Mini-Review

Thrombospondin: A Modular Adhesive Glycoprotein of Platelets and Nucleated Cells

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Platelet adhesion to the subendothelial matrix and the subsequent aggregation of the activated platelets is a special case of intercellular adhesion that is highly regulated and for which multiple, possibly redundant adhesive systems exist. The large "adhesive glycoproteins" such as fibrinogen, von Willebrand factor, and fibronectin and their receptors, which participate in platelet adhesion and aggregation, are not unique to platelets, and probably serve analogous functions in a variety of cell types and locations. Thrombospondin (TS),[1] the most abundant protein of platelet alpha-granules, has been recognized as a new member of this class of adhesive glycoproteins. Whereas other adhesive glycoproteins are contained within alpha-granules and secreted upon platelet activation, they also exist in plasma at high concentrations. Hence TS is the only member of the group that is unique to alpha-granules and whose expression in quantity at sites of platelet aggregation is absolutely dependent on platelet activation.

Since its discovery in 1971, TS has been thought to have a role in platelet aggregation (3, 4). This suspicion has recently been confirmed by the finding that both monoclonal (14) and polyclonal (22, 37) antibodies against platelet TS can block the secondary or secretion-dependent phase of platelet aggregation. This result in itself, however, does not speak directly to the mechanism of TS involvement in this complex process, since antibodies against fibrinogen (62), fibronectin (13), and the platelet glycoprotein IIb/IIIa (43), the receptor on platelets for fibrinogen and fibronectin, will all block platelet aggregation. Other agents that block aggregation are short peptide sequences related either to RGDS, the cell-binding sequence of fibronectin and fibrinogen (21), or to the COOH-terminal segment of the fibrinogen gamma chain (27). Both interfere with the binding of fibrinogen, fibronectin (58), and presumably von Willebrand factor (61) to IIb/IIIa.

In addition to its localization in platelets and its presumed synthesis in megakaryocytes (42), TS has been found, using immunofluorescence localization techniques, in a variety of extracellular locations in human tissues including blood vessel, muscle, skin, kidney, and glandular epithelium (64). The biosynthesis of TS has been demonstrated in a number of normal and transformed cells in culture, including endothelial, smooth muscle, and glial cells, type II pneumocytes, fibroblasts, keratinocytes, and macrophages (1, 25, 26, 45, 46, 50, 57, 65). Furthermore, several cell lines derived from malignancies such as HT-1080 fibrosarcoma cells and the melanoma lines C32 and G361 also make TS (54, 63). This widespread expression of TS and its appearance as a component of the extracellular matrix have led to the suggestion that TS might have a role analogous to that of fibronectin and laminin in cell attachment, motility, and perhaps differentiation. TS does not appear to be an obligatory attachment factor for cultured cells such as fibroblasts, smooth muscle cells, or endothelial cells. However, Roberts et al. (54) have reported that C32 and G361 melanoma cells attach and spread on TS-coated substrates and that attachment and spreading are blocked by different TS monoclonal antibodies (mAbs). Thus at least some types of cells appear to express receptors for TS that can mediate classical attachment and spreading functions (62a). Recently TS has been implicated in the attachment of *P. falciparum*-parasitized erythrocytes to melanoma cells, a model for endothelial cell adherence of infected red blood cells that express malaria-encoded surface antigens (55).

Modular Structure of Thrombospondin

Various experimental approaches have suggested that TS, like fibronectin, laminin, and other adhesive glycoproteins, is a multidomain or modular glycoprotein in which discrete regions of relatively stable protein structure contain binding sites for the other proteins with which TS interacts. These proteins include several found in the extracellular matrix such as fibronectin (29), heparan sulfate proteoglycans and heparin (7, 12, 35, 51), collagen (20, 47), and laminin (31). Plasma proteins important in the regulation of thrombosis such as fibrinogen (11), plasminogen (31, 59), and histidine-rich glycoprotein (38, 60) also interact with TS, and there exists preliminary evidence that TS binds to cell surface receptors on platelets (14) and melanoma cells (1a, 54). The initial approach to dissecting TS into functional domains used

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1. *Abbreviations used in this paper*: EGF, epidermal growth factor; TS, thrombospondin.

proteolysis followed by isolation of domains on affinity columns such as heparin- and fibrinogen-Sepharose (7, 11, 12, 47, 51). We produced a panel of mAbs against both native and denatured TS with the hope of identifying antibodies that might react with specific domains and block the functions of TS mediated by these domains (19).

