Controlled extracellular proteolysis of thrombospondins

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Abstract

Limited proteolysis of thrombospondins is a powerful mechanism to ensure dynamic tuning of their activities in the extracellular space. Thrombospondins are multifunctional matricellular proteins composed of multiple domains, each with a specific pattern of interactions with cell receptors, matrix components and soluble factors (growth factors, cytokines and proteases), thus with different effects on cell behavior and responses to changes in the microenvironment. Therefore, the proteolytic degradation of thrombospondins has multiple functional consequences, reflecting the local release of active fragments and isolated domains, exposure or disruption of active sequences, altered protein location, and changes in the composition and function of TSP-based pericellular interaction networks. In this review current data from the literature and databases is employed to provide an overview of cleavage of mammalian thrombospondins by different proteases. The roles of the fragments generated in specific pathological settings, with particular focus on cancer and the tumor microenvironment, are discussed.
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Introduction

Limited processing by proteases is an important post-translational mechanism modulating the function of extracellular molecules, particularly proteins containing multiple domains and active sites, such as matrix components and the matricellular proteins thrombospondins, ensuring a rapid and effective response to changes in the microenvironment [1,2]. Proteolytic processing can induce the disruption or de-novo exposure of active sites, the release of active fragments, and changes in protein conformation and localization, resulting in loss, gain or change of function of substrata. An example is the release of bioactive fragments, referred to as matrikines and matricryptins, whose biological properties differ from those of the whole molecule [3].

The matricellular proteins thrombospondins (TSPs) are major mediators of cell interactions with the environment in several processes during development and in pathological events such as cancer. Mammalian TSPs are a family of five multimeric, modular, calcium-binding glycoproteins, structurally divided into two subgroups [4]. TSP-1 and TSP-2 form homotrimers (subgroup A), and TSP-3, TSP-4 and TSP-5/COMP form homopentamers (subgroup B, Fig. 1).

A common feature of all the TSPs is a conserved C-terminal cassette – considered the signature domain of TSPs – containing variable numbers of EGF-like type 2 repeats (E), the calcium-binding TSP type 3 repeats (Ca) and a globular C-terminal region (G) structurally homologous to the L-type lectin domain. The entire C-terminal region functions as a single folded unit, stabilized by multiple interactions between the involved domains [4,5].

The amino-terminal region (N) of TSPs is more variable, with the laminin-G like domain being the most conserved feature. TSP5/COMP lacks a distinct *N*-terminal domain. In TSP-1 and TSP-2 the *N*-terminal domain is followed by an oligomerization domain, a von Willebrand Factor type C/ procollagen domain (VWFC), and three type 1 repeats (P, also called properdin or thrombospondin repeats, TSRs).

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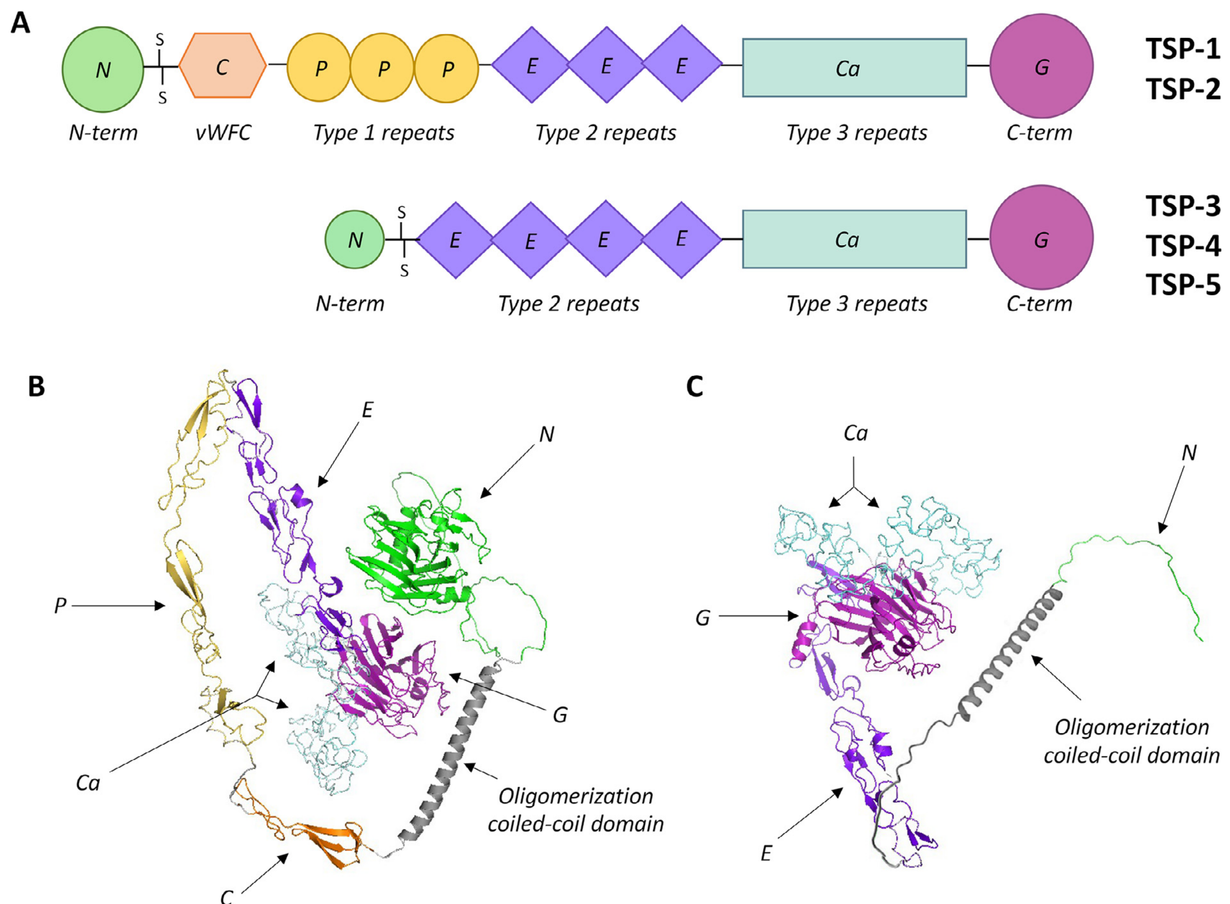

Fig. 1. Structure of TSPs. (A) Modular structure of the subgroup A (TSP-1 and TSP-2) and subgroup B (TSP-3, TSP-4 and TSP-5/COMP) monomers. TSPs in each subgroup share similar domain organization, except for a smaller *N*-terminal domain (N) in COMP. (B, C) Three-dimensional structures of monomeric TSP-1 (AF-P07996-F1, B) and COMP (AF-P49747-F1, C) based on AlphaFold predictions [165,166]. Colors were assigned to each domain as in (A) using PyMOL Molecular Graphics System (Version 2.5.4 Schrödinger, LLC). The structure is affected by oligomerization, and binding to ligands and calcium.

