production of Recombinant Protein**—The yeast strain DY150 (CLONTECH) was transformed with pYEX 4T-1 (vector), pYEX-TSP1-type 1, or pYEX-TSP2-type 1 repeat constructs. Transformants were grown at 30 °C, as recommended by the supplier, to 0.5–1.0 A600. The culture was supplemented with copper sulfate to a final concentration of 0.5 mM and further grown for 1 h. The cells were harvested, resuspended in the lysis buffer containing acid-washed glass beads, and disrupted by vigorous vortexing. Cell extract was collected, and GST and GST-tagged proteins were purified using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech).

The purified protein products were analyzed by SDS-PAGE and Western blotting as described above. The TSP1 and TSP2 sera were detected using the anti-human TSP1 antibody kindly provided by Dr. J. Lawler and anti-mouse TSP2 antibody (a kind gift of Dr. P. Bornstein, University of Washington, Seattle, WA).

**Enzymatic Assays**—Pro-MMP2 (Roche Molecular Biochemicals) was activated at 37 °C for 30 min with 1 mM p-aminophenylmercuric acetate (APMA). Alternatively, active MMP2 (Calbiochem) was used for enzymatic assays. Enzymatic assays were performed using H-labeled gelatin. The purified protein products were analyzed by SDS-PAGE and Western blotting as described above. The TSP1 and TSP2 sera were detected using the anti-human TSP1 antibody kindly provided by Dr. J. Lawler and anti-mouse TSP2 antibody (a kind gift of Dr. P. Bornstein, University of Washington, Seattle, WA).
TSP-MMP2 Interactions

RESULTS

Yeast Two-hybrid Screening—To identify proteins that bind to the TSP2 TSR, we screened a NIH 3T3 cDNA translational library using the yeast two-hybrid system. Before proceeding with the screening, the bait plasmid, which contains the TSP2 TSR was tested for its inability to activate the prototrophic reporter gene HIS3. During the library screen, protein interaction was detected by the expression of the reporter genes HIS3 and the color indicator lacZ. Thirty-nine clones showed lacZ expression on X-gal indicator filters. These clones were then counter-selected in Leu - medium supplemented with cycloheximide that favors clones carrying the library insert due to the presence of the CYH2 gene, which confers sensitivity to cycloheximide, in the pAS2–1 vector. Plasmid DNA was isolated from each of the 39 positive yeast clones. Sequencing analyses of the inserts showed that three clones encoded fibronectin, two encoded lysyl oxidase-like protein, and the remaining 34 clones represented independent genes, 16 of which could be identified on the basis of available sequence. The present study focuses on one of the known interactors identified, MMP2.

TSP1 and TSP2 TSR Bind MMP2—The isolated MMP2-encoding cDNA clone contained amino acid residues 237–633 of the mouse MMP2 sequence (Ref. 35; Fig. 1B). The NH2-terminal residue of the MMP2 cDNA clone isolated from the library localizes inside the first repeat of the fibronectin-like segment inserted in the MMP2 protein. Thus, the clone contains the next two complete 58-residue repeats.

In order to examine if the ability of TSP2 TSR to bind to MMP2 was also shared by TSP1 TSR, the corresponding TSP1 cDNA was inserted in frame in the pAS2–1 vector and tested using the yeast two-hybrid system. Similar to TSP2 TSR, the TSP1 TSR-containing plasmid construct conferred on Y190 cells the ability to transactivate the reporter genes HIS3 and lacZ (Table I).

To further map the MMP2 region that binds to the TSP1 and TSP2 TSR, we employed two prey constructs, pACT-MMP2-(237–445) and pACT-MMP2-(440–633). These constructs encode fusion proteins between the GAL4 activation domain and the NH2-terminal half of the MMP2 clone obtained, which...
includes the fibronectin-like gelatin-binding domain along with the catalytic center, and the COOH-terminal half of MMP2, which includes the hemopexin-like domain, respectively (Fig. 1B). There was no detectable interaction with the carboxyl hemopexin-like end, whereas both the TSP1 and TSP2 TSR interacted with the NH2-terminal half of MMP2 (Table I). These studies suggested that both TSP1 and TSP2 TSR interact with MMP2 and that residues within the MMP2 catalytic domain are responsible for this interaction.

