Disulfides Modulate RGD-inhibitable Cell Adhesive Activity of Thrombospondin

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Abstract. Thrombospondin (TSP) contains the Arg-Gly-Asp (RGD) sequence that is thought to be important for cell adhesion mediated by several cell-surface integrin receptors. The RGD sequence is located in the type 3 repeat region of TSP that has multiple Ca^{2+} binding sites and is subject to a complex intramolecular thiol-disulfide isomerization. TSP that we isolated from thrombin-activated human platelets using buffers containing 0.1 mM Ca^{2+}, in which Cys^{974} is the major labeled cysteine, did not have RGD-inhibitable adhesive activity. However, one of our preparations of TSP and TSP purified following alternative procedures using $\geq$0.3 mM Ca^{2+} did have RGD-inhibitable adhesive activity. Reduction of TSP with DTT, either before or after adsorption to surfaces, enhanced its adhesive activity. Reduced TSP supported robust cell spreading when coated at concentrations as low as 1 μg/ml, whereas "adhesive" TSP not treated with DTT was active at coating concentration of >20 μg/ml and supported only modest cell spreading. Lower DTT concentrations were required for enhancement of the adhesive activity of TSP if Ca^{2+} was chelated with EDTA. Cellular adhesion to DTT-treated TSP was inhibited by RGD-containing peptide and by mAb to a functional site of the $\alpha_v\beta_3$ integrin. Cell blots of reduced proteolytic fragments of TSP localized the adhesive activity to the RGD-containing type 3 repeat region. These results suggest a novel mechanism for regulation of integrin–ligand interactions in which the ligand can isomerize between inactive and active forms.

THE originally described form of thrombospondin (TSP)[1] is a major glycoprotein of platelet α-granules released upon platelet activation and a major secreted product of many cultured cells (Mosher, 1990). According to Dr. Vishva Dixit (personal communication), platelet TSP is exclusively TSP1 when analyzed by isoform-specific antibodies. However, we call TSP purified from platelets just "TSP." Recently, another closely related but clearly distinct TSP, termed TSP2, was identified from mouse and chicken cDNA libraries (Bornstein et al., 1991*a*,*b*; Lawler et al., 1991). Both TSP and TSP2 contain the cell adhesion sequence Arg-Gly-Asp (RGD) (Lawler and Hynes, 1986; Lawler et al., 1991). A number of RGD-containing extracellular proteins, such as vitronectin, fibronectin, and fibrinogen, unambiguously promote cell adhesion when coated onto substrata in a process mediated by cell surface integrin receptors (Ruoslahti and Pierschbacher, 1987). The adhesive activity of TSP, however, is more complicated. Different groups have tested TSP purified from platelet releasate for adhesive activity on various cells with contradictory and controversial results.

Opposite results have been observed for the same cell type. Lahav (1988*a*) reported that TSP inhibits endothelial cell adhesion, while others found that TSP promotes endothelial cell adhesion (Lawler et al., 1988; Murphy-Ullrich and Höök, 1989; Taraboletti et al., 1990). Lahav (1988*b*) also reported that TSP inhibits platelet adhesion, whereas Tuszynski et al. (1987) and Tuszynski and Kowalska (1991) found that TSP supports platelet adhesion.

Among the pro-adhesion papers, the proposed cell surface receptors that mediate cell adhesion to TSP-coated substrate are many. Candidates include the GPIIb/IIIa ($\alpha_{IIb}\beta_3$) (Tuszynski et al., 1989; Karczewski et al., 1989) and vitronectin receptor ($\alpha_v\beta_3$) (Lawler et al., 1988; Lawler and Hynes, 1989; Tuszynski et al., 1989) integrins. Candidates also include heparan sulfate proteoglycan (Roberts et al., 1987; Kaesberg et al., 1989), GPIV (also known as CD36) (Asch et al., 1991), and unidentified receptors that recognize the carboxyl-terminal region (Roberts et al., 1987; Kosfeld et al., 1991) or the type 1 repeat (Prater et al., 1991) of TSP. For adhesion of C32 human melanoma cells to TSP-coated substrate, Tuszynski et al. (1989) proposed that the adhesion is mediated by GPIIb/IIIa-like vitronectin receptor, inasmuch as the adhesion was sensitive to RGD-containing peptide and polyclonal antibodies against GPIIb/IIIa or vitronectin receptor. Asch et al. (1991), in contrast, proposed that the adhesion of C32 cells to TSP is mediated by the combination of heparan sulfate proteoglycan and GPIV, because

1. *Abbreviations used in this paper*; BAE, bovine aorta endothelial; HBS, Hepes buffered saline; RGD, Arg-Gly-Asp; TSP, thrombospondin purified from platelets.

heparin and mAb against GPIV together, but not alone, inhibited the adhesion.

Even for the same cell type adhering to TSP by the same presumptive receptor, different results have been obtained. Lawler et al. (1988) found that TSP supported endothelial cell attachment but not spreading and that the cell attachment was blocked by RGD-containing peptide. Since the adhesion activity was lost if dishes were coated with TSP in the presence of EGTA and could not be restored by addition of Ca^{2+} in the cell adhesion assay, Lawler et al. (1988) concluded that exposure of the RGD sequence of TSP is dependent on Ca^{2+}. Taraboletti et al. (1990) did not control $[Ca^{2+}]$ and observed that TSP strongly supported endothelial cell spreading as well as attachment and that RGD peptide mainly inhibited cell spreading rather than cell attachment.

We have attempted to reconcile some of these discrepancies. Most of our TSP preparations did not support RGD-inhibitable cell adhesion. Reduction of nonadhesive TSP with DTT substantially enhanced its adhesive activity. The cell adhesion to DTT-treated TSP was blocked by RGD-containing peptide and mAb LM609 to the $\alpha_v\beta_3$ vitronectin receptor. These results suggest that exposure of the RGD sequence in TSP is controlled by disulfides and are discussed in the context of a loop–(loop)–β-turn motif proposed for the Ca^{2+}-binding type 3 repeat region of TSP in which the RGD sequence is imbedded.

