

The Properdin-like Type I Repeats of Human Thrombospondin Contain a Cell Attachment Site

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Abstract. Thrombospondin (TS) is a modular adhesive glycoprotein that contains three domains previously implicated in the attachment of cells to TS. These include the amino-terminal heparin-binding domain, the carboxy terminal cell or platelet-binding domain, and an RGDA sequence of TS. We have characterized a mAb against human TS, designated A4.1, which inhibits the attachment of human melanoma cells (G361) to TS. The epitope for A4.1 lies within the amino terminal half of the central stalklike region of TS which is distinct from the three known cell attachment sites. This region of TS is recovered in a 50-kD peptide after chymotryptic digestion of TS in EDTA. It contains the procollagen-like domain of TS as well as three type I repeats of a 60-residue segment homologous to two malarial proteins and the complement proteins properdin, and factors C6 through C9. The purified chymotryptic fragment is an effective at-

tachment factor for G361 cells. A4.1 blocks adhesion to the 50-kD domain, as do some sulfated glycoconjugates. RGD (and RGE) peptides and mAbs against other domains of TS are not inhibitory. Peptides (19mers) based on the core homology sequence of the three type I repeats of TS are potent attachment factors for these cells, and this adhesion is also inhibited by sulfated glycoconjugates. A polyclonal antibody raised against one of these peptides inhibits adhesion of G361 cells to the peptides, to the 50-kD fragment and to intact TS. Thus a new cell-adhesion site has been identified in TS whose sequence is very similar to the site identified in region II of the circumsporozoite protein of malaria parasites (Rich, K. A., F. W. George IV, J. L. Law, and W. J. Martin. 1990. *Science (Wash. DC)* 249:1574-1577. Thus there may be a common receptor which binds TS, malarial proteins, and properdin.

THROMBOSPONDIN (TS)¹ is a multidomain, adhesive glycoprotein that functions in the attachment, migration, and proliferation of a number of cell types (Frazier, 1987). It appears early in embryogenesis (O'Shea and Dixit, 1988; O'Shea et al., 1990) and its expression is transient and tightly regulated both in vivo and in vitro (Majack et al., 1985, 1987). TS has a role in platelet aggregation (Dixit et al., 1985b), and is thought to be involved in the regulation of smooth muscle cell growth by platelet-derived growth factor (Majack et al., 1985, 1988) a scenario which has implications for atherogenesis. TS has been implicated in tumor cell metastasis (Tuszynski et al., 1987) and is synthesized by a number of highly metastatic and invasive tumors (Varani et al., 1989). Most recently, TS and a hamster homologue of TS called gp140 (Rastinejad et al., 1989) have been found to be potent inhibitors of endothelial cell migration and proliferation stimulated by angiogenic mitogens (Good et al., 1990; Taraboletti et al., 1990). The in vivo correlate of this activity is the inhibition of angiogenesis by TS and gp140 in three different assays (Good et al., 1990). These findings suggest that TS may have important roles in

processes such as vascular development, wound healing, placental development, and tumor growth.

A first step in understanding the molecular basis of TS action on cells is to define the ways in which cells can interact with this structurally and functionally complex protein. As with other adhesive glycoproteins, TS has multiple sites that bind to distinct cellular receptors. For example, the NH₂-terminal heparin-binding domain of TS has been identified as mediating the binding and internalization of TS by endothelial (Murphy-Ullrich and Mosher, 1987) and CHO cells (Murphy-Ullrich et al., 1988). Further, the spreading of cells on intact TS is blocked by soluble heparin and by A2.5 (Roberts et al., 1987), a mAb specific for the heparin-binding domain (Dixit et al., 1985a). A cell surface proteoglycan of *M*_r 70,000 has been identified that appears to act as a receptor for this region of TS (Kaesberg et al., 1989; Sun et al., 1989). The COOH-terminal platelet- or cell-binding domain was thus named because of the inhibition of platelet aggregation (Dixit et al., 1985b) and cell attachment (Roberts et al., 1987) to TS by mAb C6.7 directed against the COOH-terminal 18-kD fragment of the 180-kD TS subunit (Galvin et al., 1985). This mAb is a potent inhibitor of the attachment of human melanoma cells such as G361, C32, and many other malignant cells to TS-coated plastic

1. *Abbreviations used in this paper:* pAb, polyclonal antibody; TRAP, thrombospondin related anonymous protein; TS, thrombospondin.

wells (Roberts et al., 1987; Varani et al., 1986). The receptor with which this region of TS interacts is not clearly identified. Early reports indicated that the OKM5 antigen, now called CD36 and thought to be identical to platelet gpIV (or gpIIb), is a receptor for TS whose binding is blocked by mAb C6.7 (Asch et al., 1987). However, recent experiments in which CD36 was cloned and expressed do not support the idea that it binds TS (Oquendo et al., 1989). RGD-containing peptides partially block the adhesion of smooth muscle, endothelial, normal rat kidney, U937 (Lawler et al., 1988), and C32 cells (Tuszynski et al., 1989) to TS-coated wells, suggesting that the RGDAC sequence near the COOH-terminal end of the calcium-binding domain of TS can also act as a cell attachment site. We have found partial inhibition of G361 melanoma cell attachment to TS with RGD peptides (D. Jaye, and W. A. Frazier, manuscript in preparation) suggesting that these cells can also adhere to TS via an integrin-type receptor.

In this report we present evidence that a fourth region of TS can act as a cell attachment site. We show that mAb A4.1 which binds to the central chymotryptic fragment of the TS subunit inhibits TS-mediated cell attachment as well as mAb C6.7. Since this TS fragment does not contain any of the previously identified cell attachment sites of TS described above, we wanted to determine if mAb A4.1 was acting by indirectly hindering a known cell attachment site or sites or by blocking an as yet unknown site in the central stalk region of TS. To do this, a proteolytic fragment of TS containing the epitope for mAb A4.1 was purified and found to support the attachment of melanoma cells. We have identified a peptide sequence which is active in cell attachment that lies within the type I threefold repeats that are homologous to region II of malaria circumsporozoite (CS) proteins, thrombospondin-related anonymous protein (TRAP), and the human complement protein properdin.

Materials and Methods

Materials

mAbs were raised as previously described by fusion of mouse spleen cells with the myeloma Sp2/Ag14 (Dixit et al., 1985b). mAbs were purified from mouse ascites fluid by chromatography on protein A-Sepharose columns, elution being accomplished with the mildest acid conditions possible as determined for each antibody. Specificity of mAb A4.1 was established by Western blotting against samples of whole platelets, serum, and purified proteins. G361 cells were obtained from the ATCC (#1424) and cultured in RPMI 1640 medium supplemented with 10% FCS at 5% CO₂ (Roberts et al., 1987). Cells were passaged by mild trypsin treatment. All proteins and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Dextran sulfates were from Pharmacia Fine Chemicals (Uppsala, Sweden), *M_r* 500,000; ICN Radiochemicals (Irvine, CA) *M_r* 40,000 and Sigma Chemical Co. *M_r* 5,000. The peptides GRGDSP and GRGESP were from Immuno-Dynamics, Inc. (San Diego, CA). Human plasma fibronectin was the gift of T. Broekelmann and Dr. J. A. McDonald. Human platelet TS was purified as described (Dixit et al., 1984) and human platelet factor 4 (PF4) was obtained from the same preparations by elution of the heparin-Sepharose column with 2 M NaCl.

