Mapping of Epitopes for Monoclonal Antibodies against Human Platelet Thrombospondin with Electron Microscopy and High Sensitivity Amino Acid Sequencing

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ABSTRACT A panel of monoclonal antibodies (Mab's) has been raised against human platelet thrombospondin (TSP). One Mab, designated A2.5, inhibits the hemagglutinating activity of TSP and immunoprecipitates the NH_2 terminal 25 kD heparin binding domain of TSP (Dixit, V. M., D. M. Haverstick, K. M. O'Rourke, S. W. Hennessy, G. A. Grant, S. A. Santoro, and W. A. Frazier, 1985, *Biochemistry*, in press). Another Mab, C6.7, blocks the thrombin-stimulated aggregation of live platelets and immunoprecipitates an 18-kD fragment distinct from the heparin binding domain (Dixit, V. M., D. M. Haverstick, K. M. O'Rourke, S. W. Hennessy, G. A. Grant, S. A. Santoro, and W. A. Frazier, 1985, *Proc. Natl. Acad. Sci.* 82:3472–3476). To determine the relative locations of the epitopes for these Mabs in the three-dimensional structure of TSP, we have examined TSP-Mab complexes by electron microscopy of rotary-shadowed proteins. The TSP molecule is composed of three 180-kD subunits, each of which consists of a small globular domain (~8 nm diam) and a larger globular domain (~16 nm diam) connected by a thin, flexible strand. The subunit interaction site is on the thin connecting strands, nearer the small globular domains. Mab A2.5 binds to the cluster of three small domains, indicating that this region contains the heparin binding domain and thus represents the NH_2 termini of the TSP peptide chains. Mab C6.7 binds to the large globular domains on the side opposite the point at which the connecting strand enters the domain, essentially the maximum possible distance from the A2.5 epitope. Using high sensitivity automated NH_2 terminal sequencing of TSP chymotryptic peptides we have ordered these fragments within the TSP peptide chain and have confirmed that the epitope for C6.7 in fact lies near the extreme COOH terminus of the peptide chain. In combination with other data, we have been able to construct a map of the linear order of the identified domains of TSP that indicates that to a large extent, the domains are arranged co-linearly with the peptide chain.

Thrombospondin (TSP)[1] is essential in the aggregation of human platelets (1). We have identified a monoclonal antibody against TSP that can block the aggregation of gel-filtered human platelets stimulated with thrombin or A23187 (1). Other investigators have found that polyclonal antisera directed against either intact TSP (2, 3) or the heparin binding domain of bovine TSP (4) can inhibit platelet aggregation. These recent data have provided the first direct demonstration that TSP is necessary for platelet aggregation. TSP is secreted from platelets upon their activation by thrombin, collagen, or other activating agents (5–8) and binds to receptors on the platelet surface when divalent cations are present (9). TSP has been identified as an endogenous lectin of platelets (10), and we have characterized the agglutinating activity of the purified protein towards trypsinized, fixed human erythrocytes (11) and fixed activated platelets (12). The differential effects of heparin and fibrinogen as inhibitors of these two activities of

[1] *Abbreviations used in this paper*: Mab, monoclonal antibody; TSP, thrombospondin.

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TSP suggest that the agglutination of erythrocytes and fixed, activated platelets are in some way qualitatively distinct (11, 12).

TSP is a trimeric glycoprotein whose identical (13) M_r 180,000 subunits are linked by disulfide bonds (6). Until recently, there was some confusion about the appearance of the protein when subjected to rotary shadowing and electron microscopy. Images of rotary-shadowed TSP obtained with electron microscopy have shown molecules with both three and four globular domains connected by thinner strands within the same sample (14, 15), a result difficult to reconcile with a trimeric structure of identical chains. Recently, Lawler et al. (16) have obtained images of the protein in which one of the four large globular domains is often resolved into three smaller globular regions. Thus, when these collapse together, the molecule appears as four globular domains. The report of three globular domains is explained by the facile removal of these small domains by proteolysis (16). A variety of proteases will digest native TSP, yielding protease-resistant regions that appear to represent compact areas of protein structure that contain binding sites for ligands with which TSP interacts. Thus far, fragments have been identified that represent domains that bind heparin (13, 16–18), fibrinogen (19), type V collagen (20), fixed erythrocytes (11), and platelets (1, 12).

To begin dissecting the structure of this large protein and to relate the functional domains to the structure of the molecule, we have generated a panel of monoclonal antibodies (Mab's) against both native, calcium-replete TSP and the denatured protein. This panel has been characterized in terms of the effect of each Mab on three functional assays for TSP: hemagglutination (21), agglutination of fixed, activated platelets (21), and aggregation of live platelets (1). Mab C6.7 blocks the thrombin- and A23187-stimulated aggregation of live, washed platelets (1) and the hemagglutinating activity of purified TSP (21). In contrast, A2.5 blocks the hemagglutination activity of TSP and the agglutination of fixed, activated platelets (21). We have identified protease-resistant domains that contain the epitopes for these two antibodies. When native TSP is digested with chymotrypsin or thermolysin, a 25-kD fragment is rapidly liberated which binds to heparin-Sepharose (13) and is immunoprecipitated with Mab A2.5 (21). Heparin inhibits the binding of A2.5 to intact TSP, further confirming that this Mab recognizes the heparin binding domain of TSP. Automated protein sequencing of the isolated heparin binding domain and the intact TSP peptide chain has revealed that both have the same NH_2 terminal sequence, and hence the heparin binding domain is located at the very NH_2 terminus of TSP (13, 15, 18). The other fragment present in the digests at early times has an M_r of 140,000 and binds to fibrinogen-Sepharose (19), fixed erythrocytes (11), and fixed activated platelets (12) even though it no longer contains the heparin-binding domain. This 140-kD piece subsequently decays to yield peptides of 120 and 18 kD (1), which are disulfide linked in the absence of reductants. After separating the 120- and 18-kD chains, we have shown that Mab C6.7 binds only the small 18-kD peptide, and not the 120-kD fragment (1). Thus it would appear that Mabs A2.5 and C6.7 bind to quite distinct regions of TSP.