Materials and Methods

Standard Purification of Platelet TSP

The purification procedure for TSP that has been standard in our lab over the past 8 yr (Murphy-Ullrich and Mosher, 1985) is as follows. 8 U of 1-d-old platelet concentrates are obtained from the Badger Red Cross (Madison, WI) and washed three times with Hepes buffer containing 0.05 M Hepes, 0.15 M NaCl, and 10% of acid citrate dextrose (ACD) to give a final concentration of 0.017 M citrate/citric acid, pH 7.6. Washed platelets are resuspended in 50 ml of resuspension buffer (0.1 M Hepes, 0.15 M NaCl, 5 mM dextrose, pH 7.6) and activated with 1 U/ml thrombin in 37°C water bath for 2 min with stirring. The $[Ca^{2+}]$ in the released products (platelet releasate), determined with an ion-selective electrode, was 2.6 ± 1.0 mM (n = 4). Thrombin is inactivated with nitrophenyl p-guanidine benzoate at a final concentration of 1 mM. The reaction mixture is immediately ice-cooled and centrifuged at 20,000 g at 4°C. The supernatant is snap-frozen and kept at -70°C overnight. After thawing at 37°C and centrifugation to remove the small fibrin clot derived from platelet fibrinogen, the supernatant is applied to a heparin-agarose (Sigma Chemical Co., St. Louis, MO) column. The column is washed with TBS (0.01 M Tris-HCl, 0.15 M NaCl, pH 7.6) and eluted with 0.55 M NaCl buffered with Tris-HCl. Both washing and elution buffers contain 0.1 mM Ca^{2+}. The peak fractions, as monitored by absorption at 280 nm, are pooled and applied to a gel-filtration (model P-300; Bio-Rad Laboratories, Cambridge, MA) column equilibrated and eluted with TBS containing 0.1 mM Ca^{2+}. The peak at V_o is collected as purified TSP. Both chromatographic steps are performed at room temperature on the same day immediately after thawing. Aliquots of each preparation are snap-frozen and stored at −70°C. The yield is typically 0.6–1 mg TSP/unit of platelet or 4-8 mg/preparation.

Mapping of Free Thiols in Our Purified TSP

TSP, 12 mg pooled from several preparations, was lyophilized, dissolved in 15 ml of 8 M urea, 0.3 M Tris, pH 8, and incubated with 30 μCi 2-[^{14}C]-iodoacetic acid at 20°C for 1 h and then with 80 mg unlabeled iodoacetic acid at 20°C for 1 h. The reaction mixture was dialyzed against 0.05 M Tris buffer containing 8 M urea, pH 8.3, reduced with 10 mg dithioerythritol at 20°C for 0.5 h, and carboxymethylated by adding a surplus of unlabeled iodoacetic acid at 20°C for 0.5 h. The derived protein was dialyzed against 0.1 M NH_4HCO_3 and then digested with chymotrypsin at a 1:50 (wt/wt) enzyme to substrate ratio at 37°C for 60 h. The peptides were chromatographed by HPLC on a VYDAC C_{18} 5-μm column (0.8 × 24 cm) with a linear ethanol gradient, with solution A being 0.1% trifluoroacetic acid and solution B being 96% ethanol. The single peak that contained the radiolabel was further chromatographed on a Nucleosil C_{18} 7-μm column (0.4 × 24 cm) with the same gradient. The radioactive peptide was sequenced on a sequencer (model 470A; Applied Biosystems, Inc., Foster City, CA) after lyophilization.

Alternative Purifications of Platelet TSP

The procedure of Lawler et al. (1985) was carried out with modifications. 8 U of platelets were washed twice with pH 6.5 buffer containing 0.102 M NaCl, 3.9 mM K_2HPO_4, 3.9 mM Na_2HPO_4, 22 mM NaH_2PO_4, and 5.5 mM glucose. The platelets were resuspended in 100 ml of pH 7.6 buffer containing 0.015 M Tris, 0.14 M NaCl, 5 mM glucose, and 2 mM Ca^{2+}; activated with 1 U/ml of thrombin at 22°C for 2 min with stirring; and centrifuged in a swinging bucket centrifuge for 0.5 min at 1,000 g. The supernatant was treated with diisopropyl fluorophosphate at a final concentration of 1 mM to inactivate thrombin, and further centrifuged at 50,000 g for 40 min at 0°C. The supernatant was kept at 0°C overnight, and centrifuged to remove the small fibrin clot before application to a column of heparin-Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ) at 4°C. Stepwise elution was carried out with 0.15, 0.25, 0.55, and 2.0 M NaCl in 0.015 M Tris, 0.02% NaN_3, 2 mM $CaCl_2$, pH 7.6. The peak fractions from the 0.55 M NaCl peak were pooled and applied to gel-filtration column (model P-300; Bio-Rad Laboratories) at 4°C, equilibrated and eluted with TBS containing 2 mM Ca^{2+}. The peak at V_o was collected as purified TSP. The yields were low, largely due to loss on the heparin-Sepharose column. Less than 0.1 mg TSP/unit of platelet were obtained with a concentration lower than 100 μg/ml. Lawler, in contrast, usually obtains 0.2–0.4 mg TSP/unit of platelets (J. Lawler, personal communication).

As a third purification scheme, our routine purification (described above) was altered so that 0.3 mM Ca^{2+} was included in all buffers rather than 0.1 mM Ca^{2+}. The yields were similar to those obtained with 0.1 mM Ca^{2+}.

Other Adhesive Proteins

Fibronectin, fibrinogen, and vitronectin were purified as previously described (Mosher and Johnson, 1983; Mosher and Blout, 1973; and Dahlbäck and Podack, 1985).

Preparation of TSP-coated Wells

TSP was diluted to indicated concentrations with buffer containing 10 mM Hepes (pH 7.2), 0.135 M NaCl, 3 mM KCl, 0.5 mM $MgCl_2$ (HBS) plus 1 mM $CaCl_2$ (HBS/Ca^{2+}) or 1 mM EGTA, and added to a 24-well bacteriologic culture dish (Falcon 8111, Becton Dickinson Labware, Lincoln Park, NJ), 0.3 ml/well. The dish was incubated at 37°C for 1–1.5 h, unless otherwise indicated, and washed three times with HBS/Ca^{2+} or HBS as indicated before the cell suspension was added.

Preparation of Wells Coated with Reduced TSP

Two protocols were used. In one, TSP was diluted with buffer containing no DTT, added to wells, and incubated at 37°C for 1–1.5 h. After removing the coating buffer, the wells were incubated at room temperature for 30 min with buffer containing DTT and Ca^{2+} or EDTA. Alternatively, TSP was reduced before coating by addition of DTT to TSP in HBS containing 2 mM Ca^{2+} or EDTA. After incubation at room temperature for 30 min, the mixture was added to wells and incubated at 4°C overnight.

All the wells were washed three times with HBS/Ca^{2+} or HBS before the cell suspension was added.

A number of other proteins were tested to learn whether the enhancement of adhesion upon reduction is a specific property of TSP: bovine albumin (BSA), ovalbumin, goat IgG, and human γ-globulins from Sigma Chemical Co.; bovine thrombin from Gen Trac Inc. (Middleton, WI); and high molecular weight kininogen, antithrombin III, histidine-rich glycoprotein, and amino-terminal 70-kD fragment of fibronectin purified in our laboratory. These proteins were coated at 10 μg/ml and then treated with 20 mM DTT as was done with TSP.