To verify that the interaction detected using the yeast two-hybrid system occurs outside of yeast and to show its relevance within the context of the intact protein, we studied the interaction of purified intact human platelet TSP1 (hTSP1) or within the context of the intact protein, we studied the interaction of purified intact human platelet TSP1 (hTSP1) or purified intact human platelet TSP2 (hTSP2) and translated mouse TSP2 and human MMP2 (hMMP2) or the closely related human MMP9 (hMMP9) in vitro binding assays. Immunoprecipitation of in vitro mixture of hTSP1 and hMMP2 or hMMP9 with anti-hTSP2 or anti-hMMP9 antibody, respectively, followed by immunoblotting with anti-hTSP1 antibody demonstrated the presence of TSP1 protein when both TSP1 and hMMP2 or hMMP9 were presented in the mixture (Fig. 2A). TSP1 associated with MMP2 (left panel) or MMP9 (right panel), and thus it was immunoprecipitated with anti-MMP2 or anti-MMP9 antibody, respectively. A control TSP1 sample that was not subjected to immunoprecipitation (Fig. 2A, control) was included to confirm the size and specificity of the band detected. In order to explore in vitro interactions of TSP2 and MMP2 or MMP9, a full-length TSP2 protein was generated by in vitro transcription and translation in the presence of [35S]methionine. Radiographic analysis of the translation products showed a band of ~130–150 kDa, consistent with the expected size of the unglycosylated intact TSP2. Similar to TSP1, in vitro translated TSP2 was precipitated using anti-MMP2 or anti-MMP9 antibody from a mixture of TSP2 and MMP2 (Fig. 2B). These results confirm the interaction between MMP2 and the TSR detected in the yeast two-hybrid system and confirm that TSP1 and -2 bind to MMP2 and MMP9.

**TABLE I**

**TSR interactions with MMP2**

Interaction of TSR with MMP2 in yeast as measured by colony growth and β-galactosidase filter assay. The yeast strain Y190 was co-transformed, sequentially, with the plasmid constructs indicated. Transformed cells were plated on minimal medium lacking Trp, Leu, and His and containing 45 mM 3-azo-1,2,4-triazole. Protein-protein interaction was determined by colony growth followed by an assay for β-galactosidase activity. +, interaction; −, no interaction.

| Bait                  | Prey          | TSP1 | MMP2 | TSP1 | MMP2 |
|-----------------------|---------------|------|------|------|------|
| pAS2-1 (vector)       | −             | −    | −    | −    | −    |
| pAS2-TSP1-type 1 repeat | −           | +    | −    | +    | −    |
| pAS2-TSP2-type 1 repeat | −           | +    | −    | +    | −    |

**Fig. 2. Interaction between MMP2 or MMP9 and TSP1 or TSP2 as demonstrated by immunoprecipitation and Western blotting or fluorography.** Purified intact human TSP1 (0.3 μg) or in vitro translated TSP2 and purified MMP2 or MMP9 (0.1 μg) were incubated in PBS at 1 °C as described under “Materials and Methods.” Material bound to the beads was eluted with SDS-PAGE sample buffer containing 10 mM dithiothreitol and resolved by SDS-PAGE. The position of TSP1 and -2 is indicated (arrow). A, Western blotting. Samples immunoprecipitated with anti-MMP2 (left panel) or anti-MMP9 (right panel) antibodies were blotted with anti-TSP1 antibodies. A sample of TSP1, not subjected to immunoprecipitation, was included as a control for size of band and antibody specificity. B, fluorography of samples, in vitro translated TSP2 antibody plus MMP2 (left panel) and in vitro translated TSP2 plus MMP9 (right panel) immunoprecipitated with nonimmune rabbit serum (lane 1) and anti-MMP2 or anti-MMP9 antibodies (lanes 2).

