Effects of Anti-Thrombospondin Monoclonal Antibodies on the Agglutination of Erythrocytes and Fixed, Activated Platelets by Purified Thrombospondin†

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ABSTRACT: A monoclonal antibody (Mab) has been raised against native thrombospondin (TSP), the endogenous lectin of human platelets, that inhibits the hemagglutination of trypsinized, glutaraldehyde-fixed human erythrocytes by purified TSP. This Mab, designated A2.5, also inhibits the agglutination of fixed, activated platelets by TSP. Mab A2.5 immunoprecipitates a 25-kilodalton (kDa) peptide from chymotryptic digests of TSP that is not disulfide bonded to any other region of the TSP molecule. This fragment represents the previously characterized heparin binding domain of TSP [Dixit, V. M., Grant, G. A., Santoro, S. A., & Frazier, W. A. (1984) *J. Biol. Chem. 259*, 10100–10105]. In agreement with this assignment, heparin inhibits the binding of Mab A2.5 to TSP. Another Mab, designated C6.7, also blocks TSP-mediated hemagglutination, yet has no effect on the agglutination of fixed, activated platelets by TSP. This Mab has been shown to inhibit the thrombin-stimulated aggregation of live platelets and to immunoprecipitate an 18-kDa fragment from chymotryptic digests, which is distinct from the heparin binding domain [Dixit, V. M., Haverstick, D. M., O'Rourke, K. M., Hennessy, S. W., Grant, G. A., Santoro, S. A., & Frazier, W. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3472–3476].

Thrombospondin (TSP)[1] is a platelet α-granule protein that is secreted when platelets are activated by agents like thrombin and becomes associated with the platelet surface in the presence of divalent cations (Baenziger et al., 1972; Phillips et al., 1980; Gartner et al., 1981). TSP is composed of three apparently identical (Dixit et al., 1984a; Coligan & Slayter, 1984) glycoprotein chains, each of M_r 180 000, disulfide bonded to form a trimer. Recent work has revealed that the TSP chains are made up of several protease-resistant domains that contain functional binding sites for such macromolecules as heparin (Dixit et al., 1984a; Coligan & Slayter, 1984), fibrinogen (Dixit et al., 1984b), and type V collagen (Mumby et al., 1984). Our group (Dixit et al., 1984a), Raugi et al. (1984), and Coligan & Slayter (1984) have reported that the amino-terminal sequence of the intact TSP peptide chain and that of the heparin binding domain isolated from proteolytic digests are identical, indicating that the heparin binding domain lies at the extreme amino terminus of the 180-kilodalton (kDa)[1] TSP peptide chain. The fibrinogen binding domain is located within disulfide-linked homotrimers composed of either 140-kDa or 120-kDa peptide chains produced by thermolytic digestion (Dixit et al., 1984b). Further digestion results in a trimer of 70-kDa peptides that contain a binding site for type V collagen (Mumby et al., 1984).

For some time TSP has been thought to have a role in platelet aggregation. Activated platelets were found to express an endogenous lectin activity on their surface (Gartner et al., 1978; Jaffe et al., 1982) with properties very similar to the lectin activity of TSP itself (Haverstick et al., 1984). The best inhibitors of this activity were sugars with a free amino group such as galactosamine and mannosamine and amino acids with a net positive charge such as arginine (Gartner et al., 1978; Jaffe et al., 1982). It was further observed that these compounds were inhibitors of platelet aggregation (Gartner et al., 1978). However, these inhibition properties are reminiscent of the hemagglutinating activity of fibronectin (FN)[1] (Yamada et al., 1975), and the binding of fibrinogen to glycoproteins IIb/III on the platelet surface (Gerrard et al., 1980) is inhibited by these same compounds. Thus inhibition by amino sugars is not indicative of the exclusive involvement of TSP in platelet aggregation. We have recently characterized a monoclonal antibody (Mab)[1] against TSP that is able to inhibit the aggregation of thrombin- or A23187-stimulated platelets without affecting platelet activation or secretion (Dixit et al., 1985a). This Mab thus constitutes direct evidence for a role of TSP in the process of platelet aggregation.