In this study, we have used these Mabs in conjunction with electron microscopy and high sensitivity amino acid sequencing techniques to map the positions of the epitopes for these two antibodies on the TSP trimer. The results of the sequencing experiments also allow the assignment of the linear order of the domains of TSP within the peptide chain.

MATERIALS AND METHODS

Materials: Calcium-replete TSP was purified from the supernatant of thrombin activated platelets as described (13). The production, specificity, and biochemical characterization of the Mab's against TSP, A2.5, and C6.7, has been described (1, 21). For use in ultrastructural analyses, these antibodies were purified from ascites fluid by ammonium sulfate precipitation followed by affinity chromatography on goat anti-mouse gamma-globulin bound to Sepharose (Cappel Laboratories, Malvern, PA). In some cases it was necessary to remove traces of contaminating fibronectin on a gelatin-Sepharose column. The purity of the Mab's, both of which are IgGs, was determined by SDS PAGE. Reagents for sequence analysis were obtained from Applied Biosystems, Inc. (Foster City, CA). Reagents for SDS gels were from Bio-Rad Laboratories (Richmond, CA).

Electron Microscopy: TSP (200 μl) in Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 7.6) at a concentration of 200 μg/ml was mixed with an equal volume of glycerol and placed into an all-glass nebulizer equipped with a rubber bulb (Ted Pella, Inc., Tustin, CA) for spraying onto 3-mm-square chips of freshly cleaved mica. The mica chips were attached by Scotchbrand double-stick tape to a clean piece of cardboard which was held in a vertical position. The mouth of the nebulizer was held 2–3 in from the cardboard horizontal to the row of mica chips, and the rubber bulb was squeezed several times until a cloud of vapor could be seen emerging from the mouth and reaching to the cardboard. To examine TSP-Mab complexes, 200 μl TSP at 200 μg/ml in Tris-buffered saline and 200 μl Mab at 50 μg/ml in Tris-buffered saline were incubated at 4°C for 2 h. Glycerol was added to the TSP-Mab samples to a final concentration of 50% before they were sprayed onto freshly cleaved mica as described above. Immediately after samples were sprayed, the mica chips, still attached to a square of cardboard, were transferred to the vacuum chamber of a vacuum evaporator (model DV-502, Denton Vacuum Inc., Cherry Hill, NJ) in which they were rotary shadowed with tungsten (0.02-in-diam wire at 33 mA current) at a starting angle of 8° and then carbon coated to produce a replica. Tungsten–carbon replicas were removed from the mica chips by being floated off onto the surface of a drop of distilled water. The replicas were then picked up on 300 mesh copper grids and viewed through a Phillips 201 transmission electron microscope. Photographic negatives were reversed before printing.

Amino Acid Sequencing: Chymotryptic digests of TSP in the presence and absence of Ca^{++} (5 mM EDTA) were performed as described (1, 13, 21) and analyzed on SDS gels. Preparative digests were run under identical conditions and separated on SDS slab gels in a wide sample slot flanked by prestained molecular weight standards. Gels were stained with Coomassie Blue and the bands were excised and electroeluted as described (13). The soluble peptides were loaded onto the sample disk of the vapor phase amino acid sequencer (model 470A, Applied Biosystems, Inc.). Alternatively, the unstained SDS gels were electrophoretically transferred onto activated glass fiber filter paper which was then stained with Coomassie Blue, and the bands were cut out. These glass fiber strips containing the electroblotted peptides were placed directly on the sample disk of the sequencer (21a). Phenylthiohydantoins were identified by high-performance liquid chromatography after automated conversion. All peptides were sequenced at least twice and most were sequenced after both electroelution and electroblotting. Initial yields were comparable to those obtained for the electroelution of standard proteins, and repetitive yields with the two methods were comparable and averaged from 93 to 95%. Amounts of the peptides sequenced in each run were between 100 and 200 pmol.