Cell Adhesion Assay and Photography

Cells were harvested from near confluent cultures by brief treatment with EDTA and were immediately washed and resuspended in Hepes-buffered saline plus 4 mg/ml BSA. A 100- μ l volume of this cell suspension was added to wells containing an equal volume of TBS (pH 7.6) with 2 mM each CaCl₂ and MgCl₂. Cell viability was tested by trypan blue exclusion and

was routinely >90%. Plastic 96-well plates (Immuno Plate Maxisorp; Nunc, Roskilde, Denmark) were coated with indicated substrate proteins by incubation in 50 μ l of TBS per well at the indicated concentrations overnight at 4°C or for 2 h at 37°C. Wells were rinsed with TBS and blocked with 10 mg/ml BSA for 30 min at room temperature. After rinsing the plates, any inhibitors or other additions were made in 100 μ l TBS and cells added as above. Plates were incubated at 37°C for 1–2 h, rinsed three times with TBS, observed under Hoffman optics for qualitative assessment, and then rinsed three times with TBS. Adherent cells were photographed with a 40 \times Hoffman objective using Tri-X pan 400 ASA. Cell attachment was quantified with a colorimetric reaction using endogenous cellular phosphatase activity by adding 100 μ l of the following substrate/lysis solution to each well: 1% Triton X-100, 6 mg/ml *p*-nitrophenylphosphate, in 50 mM sodium acetate buffer, pH 5.0. Wells were incubated for 1–2 h at 37°C, the reaction stopped by the addition of 50 μ l 1 N NaOH, and read in an ELISA plate reader (Dynatech Laboratories, Inc., Cambridge, MA) with a 410-nM filter. Wells were set up in triplicate and all experiments were repeated at least three times. Percent of cell attachment was determined by comparison of color yield with that obtained from the number of cells initially plated in each well. Standard curves were linear with cell number from 100–100,000 cells.

Generation and Purification of 70- and 25-kD Domains

Proteolysis of human platelet TS was performed with TLCK-treated chymotrypsin at 1% (wt/wt) of TS in TBS, pH 7.4, 10 mM EDTA for 30–60 min at room temperature as described (Galvin et al., 1985; Dixit et al., 1986). TS fragments were purified by gel filtration on Sephacryl S-200 columns and heparin-Sepharose affinity chromatography to remove intact TS and purify the heparin-binding domain of TS (Dixit et al., 1984). The digests and purified fragments were characterized by silver-stained SDS-PAGE, and by Western blotting, with the panel of anti-TS mAbs. Purified fragments were free of intact TS, or fragments >70 kD on reducing SDS-PAGE (see Fig. 2). The 70/50-kD fragments were trimeric on unreduced SDS-PAGE.

Peptide Synthesis

Peptides based on TS amino acid sequences were synthesized by the Washington University Protein Chemistry Facility (Mark Frazier, technical director) using a solid phase peptide synthesizer (model 380A; Applied Biosystems, Foster City, CA). Peptides were cleaved and deblocked by Immudynamics, Inc. and purified by reverse phase HPLC (Waters) using acetonitrile/0.1% TFA solvent systems. Purity was tested with analytical HPLC and peptides were checked by amino acid composition and automated sequence analysis (model 477A sequencer; Applied Biosystems). Integrity of tryptophan residues was analyzed by scanning UV spectroscopy. Peptides were labeled with ¹²⁵I-Bolton Hunter reagent (ICN Radiochemicals) by incubation of 40 nmol of peptide with 0.25 mCi of reagent in 0.1 M phosphate buffer, pH 7.4 for 2 h at room temperature. Labeled peptides were purified on G25 columns equilibrated in TBS and used to quantify the amount of peptide immobilized on the plastic wells.

Generation of Polyclonal Antibodies against Peptide II

The cysteine-containing version of peptide II (see Fig. 9 for sequence) was oxidized to form large polymeric aggregates (as judged by HPLC and gel filtration). The dialyzed and lyophilized material was suspended in water and complete Freund's adjuvant. 1 mg of peptide was injected subcutaneously at 10 sites (100- μ g each) on the backs of two white New Zealand female rabbits. Each rabbit was subsequently boosted with 300 μ g of peptide material three times, and exsanguinated. Defibrinated, cell-free serum was then purified on a protein A-Sepharose column (Bio Rad Laboratories, Richmond, CA) to obtain the IgG fraction. Reactivity of the IgG fraction was determined by ELISA against peptides I, II, and III, and by Western blotting against TS and its chymotryptic digest.

Results

Characterization and Mapping of mAb A4.1

mAb A4.1 was selected by ELISA screening from hybridomas resulting from immunization with denatured TS. Screen-

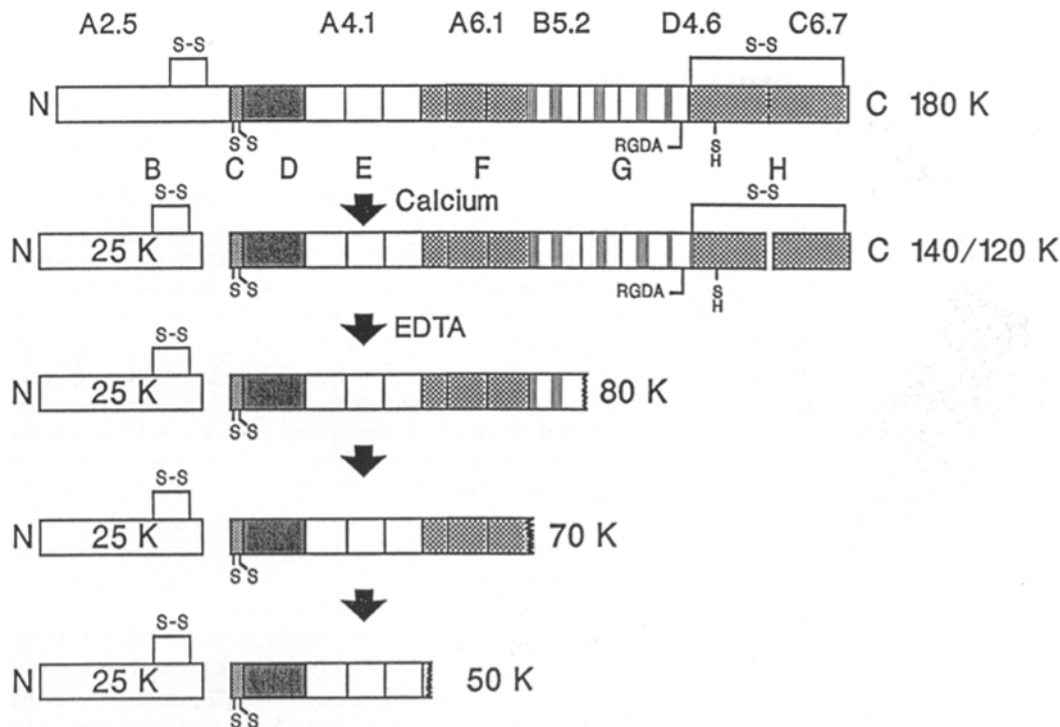


Figure 1. Schematic representation of the chymotryptic digestion of TS. In 1 mM calcium, the main products are the 25 kD, heparin-binding domain (region B), and the 120- and 20-kD fragments. Region C contains the cysteines forming interchain disulfides; region D is the procollagen-like segment; region E contains three type I repeats (see text); region F consists of three EGF-like repeats (type II); region G is the primary calcium-binding domain and is highly repetitive (type III repeats); region H is the platelet- (or cell-) binding domain. The single disulfides in region B and H are indicated as is the single free SH of TS in region H, and the RGDA sequence in region G. Above the figure are indicated the approximate locations of epitopes for mAbs. In EDTA, the digest progresses as indicated with essentially stepwise proteolysis from the COOH terminus.