**Fig. 3. Lack of TSP1 and TSP2 degradation by MMP2.** A, intact TSP1 (0.1 μg) was incubated in the absence or presence of activated MMP2 (0.1 or 0.4 μg) at 37 °C for 4 h, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membrane. The membrane was immunoblotted with anti-human TSP1 antibody. B, in vitro translated TSP2 (1 μl) was incubated in the absence or presence of activated MMP2 (0.1 or 0.4 μg) at 37 °C for 4 h and resolved by SDS-PAGE. The gel was impregnated with EN3HANCE (PerkinElmer Life Sciences) and visualized by fluorography. C, in vitro TSP2 enzymatic assay. Pro-MMP2 (Roche Molecular Biochemicals) and pro-MMP2 activated with APMA (50 ng/reaction tube) were incubated with heat-denatured [3H]collagen type 1, as described under “Materials and Methods.” At the indicated time points, the reactions were terminated, kept on ice until all samples were collected, and analyzed for trichloroacetic acid-soluble [3H]labeled products.

MMP2 enzyme preparation was functional, a parallel enzymatic assay was run using [3H]-labeled gelatin as a substrate. As expected, gelatin was degraded by activated MMP2, i.e. MMP2 plus APMA, in a time-dependent fashion (Fig. 3C). Thus, al-
though TSP1 and -2 bind to or close to the gelatin-binding region of MMP2, unlike gelatin, TSPs are not degraded as a result of this binding.

The TSR and Intact TSP1 Inhibit Collagenase Activity of Cultured Cells—The MMPs are believed to play an important role in angiogenesis, since both synthetic and natural inhibitors of MMPs inhibit this process (37–39). Given the role of thrombospondins 1 and 2 as suppressors of angiogenesis, we next explored whether TSP1 and TSP2 could, in part, achieve this effect by inhibiting metalloproteinase activity. To this end, we assessed the ability of ECV304 cells to degrade 3H-labeled collagen type IV, which is a degradation product of gelatin. Human recombinant TSP1 and TSP2 were added to the cell culture medium of ECV304 or BAEC cells, and the ability of the cells to degrade 3H-labeled collagen type IV was assessed by determining the amount of degradation in the presence or absence of recombinant fusion protein (1 μg/ml) or recombinant TSP1. Human recombinant TSP1 was added to the culture medium (50,000 dpm/well). After 16 h, the supernatant medium was collected and precipitated with trichloroacetic acid. The radioactivity in the trichloroacetic acid-soluble fraction was determined.

Cultured Cells—We assessed the ability of ECV304 cells to degrade 3H-labeled collagen type IV, which is a degradation product of gelatin. Human recombinant TSP1 and TSP2 were added to the cell culture medium of ECV304 or BAEC cells, and the ability of the cells to degrade 3H-labeled collagen type IV was assessed by determining the amount of degradation in the presence or absence of recombinant fusion protein (1 μg/ml) or recombinant TSP1. Human recombinant TSP1 was added to the culture medium (50,000 dpm/well). After 16 h, the supernatant medium was collected and precipitated with trichloroacetic acid. The radioactivity in the trichloroacetic acid-soluble fraction was determined.