RESULTS

Ultrastructure of TSP

Visualization of the TSP molecule in the electron microscope revealed it to be a trimeric structure (Fig. 1), each subunit consisting of a large globular region 16.96 ± 2.02 nm in diameter attached to a narrow arm bearing a smaller globular region, 8.09 ± 2.02 nm in diameter, at the opposite end (Fig. 2). These measurements are uncorrected for the thickness of the carbon coating. We believe that this structure corresponds to the 180-kD subunit observed upon SDS PAGE of TSP (13). To form the native trimeric molecule, the subunits appear to be attached to one another at a single junc-

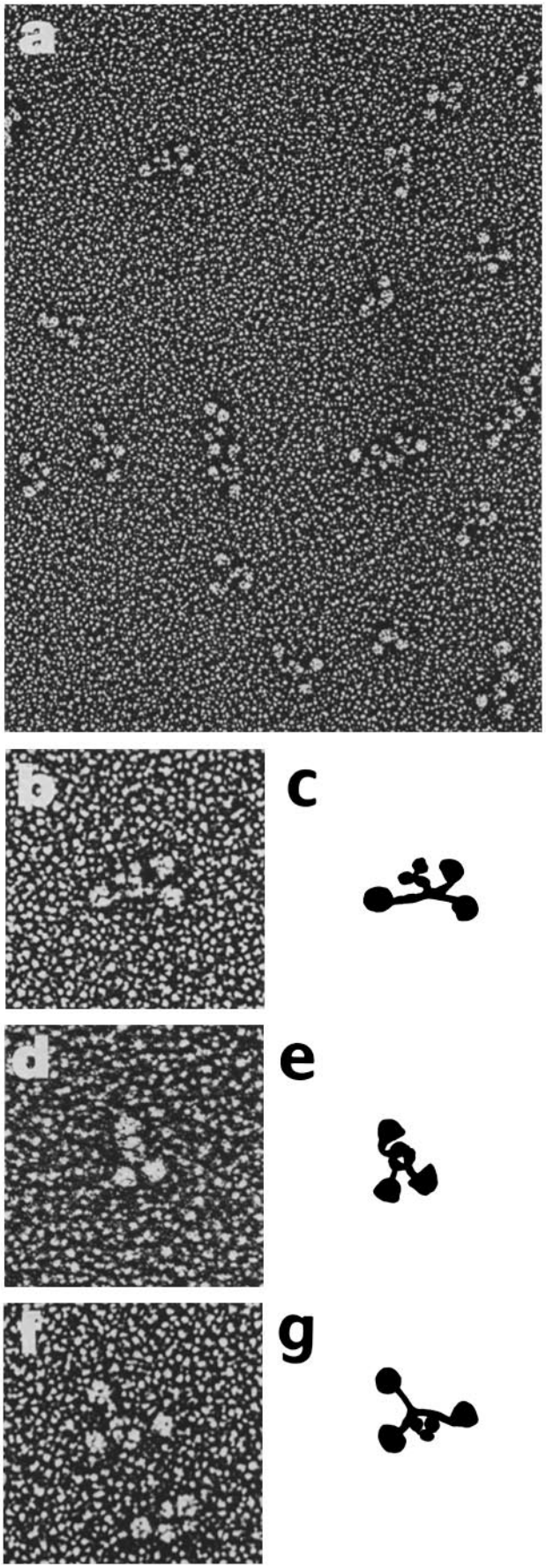

FIGURE 1 Rotary-shadowed replicas of calcium-replete TSP. The trimeric molecule consists of three large globular domains with narrow connecting strands joined together at one point and three smaller globular domains close to the junctional region. Ink drawings of the samples shown in *b*, *d*, and *f* are presented in *c*, *e*, and *g*, respectively, to assist in interpretation of the photomicrographs. (*a*) × 133,500. (*b*, *d*, *f*) × 234,500.

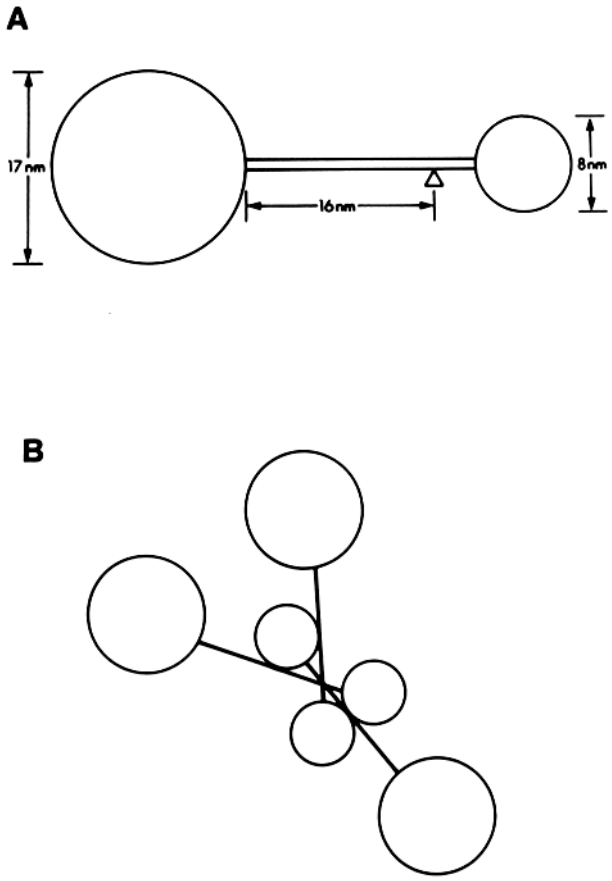

FIGURE 2 (*A*) Diagrammatic representation of the TSP subunit. The smaller of the globular domains represents the NH_2 terminus of the peptide chain. Three subunits are joined at the point indicated by the arrow to form the native trimeric molecule depicted in *B*.