Pentameric thrombospondins lack VWFC and TSRs.

Oligomerization of TSP subunits, mediated by coiled-coil regions, provides TSPs with multivalency properties playing a pivotal role in their activity, as supported by the relative transience of monomeric isoforms in TSPs' evolution [6]. Oligomerization is crucial for ECM incorporation of TSPs [7,8], for TSP-1 and TSP-2 induction of cell spreading and fascin spike organization [9], and for TSP-4 activity in matrix assembly [10].

The multiplicity and complexity of activities derive from the modular nature of these matricellular molecules, composed of multiple domains, each with a different pattern of interacting ligands and activities [11]. The cellular and molecular composition of defined biological settings dictates the bioavailability of the active sites, hence modulating the activity of TSPs.

Proteases are potent controllers of TSPs, particularly those acting in the extracellular space, as they cleave TSPs at specific sites releasing fragments which, by interacting with selective ligands, exert diverse activities in a context-dependent manner. Intracellular proteolysis of TSPs – upon their endocytic uptake – also occurs, usually as a process of complete degradation rather than limited proteolysis, contributing to regulation of their presence in the ECM [12,13].

Early studies recognized TSPs as targets for proteases. The first evidence of TSP-1 as a major inhibitor of angiogenesis [14] was the identification of a tumor suppressor antiangiogenic molecule as a fragment of TSP-1, already pointing to limited proteolytic processing of TSP-1 as an important mechanism of functional regulation. Initial studies exploited this sensitivity of TSPs to site-restricted proteolysis to characterize the domains' activity. For instance, chymotrypsin- and thrombin-generated fragments served to identify antiangiogenic sites in a large, 140 kDa C-terminal fragment [15–17] and pro-angiogenic sites in the *N*-terminal domain [18]. Site-

restricted proteolysis of TSP-1 was also instrumental in identification of the TGF-β activating site in the type 1 repeats [19], and the pro-adhesive site in the 70 kDa-core fragment of the protein [20].

This review aims to provide a broad overview of the generation of TSP fragments by proteases and to illustrate how the proteolytic degradation of TSPs acts as a powerful mechanism to control their activity through the local selective release of bioactive fragments. Following the description of the major active sites on TSPs, necessary for the comprehension of the biological consequences of proteolytic cleavage, we focus on the formation of proteolytic fragments and their activity in different settings, with the main focus on angiogenesis and tumor progression, and mentioning some other physiological and pathological settings.

Major functions of TSPs in cancer

TSPs affect several aspects of tumor progression, acting directly on tumor cell functions or indirectly by shaping the tumor microenvironment (TME), where they control extracellular matrix deposition, angiogenesis and stroma cell functions, blood vessel formation and perfusion, and immune cell activity. The expression of TSPs, frequently altered in different cancer types, and mutations in the THBS genes are correlated with alterations in the tumor cell cycle and proliferation, epithelial-mesenchymal transition and immune cell infiltration [21]. TSPs exert both pro- and anti-tumor activities, depending on tumor stage and context. TSP-1 and TSP-2, the most thoroughly investigated, have tumor-restraining activity in the early stages but can promote progression at later, advanced stages [21–23].

The role of pentameric TSPs in biological settings and other pathologies has been reported [24–26], but less is known about their involvement in cancer. TSP-3 expression correlates with tumor progression and metastasis in osteosarcoma [27]. TSP-4 is upregulated in colorectal cancer [28], promotes hepatocellular carcinoma progression [29,30], and stimulates cancer cell proliferation by enhancing the cross-talk between cancer-associated fibroblasts and tumor cells [31]. COMP promotes thyroid carcinoma cell invasiveness [21]. TSP-4 and COMP, together with TSP-2, have been proposed as potential prognostic and diagnostic markers in gastric cancer [32].

Activity of TSP-1 and TSP-2 in cancer

Activity on the tumor cells

TSPs, particularly TSP-1 and TSP-2, affect tumor cell adhesion, invasion, differentiation, proliferation, and apoptosis. Effects on extracellular matrix composition, fibrillogenesis, mechanical properties and architectural organization indirectly control tumor cell adhesion, morphology, migration, and responses to chemical and mechanical changes of the environment [33]. TSPs enhance the migration of tumor cells, a crucial event in local invasion and metastasis [34,35].

Moreover, TSPs interact with proteins involved in the regulation of tumor cell proliferation, DNA repair and programmed cell death and regulate differentiation of cancer precursor cells, cell metabolism, responses to ischemic and genotoxic stress, cell senescence, autophagy and tumor cell responses to therapy [36,37]. Finally, TSP-1 has been identified as a mediator of dormancy of disseminated metastatic cells in the vascular niche [38].

Activity on the tumor microenvironment (TME)

Angiogenesis. TSP-1 was the first member of the family to be identified as an angiogenesis inhibitor [14,39]. Among other TSPs, TSP-2 has a similar domain structure and antiangiogenic function. TSP-1 and TSP-2 act through diverse domain-specific mechanisms, which include receptor-mediated direct effects on endothelial cells (apoptosis, inhibition of endothelial cell functions, suppression of nitric oxide signaling) and indirect effects mediated by TSP interaction with angiogenic factors (FGF2, VEGF, TGF-β), and proteases [40].

The discovery of pro-angiogenic sequences in the *N*-terminal domain of TSP-1 [18,41] indicated that TSPs can have context-dependent pro- and anti-angiogenic effects on angiogenesis, depending on their concentration, association with the ECM, the spatial and temporal expression of ligands, and the availability of active domains. Besides affecting sprouting angiogenesis, a proteolytic fragment of TSP-1 was also implicated in intussusceptive angiogenesis [42] and in vascular remodeling [43]. Vascular remodeling induced by the type 3 repeats domain promoted the distribution and activity of antineoplastic drugs in preclinical models [44].

Matrix organization. TSPs play pivotal roles in matrix formation and organization. Incorporation and retention of TSPs in the ECM are mediated by the C-terminal region, specifically the L-lectin domain and RGD site [8]. TSPs bind to diverse ECM proteins, such as fibronectin, collagen, laminin, matrilins, glycosaminoglycans, and to matrix-associated factors [11,45]. Binding to fibronectin controls TSP-1 incorporation into the matrix [46] and protects TSP-1 from degradation. Conversely, retention of TSPs into the matrix is affected by proteolytic processing [47]. TSP-1 can also promote the synthesis of ECM components, either directly, or through the activation of TGF-β [48]. TSP-1 and TSP-2 modulate collagen homeostasis and ECM assembly, regulating collagen fibrillogenesis and the levels of matrix-degrading MMPs [49].