A useful tool for characterizing the fragments of TS as well as the complexes of TS with mAbs and its protein ligands is the electron microscope (19, 32). The native molecular mass of calcium-replete, trimeric TS is ~450,000 D. The mass of the subunits, based on estimates from SDS gel electrophoresis under reducing conditions, was in the range of 150,000–185,000 (36, 41). Recent sequencing of TS cDNA reveals that the true molecular mass of the subunit is somewhat less than these values, ~140,000 (see below). This is possibly due to the low isoelectric point of TS, ~4.7 (36), and the resulting low binding of SDS by the negatively charged protein. Fig. 1 *a* shows an image of a rotary-shadowed TS trimer in the presence of calcium. It is readily apparent that the protein is made up of regions of globular character as well as thin connecting strand segments. Fig. 1 *b* shows the model for TS structure that is consistent with all of the views of intact TS. Each subunit is represented as an asymmetric dumbbell consisting of a small globular domain, a connecting stalk, and a larger globular domain (19, 32). In the electron micrograph, the small globules appear in the center close to one another, whereas the larger globular domains are splayed out around them. The only connection among the three subunits appears to be on the stalks at a point quite near the small globular domains. This method also reveals a profound conformational change in TS upon the chelation of calcium ions by EDTA (10, 32). The large domains appear to unravel, resulting in longer stalks and a reduction in the size of the large domains.

This conformational change is also evident in the pattern of proteolytic cleavage of TS by a variety of proteases. We have used chymotrypsin most extensively (19), but other investigators have obtained quite similar results with thrombin, trypsin, and plasmin (30, 31, 35). In the presence of calcium, the small domains are rapidly removed and the remaining large peptide is nicked about 100 amino acids from the COOH terminus. The same cleavage occurs to liberate the small globular domain in EDTA, but the entire large globular domain is degraded resulting in a trimer of stalks each with a molecular mass of ~70 kD (10, 19, 31, 47). These proteolytic fragments are summarized in Fig. 2.

The small globular domain has been identified as the heparin-binding domain since it binds to heparin-agarose with the same affinity as TS itself. Our monoclonal antibody A2.5, which immunoprecipitates the 25-kD heparin-binding domain, decorates the cluster of three small globular domains as visualized by electron microscopy of rotary-shadowed TS–mAb complexes (19). Gartner et al. (22) reported that polyclonal antibodies specific for the heparin-binding domain of bovine TS could block the secondary phase of platelet aggregation, thus implicating the heparin-binding domain in this function of TS. Roberts et al. (52) have reported that TS interacts with sulfatides (sulfated glycolipids) via this heparin-binding domain and that heparin and fucoidin are potent inhibitors of this binding activity, whereas chondroitin sulfate and hyaluronate are not. Laminin, von Willebrand factor, and TS all bind sulfatides, but each has a slightly different specificity (53). The heparin-binding domain has been implicated in the hemagglutinating activity of TS (23, 24), and heparin is also a potent inhibitor of the binding of radiolabeled TS to human fibroblasts (44), suggesting that binding to these cells may occur via the heparin-binding domain.

