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 NH2 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 NH2 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 NH2 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.

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

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 TSP have provided further evidence that TSP is an important regulatory component of the platelet system.
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) Mr 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 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 NH2 terminal sequence, and hence the heparin binding domain is located at the very NH2 terminus of TSP (13, 15, 18). The other fragment present in the digests at early times has an Mr 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 sequenc-
The smaller of the globular domains represents the NH₂ 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.

The conformational 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...
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. 6A 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. 6B, 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
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 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).

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 NH2 terminus (25). In the case of TSP, the NH2 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

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**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 NH2 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).
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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