Immune responses. TSPs are involved in both the adaptive and the innate immune response [50]. T cells produce TSP-1 and are induced to express TSP on the cell surface by T-cell receptor (TCR) stimulation [51]. In turn, TSP-1 affects T cells activation and function, mainly through CD47 [50,52,53]. TSP-1 was associated with cytotoxic supramolecular attack particles (SMAPs) released by cytotoxic T lymphocytes [54] and NK cells [55], where it contributes to CTL target killing.

TSP-1 exerts various context-specific activities also on cells of the innate immune response [50,56], acting on macrophages [57,58], NK cells [59] and myeloid-derived precursor cells [60]. TSP-1 affects also neutrophils, as the type 3 repeat domain limits neutrophil proteolytic activity [61,62].

Main active sites of TSPs

Many active sites have been identified in each TSP domain and their ligands and function(s) have been reported (Fig. 2). In some cases, these active sequences have been used as templates for drug design applications. In most cases, activities have been reconducted to single active sites and their interaction with specific ligands [11], but there are also examples of complex functions generated by coordinated action of several pathways activated by multiple sites in different TSP domains interacting with their respective cell receptors or ligands. For each TSP domain we provide a brief overview of the main active sites, their interactions and functions.

N-terminal heparin binding domain

This domain contains the main heparin-binding site of TSPs. It contains both pro-adhesive sequence, interacting with β1 integrins [63] and de-adhesive sequences, interacting with calreticulin [64].

The *N*-terminal domain exerts pro-angiogenic effects in endothelial cells by interacting with heparan sulfate proteoglycans, integrins, and calreticulin [18,41,65].

Sequences in this domain have been reported to be active in regulating the functions of T cells and innate immune cells [51,58,66,67]. An *N*-term containing fragment produced by monocytes undergoing apoptosis promoted dendritic cell phagocytosis and clearance of apoptotic cells [68].

VWFC/procollagen homology domain and properdin type 1 repeats

The VWFC or procollagen homology domain follows the oligomerization domain. Sequences in the type 1 repeats domain of TSP-1, but not TSP-2, bind and activate latent TGF-β [19,69] and induce a number of TGF-β-dependent activities including regulation of cancer cell proliferation and apoptosis [70], and promotion of mesenchymal stromal cell proliferation [71].

The type 1 repeats of TSP-1 and TSP-2 have anti-angiogenic activity and inhibit endothelial cell functions [15] by interacting with CD36 [72] or β1 integrins [73,74]. Peptides and recombinant fragments inhibited angiogenesis and tumor growth through different mechanisms in various preclinical

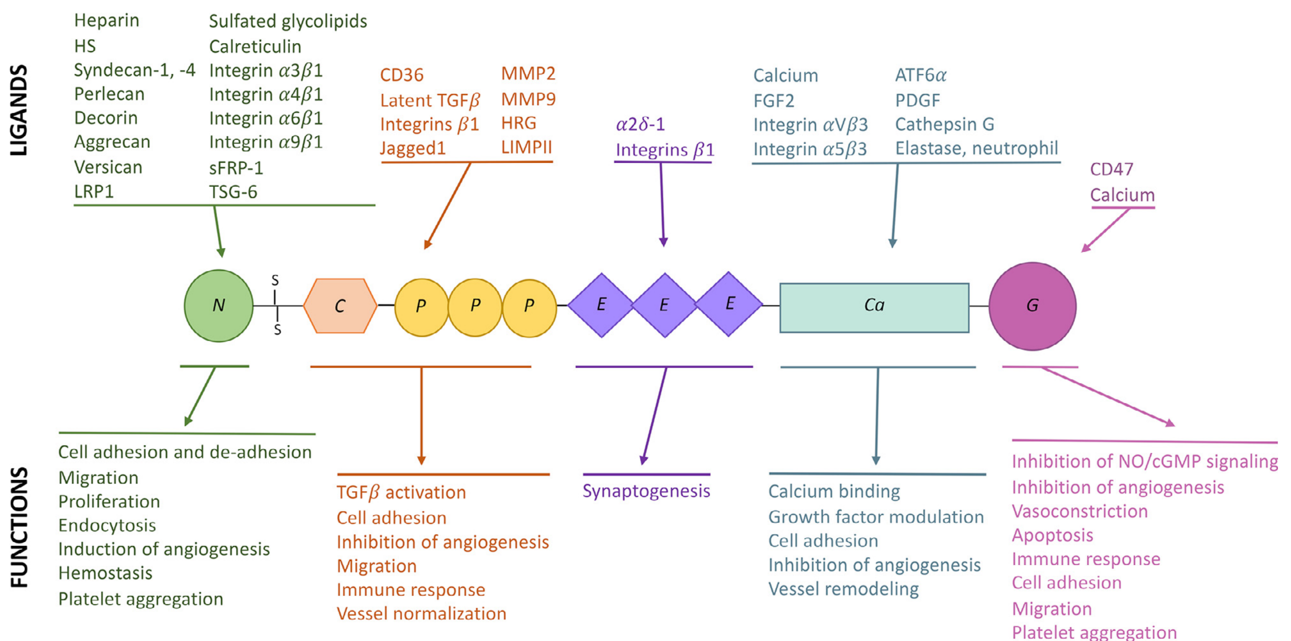

Fig. 2. Schematic representation of TSP-1 ligands and activities for each domain.

models [70,75,76]. The active sequences in the type 1 repeats have been used as models for the development of antineoplastic antiangiogenic compounds, in most cases in the form of modified synthetic peptides, including cyclic peptides [77], or D-reverse peptides, [78]. Examples of active compounds are the modified peptides ABT-510, ABT-526 and ABT-898 [79,80] and the CVX-22 and CVX-045, peptides linked to IgG1 antibody [81,82].

EGF-like type 2 repeats

The main activity of this domain is promotion of neural synapse formation. Interaction of this domain with the gabapentin receptor $\alpha 2\delta$-1 mediates the ability of astrocyte-secreted TSP to induce excitatory synapse formation [83] and is responsible for TSP-4 involvement in chronic pain [84]. Despite its similarity with EGF, this domain does not appear to bind to EGFR, but can activate EGFR signaling by an indirect mechanism involving MMP-9-mediated release of EGFR ligands [85].