The stalk-like connecting region of each TS subunit contains a binding site for collagen. The trimer of stalks is readily purified by gel filtration of a chymotryptic digest of TS performed in EDTA (20). Mumby et al. first reported that TS binds specifically to collagen (47). Their methods detected binding to type V, but not to other collagen types. This same group also found that a trimeric species composed of 70-kD fragments cleaved from the TS chains by chymotrypsin retained the ability to bind to type V collagen. We have examined the interaction of TS with pepsinized preparations of collagen types I through V using both direct, quantitative binding studies and electron microscopy of rotary-shadowed replicas of TS–collagen complexes (20). TS binds with low but significant affinity to all of these pepsinized collagens at a site at one end of the triple helical collagen monomer. The interaction with type V collagen is of much higher affinity

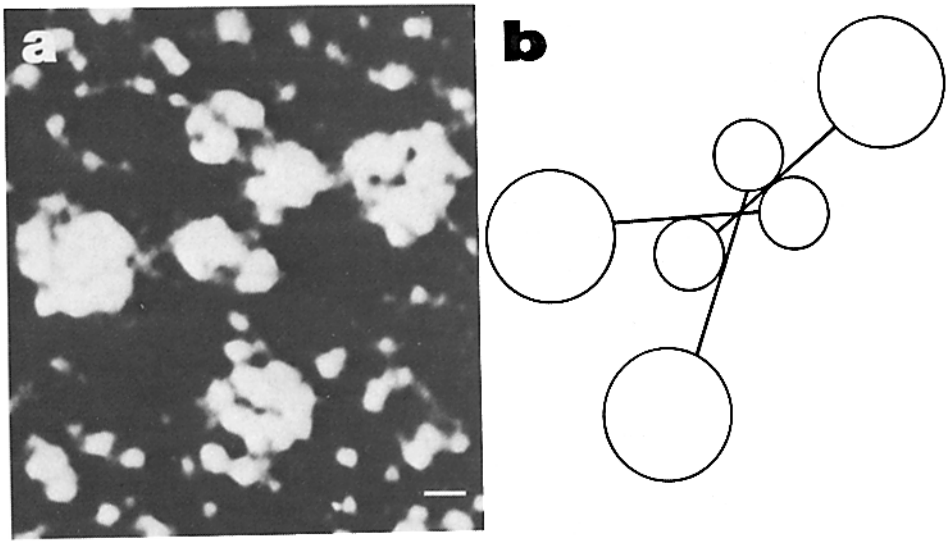

Figure 1. (*a*) Electron micrograph taken by Dr. Nancy J. Galvin in the author's laboratory of a rotary-shadowed thrombospondin trimer in the presence of calcium (1 mM). Bar, 10 nm. (*b*) A model of the three subunits approximately as they are arranged in the view of the trimer in *a*. Each subunit is one of the objects shaped like an asymmetric dumbbell.

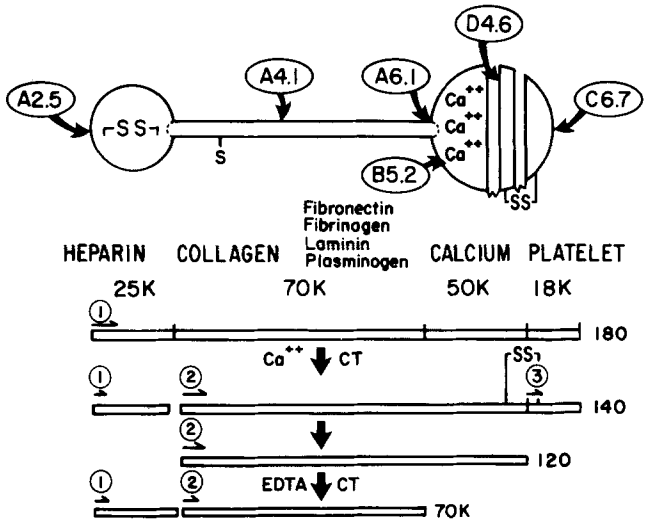

Figure 2. A model of the thrombospondin subunit based on the results of proteolysis, affinity chromatography, high sensitivity amino acid sequencing of fragments, and electron microscopy of rotary-shadowed thrombospondin–mAb complexes. A linear diagram of the peptide chain and its chymotryptic cleavage products in the presence and absence of calcium is shown in the bottom half of the figure. The ligand for each region of the subunit is indicated below the diagram. Ovals with arrows show the locations of the epitopes for the indicated monoclonal antibodies developed in the author's laboratory. Circles numbered 1, 2, and 3 indicate amino-terminal sequences obtained for the proteolytic fragments shown.