tional point that lies near the smaller globular region (Fig. 1, b–g). The length of the arm between the point of subunit attachment and the large globular head was ~15.95 ± 4.05 nm; however, this distance was difficult to measure precisely since the arms could have become folded during the drying and consequent collapse of the TSP molecules before rotary shadowing and replication. The difficulty of obtaining an accurate arm length determination is reflected in the large variability observed in the lengths of subunit arms shown in Fig. 1. These images suggest that the arms are quite flexible and can assume various orientations. The small globular regions could not be resolved in all views of the TSP molecule, and, therefore, the structure sometimes appeared to have four large globular regions, as has been reported by other laboratories (14–16). In many views, the large globular domains appear to have clefts running through them as though they might be composed of subdomains. This idea is consistent with proteolysis experiments in which stable fragments of 120, 70, and 18 kD are seen to arise from the 140-kD species (1, 13, 20, 21).

TSP-Mab Complexes

The ultrastructure of the antibody–antigen complex formed between TSP and Mab A2.5 is shown in Fig. 3. The Mab's in these photographs were identified by their slightly larger size relative to the large ball of TSP and by their somewhat triangular shape (inset, Fig. 3, *g* and *h*). In many cases, the domain structure of the antibodies can be seen, particularly the cleft separating the two Fab regions (e.g., Figs. 3*e* and 4, *c*, *e*, and *g*). Mab A2.5, which reacts with the heparin binding domain of TSP (21), was clearly attached to the area of the molecule where the small globular heads are found (Fig. 3, *a–h*). Frequently, binding of A2.5 seemed to have forced all three of the connecting strands together so that the large balls also became pushed together (Fig. 3, *a–f*) rather than splayed out randomly as in views of TSP alone (Fig. 1). This effect was seen in 54% of 218 Mab-TSP complexes with A2.5, whereas only 19% of 297 TSP molecules without a bound A2.5 antibody molecule displayed this conformation. The clustering of the large globular regions could be indicative of the conformational change in TSP produced by interaction with Mab A2.5 that has been suggested by biochemical experiments. For example, it has been noted that A2.5 inhibits not only the hemagglutinating activity of TSP (21) but also its binding to heparin (21), plasminogen, and type V collagen (unpublished data). The inhibition of heparin binding by A2.5 is presumably caused directly by steric blocking of the heparin binding site, since A2.5 immunoprecipitates this domain from digests of TSP (21). However, it is unlikely that all of the binding sites for these other functions of TSP reside on the

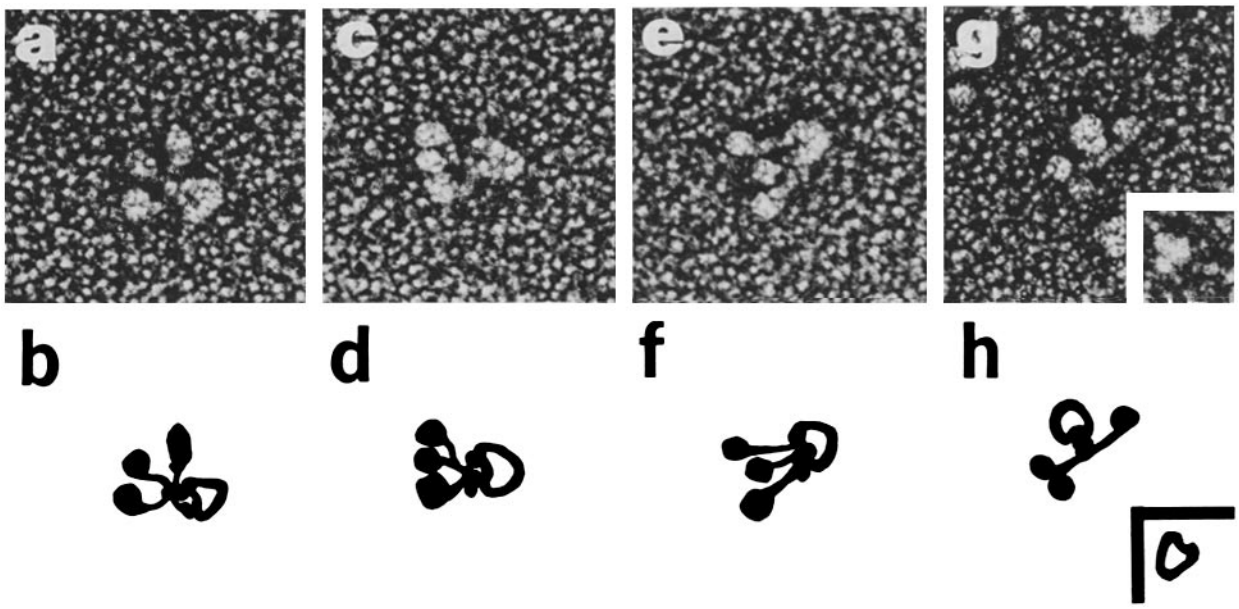

FIGURE 3 Rotary-shadowed replicas of A2.5 attached to calcium-replete TSP. The Mab can be identified by its size and triangular shape (see inset, *g* and *h*). In most examples, binding of A2.5 results in a conformational change in TSP which forces the larger globular regions together on one side of the complex (*a*, *c*, *e*). × 234,500.