Calcium-binding type 3 repeats

The distinctive feature of this domain is the presence of disulfide bonds between consecutive type 3 repeats and cooperative binding of calcium ions. The interaction with calcium and the status of disulfide bonds profoundly affect the structure of the domain, determining the exposure and availability of active sites, such as the integrin-recognition sequence RGD [86] and the binding sites for neutrophil elastase and cathepsin G [87,88].

This domain in TSP-1 and TSP-2 also interacts with FGF2, inhibits its bioavailability and function [17,89] and promotes tumor vascular remodeling [44]. The domain also interacts with PDGF, protecting it from degradation by proteases and promoting its activity on mesenchymal stromal cells [71].

C-terminal globular domain

The L-lectin domain is involved in TSP-1 retention in the ECM and cell attachment [8], and exerts several activities mediated mainly by its interaction with CD47 [50]. The 4N1K peptide has been identified as the TSP-1 binding site for CD47, although its role in CD47 recognition is still unclear since this motif is not exposed, hidden within a hydrophobic β-strand arrangement of the C-terminal domain, and either structural rearrangement or glycosaminoglycans might be involved [90].

TSP-1 interaction with CD47 affects several cell types. In endothelial cells it is involved in inhibition of nitric oxide/cGMP signaling and angiogenesis, and induction of vasoconstriction [91]. In tumor cells it mediates apoptosis [92], autophagy [93], and drug response [94]. In T cells it mediates migration [66], antigen-dependent activation and clonal expansion [52], and generation of regulatory T cells [95]. In innate immune cells it regulates response to inflammatory stimuli and tumor cell-killing and has both positive and negative effects on NK cell functions [50].

CD47 is considered a promising target for antineoplastic therapies with antibodies or peptides, with the aim of inducing immunogenic cell death of cancer cells, promoting tumor cell killing by macrophages, and overcoming drug resistance [96–98].

Proteolytic degradation of TSPs

Proteolytic enzymes cleave TSPs at distinctive sites, releasing fragments with diverse properties and activities. TSP-degrading proteases are produced by a number of cell types in specific biological settings and pathological states, associated with processes of wound healing, inflammation, immune response, bacterial infection, and angiogenesis-driven diseases. In cancer, proteases are released by both the transformed and non-transformed cells, including inflammatory cells, endothelial cells and tumor associated fibroblasts. Since these cells also release TSPs, it derives that in the TME as well as in non-tumor diseases, TSPs and TSP-degrading proteases are often found co-expressed – released by the same or different cell types– confirming the importance and biological relevance of TSP processing by proteases as a key posttranslational modification. For example, ADAM12, COMP, CTSK, FAP, and MMP2 were co-expressed with TSP-2 in breast cancer, and FAP was co-expressed with TSP-2 in lung and gastric cancer [99]. MMP1 and TSP-2 were part of a 4-gene expression signature predictive of local recurrence in patients with oral carcinoma [100], whereas MMP7, plasminogen, TIMP1 and TSP-2 were part of a panel of prognostic biomarkers in patients with pancreatic ductal adenocarcinoma [101]. TSP-1 colocalized with HTRA1 in RPE/Bruch's membrane area – the primary site for age-related macular degeneration pathogenesis [102].

Proteolysis of TSPs is tightly regulated at different levels. A main limiting step is the concentration and activation status of proteases, and the presence of their physiological inhibitors. Environment-mediated changes in TSP structure too can affect their sensitivity to protease. Conjugation with ligands (such as fibronectin or heparin), the concentration of calcium, pH, and the activity of disulfide isomerases can affect TSP structure and sensitivity to proteases [103]. TSP-1 polymorphisms are also involved: the S700 variant, associated with an increased risk of myocardial infarction, is more susceptible to trypsin and cathepsin G digestion than the N700 protein [104,105].

The data reported here were taken from the literature and public databases, including MEROPS

(https://www.ebi.ac.uk/merops/), BRENDA Enzymes (https://www.brenda-enzymes.org/), and Topfind 4.0 (https://topfind.clip.msl.ubc.ca/).

Details on the cleavage of TSPs by proteases are provided in Table 1 (a complete version of this table is presented as Supplementary Table 1). Fig. 3 gives a graphical representation of cleavage sites on TSPs. In many cases, the actual cleavage has been confirmed by the use of purified/recombinant TSPs and enzymes, and the cleavage site identified by sequencing analysis (confirmed cleavages are indicated by asterisks, in Table 1 and Fig. 3). With

Table 1. Cleavage of thrombospondins by proteases.

Substrate	Enzyme [a]		Cleavage Site [b,c]		Domain [d]	Fragments Released	Reference
TSP-1	ADAMTS-1	*	E311-L312	**	Olig - PC	trimeric-36 kDa *N* + 110 kDa C-term	[123]
	ADAMTS-3		N582-G583		E	–	[125]
	ADAMTS-7	*	–		–	140 kDa fragment	[126]
	BMP-1	*	S375-D376	**	C - P	50 kDa trimeric *N* + 120 kDa C-term	[130]
	Cathepsin G	*	–		–	25 KDa *N* + 155 KDa	[134]
		*	–		N-Olig	*N* + trimeric C-term fragment	[88]
		*	–		–	None (complete degradation)	[22]
			R255-T256	§	N	*N* + trimeric 160 kDa	[105]
	Chymotrypsin	*	–		N - Olig	25 kDa *N* + trimeric 70kDa	[150]
	Elsastase-2 (Neutrophil elastase)		–		–	*N* + trimeric 140–160 kDa	[105]
		*	–		–	25 KDa *N* + 100–155 KDa	[134]
		*	–		N - Olig	*N* + trimeric C-term	[87]
		*	–		–	None (complete degradation)	[22]
	FAP		S18-N19		N	–	[138]
	Granzyme B	*	D163-R164 D804-G805 D848-S849 D865-G866 D937-S938 D1001-E1002	^	N, Ca, G	Multiple fragments	[141]
	HTRA1	*	–		–	–	[144]
		*	V215-R216	**	N	N-term	[143]
	Kallikrein-4 (KLK4)	*	–		–	15–30 kDa N-term	[147]
	Kallikrein-15 (KLK15)		13 sites		–	–	[149]
	Kallikrein-7 (KLK7)	*	Y258-I259 Y665-L666		N E	N, C-E2	[148]
	MMP-2		–		–	–	[112]
	MMP-14		–		–	–	[116]
		*	H441-W442 P467-Q468	^	P	50 kDa N-term	[42]
		*	–		–	–	[113]
	Plasmin	*	–		–	25 KDa *N* + 155 KDa	[134]
	Thrombin	*	–		–	25 KDa *N* + 155 KDa	[134]
		*	–		N - Olig	25 kDa *N* + trimeric 85–130 kDa	[150]
	Unknown		R47-L48		N	–	[153]
	Unknown		T18-N19 R216-F217		N	–	[157]