ing of positives on Western blots of TS digested with chymotrypsin in the presence of EDTA indicated that it reacted with the 70-kD fragment of TS which we and others have previously characterized as containing the central stalk like region of TS (Fig. 1). This fragment begins with residue number 241 of the mature TS subunit and extends in a COOH-terminal direction to a site in or near the last of the three EGF-like repeats of TS (Galvin et al., 1985). It includes the two cysteines at 252 and 256 which are responsible for trimer formation, hence the fragment migrates as a 210-kD species on nonreducing SDS gels and is visualized as a trimer with rotary shadowing EM (Galvin et al., 1987). This fragment has been shown to contain the binding sites for collagens (Mumby et al., 1984; Galvin et al., 1987), plasminogen, fibronectin, laminin, and fibrinogen (Lawler et al., 1986). Binding of A4.1 to EDTA-treated TS is about twofold greater than to Ca^{2+} replete TS, indicating that its epitope lies in a region that is somewhat Ca^{2+} sensitive. Chymotryptic digestion of TS proceeds in a stepwise manner from the COOH terminus as shown in Fig. 1. We have previously mapped another mAb designated A6.1 as binding near the COOH-terminal end of the 70-kD fragment and whose binding to TS is strongly inhibited by calcium (Dixit et al., 1986). We tested mAbs A4.1 and A6.1 for mutual competition in their binding to TS and found none, suggesting that their epitopes are quite far apart. Hence, A4.1 probably binds near the NH_2 terminus of the 70-kD species. As shown in Fig. 1,

further digestion of the 70-kD species occurs with prolonged chymotrypsin treatment giving rise to a 50-kD fragment indicating further digestion from the COOH terminus. A4.1 binds to both the 50- and 70-kD fragments (Fig. 2) while A6.1 binds only to the 70-kD band proving that the A4.1 epitope is indeed NH_2 terminal to that for A6.1.

Effect of mAbs on Attachment of G361 Melanoma Cells to TS

As reported by Roberts et al. (1987), human melanomas such as G361 adhere to TS-coated plastic wells. We examined the effect of A4.1 on adhesion of these cells to TS. These results along with those of other mAbs for comparison are shown in Fig. 3. As reported previously (Roberts et al., 1987), mAb C6.7 has a dramatic inhibitory effect on the adhesion of G361 cells to TS (coated at 50 $\mu\text{g}/\text{ml}$) while A2.5 (the anti-heparin-binding domain monoclonal) has little effect on cell adhesion. mAb A4.1 is as potent as C6.7 at inhibiting cell adhesion in this assay. mAb A6.1 whose epitope lies between those of C6.7 and A4.1 (Fig. 1), shows only a slight effect on cell adhesion. We have made mAb A4.1 available to other laboratories and it has been reported that adhesion of rat smooth muscle cells (Majack et al., 1988), human keratinocytes, and squamous carcinoma cells (Varani et al., 1988) to TS is inhibited by A4.1. Since C6.7 inhibits secretion-

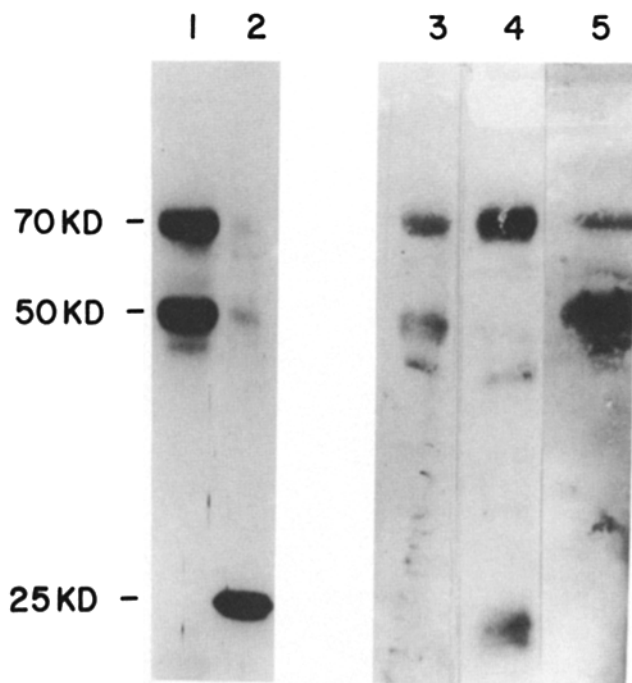


Figure 2. Purification of TS fragments. Lanes 1 and 2 show the silver-stained SDS-PAGE (reduced) of purified 70/50 kD (lane 1) and 25 kD (lane 2) fragments of TS. Western blots of the 70/50 kD shown in lane 1 were probed with mAb A4.1 (lane 3), mAb A6.1 (lane 4). Lane 5 shows the Western blot of 70/50 kD probed with polyclonal antibody raised against peptide II. The 70/50-kD sample used for the blot in lane 5 had been treated with chymotrypsin for a longer period of time resulting in an increase in the ratio of the 50- to 70-kD bands.

dependent or secondary-phase platelet aggregation (Dixit et al., 1985b) we tested A4.1 in a platelet aggregation assay and found no effect on the aggregation of washed platelets stimulated with thrombin (not shown).

Cell Attachment to Fragments of TS

To determine if the effect of mAb A4.1 on cell attachment is because of its direct blocking of an adhesive site on TS or a conformational effect transmitted to one of the other domains of TS, we prepared the fragment of TS which contains the epitope for A4.1 and tested its ability to support cell attachment. We compared the ability of G361 cells to adhere to wells coated with intact TS, the unfractionated chymotryptic digest, the purified NH₂-terminal heparin-binding domain and the 70/50-kD trimer. To ensure complete digestion we used vigorous conditions that convert some of the 70- to the 50-kD fragment, both of which bind A4.1 (above). The unfractionated digest had 30–40% (3 experiments) the attachment activity of undigested TS when coated at equal concentrations. The purified 25 kD had 10–20% the activity of TS while the purified 70/50 kD trimer had nearly the same activity as the digest, i.e., ~30% that of intact TS. These differences may be in part because of differences in the efficiency of coating of the different TS fragments. Even so, the levels of attachment to the purified 70/50 kD are quite significant and clearly indicate that a ma-

ior cell attachment site exists in this region of TS. The purified fragments were also tested for contamination by other fragments using silver-stained SDS gels and Western blotting with mAbs. In particular, no contamination of the 70/50-kD fragment by intact TS or its larger digestion products was seen (Fig. 2).

While the 70/50-kD fragment of TS is active in the attachment of cells, it is not able to support their cytoskeletal rearrangement or spreading. In many experiments, essentially no cell spreading was observed (Fig. 4 B). Roberts et al. (1987) reported that the NH₂-terminal heparin-binding domain of TS is needed for spreading of G361 cells on TS (Fig. 4 A). Interestingly, the purified heparin-binding domain alone is not a very potent attachment factor for these cells and the cells that do attach do not spread (Fig. 4 C). In an attempt to reconstruct a fully functional "molecule" immobilized on plastic, we mixed the 70/50-kD fragment of TS with the purified 25-kD heparin-binding domain and coated this mixture. Even fewer spread cells were observed on this mixed surface than were seen on either the 70/50-kD fragment or the 25-kD domain alone (not shown).