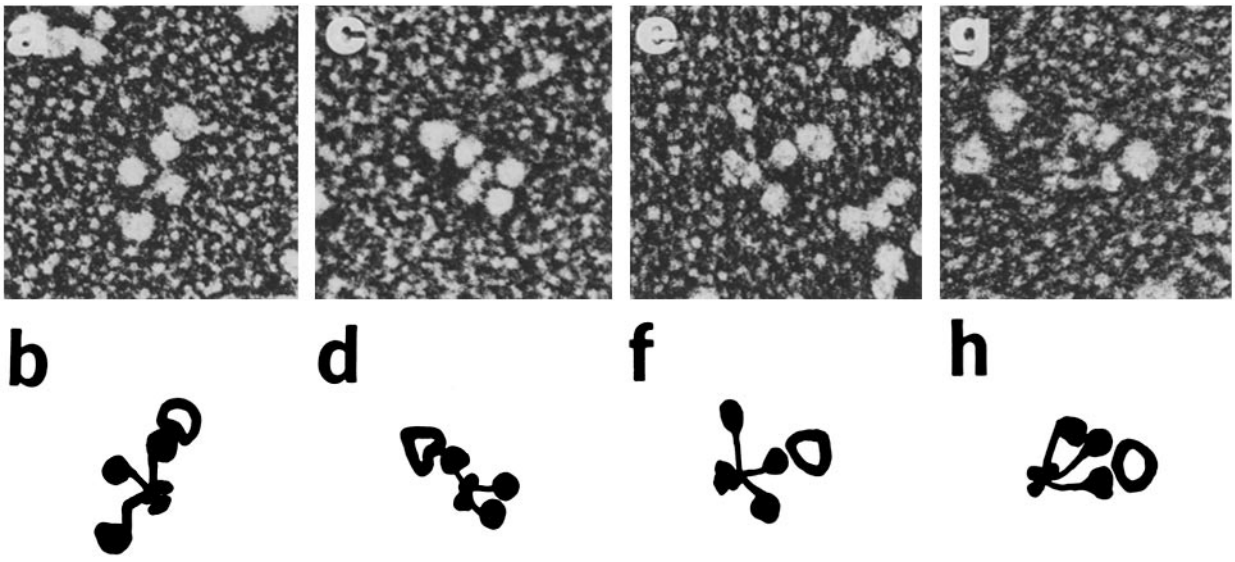

FIGURE 4 Rotary-shadowed replicas of C6.7 bound to calcium-replete TSP. The Mab is attached to the outer surface of the large globular domain. × 234,500.

relatively small (25 kD) heparin binding domain. In fact, it is clear that the binding site for type V collagen is not on the heparin binding domain (20) and the 140-kD fragment, which lacks the heparin binding domain binds to erythrocytes and platelets (11, 12).

The complex formed between TSP and Mab C6.7 is shown in Fig. 4. In this case, the antibody binds to the large globular domain of the TSP molecule, at the opposite end of the subunit from the A2.5 binding site. Usually, binding of C6.7 did not affect the spreading of the long arms of TSP on the mica surface, but, occasionally, where the antibody appeared to be cross-linking two large globular heads on the same molecule, the arms were again all lying close together (Fig. 4, *g* and *h*). Thus, it seems possible that C6.7 could also produce a conformational change in TSP by cross-linking domains on different subunits of the same trimer. However, this appears to be a relatively rare event, even at the ratio of ~1 mol TSP to 1 mol Mab used in these studies. Only 10% of the TSP-C6.7 complexes (177 scored) showed cross-linking of the large globular regions.

Sequencing of TSP Fragments

It has been established that the heparin binding domain of TSP, and hence the epitope for Mab A2.5, lies at the NH_2 terminus of the TSP subunit (13, 15, 16, 18). The results presented above suggest that the distant epitope for Mab C6.7 might lie near the COOH terminus of the molecule. Digestion of TSP with chymotrypsin in the presence of Ca^{++} results in rapid liberation of the heparin binding domain from the NH_2 terminus with production of a 140-kD fragment (Fig. 5). This large fragment is subsequently cleaved to yield a 120- and an 18-kD piece linked to it by disulfide bonds, which contains the C6.7 binding site (1). In Fig. 5 it is seen that the 18-kD fragment becomes more intense in parallel with the 120-kD species while the 140-kD fragment disappears. We have previously determined the amino acid sequence at the NH_2 terminus of this 18-kD peptide, but to order it within the 140-kD fragment, more data were needed. We reasoned that if the 18-kD peptide were liberated from the COOH terminus of the 140-kD species, then the 120-kD peptide might have the same NH_2 terminus as the 140-kD peptide if further digestion had not occurred at that end of the chain. Since the only means of preparing pure samples of these peptides is SDS gel electrophoresis, we undertook the microsequencing of these very large peptides eluted from SDS slab gels. Fig. 6*A* is a schematic diagram of the chymotryptic digestion of TSP in the presence of Ca^{++} ion. The sequences obtained for the 140- and the 120-kD peptides are indicated in Fig. 6 and are identical for the 11 residues that could be interpreted with confidence. This common sequence is Ile-Gly-His-Lys-Thr-Lys-Asp-Leu-Gln-Ala-Ile-. The sequence of the 18-kD peptide, which has been previously determined (1), is shown in Fig. 6. Thus the 18-kD fragment containing the C6.7 epitope must be removed from the COOH terminus of the 140-kD chain to generate the 120 kD species.