(*continued*)

Table 1 (*Continued*)

Substrate	Enzyme [a]		Cleavage Site [b,c]		Domain [d]	Fragments Released	Reference
TSP-2	ADAMTS-1	*	E306-L307	**	Olig	30 and 42 kDa	[123]
	FAP		G19-D20 E306-L307		N Olig	–	[138]
	Granzyme B	*	D176-E177 D300-N301 D791-N792 D852-L853 D867-G868 D1003-E1004	^	N, Ca, G	Multiple fragments	[141]
	MMP-2		–		–	–	[115]
			E288-L289		Olig	–	[114]
		*	G258-V259 Q541-M542	**	Olig P	Multiple fragments	
	MMP-9	*	–		–	Multiple fragments	[114]
	MMP-14		–		–	–	[116]
TSP-4	*Unknown*		R158-A159 R827-A828		N G	–	[153]
	Unknown		R827-A828		G	–	[157]
COMP	ADAMTS-4		D530 - F531 V552 - V553		Ca- G	–	[119]
		*	–		–	110 kDa C-term fragment	[129]
	ADAMTS-7	*	–		–	51–100 kDa fragments	[127]
	ADAMTS-12	*	–		–	100 kDa main fragment	[128]
	Caspase 6		D273-G274		Ca	–	[158]
	HTRA1		S77-V78 H90-C91 N194-S195 G203-S204 E523-N524 Y574-T575		Olig E Ca G	–	[145]
	MMP-9	*	–		–	Multiple fragments	[117]
	MMP-12		–		–	–	[119]
	MMP-13	*	–		–	Multiple fragments	[117]
	MMP-19	*	–		–	60 kDa fragment	[118]
	MMP-20	*	–		–	60 kDa fragment	[118]

[a] * Evidence of cleavage obtained with purified/recombinant enzyme and substrate.
[b] Cleavage site: P1-P1′ aminoacids are indicated. Residue numbers refer to UniProt sequences. Release of signal peptide is not shown.
[c] ** Cleavage site confirmed by sequencing fragments generated by purified/recombinant enzyme and substrate. § cleavage site confirmed with synthetic peptides covering the region of interest. ^ putative cleavage site.
[d] Cleaved domain: *N* (*N*-terminal), Olig (oligomerization site), PC (procollagen, VWFC), P (Properdin-like type I repeats), E (EGF-like type II repeats), Ca (Calcium-binding type III repeats), G (C-terminal globular domain).

few exceptions, the identified cleavage sites are exposed in the native molecule, as they are found at domain boundaries, disordered regions and surface loops (Supplementary Table 1). Cleavage has often been described or validated in cellular systems or in vivo, confirming the accessibility of the cleavage site in physiological conditions and further supporting the biological importance of the described proteolytic event.

The TSP-cleavage activity of each protease is summarized in Fig. 4. Proteases are classified according to MEROPS.

Metallo peptidases - Matrix metalloprotease family

MMPs. Matrix metalloproteases (MMPs) are a large family of secreted or membrane-bound zinc-

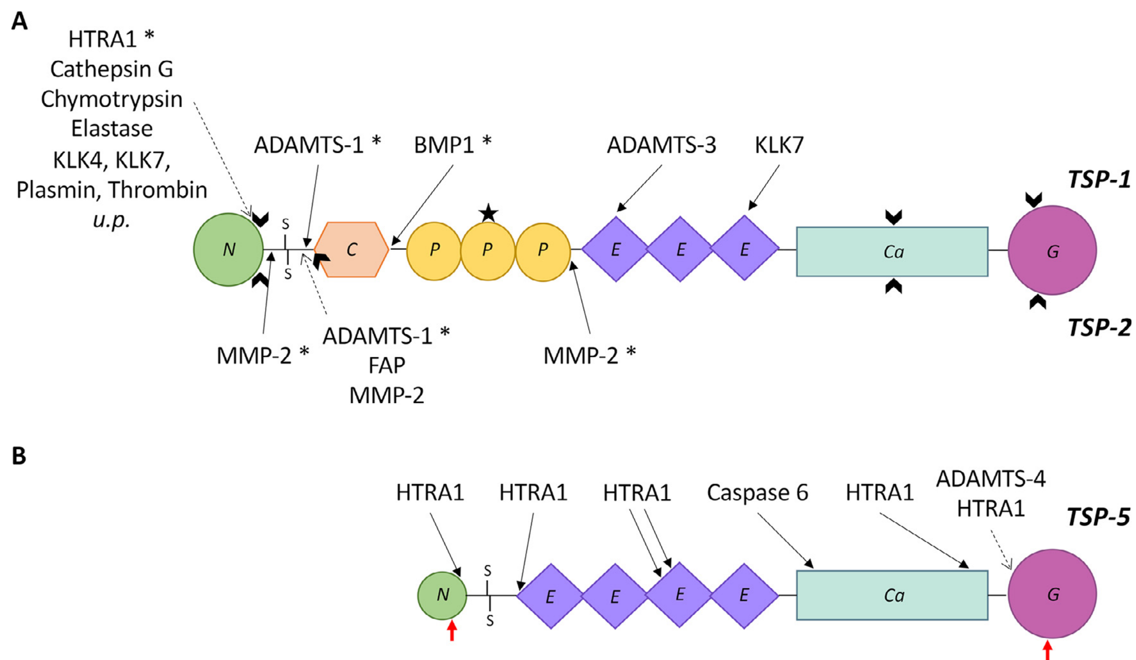

Fig. 3. Protease cleavage sites on TSPs. Graphic representation of cleavage sites for the proteases indicated on TSP-1 (A, top) and TSP-2 (A, bottom) and TSP-5/COMP (B). Red arrows in B indicate unknown protease cleavage sites on TSP-4 (no cleavage reported for TSP-3). Cleavage at sites close to the N-term/signal peptide are not indicated. Asterisks indicate cleavage sites confirmed by sequencing of fragments generated by purified/recombinant enzyme and substrate. Dotted line, multiple cleavage sites in the same region. Star, in silico prediction of the MMP-14 cleavage sites. Arrow heads, in silico prediction of the Granzyme B cleavage site on TSP-1 and TSP-2. u.p., unknown protease.

dependent endopeptidases [106]. MMPs are responsible for the proteolytic processing of a wide range of ECM substrates, but also of growth and angiogenic factors, cytokines, other proteases, receptors, and adhesion molecules, with consequent complex biological activities.