Cell Adhesion Assay

Nearly or freshly confluent bovine aorta endothelial (BAE) cells (Murphy-Ullrich and Mosher, 1987) between passage 5 and 13 were removed from

tissue culture plates with 0.1% trypsin and 0.02% EDTA at a 1:2 (trypsin/EDTA) ratio at 37°C (Lawler et al., 1988; Murphy-Ullrich and Höök, 1989). Trypsin was inactivated by addition of soybean trypsin inhibitor. After washing once with HBS/Ca^{2+}, the cells were resuspended in HBS/Ca^{2+} containing 0.2% BSA, which had been inactivated by heating to 70°C for 1 h, and incubated in TSP-coated wells at 37°C for 1.5 h (Lawler et al., 1988). In some experiments, Gly-Arg-Gly-Asp-Ser (GRGDS) (Biotechnology Center, University of Wisconsin) or Arg-Gly-Glu-Ser (RGES) (Peptide/Oligonucleotide Facility, University of California-San Diego, a kind gift from Dr. B. L. Allen-Hoffmann, University of Wisconsin) or LM609 mAb (a kind gift from Dr. David Cheresh, Scripps Clinic, La Jolla, CA) was added at the beginning of the incubation. After incubation, the wells were washed three times with HBS/Ca^{2+}, and the attached cells were fixed with 4% paraformaldehyde, stained with amido black, and counted under a microscope. To avoid bias, a 1.4-mm-wide strip on each well along its diameter (1.5 cm) was counted (total area of 21 mm^2). Cells did not attach to nonprecoated wells.

Adhesion assays were also done with C32 human melanoma cells, U937 human monocyte cells, and NRK cells, all obtained from the American Type Culture Collection (Rockville, MD).

Cell Blotting of TSP and Its Fragments on Nitrocellulose Paper

Thrombin digestion of TSP was performed with 4 U/ml thrombin (a kind gift from Dr. John Fenton, New York State Department of Health, Albany, NY) and 2 mM EDTA at room temperature for 20 h. Trypsin digestion was either with 1:20 (wt/wt) TPCK-trypsin (Sigma Chemical Co.) and 2 mM EDTA at 0°C for 20 h, or with 1:5 (wt/wt) trypsin and 2 mM Ca^{2+} at 0°C for 24 h. Chymotrypsin digestion was either with 1:30 (wt/wt) chymotrypsin (Sigma Chemical Co.) and 2 mM EDTA at 37°C for 20 min, or with 1:5,000 (wt/wt) chymotrypsin and 2 mM EDTA at 0°C for 32 h. The digestions were stopped by addition of reducing sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and heated.

Cell blotting was performed following a previously published procedure (Hayman et al., 1982) with modifications. Briefly, TSP (4 μg/well) or its protease digestion mixtures (8 μg/well) were electrophoresed by SDS-PAGE. The proteins were electrophoretically transferred to nitrocellulose paper (Schleicher & Schuell, Keene, NH) in a Tris-glycine transfer buffer containing 10% methanol. The transferred nitrocellulose paper was stained with ponceau S (Harlow and Lane, 1988) to visualize and mark the protein bands. The paper was then incubated with 0.5% BSA at 37°C for at least 30 min to block the surface. BAE cell suspensions (8×10^5 cells/ml, generated as in cell adhesion assay) were carefully layered on the paper (~1 ml/10-cm^2 paper) spread on a flat parafilm surface. After incubation at 37°C for 1 h, the paper was washed three times with HBS/Ca^{2+} to remove nonadherent cells. The paper-adherent cells were fixed with paraformaldehyde, stained with 1:5 diluted trypan blue (Gibco Laboratories, Grand Island, NY) for 15 min, briefly destained with H_2O, and air dried. The paper was examined under a microscope to insure that staining was due to the adherent cells. Duplicate sample sets were directly stained with amido black after transfer to nitrocellulose paper to give a permanent record of transferred proteins or immunoblotted with MA-1 mAb (a kind gift from Dr. J. Lawler, Harvard Medical School, Boston, MA).

Reduction and Carboxymethylation of TSP

TSP was treated with 2 mM Ca^{2+} or 2 mM EDTA for 10 min. DTT was added to final concentrations of 0, 0.2, 0.6, 2, 6, or 20 mM. The samples were incubated for 1 h under argon gas. Iodoacetamide (Sigma), 0.5 M in TBS, was added to a final concentration of 50 mM with a final pH >8. The samples were incubated in the dark for 3 h under argon gas. The procedures were performed at room temperature. The samples were divided into two aliquots, dialyzed at 4°C either against TBS with 2 mM Ca^{2+} (for DTT + Ca^{2+}-treated samples) or TBS (for DTT + EDTA-treated samples) for SDS-PAGE and cell adhesion studies, or against 1% acetic acid followed by lyophilization for amino acid analysis.

Amino Acid Analysis

Amino acid analysis was performed as previously described (Öhlin and Stenflo, 1987) on a Beckman 6300 amino acid analyzer.

Results

Different Preparations of Platelet TSP Have Different Adhesive Activities

Seven of the eight preparations of our standard TSP did not have adhesive activity for trypsinized cells when coated on nontissue culture dishes. One preparation of our TSP (BR220), however, did support adhesion of C32, NRK, and BAE cells, but not of U937 cells. Cell adhesion was sensitive to the presence of RGD-containing peptide in the adhesion assay or the presence of EGTA in TSP coating buffer (Fig. 1 *A*), as Lawler et al. (1988) described. There was no obvious difference between the adhesive and nonadhesive TSP preparations in details of the purification procedure or in immunoblots for fibronectin, fibrinogen, or vitronectin, potential contaminants with adhesive activity. Furthermore, the adhesion of BAE cells to TSP-BR220 was not affected by polyclonal antibody against vitronectin added at an amount that abolished the cell attachment to wells coated with 0.2 μg/ml vitronectin (data not shown).

TSP was also purified following Lawler's procedure with the modifications described in Materials and Methods and by a modification of our standard method using buffers with 0.3 mM Ca^{2+} rather than 0.1 mM Ca^{2+}. These alternatively purified TSPs were adhesive, and cell adhesion was sensitive to RGD-containing peptide and to the presence of EGTA in the coating buffer (Fig. 1 *B*). Although the alternatively purified TSP purified by Lawler's method was contaminated with fibrinogen (~5% as estimated by SDS-PAGE and immunoblotting), polyclonal antibody against fibrinogen did not block cell adhesion whereas the same amount of antibody abolished cell adhesion to fibrinogen-coated wells (data not shown).