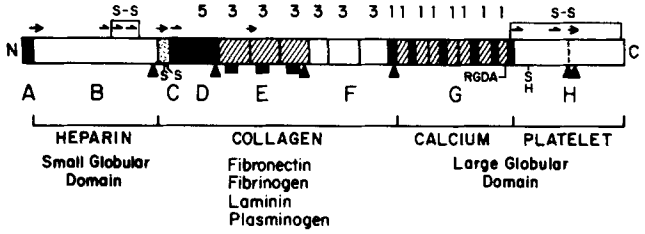

Figure 3. Schematic representation of the amino acid sequence of the thrombospondin peptide chain of 1,170 amino acids. For scale, note that the heparin-binding domain, region B, is 240 amino acids long. Numbers above the bar indicate the number of disulfide bonds in each segment; arrows above mark known peptide sequences used to confirm and align the nucleotide sequence. The extreme NH_2- and COOH-terminal disulfide bonds are indicated as such above the bar. Filled arrowheads below mark potential N glycosylation sites and the two S's below show the proposed interchain disulfide crosslinking sites contained within region C. The locations of the RGDA sequence and the free sulfhydryl are shown below the bar. The vertical dashed line in region H is the site of chymotryptic cleavage of the COOH-terminal fragment which contains the epitope for the platelet aggregation blocking mAb C6.7.

and stability and occurs at a site at the end of the pepsinized collagen molecule and at an additional site about two-thirds of the distance along the collagen triple helix from the lower affinity end site (20). The trimer composed of 70-kD chymotryptic peptides, which constitute the stalk region of TS, was also examined and found to bind to the same sites on all the collagen types as intact TS. A significant difference between the binding of intact TS and the trimer of stalks was found in the effect of calcium. Binding of intact TS was markedly lowered by increasing calcium levels from 10 μM to 1 mM, whereas the binding of the trimer of 70-kD fragments was completely insensitive to increasing calcium concentrations (20). This suggests that either the large globular domain in its calcium liganded state can interfere with the access of collagen molecules to their binding site on the stalk region of TS or that the globular state of the large domain exerts an indirect or conformational influence on the collagen-binding site.

TS also binds to fibronectin (29, 31), fibrinogen (11, 29, 31), plasminogen (31, 59), and laminin (31). The dissociation constants for the binding of TS to all of these proteins is in the range of 40 to 150 nM. Lawler et al. (31) have investigated the binding of the isolated trimer of 70-kD fragments described above to these proteins immobilized on plastic wells and found that the 70-kD stalk region also appears to contain the binding sites for fibrinogen, fibronectin, plasminogen, and laminin. Sequencing studies (below) reveal that this 70-kD region is composed of at least four distinct regions of protein structure. Thus, some or all of these proteins could have distinct binding sites within the stalk region of TS.

The model for the structure of the TS subunit that emerged from the summation of these studies mentioned above along with the results of microsequencing of the isolated domains (19) is shown in Fig. 2. A primary conclusion is that the subunit of TS is a colinear representation of the polypeptide chain, another hallmark of modular proteins like fibronectin. This allows for evolution by addition or deletion of exons without perturbing the structure of adjacent regions of the protein.

Amino Acid Sequence of Thrombospondin from cDNA Clones

The complete amino acid sequence of TS allows one to think in more precise terms about the structures of the TS domains, and also defines for the first time several interesting regions of TS structure. We have determined the complete amino acid sequence of human TS from cDNA clones isolated from a human fibroblast library. We first isolated a clone designated 6A that encodes the heparin-binding domain and part of the collagen-binding domain of TS (15). This clone was isolated by screening the human fibroblast cDNA library with degenerate synthetic oligonucleotides based on the sequence of TS tryptic peptides. The nucleotide sequence was confirmed as being that of TS since it predicted the amino acid sequences of six TS peptides including those found at the NH_2 terminus of the mature peptide chain and the collagen-binding domain. Using radiolabeled restriction fragments from 6A, we rescreened the human fibroblast library and recovered two cDNAs each ~4.2-kb long. One of these has been sequenced in its entirety and contains an open reading frame of 3,510 nucleotides encoding the entire TS peptide chain.