Different MMPs are differently expressed (upregulated or downmodulated) in cancer and can exert pro- and anti-tumor activities [1,107,108]. MMPs can be produced by both the tumor and the stroma cells. For instance, MMP9 is produced by neutrophils and tumor associated macrophages. MMP-14 is expressed by cancer but also by cancer-associated fibroblasts, macrophages, endothelial cells, and bone marrow derived mesenchymal stromal cells [107,109]. MMPs contribute to focal degradation of the ECM, promoting invasion of both normal (endothelial cell and immune cells) and cancer cells. They support tumor cell invasion, metastatic dissemination, and angiogenesis. In addition, MMPs can also shape the microenvironment of premetastatic niches and are implicated in the reawakening of dormant disseminated tumor cells [110].

Interestingly, several MMPs have been detected intracellularly, in different compartments, where they are activated and able to cleave intracellular substrata [111]. In view of the intracellular presence and activities of TSPs, the possibility that TSPs may be processed intracellularly warrants further investigation.

The group of C.M. Overall made a significant contribution to the identification of substrates and cleavage product of MMPs in physiological contexts. They identified TSP-1 as a substrate of MMP-2 in fibroblasts [112] and MMP-14 (MT1-MMP) in breast cancer cells overexpressing these MMPs [113]. Using an iTRAQ-TAILS approach for *N*-terminomics analyses, by labeling and blocking primary amines, these authors also characterized the substrate degradome of MMP-2 and MMP-9, confirming that TSP-2 is a substrate of MMP-2 (validated by Edman sequencing of MMP-2-generated TSP-2 fragments) and identifying multiple cleavage sites in different regions of the molecule [114]. Interestingly, this study found some differences in cleavage sites when analyzing fragments generated in a cell system or obtained by incubating recombinant TSP-2 with MMP-2. This points to a complex critical role of the microenvironment in controlling the release of proteolytic TSP fragments. MMP-2 cleavage sites were also found in the type 2 repeats of murine TSP-2 [115].

By comparing the proteomic profile of TNFα-activated endothelial cells from animals expressing or not MMP-14 (MT1-MMP), the group of A.G. Arroyo identified TSP-1 and TSP-2 among the substrates

PROTEASE	TSP-1	TSP-2	COMP	*Reference*
Metallo Peptidases				
Matrix metalloproteases				
MMP-2	■	■		*[112, 114, 115]*
MMP-9		■	■	*[114, 117]*
MMP12			■	*[119]*
MMP-13			■	*[117]*
MMP-14	■	■		*[42, 113, 116]*
MMP-19			■	*[118]*
MMP-20			■	*[118]*
Astacin/adamalysin				
ADAMTS-1	■	■		*[123]*
ADAMTS-3	■			*[125]*
ADAMTS-4			■	*[119, 129]*
ADAMTS-7	■		■	*[126, 127]*
ADAMTS-12			■	*[128]*
BMP-1	■			*[130]*
Serine Peptidases				
Cathepsin G	■			*[22, 88, 105, 134]*
Chymotrypsin	■			*[150]*
Elastase-2, neutrophil	■			*[22, 87, 105, 134]*
FAP	■	■		*[138]*
Granzyme B	■	■		*[141]*
HTRA1	■		■	*[143 - 145]*
Kallikrein-4 (KLK4)	■			*[147]*
Kallikrein-7 (KLK7)	■			*[148]*
Kallikrein-15 (KLK15)	■			*[149]*
Plasmin	■			*[134]*
Thrombin	■			*[134, 150]*
Cysteine Peptidases				
Caspase 6			■	*[158]*

Fig. 4. Cleavage activity of proteases for the TSPs indicated. Proteases are classified according to MEROPS.

degraded by this protease during inflammation [116]. They identified positions H441-W442 and P467-Q468 in TSP-1 as putative sites for MMP14 cleavage. Cleavage of TSP-1 at these sites would disrupt the CD36-binding sequence and release a C-terminal fragment containing the CD47 and $\alpha v\beta 3$ integrin binding motifs, which mediate NO production in endothelial cells, vessel dilation and intussusceptive angiogenesis in a model of inflammatory bowel disease [42]. The E123CaG-1 recombinant fragment, containing the TSP-1 region predictably released after MMP14 processing, retained the activity. The lack of activity of the whole TSP-1 molecule [42] indicated that in conditions of increased MT1-MMP and TSP-1 expression (such as during inflammation, vascular diseases and cancer), release of the C-terminal TSP-1 fragment would favor integrin $\alpha v\beta 3$- and CD47-mediated cell functions over functions mediated by CD36 or other receptors, underlining the importance of MMPs in TSP-1 activity through the release of fragments which interact with specific sets of receptors.

In articular cartilage, several MMPs were responsible for the release of COMP fragments similar to those typically found in synovial fluid from patients with rheumatoid arthritis and osteoarthritis. COMP is cleaved by several MMPs, mainly MMP-9, MMP-12, MMP-13, MMP-19 and MMP-20 [117–119], indicating a role for these MMPs in rheumatoid arthritis and osteoarthritis.

Metallo peptidases - astacin/adamalysin family

ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) are zinc metalloproteinases. The severe disorders or birth defects associated with mutation in ADAMTS genes in mammals demonstrate their important role on embryonic

development and physiological processes [120]. In cancer, their expression and activity are often deregulated. ADAMTS proteins, secreted by both tumor cells and stromal cells, cleave and regulate the activity of ECM components, growth factors and receptors, finally affecting tumor cell behavior, the tumor vasculature and the immune microenvironment. These proteases exert tumor-suppressive, pro-tumorigenic and immunosuppressive functions depending on the nature of available substrates and interacting factors [121,122].

The proteolytic degradation of TSP-1 and TSP-2 by ADAMTS is important to control their activity. ADAMTS-1 cleaves both TSPs, releasing different antiangiogenic fragments identified in vitro and detected in wound-healing models in vivo [123]. ADAMTS-1 cleaves TSP-1 between the coiled-coil oligomerization domain and the VWFC domain (residues E311-L312), releasing a trimeric *N*-terminal fragment predicted to be unstable, and monomeric 110 to 125 kDa C-terminal fragments that can remain in the inflamed tissue or be released in the circulation. These fragments were important in the control of angiogenesis in wound healing [123], and in the formation of colon and renal cancer metastasis to the liver, but not to the lungs, where TSP-1 was cleaved less efficiently [124].

In human fibroblasts, N terminomics analysis identified TSP-1 as a substrate of ADAMTS-3, but not ADAMTS-2 or ADAMTS-14 [125]. ADAMTS-7-generated fragments of TSP-1 inhibited reendothelialization after injury of the carotid artery [126], confirming the biological importance of the TSP-ADAMTS axis in the regulation of vascular remodeling in different settings.