Cys^{974} Is the Only Labeled Thiol in Our Purified TSP

These results suggested that some of the controversies in the literature result from differences in TSPs. The RGD sequence of TSP is located in the type 3 repeat region (Lawler and Hynes, 1986). This region has multiple Ca^{2+}-binding sites and is rich in cysteine. When TSP in platelet releasate

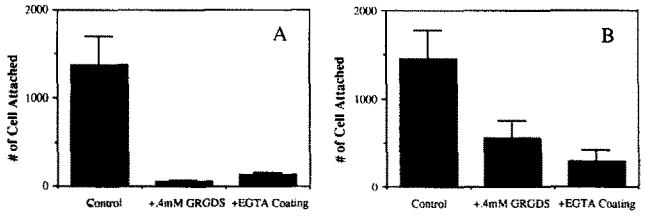

Figure 1. Attachment of BAE cells to TSP. A preparation of TSP purified by our standard procedure (*A*) and TSP purified by an alternative procedure (*B*) were diluted to 20 μg/ml with HBS/1 mM Ca^{2+} (*Control*) or HBS/1 mM EGTA (*+EGTA Coating*), coated on nontissue culture dishes, and assayed for cell adhesive activity as described in Materials and Methods. GRGDS was added to some wells at a final concentration of 0.4 mM (+0.4 mM GRGDS). The cell suspension was added at 50,000 cells/2.1-cm^2 well. The number (#) of cells attached to 12% of the area of the well was quantified. Bars represent means of triplicate assays, and brackets represent standard deviations.

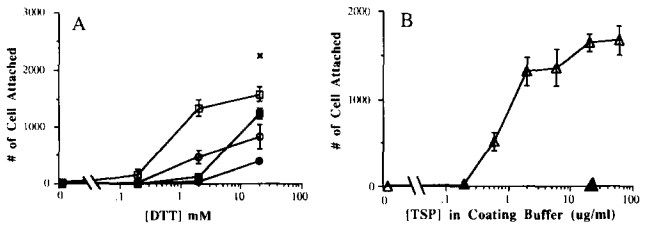

Figure 2. [DTT]- and [TSP]-dependent BAE cell attachment to DTT-treated TSP. (*A*) DTT dose response curve for activity. Squares represent results when TSP was coated to wells first (5 μg/ml, 37°C, 1 h) and then incubated with DTT in the presence of 2 mM Ca^{2+} (*solid squares*) or EDTA (*open squares*). Circles represent results when TSP was treated with DTT first in HBS containing 2 mM Ca^{2+} (*solid circles*) or EDTA (*open circles*) and then coated on wells at 4°C overnight. The cross (X) represents the result of cell attachment to fibronectin coated at 5 μg/ml. (*B*) [TSP]-dependent cell attachment. Wells were coated with TSP (37°C, 1 h) at the indicated concentrations and incubated with 20 mM DTT in HBS at room temperature for 30 min (*open symbols*) or with HBS without DTT as control (*closed symbol*). In both experiments, 25,000 cells/well were allowed to attach for 1.5 h at 37°C. Each point represents mean of triplicate samples, and brackets represent standard deviations.

was analyzed, 12 free cysteines were identified in the type 3 repeat region and adjacent carboxyl-terminal globular region, although there was a stoichiometry of one free thiol per TSP subunit (Speziale and Detwiler, 1990). An interpretation of these results is that the position of the free thiol may vary through thiol-disulfide isomerization so that each of the labeled cysteines is a free thiol in a small fraction of TSP molecules. When our nonadhesive TSP purified in 0.1 mM Ca^{2+} was labeled with ^{14}C-iodoacetic acid to derivatize free thiols, followed by chymotrypsin digestion and separation of peptides by HPLC, only a single peak (>5-fold higher than background) was found to contain radio-derivatized thiol (peak 19 of VYDAC C_{18} 5 μm column and peak 6 of Nucleosil C_{18} 7-μm column, data not shown). Sequencing of this peptide yielded VQTVNCDPGLAVGYDEF, corresponding to residues 969–985 in TSP (Lawler and Hynes, 1986). Thus, only Cys^{974} was found to be labeled.

TSP Reduced with DTT Is Adhesive

Because our nonadhesive TSP was only labeled at Cys^{974}, we hypothesized that nonadhesive preparations of TSP lacked thiol-disulfide isomer(s) in which the RGD adhesive sequence is exposed. To test this possibility, nonadhesive TSP was reduced with various concentrations of DTT and tested for adhesive activity. Fig. 2 *A* shows the DTT dose-response curves for cell adhesion. TSP, 5 μg/ml, was either coated on the well first then reduced with up to 20 mM DTT in the presence of Ca^{2+} or EDTA, or reduced first and then coated on wells. Reduction of TSP with DTT by either route made the TSP adhesive. The presence of Ca^{2+} or EDTA when TSP was treated with DTT significantly changed the response to DTT. In the presence of Ca^{2+}, generation of adhesive activity required 20 mM DTT, whereas in the presence of EDTA, activity required only 2 mM DTT. Although more cells attached to wells on which TSP was coated first and then reduced compared to wells coated with TSP that had been incubated with DTT before coating, the response patterns to DTT were the same.

When nonadhesive TSP was precoated and treated with DTT at a fixed concentration of 20 mM, cell attachment was dependent on the concentration of TSP in the coating buffer (Fig. 2 *B*). Under these conditions, half maximal cell adhesion was seen in wells coated with 1 μg/ml TSP.

Cells were well spread on DTT-treated TSP (Fig. 3 *A*) compared to those attached to the adhesive TSP (BR220) described above (Fig. 3 *B*). It should be noted that the concen-