A time course analysis of supernatant medium collected from BAEC (Fig. 5A, lanes 1–3) showed that the BAEC cells secreted a substantial and small amount of MMP2 and MMP9, respectively. Small amounts of low M ω, MMP9 and MMP2, in particular the intermediate (64 kDa) and active (62 kDa) MMP2 species, were also detected. In the presence of TSP1, the generation of the low M ω species was inhibited (Fig. 5A, lanes 1–3 with lanes 4–6). To further examine the ability of TSP1 to inhibit MMP2 activation, we studied thrombin-induced gelatinase activity by MMP2 in BAEC, in the presence and absence of TSP1 (Fig. 5B). Thrombin-treated BAEC showed higher gelatinase activity than untreated cells. This thrombin-induced enhanced rate of gelatin degradation was inhibited in the presence of TSP1. These observations, together with the discrepancy between the ability of TSR recombinants and purified TSP1 to suppress collagenase activity in cell culture and the lack of direct inhibition of collagenase activity in in vitro assays with purified proteins (Fig. 4) suggested that TSP protein may be involved in
shown by its ability to degrade $^{3}$H-labeled gelatin. The level of presence, of MMP3, pro-MMP9 was gradually activated as enzyme assay in solution (Fig. 6). In the presence, but not in the activation of pro-MMP9 by MMP3 was examined using a gelatin-in vitro

that MMP2 and MMP9 enzyme secretion by BAEC cells cultured in the absence (control) or presence of TSP1 protein (TSP1-treated). BAEC were seeded in 48-well plates and allowed to grow in 10% fetal bovine serum-containing medium for 16–48 h. The monolayer was washed twice with PBS, and serum-free medium added. After a 16-h incubation, the medium was removed and washed once with PBS, and fresh serum-free medium without or with TSP1 (25 $\mu$g/ml) added. At the indicated time points, 15-$\mu$l aliquots of supernatant medium were collected and analyzed for MMP2 and MMP9 enzyme levels by gelatin zymography. A, inhibition of thrombin-induced gelatinase activity by TSP1 (3 $\mu$g/ml). BAEC were cultured in six-well plates as described for Fig. 4C in the presence of heated rat $^{3}$H-collagen type 1 substrate. After preincubating for 30 min in the presence or absence of TSP1, thrombin (100 nM) was added to the wells. After a 24-h incubation, the radioactivity in the trichloroacetic acid-soluble fraction was determined.

regulation of metalloproteinase activity by inhibiting zymogen activation.

MMP2 and MMP9 share many similarities including the presence of a gelatin-binding region inserted in the catalytic domain in contrast to other MMPs. Since BAEC cells secrete low levels of MMP9 and the generation of the active species could not easily be demonstrated, we took advantage of the known ability of MMP3 to activate MMP9 in vitro by cleaving the pro-MMP9 protein (40, 41). A time course of in vitro activation of pro-MMP9 by MMP3 was examined using a gelatinase assay in solution (Fig. 6). In the presence, but not in the absence, of MMP3, pro-MMP9 was gradually activated as shown by its ability to degrade $^{3}$H-labeled gelatin. The level of pro-MMP9 activation by MMP3 was inhibited in the presence of TSP1. In addition, MMP3-dependent activation of MMP9 was inhibited by TSP1 in a dose-dependent manner (Fig. 7). These results plus the patterns of MMP activation observed in BAEC in tissue culture (Fig. 5) suggest that inhibition of the accumulation of trichloroacetic acid-soluble $^{3}$H-gelatin was due to reduced generation of active MMP2 and MMP9 species.

DISCUSSION

Thrombospondins 1 and 2 share a number of structural similarities and have been reported to bind to a number of cell surface membrane and matrix proteins (17, 42). In addition, unlike TSPs 3, 4, and 5, TSPs 1 and 2 inhibit angiogenesis. The anti-angiogenic region has been localized to the second and third repeats of the TSR (43). Despite these common features, the TSP1 and TSP2 knockout mice exhibit different phenotypes. Thus, the lack of TSP2 expression in the presence of normal levels of TSP1 expression was associated with disordered collagen fibrillogenesis and an increase in blood vessel count in the skin and other tissues (26). Furthermore, these tsp2−/− mice demonstrate accelerated wound healing and altered foreign body reaction in the context of increased angiogenesis (26, 44). These and other changes are consistent with increased MMP activity.2 On the other hand, lack of TSP1 expression resulted in abnormalities primarily in the lungs with an increase in neutrophils and macrophages staining for hemosiderin, suggestive of diffuse alveolar hemorrhage (32).