The agglutination of red cells by TSP has been an attractive model for TSP function, since it represents a more readily controlled and available system than live platelets. In addition, it has become clear that TSP is synthesized and secreted by a large number of different cells including endothelial cells (McPherson et al., 1981; Mosher et al., 1982; Raugi et al., 1982), fibroblasts (Raugi et al., 1982; Jaffe et al., 1983), smooth muscle cells (Raugi et al., 1982), and type II pneumocytes (Sage et al., 1983). This information suggests a potential role for TSP in cell–cell and cell–substratum interactions beyond its role in platelet aggregation. To further explore the red cell model for TSP action, we have characterized the hemagglutinating activity of purified, calcium-replete TSP using trypsinized, glutaraldehyde-fixed human erythrocytes (Haverstick et al., 1984). Both calcium and

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[1] Abbreviations: TSP, thrombospondin; FN, fibronectin; Mab, monoclonal antibody; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium docecyl sulfate–polyacrylamide gel electrophoresis; kDa, kilodalton; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; RIA, radioimmunoassay; IgG, immunoglobulin G.

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magnesium are required at concentrations of 2 mM each for optimal expression of this activity, and heparin is a potent inhibitor, while other glycosaminoglycans are essentially without effect (Haverstick et al., 1984). We have also reported that purified TSP agglutinates fixed, thrombin-activated platelets and that this activity also requires both 2 mM Ca^{2+} and 2 mM Mg^{2+} (Haverstick et al., 1985). However, this assay appears to be more complex than the hemagglutination assay in that heparin can only partially inhibit the agglutination of the fixed, activated platelets, and fibrinogen, which has no effect on hemagglutination, is also a partial inhibitor in the fixed platelet assay (Haverstick et al., 1985).

We report here that a Mab, designated A2.5, which was raised against native, Ca^{2+}-replete TSP, inhibits the hemagglutination of both fixed, trypsinized human erythrocytes and fixed, activated platelets by TSP. Mab A2.5 specifically immunoprecipitates a 25-kDa fragment from chymotryptic digests of TSP, which represents the heparin binding domain of TSP, and heparin inhibits the binding of this Mab to TSP.

Materials and Methods

Materials. Calcium-replete TSP was purified from the supernatant of thrombin-activated platelets as described (Dixit et al., 1984a). Radiochemicals were from New England Nuclear (Boston, MA), except for ^{75}Se-labeled methionine (20–50 Ci/mmol; 1 mCi/mL), which was from Amersham, Chicago, IL. Mab A3.3 against human fibronectin has been previously characterized (Dixit et al., 1985b). Human von Willebrand factor (Santoro & Cowan, 1982) and human FN (McDonald & Kelley, 1980) were purified as described, and human fibrinogen was from Kabi (Sweden). Reagents for SDS–PAGE[1] were from Bio-Rad (Richmond, CA).

Production of Mabs against Human TSP. Mabs were produced essentially as described by Galfre et al. (1977). Balb/c mice were immunized with either native (not exposed to EDTA) or reduced and alkylated TSP. Three days after the final boost, spleen cells were fused with myeloma line SP2/Ag14, and those hybridomas were selected that produced an antibody which bound to TSP immobilized on PVC wells. Positives were subcloned in soft agar and screened for reaction against calcium-replete TSP, TSP treated with EDTA,[1] and reduced and alkylated TSP. Selected subclones were propagated by intraperitoneal injection into pristane-primed Balb/c mice, and ascites fluid was obtained about 10 days later. Mabs were purified from ascites fluid by ammonium sulfate precipitation, followed by gel filtration on Sephacryl S-200 or affinity chromatography on a goat anti-mouse γ-globulin (Cappel) affinity column. Mabs were typed with an ELISA kit from Zymed Laboratories (South San Francisco, CA). To determine specificity, the solid-phase radioimmunoassay was performed as described (Dixit et al., 1985a,b) with immobilized antibodies and TSP iodinated with the Bolton–Hunter reagent or $Na^{125}I$ and Iodobeads (Pierce, Rockford, IL). Immunoprecipitations were performed as described (Dixit et al., 1985a). Immune complexes were precipitated with rabbit anti-mouse IgG (DAKO Corp., Santa Barbara, CA), followed by protein A–Sepharose (Pharmacia, Uppsala, Sweden). The complexes were washed on protein A–Sepharose 8–10 times with 0.02 M Tris-HCl, pH 7.6, 0.15 M NaCl (Tris-buffered saline) containing 2 M urea, 0.1 M glycine, and 1% Triton X-100. SDS–PAGE and autoradiography were performed as described (Dixit et al., 1985a).