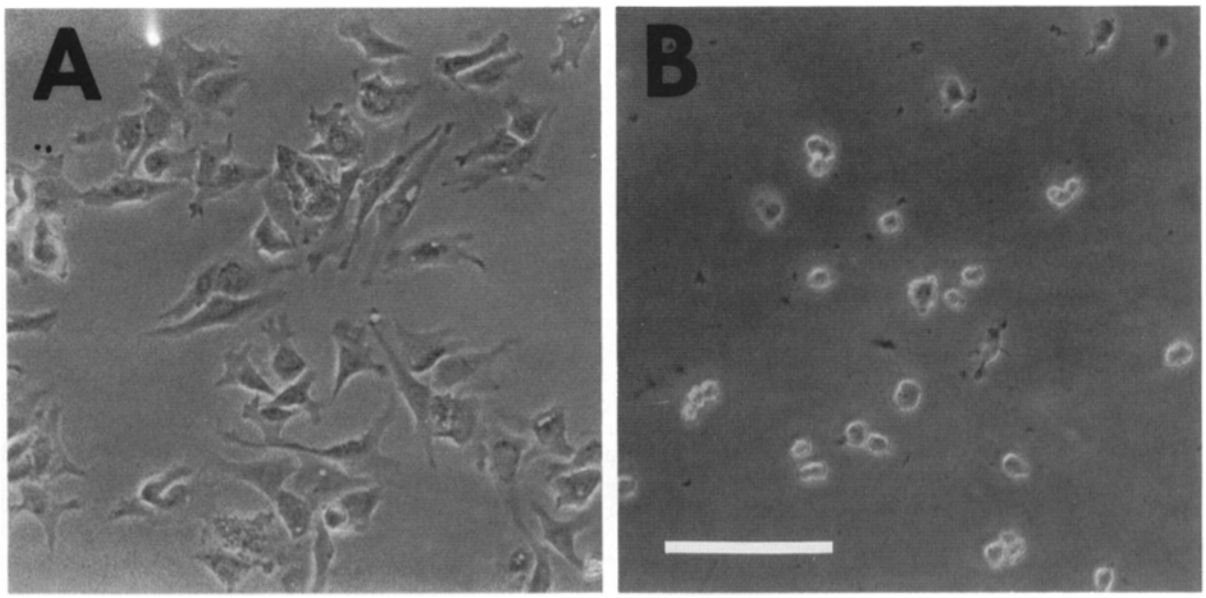

Figure 3. Spreading of cells on nonadhesive TSP subsequently treated with DTT as compared to the adhesive preparation of TSP. (*A*) BAE cells spread on nonadhesive TSP coated at 6 μg/ml and then treated with 20 mM DTT in HBS at room temperature for 0.5 h. (*B*) BAE cells attached to adhesive TSP (preparation BR220), coated at 20 μg/ml. In both cases, TSP was coated at 37°C for 1 h, and cells were allowed to attach for 1.5 h at 37°C. The attached cells were fixed with paraformaldehyde before photography. Bar, 40 μm.

trations of TSP in coating buffers in this experiment were different: nonadhesive TSP was coated at 6 μg/ml before it was treated with 20 mM DTT, whereas adhesive TSP was coated at 20 μg/ml, a usual dose described in the literature. Treatment of adhesive TSP with DTT caused the TSP to be more adhesive so that cells spread as in Fig. 3 *A* on adhesive TSP coated at concentrations of 3 or 10 μg/ml and then were treated with DTT (not shown).

Adherent cells were viable as judged from their ability to exclude trypan blue (data not shown). BAE cells pretreated with cycloheximide, 10 μg/ml, spread as well as non-cycloheximide-treated cells (data not shown).

Similar results as those described above for BAE cells were obtained when the experiments were done with C32 melanoma cells (data not shown).

Cellular Adhesion to DTT-Reduced TSP Is Sensitive to RGD-containing Peptide and a Monoclonal Antibody to the Vitronectin Receptor

Cell adhesion to DTT-treated TSP was sensitive to the presence of GRGDS peptide: 0.025 mM GRGDS reduced cell adhesion to 50 ± 6.2% of control wells in which no peptide was added, and 0.1 mM GRGDS reduced to 12 ± 4.5%, whereas 0.1 mM RGES only slightly reduced the cell adhesion (85 ± 3.2%). These results support the hypothesis that the enhanced adhesive activity of DTT-treated TSP is due to the exposure of its RGD sequence.

Inhibition of adhesion by the RGD-containing peptide suggests that integrin receptor(s) are involved. Lawler et al. (1988) and Tuszynski et al. (1989) showed that cellular adhesion to their adhesive TSP was sensitive to antivitronectin receptor ($\alpha_v\beta_3$) polyclonal or monoclonal antibodies. LM609 mAb, which recognizes a functional site on intact vitronectin receptor and inhibits attachment of endothelial cell to vitronectin and von Willebrand factor (Cheresh, 1987), was tested for its inhibitory effect on the cell adhesion. LM609, 0.1 mg/ml, reduced the cell adhesion to 33 ± 4.5% of control value, while the control mAb 8E6 to vitronectin did not have an inhibitory effect (109 ± 9.3%).

Reduction and Derivatization of Cysteines Is Less in the Presence of Ca^{2+}

Because the DTT dose response curve for TSP adhesive activity was different in the presence of Ca^{2+} than in the presence of EDTA, we quantified how the presence of Ca^{2+} or EDTA influenced the reduction of TSP with increasing concentrations of DTT. To this end, TSP was treated with DTT at different concentrations in the presence of 2 mM Ca^{2+} or EDTA, reacted with iodoacetamide to carboxyamidomethylate free cysteine residues, dialyzed, and subjected to amino acid analysis. As shown in Fig. 4, there was a DTT dose-dependent increase in content of carboxymethyl-cysteine (CM-Cys). The amount of CM-Cys detected in each sample is expressed as its ratio to alanine detected in the same sample. There was no detectable CM-Cys in samples not treated with DTT. The maximum ratio of CM-Cys/Ala obtained was 1.02 in the presence of EDTA, and 0.59 in the presence of 2 mM Ca^{2+}, whereas the Cys/Ala ratio predicted from amino acid sequence is 1.4.

The DTT-treated and carboxyamidomethylated TSP was also subjected to SDS-PAGE analysis under nonreduced conditions (Fig. 5). TSP derivatized after exposure to <2 mM DTT remained trimeric and moved with decreasing mobility. This was more obvious in samples treated in the presence of EDTA than in the presence of Ca^{2+}. This phenomenon suggests that in the presence of EDTA, intrachain disulfides in TSP molecule are more vulnerable to reduction with DTT. Monomeric subunits were seen in TSP derivatized after exposure to >2 mM DTT. After exposure to 20 mM DTT, all the TSP was monomeric. The presence of Ca^{2+} or EDTA did not facilitate or change the production of monomer. Electrophoretic analysis under the reduced condition showed no difference between the samples (not shown).

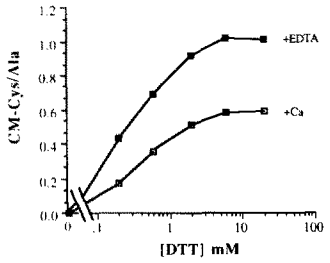

Figure 4. Derivatization of carboxymethyl-cysteine, the effect of [DTT], Ca^{2+}, and EDTA. TSP was treated with different concentrations of DTT in the presence of 2 mM of Ca^{2+} (*open symbols*) or EDTA (*closed symbols*). The free cysteine residues were then carboxyamidomethylated with iodoacetamide as described in Materials and Methods. Samples were dialyzed against 1% acetic acid, lyophilized, and subjected to amino acid analysis. The amount of carboxymethyl-cysteine (*CM-Cys*) detected in each hydrolysate was normalized by the amount of alanine (*Ala*) in the same sample.

After dialysis against TBS, there were visible aggregates in the carboxyamidomethylated TSP samples, especially in those treated with higher concentrations of DTT and in the presence of Ca^{2+}. Chemical cross-linking with glutaraldehyde confirmed that most protein was aggregated (not shown). TSP treated with DTT but not carboxyamidomethylated also could be cross-linked to multimers, but to a lesser extent. Most probably due to the degree of aggregation, fewer cells were attached to wells coated with carboxyamidomethylated TSP than to those coated with TSP treated with equivalent concentrations of DTT but not carboxyamidomethylated and dialyzed (data not shown).