Fig. 5 shows the concentration dependence of cell attachment to the purified 70/50-kD trimer. A number of potential inhibitors of cell binding to the 70/50-kD trimer were tested. The panel of mAbs was tested with the result that only A4.1 and A6.1 inhibited cell attachment (Fig. 6). As expected, cell attachment to the heparin-binding domain (25 kD) was inhibited by heparin, fucoidin, and dextran sulfates of mol wts 5,000, 40,000, and 500,000 (not shown) (Roberts et al., 1987). Binding of cells to the 70/50-kD trimers was also partially inhibited by heparin, heparan sulfate, fucoidin, and a panel of dextran sulfates of different molecular weights (Fig. 7). The order of potency found here is similar to that reported recently by Holt et al. (1990) for the competition of binding of the homologous proteins properdin and antistasin to immobilized sulfatides. While the heparin and sul-

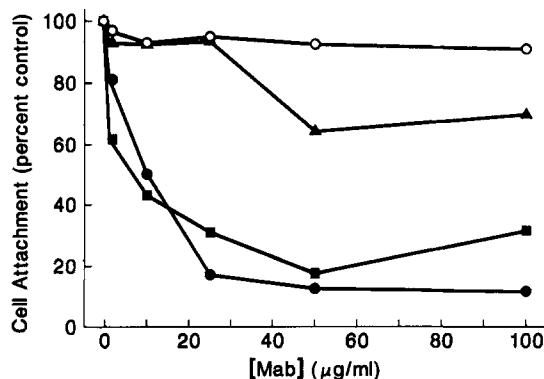


Figure 3. Inhibition by mAbs of G361 cell attachment to TS. Cells were incubated for 1 h at 37°C in wells coated with 50 µg/ml TS. Attachment was quantitated with a colorimetric, endogenous cellular phosphatase assay (see Materials and Methods). Control wells had an absorbance at 410 between 1 and 1.5 in five separate experiments. Wells coated with BSA were used as negative controls and these values were routinely less than 1% of the positive controls. The mAbs were added to cells at the indicated concentrations: mAb A2.5 (○); mAb A6.1 (▲); mAb C6.7 (■); mAb A4.1 (●).

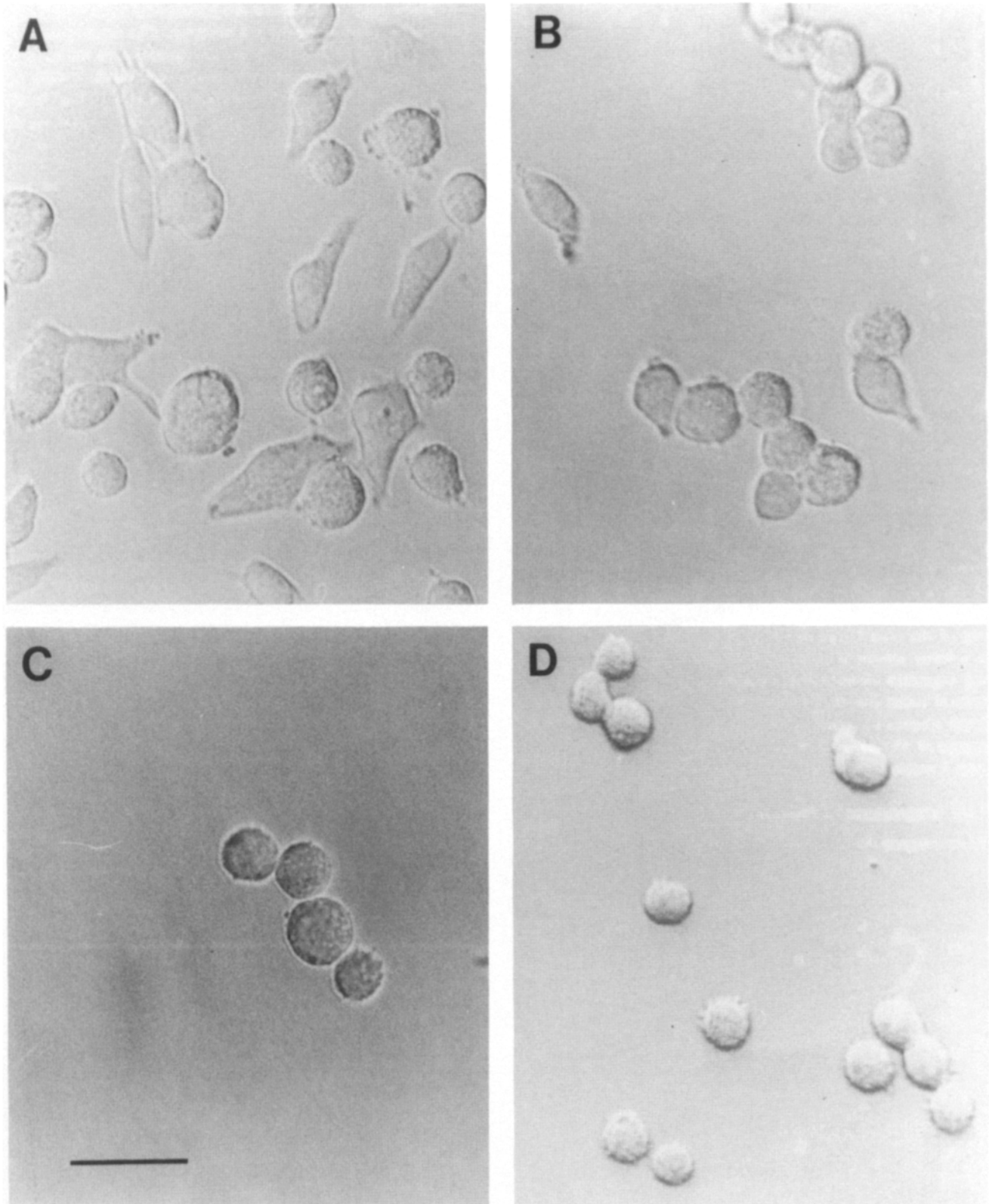


Figure 4. Attachment of G361 cells to TS fragments and peptides. Wells were coated with 100 $\mu\text{g}/\text{ml}$ of TS: (A) 70/50 kD; (B) 25 kD; (C) or 100 μM of Peptide III (D), for 2 h at 37°C. After blocking with BSA, cells were allowed to attach for 90 min and photographed with a Hoffman 40 \times objective. Bar, 50 μm .

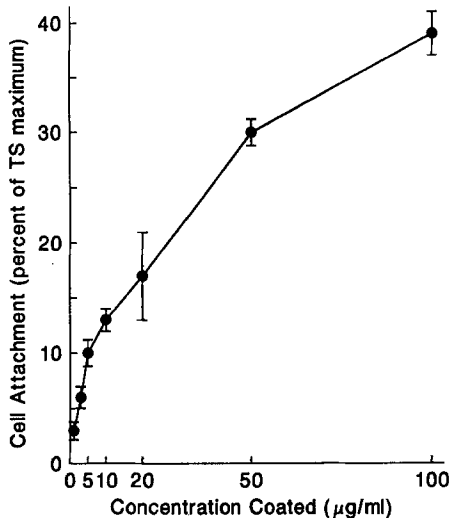


Figure 5. Attachment of G361 cells to the 70/50 fragment of TS. The purified 70/50 kD was coated at indicated concentrations. The assay was performed as described and results expressed as percent maximum adherence to 10 µg/ml TS.

fatide-binding activity of TS has been previously ascribed to the NH₂-terminal 25-kD domain of TS (Dixit et al., 1984; Roberts et al., 1985), it seems that the 70-kD fragment of TS, and specifically the properdin-like type I repeats, may contain another binding site for these compounds. In fact, two of the type I repeats of TS contain the BBXB (where B is a basic amino acid) consensus glycosaminoglycan-binding sequence identified in a number of heparin-binding proteins (Cardin and Weintraub, 1989). The effects of these sulfated glycoconjugates on cell attachment to the 70/50-kD region of TS serves to focus attention on the properdin-like type I repeats of TS as potential cell-binding sites.