To further define protein interactions that might underlie angiogenesis inhibitor actions of TSP2, we used the yeast two-hybrid system to identify proteins that interact with the TSP2 TSR. This technique, which is capable of detecting very weak interaction, $K_d$ of $10^{-6}$ (45), permitted us to identify 36 independent clones. Since the interactions include TSP1-interacting proteins that were not identified previously, the yeast two-hybrid analysis suggests that the list of known proteins that interact with TSP1 is far from complete.

One of the proteins identified in the screen was MMP2. This enzyme is of particular interest because of its demonstrated involvement in regulation of angiogenesis. Specifically, a num-

\[ 2 \text{ P. Bornstein, personal communication.} \]
ber of studies have documented that activation of MMP2 is associated with robust new vessel growth, while inhibition of its activity leads to depressed angiogenesis (46, 47). Given the high degree of sequence homology between the TSP of TSP1 and TSP2, we have investigated whether TSP1 TSR also interacts with MMP2. Similar to TSP2, the yeast two-hybrid assay demonstrated TSP1-MMP2 binding. The specificity of the yeast two-hybrid interactions between TSP1, TSP2, and MMP2 was confirmed in co-immunoprecipitation experiments with both thrombospondins and the MMP2.

To further clarify the nature of TSP-MMP2 interaction and the effect it has on the MMP2 activity, we have determined the region of MMP2 involved in TSP binding. Yeast two-hybrid analysis determined that the MMP2 site binding to TSP1 and TSP2 TSR localizes to amino acid residues 237–445 of MMP2. This region of MMP2 encompasses the catalytic and the gelatin-binding domains. Structural similarities between MMP2 and MMP9 catalytic domains motivated us to investigate TSP binding to the latter enzyme. Co-immunoprecipitation studies with purified TSP1 and MMP9 protein confirmed direct interaction between the two proteins. Binding to the catalytic region of MMP2 might suggest that these proteins serve simply as MMP2 substrates. However, direct studies of TSP1 and TSP2 stability suggested that the proteins were not degraded even in the presence of high levels of activated MMP2.

The addition of purified intact TSP1 or TSP1 and TSP2 TSR fusion proteins to cells in culture inhibited collagenase activity, demonstrating the inhibitory nature of MMP-TSP interaction. However, the addition of TSP2 did not inhibit enzymatic activity of a purified, activated MMP2 in an in vitro assay. Studies were conducted to investigate the role of TSP-MMP interaction in inhibiting activation of pro-MMP2 or pro-MMP9 proteins. Partial support for this hypothesis comes from observation of decreased amounts of active MMP9 and MMP2 species in BAEC cells treated with TSP1. The presence of TSP1 inhibited endogenous and thrombin-induced activation of MMP2. Previous studies (48) reported that thrombin represents a physiological activator of MMP2 in human endothelial cells. Consistent with our observation, endothelial cells from TSP1 null mice show lower levels of active MMP2. To obtain a more direct confirmation for the proposed hypothesis, we studied the effect of purified TSP1 on activation of pro-MMP9 by MMP3. In these assays, we found that the rate of MMP3-dependent activation of MMP9, as measured by radiometric gelatinase activity in solution, was reduced in the presence of TSP1. Taken together, these observations suggest that TSP1 and -2 can inhibit generation of active gelatinase (MMP2 and MMP9) species.

In summary, TSP1 and TSP2 regulate MMP2 and MMP9 enzyme activity by inhibiting conversion of the MMP zymogen to the activated form. Such a regulatory mechanism might be biologically significant. The ability of the TSPs to modulate MMP2 and MMP9 enzymes at the activation step might represent an enzymatic control point that would not only contribute to the simultaneous regulation of the degradation of various downstream substrates but also inhibit several possible upstream activators. This regulation of metalloproteinase activity by the TSPs probably contributes to the anti-angiogenic activity of these proteins.

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