Cell Blots of Reduced Proteolytic Fragments of TSP Localize the Adhesive Activity to Its RGD-containing Type 3 Repeat Region

Cell blots of reduced TSP and its proteolytic fragments were performed to map the DTT-enhanced adhesive activity within TSP. TSP was digested by thrombin, trypsin, or chymotrypsin using published conditions that control the extension of proteolysis at the carboxyl-terminal end of the molecule (Lawler et al., 1985, 1986). The digestion mixtures were analyzed by SDS-PAGE under reducing conditions and transferred electrophoretically to nitrocellulose paper. The paper was then incubated with BSA to block its surface, and with BAE cell suspensions to allow cells to attach on proteins that have the adhesive activity. The adherent cells were stained with trypan blue.

Fig. 6 *A* shows that cells adhered to intact TSP and 120–140-kD thrombin-generated fragments which are known to contain the Ca^{2+}-sensitive type 3 repeat region recognized by MA-1 mAb in experiments by Lawler et al. (1985, 1986). Adherent cells were well-spread and polygonal when viewed with a dissecting microscope. Adherence of cells to these

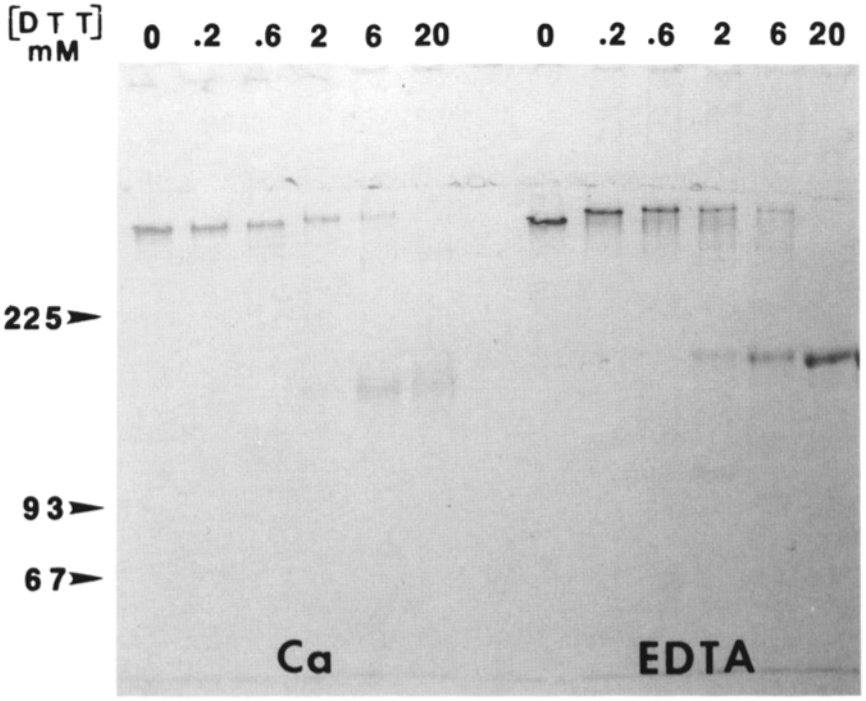

Figure 5. SDS-PAGE analysis of DTT-treated and carboxyamidomethylated TSP. TSP samples, treated with various concentrations of DTT in the presence of Ca^{2+} or EDTA and then alkylated with iodoacetamide, were electrophoresed on a 5% polyacrylamide gel with SDS under nonreduced condition. The protein was stained with Coomassie blue.

bands was blocked by the RGD-containing peptide. The 85-kD fragment generated by trypsin digestion in the presence of EDTA, and the 70-kD fragment generated by chymotrypsin digestion in a high enzyme-to-substrate ratio did not support cell adhesion. Both fragments did not contain the MA-1 epitope in experiments reported by Lawler et al. (1985, 1986).

Fig. 6 *B* shows that, when TSP was digested by trypsin and chymotrypsin under conditions that spare the MA-1 epitope, the cell blot patterns were changed. Trypsin digestion of TSP in the presence of Ca^{2+} generates a major 85-kD fragment that contains the MA-1 epitope (Lawler et al., 1985); this fragment supported cell adhesion. Chymotrypsin digestion of TSP in a low enzyme-to-substrate ratio generates fragments in a wide molecular weight range, most containing the MA-1 epitope (Lawler et al., 1986). These same fragments also supported cell adhesion.

To confirm the correlation between the presence of MA-1 epitope and the cell adhesive activity of the reduced TSP fragments, duplicated sample sets were immunoblotted with MA-1 mAb. The immunoblot patterns (not shown) were the same as those of cell blot pattern.

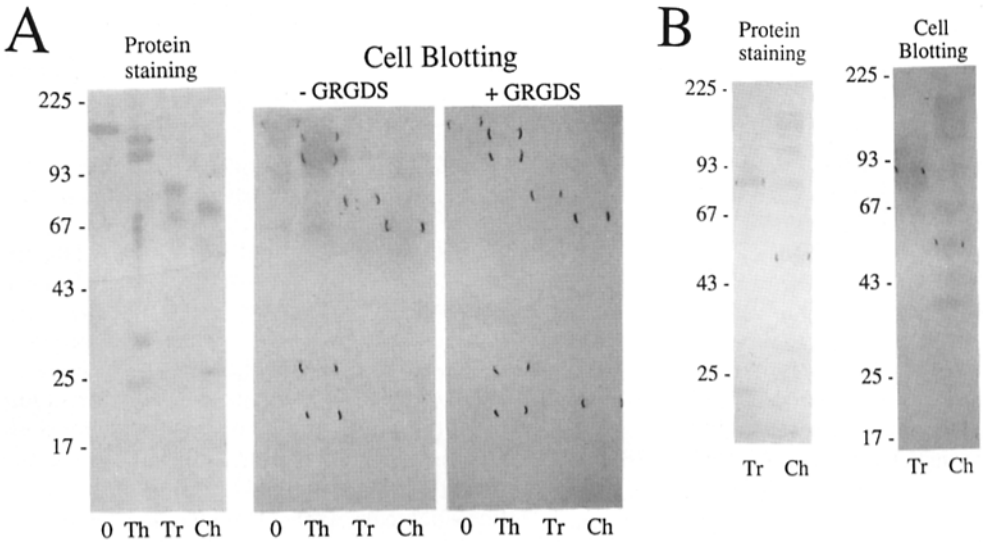

Figure 6. Cell blots of reduced TSP fragments. Reduced TSP (4 μg/lane) and the protease digestion mixtures (8 μg/lane) were separated by SDS-PAGE and then electrophoretically transferred to a nitrocellulose paper. The paper was either stained with amido black for a permanent record of protein bands (protein staining) or stained temporarily for protein with ponceau S and then incubated with BSA/TBS followed by a BAE cell suspension (cell blotting). Pen marks in the blotting panels indicate bands that stained with ponceau S. Samples analyzed in A; *0*, intact TSP; *Th*, thrombin digest; *Tr*, trypsin (1:20 wt/wt) digest in 2 mM EDTA, 0°C, 20 h; *Ch*, chymotrypsin (1:30 wt/wt) digest in 2 mM EDTA, 37°C, 20 min. Adhesion of BAE cells was performed without (*−GRGDS*) or with (*+GRGDS*) 0.4 mM GRGDS in the incubation. Samples analyzed in *B*: *Tr*, trypsin (1:5 wt/wt) digests in 2 mM Ca^{2+}, 0°C, 24 h; *Ch*, chymotrypsin (1:5,000 wt/wt) digest in 2 mM EDTA, 0°C, 32 h. The papers for protein staining shrunk before photography due to exposure to ethanol and therefore do not line up exactly with the papers for cell blotting.