Cell Attachment to Peptides

To investigate this idea, we synthesized peptides based on the

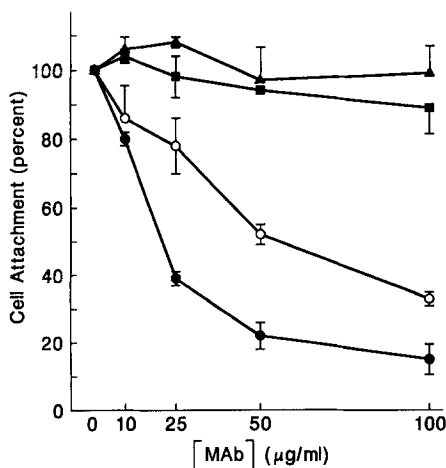


Figure 6. Effect of mAbs on attachment of G361 cells to the 70/50-kD fragment. In the standard assay mAbs were included at the indicated concentrations. The mAbs are: A2.5 (▲); C6.7 (■); A6.1 (○); A4.1 (●). Control attachment (100%) is that with no added mAb.

core homology of the three properdin-like type I repeats of TS with properdin and the CS proteins. The sequences of the three segments of TS are shown compared with the homologous regions of properdin (Goundis and Reid, 1988), antistasin (Nutt et al., 1988), and a CS protein in Fig. 8. The TS peptides were synthesized with alanines replacing the cysteines, and peptide II was also synthesized with the cysteines intact.

We first tested these three peptides along with GRGDSP and its control, GRGESp as potential inhibitors of cell adhesion to TS and 70/50-kD coated wells. GRGDSP at 1.5 mM inhibited attachment of G361 cells to intact TS by 25% ($\pm 4\%$) while GRGESp was inactive (not shown). However, GRGDSP was completely inactive against attachment to the 70/50-kD trimers as expected since there is no RGD sequence within the 70-kD region of TS. The three type I peptides caused a stimulation of cell attachment to both TS and the 70/50-kD fragment. We suspected that this effect was because of the peptides binding to the blocked wells. Thus, we coated the peptides directly on the plastic wells to test their activity as cell attachment factors. As shown in Fig. 9, peptides II and III were very potent at attaching cells, while peptide I was nearly inactive. Restoring the two cysteines in the peptide II sequence caused about a twofold increase in cell attachment activity. Using radioiodinated peptides, we compared the binding of the four type I peptides to the plastic wells. Peptides II and III bound to the plastic to identical extents and peptide II with cysteines bound 50% better, thus explaining the increased cell attachment (above). Peptide I which bound cells very poorly, was in fact adsorbed to wells more efficiently than peptides II and III. Cell attachment to the peptides was blocked by the same sulfated glycoconjugates that inhibited attachment to the 70/50-kD trimers (Fig. 7), indicating that the peptides probably represent the cell attachment site expressed within the 70-kD region of TS. Interestingly, heparin and heparan sulfate are more potent against peptide III-mediated cell attachment than peptide II-mediated attachment. This may reflect the presence of a complete BBXB glycosaminoglycan-binding sequence at the COOH-terminal end of peptide III.

We raised polyclonal antibodies (pAbs) against the cysteine-containing form of peptide II in rabbits. These pAbs reacted with peptides II and III having essentially no reaction with peptide I in ELISA assays (not shown). The pAbs reacted with both the 50- and 70-kD species on Western blots (Fig. 2) thus establishing that the 50-kD fragment of TS does in fact contain the properdin-like sequences. On Western blots of unreduced SDS gels, the pAb stains the trimeric species which migrate in the 150–200-kD size range (not shown). The effect of the pAb on cell attachment to peptides II and III, the 70/50-kD trimer and intact TS is shown in Fig. 10. As expected, the most potent effect is on cell attachment to peptide II. However, cell attachment to peptide III, the 70/50-kD trimers and intact TS is also substantially inhibited (Fig. 10). G361 cells adhere quite avidly to platelet factor 4, and this attachment is unaffected even at the highest concentrations of the pAb (Fig. 10).

Discussion

The data presented here indicate that a previously undetected site on TS can function in the attachment of melanoma (and

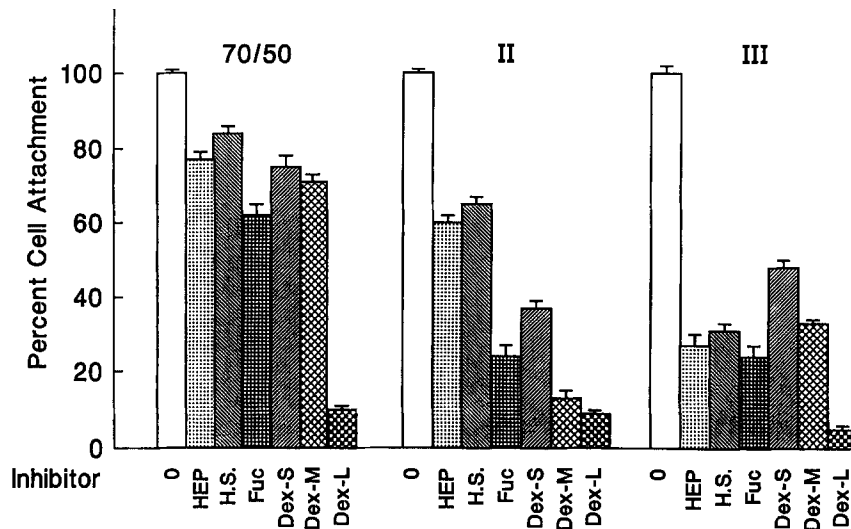


Figure 7. Effect of sulfated glycans on G361 cell attachment to 70/50-kD fragment and peptides. 70/50-kD fragment was coated at 25 $\mu\text{g/ml}$ and the assay performed as described. Peptides were coated at 100 μM . The following inhibitors were included with cells at 50 $\mu\text{g/ml}$: (O) no inhibitor; (HEP) heparin; (H.S.) heparan sulfate; (FUC) fucoidin; (DEX-S) dextran sulfate, M_r 5,000; (DEX-M) dextran sulfate, M_r 40,000; (DEX-L) dextran sulfate M_r 500,000.

probably other) cells to TS. This site is defined by the mAb A4.1, the 50-kD proteolytic fragment of TS and is pinpointed by the activity of peptides II and III as cell attachment factors. The inhibition of cell binding to the 70/50-kD fragment and intact TS by mAb A4.1 and by the pAb against peptide II suggests that peptides II and III are functional in the larger context of TS structure. The biological importance of this site within TS is indicated by the fact that human melanoma G361 cells, human keratinocytes, squamous carcinoma cells (Varani et al., 1988), and rat aortic smooth muscle cells (Majack et al., 1988) all adhere to TS in a manner inhibited by mAb A4.1. In agreement with earlier results with larger fragments of TS (Roberts et al., 1987), we find that the 70/50-kD fragment of TS does not support spreading of these cells.