In the absence of Ca^{++} (5 mM EDTA), the 140- and 120-kD species are not stable in chymotryptic digests (Fig. 5). Instead, the largest stable fragment found is 70 kD and is thought to represent the type V collagen binding domain of TSP (20). Since the 25-kD heparin binding domain is produced in either the presence or absence of Ca^{++} (13–17), a cleavage must occur at roughly the same site at the COOH terminus of the heparin binding domain. To determine if this cleavage might be the same one as that which gives rise to the 140/120-kD fragments (above), we electroblotted the 70-kD fragment and subjected it to sequence analysis. These results, shown in Fig. 6*B*, indicate that the 70-kD fragment has an NH_2 terminal sequence identical to that of the 140/120-kD peptides. The 80-kD peptide shown in Fig. 6 is a transient intermediate in the digest that decays to the 70 kD species (Fig. 5). We infer that it must also have the same NH_2 terminal sequence as the 70-kD species, but this has not been directly determined. We also analyzed the 25-kD species produced in the presence of EDTA and found that it is in fact identical to the heparin binding domain obtained in the presence of Ca^{++} (13). Thus, even in the presence of EDTA, which induces dramatic conformational changes in TSP (14–17), the precise cleavage site between the heparin binding domain and the rest of the peptide chain is maintained.

In summary, these results indicate that generation of the 140-kD fragment is a primary event in the proteolysis of TSP and that all subsequent cleavages that give rise to the smaller fragments observed in either the presence or absence of Ca^{++} occur at the COOH terminus of the 140-kD peptide chain. The main conclusion for the Mab mapping studies is that whereas the heparin binding domain lies at the very NH_2 terminus of the TSP chain, the 18-kD fragment that contains the epitope for C6.7, and by inference, the binding site(s) for platelets or platelet-associated proteins, resides at the COOH terminus of TSP. This conclusion is also consistent with the electron micrographs of TSP-C6.7 complexes in which this Mab binds to the opposite end of the subunit from Mab A2.5 that binds to the NH_2 terminal heparin binding domain.

DISCUSSION

Electron microscopy of tungsten-shadowed, carbon-coated replicas of calcium-replete TSP has shown it to be a trimer composed of subunits that contain a small globular domain, a thin connecting strand, and a larger globular domain (reference 16 and this study). We have used a Mab directed against the NH_2 terminal heparin binding domain (21) to identify the small globular region as the heparin binding domain and hence mark the NH_2 terminus of the peptide chain as it exists in the three-dimensional configuration of the protein. Lawler et al. (16) have independently arrived at precisely the same model of TSP structure. They also used a monoclonal antibody directed against the heparin domain to identify the small domain, which they refer to as globular region N. It appears, however, that their Mab, called MA-II, has no discernible effects on TSP function and does not cause the clustering of the large globular domains, referred to as globular region C (16), which we have noted to result from the binding of our Mab A2.5 to TSP (Fig. 4). We suggest that A2.5 does this by binding to two of the heparin binding domains simultaneously, thus cross-linking two subunits of the trimer at their NH_2 termini. A2.5 inhibits both the hemagglutination activity of TSP and the agglutination of fixed, activated platelets by purified TSP (21) yet has no effect on the aggregation of live platelets (1). It also inhibits the binding of TSP to sulfatides (22), plasminogen, and type V collagen (unpublished data). These multiple, diverse effects of Mab A2.5 can be explained by its dramatic conformational reorientation of the TSP molecule (Fig. 3). Thus, any function mediated by a site on the large globular domain could be