Treatment of Most Proteins with DTT Does Not Make the Proteins Adhesive

The "cell blotting" experiments indicated that cells bind specifically to the RGD-containing region of TSP. To test specificity further, a number of proteins that do not contain RGD were coated on bacteriologic culture dishes at 10 μg/ml and treated without or with 20 mM DTT. DTT treatment did not enhance cell adhesion to BSA, ovalbumin, goat IgG, human γ-globulin, high molecular weight kininogen, antithrombin III, histidine rich glycoprotein, and the amino-terminal 70-kD fragment of fibronectin. In contrast, the adhesive activity of bovine thrombin, which has an RGD sequence that supports cell adhesion in certain derivatives of thrombin (Bar-Shavit et al., 1991), was enhanced more than 10-fold by treatment with DTT (data not shown).

Discussion

The inconsistencies in the literature about the adhesive activity of TSP were reproduced in our experiments. Most preparations of our standard TSP did not have RGD-inhibitable adhesive activity for trypsinized cells, although these preparations did have heparin-inhibitable adhesive activity for cells that are allowed to recover from trypsinization and thus to display cell surface proteoglycans (Kaesberg et al., 1989). However, a single preparation of our TSP and TSP purified following alternative procedures did have the RGD-inhibitable adhesive activity. These findings indicate that at least part of the controversies in the literature are due to differences in TSPs, with some TSPs being adhesive and some not, and suggest that exposure of the RGD sequence in TSP is regulated.

Because the RGD sequence of TSP is located in the type 3 repeat region, which contains multiple Ca^{2+}-binding sites and because TSP adsorbed to plastic in the presence of EGTA did not support cell adhesion upon Ca^{2+} restoration, Lawler et al. (1988) proposed that exposure of the RGD sequence is dependent on Ca^{2+}. This region of TSP undergoes a major conformational change upon chelation of Ca^{2+} as ascertained by rotary shadowing (Lawler et al., 1985; Galvin et al., 1985; Dixit et al., 1986), immunological reactivity (Dixit et al., 1986), susceptibility to proteolysis (Lawler et al., 1982; Lawler and Simons, 1983; Lawler et al., 1985), circular dichroism spectroscopy (Lawler and Simons, 1983), electron spin resonance spectroscopy (Slane et al., 1988), and other techniques (Lawler et al., 1982). A Hill plot of ellipticity versus $[Ca^{2+}]$ revealed a Hill coefficient of 12.3, indicative of a highly cooperative interaction between ~0.1 and 0.3 mM Ca^{2+} (Lawler and Simons, 1983). Possible differences between Lawler's TSP purification procedure and our standard procedure are Lawler's addition of 2 mM Ca^{2+} to the platelet suspension before activation by thrombin and the addition of 2 mM rather than 0.1 mM Ca^{2+} to the buffers used in later purification steps. Our platelet releasate contained >1 mM Ca^{2+} when measured by Ca^{2+}-ion electrode due to endogenous Ca^{2+} released from platelets upon activation (Detwiler and Feinman, 1973). Incubation of our nonadhesive TSP with 2 mM Ca^{2+} before it was coated on plastic well did not make it adhesive (data not shown). Nevertheless, when we used 0.3 rather than 0.1 mM Ca^{2+} during all later steps, we obtained adhesive TSP. These results indicate that Ca^{2+} preserves TSP in an "adhesive" conformation but alone is not sufficient to regulate the exposure of the RGD sequence.

Recently, Speziale and Detwiler (1990, 1991) studied the number and position of free thiols in freshly secreted platelet TSP by derivatization of thiols with radiolabeled N-ethylmaleimide or iodoacetamide, followed by digestion and sequencing of peptides containing derivatized thiols. Although there was a stoichiometry of one free thiol per TSP subunit, the derivatized cysteine residues were mapped at 12 different positions. Of the 12 radiolabeled thiols, 10 were in the type 3 repeat region, which contains 17 of the total of 69 cysteines in each subunit of TSP. The remaining two labeled thiols were in the adjacent carboxyl-terminal globular (C-globe) region. The number of reactive thiols was a function of $[Ca^{2+}]$. With increased $[Ca^{2+}]$, the number of derivatized thiols decreased in the same cooperative manner as described above for the change in circular dichroism. Thus, the type 3 repeats the adjacent C-globe region of TSP may participate in a complex intramolecular thiol-disulfide isomerization that is modulated by Ca^{2+}. In our purified nonadhesive TSP, only Cys^{974} in the C-globe region was labeled. These considerations led us to hypothesize that the nonadhesive TSP that we purified was only one of the possible isomers of TSP and that the exposure of the RGD sequence in TSP is controlled by disulfides. To test this hypothesis, TSP was treated with reducing agents to break disulfides and tested for its adhesive activity. Several interesting phenomena were observed.

First, DTT-treated TSP acted as a potent adhesive molecule. In previous work when TSP was reported to be adhesive, it was coated to plastic wells at ≥20 μg/ml (Tuszynski et al., 1987; Lawler et al., 1988) and supported cell attachment but not robust cell spreading (Lawler et al., 1988). In contrast, cells were well spread in dishes onto which TSP had been coated at concentrations as low as 1 μg/ml and then treated with DTT.

Second, the adhesion of cells to DTT-reduced TSP was inhibited by RGD peptide and sensitive to a mAb which recognizes a functional site of the $\alpha_v\beta_3$ vitronectin receptor as previously reported (Lawler et al., 1988).

Third, the DTT concentrations needed to generate adhesive activity were 10-fold higher in the presence of Ca^{2+} than in the presence of EDTA. Ca^{2+} also protected disulfides from reduction as ascertained by gel electrophoresis and quantification of carboxymethyl-cysteine content after carboxyamidomethylation. Such a protective effect has been observed previously by Turk and Detwiler (1986) and Speziale and Detwiler (1990).

Finally, cell blots of reduced TSP fragments localized the RGD-inhibitable adhesive activity at its Ca^{2+}-sensitive and RGD-containing type 3 repeat region.