An interesting and significant facet of the type I peptide sequences is their remarkable degree of conservation in a number of other proteins. As shown in Fig. 8, the three TS peptides are most closely related to two classes of proteins synthesized by different stages of *Plasmodia* species, the causative agent in malaria. The CS proteins are of particular

interest in that they are thought to mediate binding of the sporozoites to liver, the first stage in the cycle of infection. In particular, region II of the CS protein, which is contained within the sequence shown in Fig. 8, is thought to be part of the recognition site. Support for this idea comes from recent work of Rich et al. (1990) who found that a peptide sequence in region II of the *Plasmodia vivax* CS protein is an attachment factor for T lymphocytic and myeloid cell lines. These authors used shorter peptides to narrow down the recognition site to the sequence VTG of region II. Interestingly, TS type I peptides II and III contain this sequence and both are active in melanoma cell attachment, while peptide I does not contain this sequence (Fig. 8) and is not active (Fig. 9). This raises the possibility that the same receptor is present on G361 cells, the cell lines identified by Rich et al. (1990), and perhaps hepatocytes as well. If this is indeed the case, our data suggest that the cysteine is not a required determinant of this site since our peptides contain alanine at these positions. In fact Rich et al. (1990) remarked that the oxidation state of the Cys is not critical, suggesting that the recognition sequence is probably VTG. The VTG se-

Peptides I, II, III of Thrombospondin and Related Sequences

Protein	Sequence	Residues
Thrombospondin		
I	S E W T S C S T S C G N G I Q R G R	368-86
II	S P W S S C S V T C G D G V I T R I R	424-42
III	S P W D I C S V T C G G G V Q K R S R	481-99
<i>P. falciparum</i>		
C.S. Protein	D E W S P C S V T C G N G I Q V R I K	344-62
TRAP	T E W S P C S V T C G K G T R S R K R	245-63
Properdin		
1	S L W G P C S V T C S E G S Q L R H R	57-75
2	G P W G P C S V T C S K G T Q I R Q R	116-34
Antistasin		
1	S G V - R C R V H C P H G F Q - R S R	29-45
2	R I D I N C R K T C P N G L K - R D K	83-100

Figure 8. Peptides used in this study and their homologues. The most highly conserved regions of the three TS type I repeats were selected for peptide synthesis. The sequences of these three peptides as they occur in TS (along with residue number in the TS sequence) are aligned in the first three lines. The peptides used for most cell-binding studies contained alanine residues in place of the two cysteines. Related amino acid sequences found in four other proteins are shown below their TS homologues. The circumsporozoite protein (CS protein) and the TRAP contain regions displaying the highest similarity to the TS repeats. The first two of six type I repeats of properdin are shown along with the two type I units of antistasin (see text for references to the sequences).

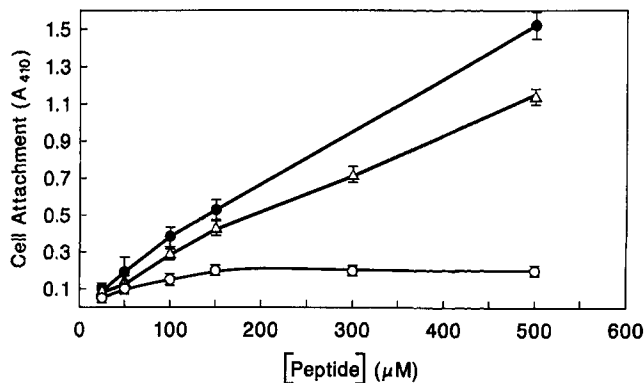


Figure 9. Attachment of G361 cells to synthetic TS peptides. Peptides I, II, and III of TS (see Fig. 8) were coated on plastic wells at the indicated concentrations and the assay was performed as described. Peptide I (○); peptide II (△); peptide III (●). In this case, the actual absorbance at 410 nm is plotted. The value for intact TS (10 μg/ml) in this experiment was 1.9 ± 0.03 .

quence also occurs in TRAP (Robson et al., 1988) which is expressed on the surface of parasitized erythrocytes at a later stage of infection.

Our finding that sulfated glycoconjugates inhibit cell attachment to the type I peptides, particularly peptide III, may indicate that a two domain-binding site is represented on these peptides. VTCG could represent a recognition site for a receptor and the BBXB sequence downstream would be a likely candidate for the glycoconjugate-binding site. This would be particularly relevant if the receptor contained a glycosaminoglycan chain in proximity to the VTCG-binding site. In this regard, it should be noted that all of the species of CS proteins and TRAP contain a cluster of positively charged amino acids downstream of the VTCG site and some contain a BBXB motif. Our preliminary data suggests that even though sulfated glycans inhibit cell attachment to the peptides, treatments of cells which reduce the numbers of glycosaminoglycan chains on their surface do not cause reduced attachment. Cells grown in β-D-xyloside (Hamati et al., 1989) or treated with heparitinase (Sun et al., 1989), show no deficit in their attachment to peptides II and III (Prater, C. A., and W. A. Frazier, unpublished data). This suggests that cell surface glycosaminoglycans are not the receptor for the peptides, but does not rule out their involvement as a modulator of receptor-peptide interactions.

Homologues of the type I repeats of TS are also found in the human protein properdin (Goundis and Reid, 1988). This protein consists of little else besides six copies of the TS type I motif. Properdin is thought to stabilize complex formation between factors C3b and the protease Bb of the complement system leading to the formation of the convertase which activates C3 and C5. Goundis and Reid (1988) who sequenced properdin cDNA and noted the homology between TS and the complement proteins C6 through C9 (Haefliger et al., 1989), speculated that the type I repeats may be involved in a membrane-binding event. This could occur directly to a unique receptor for type I motifs or indirectly by first binding to C3b (or its derivatives) which would then bind to CR1, CR2, or CR3. We have tried to detect the binding of radioiodinated TS to C3b immobilized on plastic wells and found no specific interaction (Prater,

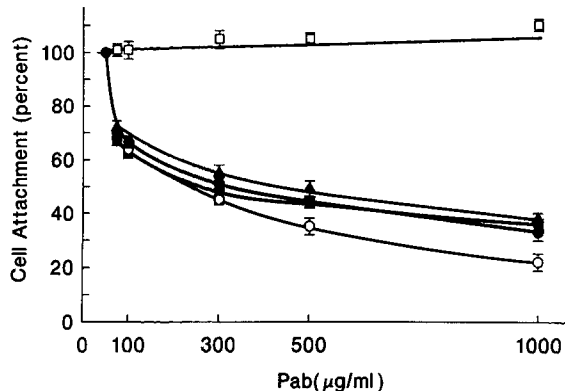


Figure 10. Effect of anti-peptide pAb on attachment of G361 cells to TS, TS fragments, and peptides. Wells were coated with TS (■) at 10 μg/ml, 70/50 kD (●) at 25 μg/ml; or platelet factor 4 (□) at 25 μg/ml. Peptide II (○) and peptide III (▲) were coated at 100 μM. Assays were performed as described with pAb added to cells at the indicated concentrations.

C. A., and W. A. Frazier, unpublished data). Thus it seems likely that some direct interaction may occur between the type I repeats of TS (and perhaps its homologues) and a receptor on the surface of G361 cells, liver, and some circulating cells (Rich et al., 1990).

Another protein that contains more distantly related TS type I repeats is the leech protein antistasin, so named for its anticoagulant properties (Nutt et al., 1988). Holt et al. (1990) have recently shown that TS, properdin, and antistasin all bind to sulfatide [Gal(3-SO₄)/β1-ICer], and this binding has a very similar profile of inhibition by sulfated glycoconjugates for all three proteins. Since the only homologous sequence among these three proteins is their TS type I repeat structures, Holt et al. (1990) suggest that this sequence forms the binding site for sulfatides and glycosaminoglycans. These observations along with our data suggest that TS has a second "sulfated glycan binding" domain distinct from the previously characterized NH₂-terminal 25-kD heparin-binding domain. It should be noted that antistasin type I repeats do not contain the VTCG sequence found in TS, properdin, and the CS proteins (Fig. 8).