Fig. 3 summarizes the information contained in the deduced amino acid sequence of TS. Each subunit is composed of 1,152 amino acids plus an 18-residue signal peptide. This amino acid sequence translated from the cDNA contains all of the remaining TS peptides that we had sequenced previously and confirms the order of the domains originally proposed based on limited amino acid sequence data (19) (Fig. 2). Using our protein sequence data to confirm the identity of the clones, Lawler and Hynes (33) cloned a series

of overlapping cDNAs from a human endothelial cell library with antibody selection. This composite sequence is identical to the one we have obtained except at one nucleotide, which results in amino acid number 66 being an alanine residue in the fibroblast sequence and a threonine residue in the endothelial sequence. Kobayashi et al. (28) have also reported the sequence of the first 379 amino acids of human umbilical vein TS, and their sequence is identical with our fibroblast sequence. Northern analysis indicates an mRNA size for TS of over 6 kb (15, 28, 33). Preliminary Southern blotting experiments are consistent with there being only one TS gene (15). However, more thorough analysis will be required to determine this number with certainty.

The amino acid sequence of TS reveals several striking features that help to explain the previously deduced properties of the molecule. The most fascinating aspect of the structure is that it has obviously been assembled from a variety of ancestral precursors presumably by "exon shuffling." This is particularly evident in the stalk-like, central portion of the peptide chain, which is extremely rich in cysteine (Fig. 3), all of which, with one exception, are presumably involved in disulfide bonds. Given the modular nature of this region, it is quite likely that the disulfide bonds are limited to pairing within each region rather than introducing the complications of maintaining intersegment (probably inter-exon) connections during evolution. This notion is made more tenable because the majority of the cysteines align with those in homologous proteins (15, 28, 33). In contrast to regions D through G, the amino-terminal heparin-binding domain (region B) and the COOH-terminal cell (platelet) adhesion domain (region H) are unique in that they show no significant homology with other proteins. Each region contains only a single intradomain disulfide bond, consistent in both cases with our previous observations (12, 14).

The heparin-binding domain is followed by the sequence that we had determined for the NH_2 terminus of the collagen-binding domain or stalk region of TS (19, 20). The stalk begins with region C, which contains only 21 amino acids and two cysteines, which are probably involved in the intersubunit disulfide bonds. The sequence is C-G-I-S-C, and this spacing of the cysteines also occurs in all three fibrinogen chains at both sites at which disulfides connect the three chains (48). Note that to form a symmetric trimeric structure each peptide chain must contribute at least two cysteines. Region D is composed of 97 residues including 10 cysteines, all of which align with cysteine residues in the NH_2-terminal propeptide of alpha-(1)-I human procollagen (6). In fact this part of TS is 35% identical with exon 50 of the human type I collagen and appears to represent the precise incorporation of a progenitor of exon 50 into the ancestral TS gene. Exon 51, which encodes the signal peptide of the procollagen, is also represented at the NH_2 terminus of region D, but the homology is somewhat less striking. All 10 of the cysteine residues in the type I collagen NH_2-terminal propeptide are intrachain, paired within the propeptide (49). Since all 10 of these cysteines are conserved in TS, we suggest that they form five intrasegment disulfide bonds.

Recently it has been demonstrated that the purified NH_2 terminal propeptide of type I collagen can inhibit the synthesis of procollagen in fibroblasts by lowering the levels of type I procollagen mRNA (66). Perhaps this region of TS has functions other than collagen binding related to regulation of gene expression. In this vein, it has been noted that the addition of TS to the medium of smooth muscle cells causes an enhanced sensitivity to epidermal growth factor (EGF) stimulation and this effect was blocked by heparin (40). An autocrine role for TS synthesized by these cells was proposed in which TS and heparin modulate the growth of smooth muscle cells. In turn, it has been reported that platelet-derived growth factor rapidly stimulates the synthesis of TS (39) by smooth muscle cells by increasing the level of TS mRNA (28). Thus stimulation of smooth muscle cells by platelet-derived growth factor from platelets could have the effect of making them more sensitive to EGF.