Our results, together with those by Speziale and Detwiler (1990), strongly suggest that the exposure of RGD sequence in TSP is regulated by the disulfide arrangement in its type 3 repeats and C-globe region. This possibility prompted us to analyze closely the amino acid sequence in these regions. When amino acid sequence and the homologous alignment in the type 3 repeats were first described by Lawler and Hynes (1986), residues were aligned into eight repeats, called 3A and 3B, to maximize the homology among the repeats. A Ca^{2+}-binding loop sequence (DXDXDGXXD-XXDX or slight modifications thereof) appeared twice in the five full type 3A repeats and once in the two partial type 3B repeats. This sequence has been noted in several Ca^{2+}-bind-

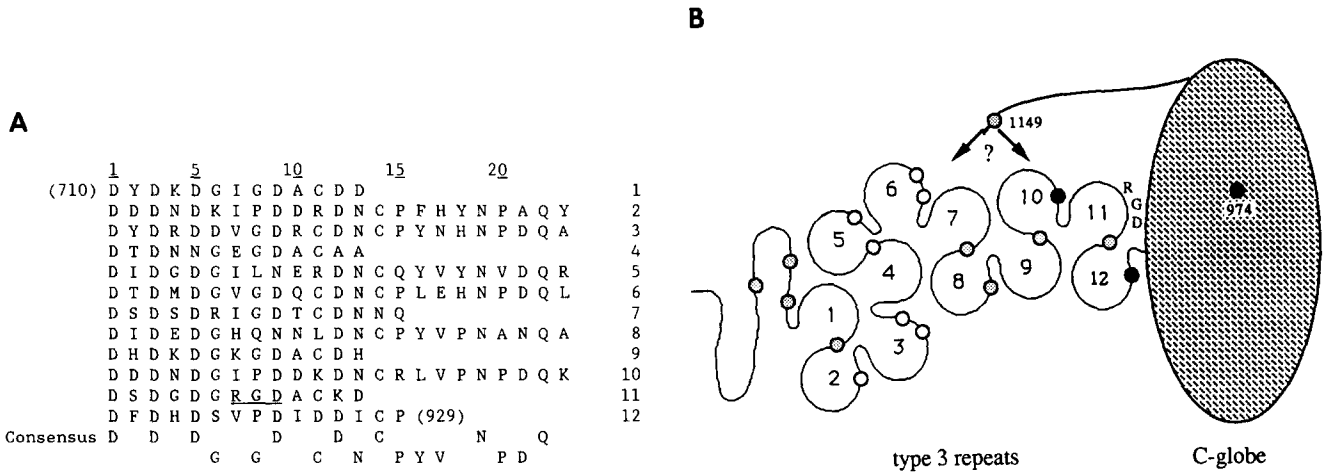

Figure 7. Alignment of the type 3 repeats and schematic diagram of the type 3 repeat and adjacent C-globe regions. (*A*) The type 3 repeats are aligned according to homologies in the 12 Ca^{2+}-binding loops. (*B*) The 12 Ca^{2+}-binding loops are numbered. Small circles represent the 19 cysteines in the type 3 repeat region and C-globe. Open circles, lightly shaded circles, and heavily shaded circles represent cysteines not labeled, lightly labeled, and heavily labeled, respectively, in the experiments of Speziale and Detwiler (1990). Note that the three heavily labeled cysteines are in proximity to the RGD adhesive sequence in loop 11. The uncertain arrangements of disulfides in this region of TSP are indicated by the question mark for the pairing of Cys-1149 at the extreme carboxyl terminus and are discussed in the text. The epitope for MA-1 mapped to a part of TSP carboxyl-terminal to residue 892 in the 10th line when a panel of fusion proteins were tested (Lawler and Hynes, 1986).

ing proteins (Dang et al., 1985). These proteins adopt a characteristic helix-loop-helix conformation (Kretsinger, 1976; Sudhakar Babu et al., 1985), with the Ca^{2+} bound in the loop between the helices. The coordination of Ca^{2+} can be represented by an octahedron with six vertices. Five vertices are coordinated with oxygen atoms from side chains, and a peptide oxygen coordinates at the sixth (Kretsinger, 1976, 1980).

If the 12 loop sequences in this region are aligned, a striking pattern emerges (Fig. 7A). DXDXDGXXDXXDX loop sequences are followed six times by a 10-amino acid extension, tentatively termed the break sequence. The boundaries of both loop and break sequences line up, with the exception at line 7, in which the extension of the loop sequence is only 2 amino acids. The cysteines are aligned either in the loop sequence (short lines) or in the break sequence (long lines), with the exceptions of lines 3 and 6, in which the cysteines are vicinal. The alignment suggests that of the 12 loops, 10 are in pairs forming 5 loop-loop structures that are followed by a break sequence, whereas loops 3 and 6 are separated by break sequence after a single loop.

Inasmuch as there are homologies among the break sequences, it is likely that these sequences share a common secondary structure. As pointed out by Lawler et al. (1988), the algorithm of Chou and Fasman (1978) predicts that the conserved NPDQ in the break sequence has a strong tendency to form β-turn, with a calculated probability 15-fold greater than average. Thus, we propose that this entire Ca^{2+}-binding region is composed of loop–(loop)–β-turn–repeats (Fig. 7 *B*). Loop 11, containing the RGD, and loop 12, as Lawler et al. (1988) proposed, forms the last of the double loop structures followed by the bulky C-globe region.

Fig. 7 *B* also shows the relative labeling of cysteines in the type 3 repeats and the C-globe region (Speziale and Detwiler, 1990). The cysteines that are fractionally labeled may be free or participate in more than one type of disulfide. For instance, Cys^{912} in loop 11 may form a disulfide with Cys^{928} next to loop 12 or with Cys^{892} next to loop 10; Cys^{892} may form a disulfide with Cys^{912} or with Cys^{876} in loop 9, etc. Cys^{974}, which is the only labeled cysteine in our TSP and is replaced by a serine in TSP2 (Lawler et al., 1991), may play a critical role in the isomerization either by forming a disulfide with Cys^{1149} or with a cysteine in the type 3 repeats. Cys^{1149}, which is the fourth residue from the carboxy terminus of TSP and is thought to be involved in a disulfide (Dixit et al., 1985), may be important in maintaining the C-globe structure and the exposure of the RGD sequence. One or more of the many potential isomers of TSP may have an exposed RGD adhesive sequence and mimic our reduced TSP. Such isomers may be only a small percentage of the TSP molecules or subunits released from platelets.

It will be of great interest to identify factors in addition to Ca^{2+} and chemical reducing agents that modulate exposure of the RGD sequence. One possible modifier of TSP is type V collagen, which interacts more strongly with Ca^{2+}-depleted TSP than with Ca^{2+}-replete TSP (Galvin et al., 1987). TSP2 has the loop-(loop)-break structure, the RGD sequence in loop 11, and the C-globe (Lawler et al., 1991). However, there is a serine at 974 rather than cysteine. It also will be of interest to learn if TSP2 has a regulated RGD or whether this adhesive sequence is constitutively exposed or constitutively cryptic.

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