Other adhesive glycoproteins have multiple heparin or glycosaminoglycan binding domains. For example, fibronectin has at least two well-characterized heparin-binding sites, one active at physiological salt concentrations and another site which is effective at binding heparin at lower ionic strength (Ruoslahti, 1988). However, this second site may have higher affinity for another glycosaminoglycan or sulfatide. Laminin has three characterized heparin-binding regions, two in the B chain and one in the A chain, each of which has been modeled as a peptide sequence (Kouzi-Koliakos et al., 1989). Type IV collagen has also been found to display three distinct affinities for heparin (Tsilibary et al., 1988) corresponding to three sites distributed at three distinct locations along the type IV molecule. Thus it appears that multiple heparin-binding sites are commonly present in extracellular adhesive glycoproteins. While heparin affects the assembly of laminin polymers (Kouzi-Koliakos et al., 1989) and type IV collagen polymers (Tsilibary et al., 1988), little is known about the functions of these binding sites *in vivo*, or even the precise nature of their physiological li-

gands. It seems clear that for most of these sites heparin is an artificial probe that is conveniently available, and is probably not the relevant ligand in vivo.

Two other regions at which cells attach to TS are found near the COOH terminus of the peptide chain. These are the RGDA sequence at the COOH terminus of the calcium-binding domain and the COOH-terminal "cell-binding" domain. The receptor for the RGDA site appears to be related or identical to the vitronectin receptor, a β_3 integrin (Lawler et al., 1988). It is likely that the exposure or conformation of the RGD sequence is regulated by calcium (Frazier, 1987; Lawler et al., 1988). The identity of the receptor for the COOH-terminal cell-binding domain of TS is somewhat controversial. Asch et al. (1987) have reported that platelet glycoprotein IV (or IIIb) is the TS receptor, and that this protein is the same as the antigen CD36 defined by mAb OKM5. However, expression of CD36 does not lead to TS binding while another function ascribed to CD36, i.e., binding of malaria-parasitized red cells, is present (Oquendo et al., 1989). It is this COOH-terminal domain of TS with which mAb C6.7 reacts to block adhesion of a number of cell types to TS-coated surfaces and to inhibit secondary phase (secretion dependent) platelet aggregation (Dixit et al., 1985b). The fact that mAb A4.1 does not affect platelet aggregation is consistent with a separate cell-binding site in the TS type I repeats whose receptor is not present on platelets.

The functional consequences of cellular interaction at these four sites on TS remain to be elucidated. One can speculate that the different receptors that bind to these sites are linked to different signal transduction mechanisms or perhaps to different cytoskeletal proteins. Taraboletti et al. (1987) reported that the amino terminal heparin-binding domain of TS is required for melanoma cell spreading while the COOH-terminal cell-binding domain is necessary for their attachment, but will not allow spreading to occur. This suggests a differential interaction of these TS domains with the cytoskeletal apparatus. Furthermore, TS appears to have opposite effects on cellular proliferation in different cell types. Rat aortic smooth muscle cells respond to PDGF treatment by making and secreting TS very rapidly, and this extracellular TS seems to be permissive or stimulatory for cell division in response to the mitogen (Majack et al., 1985, 1988). Bovine capillary endothelial cells chemotax toward bFGF which is also a mitogen for these cells and a potent angiogenic stimulus in vivo (Rastinejad et al., 1989; Folkman and Klagsbrun, 1987). TS blocks the migration and proliferation of BCEC in response to bFGF in vitro (Good et al., 1990; Taraboletti et al., 1990). These effects would appear to account for the finding that TS is a potent inhibitor of angiogenesis in three in vivo assays (Good et al., 1990). These opposite effects of TS on smooth muscle cells and bovine capillary endothelial cells may reflect the presence of different sets of TS receptors on these cell types that recognize different sites on TS. The situation may be even more complex. We have recently found that an isoform of TS exists that is derived by alternative splicing of TS mRNA to yield a protein that contains the NH₂-terminal heparin-binding domain of TS but none of the other three cell attachment sites (Paul et al., 1990). Preliminary evidence suggests that other TS isoforms exist that may lack other cell attachment sites thus setting the stage for a complex set of protein variants which can interact with as many as four cellular receptors.

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References

- Asch, A. S., J. Barnwell, R. L. Silverstein, and R. L. Nachman. 1987. Isolation of the thrombospondin membrane receptor. *J. Clin. Invest.* 79:1054-1061.
- Cardin, A. D., and H. J. R. Weintraub. 1989. Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis*. 9:21-32.
- Dixit, V. M., G. A. Grant, S. A. Santoro, and W. A. Frazier. 1984. Isolation and characterization of a heparin-binding domain from the amino terminus of platelet thrombospondin. *J. Biol. Chem.* 259:10100-10105.
- Dixit, V. M., D. M. Haverstick, K. M. O'Rourke, S. W. Hennessy, G. A. Grant, S. A. Santoro, and W. A. Frazier. 1985a. Effects of anti-thrombospondin monoclonal antibodies on the agglutination of erythrocytes and fixed, activated platelets by purified thrombospondin. *Biochemistry*. 24:4270-4275.
- Dixit, V. M., D. M. Haverstick, K. M. O'Rourke, S. W. Hennessy, G. A. Grant, S. A. Santoro, and W. A. Frazier. 1985b. A monoclonal antibody against human thrombospondin inhibits platelet aggregation. *Proc. Natl. Acad. Sci. USA*. 82:3472-3476.
- Dixit, V. M., N. J. Galvin, K. M. O'Rourke, and W. A. Frazier. 1986. Monoclonal antibodies that recognize calcium-dependent structures of human thrombospondin. *J. Biol. Chem.* 261:1962-1968.
- Folkman, J., and M. Klagsbrun. 1987. Angiogenic factors. *Science (Wash. DC)*. 235:442-447.
- Frazier, W. A. 1987. Thrombospondin: a modular adhesive glycoprotein of platelets and nucleated cells. *J. Cell Biol.* 105:625-632.
- Galvin, N. J., V. M. Dixit, K. M. O'Rourke, S. A. Santoro, G. A. Grant, and W. A. Frazier. 1985. Mapping of epitopes for monoclonal antibodies against human platelet thrombospondin with electron microscopy and high sensitivity amino acid sequencing. *J. Cell Biol.* 101:1434-1441.
- Galvin, N. J., P. M. Vance, V. M. Dixit, B. Fink, and W. A. Frazier. 1987. Interaction of human thrombospondin with types I-V collagen: direct binding and electron microscopy. *J. Cell Biol.* 104:1413-1422.
- Good, D. J., P. J. Polverini, F. Rastinejad, M. M. Le Beau, R. S. Lemons, W. A. Frazier, and N. P. Bouck. 1990. A tumor suppressor dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc. Natl. Acad. Sci. USA*. 87:6624-6628.
- Groundis, D., and K. B. M. Reid. 1988. Properdin, the terminal complement components, thrombospondin and the circumsporozoite protein of malaria parasites contain similar sequence motifs. *Nature (Wash. DC)*. 335:82-85.
- Haeffliger, J. A., J. Tschopp, N. Vial, and D. E. Jenne. 1989. Complete primary structure and functional characterization of the sixth component of the human complement system. *J. Biol. Chem.* 264:18041-18051.
- Hamati, H. F., E. L. Britton, and D. J. Carey. 1989. Inhibition of proteoglycan synthesis alters extracellular matrix deposition, proliferation, and cytoskeletal organization of rat aortic smooth muscle cells in culture. *J. Cell Biol.* 108:2495-2505.
- Holt, G. D., M. K. Pangburn, and V. Ginsburg. 1990. Properdin binds to sulfatide [Gal(3-SO₄) β 1-Cer] and has a sequence homology with other proteins that bind sulfated glycoconjugates. *J. Biol. Chem.* 265:2852-2855.
- Kaesberg, P. R., W. B. Ershler, J. D. Esko, and D. F. Mosher. 1989. Chinese hamster ovary cell adhesion to human platelet thrombospondin is dependent on cell surface heparan sulfate proteoglycan. *J. Clin. Invest.* 83:994-1001.
- Kouzi-Koliakos, K., G. G. Koliakos, E. C. Tsilibary, L. T. Furcht, and A. S. Charonis. 1989. Mapping of three major heparin-binding sites on laminin and identification of a novel heparin-binding site on the B1 chain. *J. Biol. Chem.* 264:17971-17978.
- Lawler, J., and R. O. Hynes. 1986. The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium binding sites and homologies with several different proteins. *J. Cell Biol.* 103:1635-1648.
- Lawler, J., J. E. Connolly, P. Ferro, and L. H. Derick. 1986. Thrombin and chymotrypsin interactions with thrombospondin. *Ann. NY Acad. Sci.* 485:271-287.
- Lawler, J., R. Weinstein, and R. O. Hynes. 1988. Cell attachment to thrombospondin: the role of ARG-GLY-ASP, calcium, and integrin receptors. *J. Cell Biol.* 107:2351-2361.
- Majack, R. A., S. C. Cook, and P. Bornstein. 1985. Platelet-derived growth factor and heparin-like glycosaminoglycans regulate thrombospondin synthesis and deposition in the matrix by smooth muscle cells. *J. Cell Biol.* 101:1059-1070.
- Majack, R. A., J. Mildbrandt, and V. M. Dixit. 1987. Induction of thrombospondin messenger RNA levels occurs as an immediate primary response to platelet-derived growth factor. *J. Biol. Chem.* 262:8821-8825.
- Majack, R. A., L. V. Goodman, and V. M. Dixit. 1988. Cell surface throm-