The remainder of the stalk region is made up of two different threefold repeated segments. Region E consists of three repeats of ~60 residues, each homologous with one another and with a region of a malaria circumsporozoite protein. Region F is a threefold repeated segment, again of ~60 residues, homologous to regions h, i, and j of the EGF precursor (16). Lawler and Hynes (33) have designated these type I and type II TS repeats. Both types of segments have six cysteine residues within ~60 amino acids. Particularly for the EGF-related segments, it is likely that the disulfide pairing pattern is contained within each 60 residue segment. All of the 18 cysteines align with those in the EGF precursor, allowing for one insertion that would not interrupt the disulfide bonding pattern. Hence the EGF structure is probably a good model for this part of the stalk, and these "growth factor" repeats would be consistent with the average thickness of the stalks calculated to be ~1.5 nm. It should be emphasized that the overall identity with the EGF repeats is on the order of 25%, so that it is very unlikely that TS retains any ability to interact with the EGF or related receptors.

The type I repeats of region E are interesting considering previous information regarding the possible involvement of TS in the pathogenesis of malaria (55). The sequences in region E of TS exhibit homology with a segment of the circumsporozoite protein designated region II (8). In contrast to most regions of this highly variable protein, region II is well conserved among all species of malaria for which sequence data are available. In the area of greatest similarity with TS, 15 of 17 continuous residues are identical and these overlap with the 13 residues of region II in the malaria protein. The sporozoite is the initial infective stage of malaria, which is introduced into the bloodstream from the mosquito salivary gland, and homes to and colonizes the liver. From here, merozoites are liberated and infect erythrocytes. These infected red cells are sequestered along venular endothelium, thus removing them from the circulation. In this later sequestration event TS-mediated adherence was proposed to play a role (55). An interesting hypothesis emerges from these considerations. If region II is actually the determinant on the sporozoite that is recognized by liver cells, then the receptor in liver may be designed to bind TS by recognizing its type E repeats. If this were in fact the case, it would represent another instance of the appropriation of a host recognition system by a parasite to gain entry, in this case, to the liver. Using synthetic peptides based on the malaria-like sequences we are currently testing this hypothesis. However, at present, the homology between the type E repeats of TS and the circumsporozoite protein region II does not help to explain the proposed involvement of TS in the sequestration event. The data suggest that the parasitized red cells should express a protein

that recognizes TS (55), perhaps a homologue of the putative liver receptor.

Region G of the TS sequence is readily identified as the primary calcium-binding domain. Electron microscopy (10, 32), proteolysis (10, 20, 30–32, 34) and monoclonal antibodies that recognize this region of the structure (10) all suggest that it is compact in the presence of calcium and becomes extended or loosened when calcium is removed. This domain provides the most interesting challenge in terms of deducing a testable model for its structure. Four calcium binding sites per subunit have been detected with optical methods and proteolysis (34) and these have dissociation constants in the range of 50–120 μM. We have used two mAbs that recognize epitopes whose exposure is modulated by calcium concentration to detect conformational transitions in TS (10). One of these, A6.1, binds over a broad range of calcium concentrations probably reflecting a number of calcium-binding sites with dissociation constants ranging from above 100 μM to less than 1 μM. The other mAb, D4.6, detects a conformational change in TS which is coupled to an extremely tight binding site or sites with a dissociation constant below 100 nM (10). Thus there probably exist in TS several (five or more per subunit) calcium-binding sites that exhibit a broad range of affinities and should have differing, but perhaps related structures.