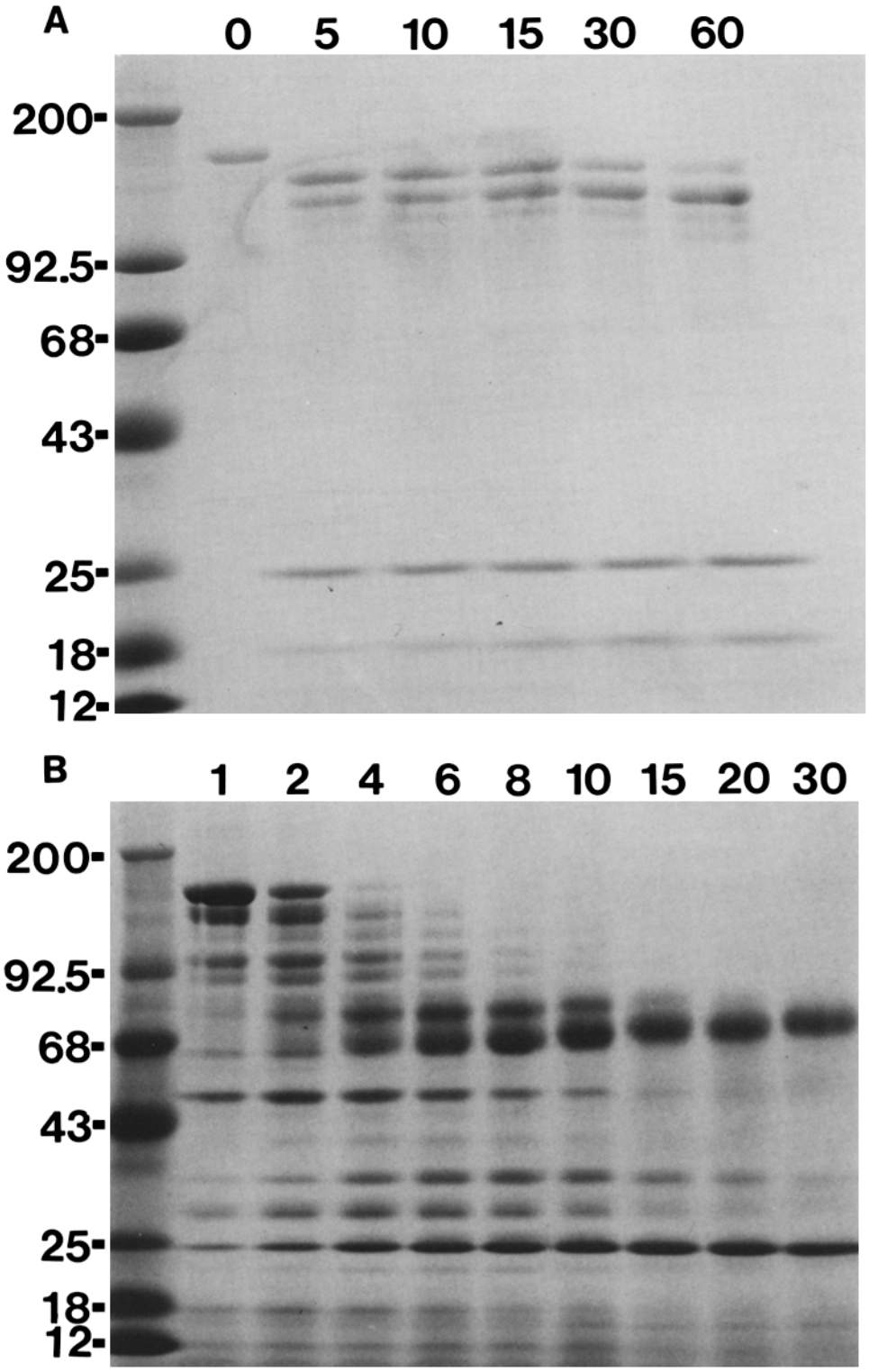

FIGURE 5 The time course of digestion of TSP by 0.5% (wt/wt) chymotrypsin in the presence (*A*) and absence (*B*, 5 mM EDTA) of calcium. The SDS gels were run as described in Materials and Methods and stained with Coomassie Blue. The numbers above the gel lanes indicate the time of digestion in minutes.

hindered by their crowding together induced by A2.5.

In view of the possibility that A2.5 can inhibit functions mediated by sites on TSP distant from the heparin binding domain, one should perhaps interpret with caution the recent report that polyclonal antisera raised against the purified heparin binding domain of bovine TSP can inhibit the aggregation of bovine platelets (23). The authors of that report suggest that the effect of the antiserum indicates an important role for the heparin binding domain in platelet aggregation. The possibility must be considered however, that the polyclonal antiserum can also cause the reorientation of the TSP domains observed here with Mab A2.5, and hence could exert its effects on platelet aggregation by this indirect mechanism.

The primary purpose of this study was to map the epitope for our Mab C6.7, which has the property of inhibiting the aggregation of thrombin- or A23187-stimulated platelets (1). The micrographs in Fig. 4 clearly reveal that this Mab binds to the outer face of the large globular region, referred to as globular region C by Lawler et al. (16). C6.7 also inhibits the hemagglutinating activity of TSP but has no effect on the

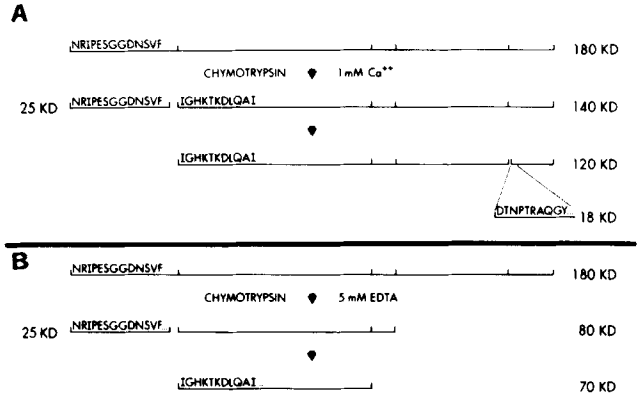

FIGURE 6 Schematic representation of the cleavage of the 180-kD peptide chain of TSP by chymotrypsin in the presence (*A*) and absence (*B*) of Ca^{++}. The NH_2 terminal sequence of each fragment is indicated by the single letter amino acid code (the sequence of the 140/120- and 70-kD peptides using the three letter abbreviation system is given in the text).

agglutination of fixed platelets (21). Lawler et al. (16) also reported a Mab called MA-I which binds to the large globular region. No functional effects have been reported for this Mab which can be seen in their micrographs of TSP-MA-I complexes to bind to a different site on the large globular region than our MAb C6.7. C6.7 binds to a site directly opposite the point at which the connecting strand enters the large domain, and MA-I binds to a site about midway between the point of connecting strand entry and the epitope for C6.7 (see Fig. 14 in reference 16). If in fact the epitope recognized by C6.7 represents the platelet binding site of TSP or the site at which it interacts with some other protein that functions in bridge formation between aggregating platelets, this site is ideally located to bind in an unhindered fashion to this putative TSP receptor.