The type 2 repeats domain of COMP binds the four C-terminal TSP-1-like repeats of ADAMTS-7 [127] and ADAMTS-12 [128]. Both proteases degrade COMP, generating fragments with a molecular mass similar to the COMP fragments in the synovial fluid of patients with arthritic diseases [127,128]. ADAMTS-4 too was reported to degrade COMP in vitro [129], and in a proteomic analysis of ECM proteolyzed peptides released from human articular cartilage [119]. This suggests that these proteases, upregulated in the cartilage and synovium of arthritic patients, are responsible for COMP degradation associated with loss of articular cartilage in arthritic diseases such as osteoarthritis and rheumatoid arthritis.

BMP-1. Bone morphogenetic protein 1 (BMP-1) is involved in the maturation of matrix structural proteins and enzymes, controlling matrix assembly during cartilage and bone formation, wound healing and tissue repair and fibrosis. Among several other substrates, including ECM components, cytokine and growth factor precursors, BMP-1 cleaves TSP-1. Cleavage occurs at a site between the VWFC and the type 1 repeats domains (residues S375-D376, a site conserved in TSP-1 in various species but absent in TSP-2) and results in the release of a trimeric 50 kDa fragment and a soluble monomeric 120 kDa fragment [130]. The 120 kDa fragment did not support cell adhesion but efficiently activated TGF-β and promoted the differentiation of primary keratocytes into myofibroblasts, with a substantial role in corneal wound healing and scar formation [130]

Serine peptidases

Serine proteases account for about one third of human proteases. They are involved in development and in major physiological processes. Deregulation of expression, activation or proteolytic activity have been associated with pathological conditions, including response to bacterial infections, inflammation, arthritis, angiogenesis-driven diseases, and cancer, where they contribute to extracellular matrix remodeling and TME organization, and mediate the activity of immune cells [131,132].

Neutrophil serine proteases. Proteases released by neutrophils, particularly elastase (ELANE), cathepsin G and MMP-9, can support cancer progression by acting in the TME of the primary tumor, but also by contributing to the premetastatic niche at distant organs [133]. Neutrophil serine proteases released locally during inflammation rapidly degrade a number of glycoproteins, including TSP, FN, and VWFC, in a native subendothelial matrix [134]. TSP-1 is very sensitive to cathepsin G, elastase 2 (neutrophil elastase), and plasmin, which released soluble fragments of approximately 150 kDa (C-term) and 24–27 kDa (HBD) [134]. Neutrophil cathepsin G cleaves platelet- or endothelial cell-derived TSP-1 at residues R255-T256, releasing a trimeric C-terminal fragment [105]. This fragment was more potent than intact TSP-1 in promoting platelet adhesion to collagen and string formation under flow [105]. The formation of neutrophil extracellular trap (NETs) was associated with increased generation of the 160 kDa fragments, and protection of the TSP-1 from further degradation [105]. A recent study confirmed that NETs, formed by neutrophils in the lung metastatic niche, could degrade TSP-1 through a process requiring elastase [135]. Thus, neutrophil-mediated degradation of the antiangiogenic TSP-1 [60] might contribute to the pro-metastatic role of an inflammatory environment in the lung [22,135].

In a feedback mechanism of regulation, sequences in the type 3 repeats of TSP-1 have been reported to inhibit the catalytic activity of serine proteases, including plasmin, cathepsin G, and elastase [87,88,136], suggesting a mutual functional control between TSP-1 and neutrophil proteases, and a role for TSP-1 in restraining neutrophil response in different phases of inflammation, hemostasis and response to bacterial infections [61,137].

FAP. Fibroblast activation protein-alpha (FAP) is a cell surface, constitutively active serine protease, with both dipeptidylaminopeptidase and endopeptidase activities. It is upregulated in activated fibroblasts during tissue remodeling, fibrosis, atherosclerosis, arthritis and cancer. A recent TAILS-based degradomic analysis in mouse embryonic fibroblasts expressing or not FAP identified FAP cleavage sites in several ECM-associated proteins, including TSP-1 and TSP-2. FAP cleaved murine TSP-2 at E306-L307 in the oligomerization domains, the same site cleaved by ADAMTS-1 [138].

Granzyme B is mainly produced by immune cells (NK cells, cytotoxic T cells), but is also expressed by other cell types, including different tumor cell types. Besides its role in immune cell-mediated death of infected or cancerous cells, granzyme B has non-cytotoxic activities in processes such as inflammation, angiogenesis, fibrosis, and extracellular matrix remodeling, related to the ability to cleave extracellular matrix components, receptors, cytokines, and angiogenic factors. Its presence in cancer tissues has been associated with either favorable clinical outcome or poor prognosis and therapy resistance [139,140]. A recent study indicated that TSP-1 and TSP-2 are substrates of granzyme B in age-related macular degeneration (AMD) [141]. Cell-free cleavage assays showed the formation of multiple TSP-1 and TSP-2 fragments, in agreement with the in-silico prediction of several cleavage sites in the *N*-term, Ca and G domains of both TSP-1 and TSP-2. Since aspartates in some of the putative cleavage sites in the Ca domain (position 848, 865, 937 in TSP-1 and 791, 852, and 867 in TSP-2) are involved in the coordination of calcium ions [86,142], calcium concentrations might modulate the accessibility of these sites and hence cleavage. TSP-1 levels and inhibitory activity in an explant choroid sprouting assay were reduced by granzyme B, suggesting that degradation of the anti-angiogenic TSP-1 by extracellular granzyme B may contribute to AMD-related choroidal neovascularization.

HTRA1. The serine peptidase high-temperature requirement protein A1 (HTRA1) is considered a key factor in several vascular diseases including AMD. It cleaves TSP-1, releasing an *N*-terminal pro-angiogenic fragment [143,144]. TAILS analysis identified the cleavage site at V215-R216, with release of the proangiogenic 25−30 kDa fragment [143]. In line with the reported ability of TSP-1 to inhibit serine protease activity, the proteolytic activity of HTRA1 was completely inhibited in the presence of TSP-1 [143], pointing to mutual regulation between HTRA1 and TSP-1 in matrix remodeling and neovascularization in AMD.

HTRA1 is the most abundant protease in the osteoarthritis cartilage. Degradomic analysis of HTRA1-treated cartilage identified several COMP fragments, cleaved at multiple sites, corresponding to COMP fragments identified in osteoarthritic cartilage [145].

Kallikreins. Kallikrein-related peptidases (KLKs) are la large family of secreted serine proteases, that play a role in processes spanning form skin homeostasis to neural development, angiogenesis, and innate immunity [131]. Although usually expressed by cancer cells, KLKs can be expressed also by cells in TME, including endothelial and immune cells (neutrophils and T cells) [146]. They are released in the TME, where their proteolytic activity controls TME organization, metastasis, angiogenesis, immune suppression, and resistance to therapies in several cancer types [131,146].