Region G of TS consists of seven highly conserved repeats of 23 amino acids containing two cysteine residues, one near each end. In some cases, this unit is separated by up to 15 amino acids from its next repeat by a segment of variable length that is related to the 23 amino acid segment. If the highly conserved 23 residue segments are called A and the less well conserved, but still related, intervening segments of variable length called B, then the overall structure of this region is B (A) A B A A B A A B A B A B (A). Partial repeats of the A structure are in parentheses. The concensus sequences of the A and B segments are

D N C (P) h h x N x (D) Q x D (OH) D x D G x G D x C (A repeat)
x x D x D x D G x (P) B x x (B repeat),

where the capital letters are the single letter code for an amino acid, which always occurs at that position, parentheses indicate a strong preference for the amino acid (OH stands for thr, ser, or tyr), the h represents any hydrophobic residue, and x means no obvious preference in that position. The B repeats are obviously related to the A structures and may have been derived from a common evolutionary precursor. It is clear that this region has been built up through a series of duplication and deletion events, and the degree of relatedness among specific A and B repeats implies a very complex pathway of evolution. The degree of overt homology between region G of TS and calcium-binding proteins like calmodulin is extremely small, limited to the spacing of the acidic, presumably calcium-binding, residues. If TS and calmodulin are derived from a common ancestor, the divergence must have occurred very long ago.

A major difference between these putative calcium-binding sites of TS and those of the intracellular calcium-binding proteins is the presence of the cysteine residues. In calmodulin, for example, the calcium-binding residues are bounded by the E and F helices, which both stabilize and participate in the binding of calcium and form the so-called "E-F hand" structure (2). Given the locations of the cysteines in TS, it is reasonable to suggest that the calcium-binding loop is closed and constrained by disulfide bonds formed between the cysteines at the beginning and end of each type A segment. A direct test of whether the type A elements can bind calcium and if the binding is affected by the presence or absence of a disulfide closure of the loop is being conducted using synthetic peptides representing several of the type A sequences from TS. If these type A repeats bind calcium in the closed disulfide bonded loop form, this would suggest that the correct model for the disulfide connectivity of this region is that which allows only pairing within each type A structure. This model is also consistent with the observed extensibility of this region since the type B segments can provide more than enough peptide chain to account for the apparent increase in length when calcium is removed (10, 32). It is as though this region of TS provides a way of varying the length of the connecting region between the binding domains for heparin and collagen at the NH_2 terminus of the subunit and those for platelets and cells at the COOH terminus.

Given the wide participation of the RGD (Arg-Gly-Asp) sequence in cell–matrix interactions (56), it was of great interest to determine whether TS contained a potentially active RGD sequence somewhere within its structure. In fact one RGDA tetrapeptide, an active analogue of RGDS, is found at the very end of the calcium-binding domain (Fig. 3). An RGDA sequence exists in the slime mold lectin *Discoidin I* where it is bound by a receptor on the surface of aggregating *Dictyostelium* cells (18). Despite the existence of this RGDA sequence in TS, accumulating evidence has failed to implicate RGD recognition in TS function. This may be for two reasons. First, the RGDA sequence in TS is immediately followed by a cysteine residue that is almost certainly disulfide bonded to another. If the above model for the calcium-binding domain is correct, the RGDA sequence would be contained within a putative calcium-binding loop. Thus interference from the disulfide bond may prevent the binding of the RGDA sequence to any of the receptors that can bind it.

An alternative possibility, and one that is perhaps more interesting in light of the large effects of calcium on this region of the TS structure, is that the exposure of the RGDA sequence is regulated by the number of calcium ions bound to TS. Since the RGDA occurs within a proposed calcium-binding loop, the presence of the bound ion could block the ability of the sequence to interact with a receptor. If such a mechanism were at work in TS, one must then ask what relevance this might have to TS function. Being a matrix glycoprotein, TS is synthesized and perhaps cosecreted with other matrix components from vesicles. These intracellular compartments would provide an environment in which the calcium concentration could be closely regulated. This may be important for the proper folding of the protein. Furthermore, if RGD receptors were also being synthesized by the same cell, these would be inserted into the membranes of vesicles with their binding domains facing the lumen where soluble components containing RGD sequences could be bound. In this type of scenario it is easy to imagine that regulation of calcium levels could have the effect of controlling the extent or the order in which TS and perhaps other matrix components, many of which bind calcium, would be brought into juxtaposition and assembled. A further consideration is that

some types of matrix may exist in regions to which calcium access from the circulation is limited, thus resulting in lower than "physiological" ambient calcium concentrations. This effect of sequestration could be augmented by the high calcium-binding capacity of many matrix constituents such as proteoglycans and glycosaminoglycans. In this way local calcium levels could vary considerably with concomitant effects on TS functions such as collagen binding (20).