- bospondin is functionally essential for vascular smooth muscle cell proliferation. *J. Cell Biol.* 106:415-422.
- Mumby, S. M., G. J. Raugi, and P. Bornstein. 1984. Interactions of thrombospondin with extracellular matrix proteins: selective binding to type V collagen. *J. Cell Biol.* 98:646-652.
- Murphy-Ullrich, J. E., and D. F. Mosher. 1987. Interactions of thrombospondin with endothelial cells: receptor-mediated binding and degradation. *J. Cell Biol.* 105:1603-1611.
- Murphy-Ullrich, J. E., L. G. Westrick, J. D. Esko, and D. F. Mosher. 1988. Altered metabolism of thrombospondin by Chinese hamster ovary cells defective in glycosaminoglycan synthesis. *J. Biol. Chem.* 263:6400-6406.
- Nutt, E., T. B. Gasic, J. Rodkey, G. J. Gasic, J. W. Jacobs, P. A. Friedman, and E. Simpson. 1988. The amino acid sequence of antistasin. *J. Biol. Chem.* 263:10162-10167.
- Oquendo, P., E. Hundt, J. Lawler, and B. Seed. 1989. CD36 directly mediates cytoadherence of plasmodium falciparum parasitized erythrocytes. *Cell.* 58:95-101.
- O'Shea, K. S., and V. M. Dixit. 1988. Unique distribution of the extracellular matrix component thrombospondin in the developing mouse embryo. *J. Cell Biol.* 107:2737-2748.
- O'Shea, K. S., J. S. T. Rheinheimer, and V. M. Dixit. 1990. Deposition and role of thrombospondin in the histogenesis of the cerebellar cortex. *J. Cell Biol.* 110:1275-1283.
- Paul, L. L., J. M. Boeing, M. B. Finn, and W. A. Frazier. 1990. Identification of a human thrombospondin isoform generated by alternative splicing. *J. Cell Biol.* In press.
- Rastinejad, F., P. J. Polverini, and N. P. Bouck. 1989. Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. *Cell.* 56:345-355.
- Rich, K. A., F. W. George IV, J. L. Law, and W. J. Martin. 1990. Cell adhesive motif in region II of malarial circumsporozoite protein. *Science (Wash. DC)*. 249:1574-1577.
- Roberts, D. D., D. M. Haverstick, V. M. Dixit, W. A. Frazier, S. A. Santoro, and V. Ginsburg. 1985. The platelet glycoprotein thrombospondin binds specifically to sulfated glycolipids. *J. Biol. Chem.* 260:9405-9411.
- Roberts, D. D., J. A. Sherwood, and V. Ginsburg. 1987. Platelet thrombospondin mediates attachment and spreading of human melanoma cells. *J. Cell Biol.* 104:131-139.
- Robson, K. J. H., J. R. S. Hall, M. W. Jennings, T. J. R. Harris, K. Marsh, C. I. Newbold, V. E. Tate, and D. J. Weatherall. 1988. A highly conserved amino-acid sequence in thrombospondin, properdin, and in proteins from sporozoites and blood stages of a human malarial parasite. *Nature (Lond.)*. 335:79-82.
- Ruoslahti, E. 1988. Fibronectin and its receptors. *Annu. Rev. Biochem.* 57:375-413.
- Sun, X., D. F. Mosher, and A. Rapraeger. 1989. Heparan sulfate-mediated binding of epithelial cell surface proteoglycan to thrombospondin. *J. Biol. Chem.* 264:2885-2889.
- Taraboletti, G., D. D. Roberts, and L. A. Liotta. 1987. Thrombospondin-induced tumor cell migration: haptotaxis and chemotaxis are mediated by different molecular domains. *J. Cell Biol.* 105:2409-2415.
- Taraboletti, G., D. Roberts, L. A. Liotta, and R. Giavazzi. 1990. Platelet thrombospondin modulates endothelial cell adhesion, motility and growth: a potential angiogenesis regulatory factor. *J. Cell Biol.* 111:765-772.
- Tsilibary, E. C., G. G. Koliakos, A. S. Charonis, A. M. Vogel, L. A. Reger, and L. T. Furcht. 1988. Heparin type IV collagen interactions: equilibrium binding and inhibition of Type IV collagen self-assembly. *J. Biol. Chem.* 263:19112-19118.
- Tuszynski, G. P., T. B. Gasic, V. L. Rothman, K. A. Knudsen, and G. J. Gasic. 1987. Thrombospondin, a potentiator of tumor cell metastasis. *Cancer Res.* 47:4130-4133.
- Tuszynski, G. P., J. Karczewski, L. Smith, A. Murphy, V. L. Rothman, and K. A. Knudsen. 1989. The GPIIb-IIIa-like complex may function as a human melanoma cell adhesion receptor for thrombospondin. *Exp. Cell Res.* 182:473-481.
- Varani, J., V. M. Dixit, S. E. G. Fligiel, P. E. McKeever, and T. E. Carey. 1986. Thrombospondin-induced attachment and spreading of human squamous carcinoma cells. *Exp. Cell Res.* 167:376-390.
- Varani, J., B. J. Nickoloff, B. L. Riser, R. S. Mitra, K. O'Rourke, and V. M. Dixit. 1988. Thrombospondin-induced adhesion of human keratinocytes. *J. Clin. Invest.* 81:1537-1544.
- Varani, J., B. L. Riser, L. A. Hughes, T. E. Carey, S. E. G. Fligiel, and V. M. Dixit. 1989. Characterization of thrombospondin synthesis, secretion and cell surface expression by human tumor cells. *Clin. Exp. Metastasis*. 7:265-276.