PROTOMAP approaches identified TSP-1 among the targets of KLK4 and KLK7 [147,148]. KLK4 degraded TSP-1 in the conditioned media of prostate cancer cells and in the matrix deposed by osteoblasts, releasing proangiogenic *N*-terminal fragments, potentially contributing to the tumor promoting activity of KLK4 in this tumor type [147]. In an ovarian cancer model KLK7 was able to cleave TSP-1 at two distinct sites, one at Y258-I259 releasing the *N*-terminal 28 kDa fragment and a second one at Y665-L666 in the type 2 repeats domain, conceivably releasing fragments spanning from the VWFC domain to the type 2 repeats and a fragment comprising the type 3 repeats and the globular C-term [148]. In a prostate cancer model, KLK15 was reported to degrade a number of ECM-related proteins, including TSP-1, acting at different sites and releasing multiple fragments [149].

Thrombin, plasmin and chymotrypsin. TSP-1 is cleaved by thrombin, plasmin and chymotrypsin within the VWFC homology domain, releasing the *N*-terminal domain [134,150]. Early studies used proteolytic cleavage of TSPs as a tool to identify active domains. Thrombin-generated fragments of TSP-1 showed opposite effects in angiogenesis. The 25 kDa *N*-terminal fragment promoted angiogenesis, increasing endothelial cell invasiveness, through activation of MMP-2 and reduction of TIMP-2 synthesis [18] whereas the 140-kDa C-terminal fragment inhibited angiogenesis through multiple mechanisms and active sites, including interaction with CD36, CD47, angiogenic factors and proteases [15,16,151].

A chymotrypsin-like serine protease was responsible for the release of a 26-kDa fragment, corresponding to the heparin-binding domain, in apoptotic monocytes [152]. This fragment promoted clearance of apoptotic cells by inducing a phagocytic state in dendritic cells [68].

"Orphan" fragments (for which the protease responsible has not been identified)

Several proteomic studies have identified fragments of TSPs associated with specific diseases,

although the proteases responsible for cleavage have not been identified. Analytical approaches based on labeling and enriching for N- or C-terminal peptides have revealed proteolytic fragments of TSPs, including TSP-1 and TSP-4, in vivo in human serum [153].

The release of platelet TSP-1, associated with a process of wound healing, was accompanied by proteolytic degradation, with the generation of a 140 kDa fragment detectable in the circulation up to three days after surgery [154]. Similarly, a 160 kDa TSP-1 fragment was detected in human plasma one day after surgical resection of liver metastasis [105]. A high-molecular-weight C-terminal fragment was observed, associated with vessels, in diabetic rats [155].

A C-terminal 60 kDa fragment of TSP-1, which included the calcium-binding type 3 repeats, was identified as a component of cytotoxic supramolecular attack particles (SMAPs) released by cytotoxic T lymphocytes [54] and NK cells [55]. The TSP-1 fragment was associated with the SMAP glycoprotein shell that surrounds a cytolytic core made of perforin and granzyme-B. The TSP-1 fragment contributed to CTL target killing. The finding that SMAPs are preassembled in specialized lysosomes within the cell [54] adds to the growing evidence of intracellular interactions and activities of typically extracellular TSP-1 [156].

Fragments of TSP-1 and TSP-4 were detected by a subtiligase-based enrichment approach in apoptotic cells exposed to different chemotherapeutic drugs. Cleavage was not due to the activity of the cysteine protease caspases [157]. However, evidence that a TSP family member is a substrate of caspases was provided by the group of JA Wells, who identified COMP proteolytic fragments in leukemia cells exposed to exogenous caspase-6, but not caspase-2 [158].

Conclusions

Controlled proteolytic cleavage of thrombospondins is a critical mechanism to tune their functions in different biological settings. The functional consequences are manifold, including alteration in TSP-interaction with the ECM (e.g. following trypsin digestion), modulation of the activity and availability of growth factors and cytokines (e.g. activation of TGF-β by a BMP-1-generated fragment of TSP-1), disruption/exposure of active TSP sequences (e.g. disruption of CD36-binding sequence of TSP-1 by MMP14 and release of a fragment active in inflammatory bowel disease, where entire TSP-1 was inactive), local release of active TSP fragments (e.g. proangiogenic / antiangiogenic fragments released by ADAMTS-1, HTRA-1, KLK4, granzyme B, and thrombin), and alteration in the composition and function of multi-molecular interaction networks by physical separation of the TSP binding sites for different ligands.

In a mechanism of mutual functional control, proteases cleave and regulate the activity of TSPs and, in turn TSPs can act as inhibitors of proteolytic enzymes, as in the case of TSP-1 interaction with HTRA1 during matrix remodeling and neovascularization in AMD [143]. In other cases, the inhibitory effect of TSPs can have a protective effect. For example, TSP-1 inhibition of neutrophil elastase and cathepsin G might control the innate immune response and protect against proteolytic tissue injury in microbial infections [61,62]. Inhibition of proteases can also contribute to TSP-1 antiangiogenic activity, through the reduction of MMP-9-mediated release of matrix-bound VEGF [159].

TSPs can control proteolysis directly, as reported for TSP-1, which inhibits MMP-2 [160], neutrophil elastase and cathepsin G [87,87] or for COMP, which inhibits thrombin [161]. TSP inhibition of proteolytic processes can also be indirect, through binding of the substrates, such as VWFC, protected from cleavage by ADAMTS13 [162,163], or PDGF, protected by TSP-1 from proteolytic degradation by thrombin and proteases produced by mesenchymal stromal cells [71].

Finally, TSPs can also modulate the activity of proteases by affecting the expression of proteases and protease inhibitors. For instance, TSP-1 reduces the expression of tissue inhibitors of metalloproteinases-1 (TIMP1) in macrophages [164] and TIMP-2 in endothelial cells [18].

In conclusion, the relationship between TSPs and proteases is extremely complex, strictly controlled and regulated by mutual influences. It forms a complex, multiplayer system that dynamically tunes the activity of matricellular proteins and proteases in physio-pathological settings, and particularly in cancer. Deeper knowledge of the occurrence and activity of proteolytic fragments of TSPs might improve our understanding of disease pathogenesis and progression. The development and wider use of proteomic-degradomic approaches will be fundamental to define the profile of proteolytic fragments of TSPs, and of other matricellular and matrix proteins in specific diseases. The identification of fragments with beneficial activity would lay the ground for the design of new therapeutic tools.

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Declaration of Competing Interest

None.

Supplementary materials

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