Finally, the extreme COOH-terminal portion of TS, which is proposed to have a role in platelet function and cell adhesion, appears to be unique in structure. Only two cysteine residues exist in this domain and our previous studies indicate that the one nearest the COOH terminus of TS must be paired with a Cys upstream of the chymotryptic cleavage site (Fig. 3, *dashed line*). The removal of this COOH-terminal chymotryptic fragment from the rest of TS requires both denaturation and reduction (14). Thus the most COOH-terminal cysteine of TS could be paired with either the last one in the calcium-binding domain (which occurs in the last partial type A repeat and its partner is absent), or the first and only other one in the COOH-terminal domain. The one of these two cysteines that remained unpaired would then presumably be the one identified by Danishefsky et al. (9) as the free sulfhydryl group of TS. To resolve this question, D. F. Mosher and K. Skorstengaard (personal communication) treated intact TS with radiolabeled iodoacetic acid in 8 M urea, then digested the labeled TS and isolated the peptide bearing the label. Sequence analysis indicated that the alkylated Cys residue is number 974 in the TS sequence, or the one that occurs at the beginning of the COOH-terminal domain. Thus, barring disulfide exchange, it appears that the most COOH terminal cysteine of TS is paired to the last Cys of the calcium-binding domain, which occurs in its last imperfect type A repeat.

Prospects

The amino acid sequence of TS has already provided a wealth of new ideas and speculations about how TS accomplishes its known functions and has even suggested new functions for TS. Further, the availability of cDNA clones to be used as probes for cloning TS genes will lead to the description of an undoubtedly complex intron–exon structure as well as the 5′ transcriptional regulatory region of the gene. There are already some tantalizing hints of the kinds of signals that regulate TS gene expression, such as platelet-derived growth factor, which causes a very rapid increase in levels of TS mRNA and subsequent synthesis in smooth muscle cells (28, 39). The release of TS from the cell matrix by heparin suggests another mode of rapid regulation of TS function (39). The rapidity with which TS levels or locations are altered in these systems as well as in the case of secretion by activated platelets may indicate that it has a function even in nonplatelet sites, which necessitates its rapid modulation. This idea is further supported by binding studies using iodinated TS that found that it binds to fibroblasts and is rapidly internalized and degraded in contrast to fibronectin, which is slowly incorporated into the matrix (44). As yet it is unclear just what this function might be, although from this early work it seems as though a role in regulation of gene expression through rapid alteration of the properties of the extracellular matrix is not an unreasonable suggestion. Another area that needs to be addressed is the possible role of TS in development, since it is now abundantly clear that other adhesive glycoproteins function in a highly regulated fashion in cell adhesion and in the cell migrations that form tissues and organs (17). Another use of the cDNA will be in expressing first, the major domains of TS, and then selectively altered or mutagenized pieces of the protein. Tests of the in vivo functions of TS will require the use of transgenic animals, particularly to explore the role of TS in hemostasis.

Despite our ever widening knowledge about the distribution of TS in a variety of tissues and cells, several factors focus our attention on the role of TS in hemostasis. Not only is TS the most prevalent protein in platelet alpha-granules (4, 36) and involved in platelet aggregation (14, 22, 37), but it is implicated in processes like smooth muscle cell proliferation (39, 40) and perhaps wound healing. The finding that TS becomes incorporated, perhaps transiently, into blood clots (5) is also intriguing and may be related to the role of the clot as a specialized matrix that can promote the healing process as well as halt bleeding. Much remains to be learned about the possible involvement of TS in atherosclerosis (64). Therapies that rely on targeting plasminogen activators to sites of clot formation currently focus on the unique localization of fibrin in the clot. However, TS in clots may present another site to which therapeutic agents can be targeted. The ability of TS to bind plasminogen may also be of relevance here (31, 59, 60). The next few years should provide us with answers to many of the questions raised here and will raise intriguing new ones as the functions of this interesting protein are explored.

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