Since cross-linking of the large globular regions of TSP by C6.7 was only rarely seen at Mab concentrations comparable to those used in the platelet aggregation studies (1), we infer that the inhibitory action of C6.7 in platelet aggregation is due to its direct interference with a platelet binding site in or near the 18-kD domain immunoprecipitated by Mab C6.7 (1). We have previously shown that trimers composed of 140-kD chains, and thus devoid of the heparin binding domain, can bind to erythrocytes (11) and fixed, activated platelets (12). Mab C6.7 precipitates those 140-kD chains, and, after cleavage to produce the 120- and 18-kD species and separation of the chains, C6.7 precipitates the 18-kD peptide (1).

The results of the sequencing experiments presented here establish that both the 120-kD fragment and the 140-kD species from which it is derived have identical NH_2 termini for 11 residues (Fig. 6). This means that the 18-kD fragment to which C6.7 binds must be cleaved from the COOH terminal end of the 140-kD peptide, as shown in Fig. 6. We (13) and others (15, 18) have previously shown that the heparin binding domain and the intact 18-kD TSP peptide chain have identical NH_2 terminal sequences, so the 140-kD fragment must be preceded by the heparin binding domain. The heparin domain has a size of 25–35 kD depending on the protease used to cleave it (13, 15–18), which suggests that the 25-kD form that we isolate from chymotryptic digests is followed by about another 10 kD of peptide in a rather extended conformation. In the absence of Ca^{++}, both the 140- and 120-kD species are unstable to chymotrypsin, and a 70-kD fragment generated from these digests has been shown by Mumby et al. (20) to contain a binding site(s) for type V collagen. This peptide also has the same sequence at its NH_2 terminus as the 140- and 120-kD peptides generated from Ca^{++} replete TSP. Hence, all proteolysis of the subunit except for the initial cleavage that liberates the heparin binding domain proceeds from the COOH terminus. Therefore, the order of domains within the TSP peptide chains would be

NH_2-heparin-type V collagen-[ ]-fibrinogen-platelet-COOH
25–35 kD 70 kD 10 kD 40 kD 18 kD

The positioning of the fibrinogen binding region is inferred from the fact that both the 140- and 120-kD peptides appear to bind to fibrinogen (19), and this region must be distal to the 70-kD type V collagen binding domain (20). It is not yet known if the fibrinogen binding region might overlap the type V collagen binding region or the 18-kD peptide to some extent. The 10-kD "spacer region" between the type V collagen domain and the 40-kD fragment that we infer to contain the fibrinogen binding site is the COOH terminal peptide, which is cleaved from the transient 80-kD species by chymotrypsin in EDTA as shown in Figs. 5 and 6.

This model for the linear order of the TSP domains implies that, at least to some extent, the folded TSP subunit is a co-linear representation of the TSP peptide chain. This is an interesting result since the adhesive glycoprotein fibronectin, which is also involved in platelet aggregation (24), has several functional domains with binding activities similar to those of TSP, and the domains of fibronectin are arranged in a strictly co-linear order along the peptide chain, beginning with a heparin binding domain at the NH_2 terminus (25). In the case of TSP, the NH_2 terminal heparin binding domain is a compact globular structure that contains no disulfide bonds (13). Once the peptide chain exits this domain it does not fold back and reenter it but rather proceeds through the connecting region where interchain disulfides cross-link the subunits into the trimer. This region, which binds type V collagen (20), remains as the protease-resistant "core" when TSP is digested with chymotrypsin in the absence of Ca^{++}. At the ends of these arms, which may be composed of more than one strand of peptide chain folded back and forth or arranged in a helical conformation, the peptide chain enters the large globular domain. Part of the 70-kD peptide is probably involved in forming part of this globular structure, but apparently can adopt a more extended conformation when Ca^{++} is removed. Lawler et al. have reported (16) and we have confirmed that upon EDTA treatment of TSP, the connecting strands become longer, and the large globular domains become smaller, as though they are unrolling. Distal to the 70 kD region, the peptide chain must fold and become cross-linked with disulfides in a complex pattern, since the 18-kD region containing the epitope for C6.7 cannot be obtained from digests without both reduction and complete denaturation (1) to allow separation of it from other regions of the peptide chain with which it interacts. However, this large globular region is probably not a single compact domain, since our images of TSP as well as those of Lawler et al. (16) suggest that the large globular region is divided into subregions by clefts running across its surface. One of these putative subdomains may represent the fibrinogen binding region. Further work is needed to localize the fibrinogen binding region, which, according to the above

model, should be in the large globular domain near the epitope for C6.7. Since fibrinogen has been suggested to be the platelet-bound receptor for TSP (10, 26, 27), C6.7 may exert its effects on platelet aggregation by interfering with the TSP-fibrinogen interaction.

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