Controlled Proteolysis of TSP. Digestion of TSP with chymotrypsin was performed at an enzyme to substrate ratio of 0.5% w/w in Tris-buffered saline containing either 1 mM $CaCl_2$ or 5 mM EDTA. The extent of digestion was varied by using either 25 or 37 °C and by varying the time of digestion from 30 to 60 min. Proteolysis was terminated with 5 mM phenylmethanesulfonyl fluoride (PMSF).[1] Digests were always characterized by SDS–PAGE since, while the pattern of fragments obtained is quite reproducible, the time course can vary when low concentrations of labeled TSP are used. In particular, the 18-kDa species (see below) is quite labile to digestion for prolonged times, while the 25-kDa species is very stable.

Agglutination Assays. The hemagglutination assay of TSP using trypsinized, glutaraldehyde-fixed human erythrocytes was performed as described previously (Haverstick et al., 1984). As noted earlier, both 2 mM Ca^{2+} and 2 mM Mg^{2+} were required for optimal expression of TSP's hemagglutinating activity, and these ions were included in the assay. The agglutination of fixed, thrombin-activated platelets by TSP was also performed as described (Haverstick et al., 1985) in microtiter well plates.

Solid-Phase Binding Assay Using [^{75}Se]Selenomethionine-Labeled Mabs. Clonal hybridomas were grown in methionine-free medium (Cancer Center, Washington University School of Medicine) containing 10 μCi of [^{75}Se]-selenomethionine/mL. Ten to twenty million cells were grown in 1 mL of medium overnight, the medium was collected, BSA was added for a final concentration of 0.1%, and the solution was dialyzed against Tris-buffered saline containing 1 mM Ca^{2+} and 0.1% BSA to remove free methionine. For the solid-phase assay, TSP at 10 μg/mL was adsorbed to PVC wells for 12 h at 4 °C. Wells were blocked with 1% w/v BSA. Test solutions of the various unlabeled antibodies (supernatant medium from hybridomas grown in the absence of radiolabeled methionine) were added at the indicated dilutions together with a constant volume of the biosynthetically labeled antibody to each well. The plates were incubated for 4 h at 37 °C and extensively washed with Tris-buffered saline containing 1 mM $CaCl_2$ and 3% BSA, and the individual wells were counted in a Beckman γ counter using the ^{22}Na channel. Glycosaminoglycan inhibition of Mab binding to TSP was performed with the usual solid-phase RIA in which the Mab was immobilized on PVC wells and the binding of iodinated TSP was challenged with the indicated concentrations of glycosaminoglycan. Plates were incubated for 4 h at 37 °C and washed extensively with Tris-buffered saline and 1% BSA before counting.

Results

Specificity of the Mabs. The specificity of Mab A2.5 was established by both immunoprecipitation and competitive radioimmunoassay. This approach was necessitated since Mab A2.5 did not identify TSP on immunoblots. Thus the supernatant obtained from thrombin-activated platelets was radioiodinated (Figure 1A, lane 2) and subjected to immunoprecipitation with Mab A2.5 (Figure 1A, lane 3) and a control Mab (Figure 1A, lane 4). It can be seen that Mab A2.5 immunoprecipitated a protein of M_r 180 000 that corresponds to purified TSP (Figure 1A, lane 1). In order to ensure that Mab A2.5 did not react with a platelet protein that did not iodinate well, a competitive radioimmunoassay was performed (Figure 1B). In this experiment Mab A2.5 was immobilized on PVC wells, and the binding of iodinated TSP was challenged with purified TSP itself, the crude platelet supernatant, and the supernatant after affinity adsorption with heparin–Sepharose, which removes TSP, platelet factor 4, and β-thromboglobulin (Dixit et al., 1984a). Also tested as competitors were purified FN, fibrinogen, and von Willebrand factor. It can be seen in Figure 1B that only unfractionated

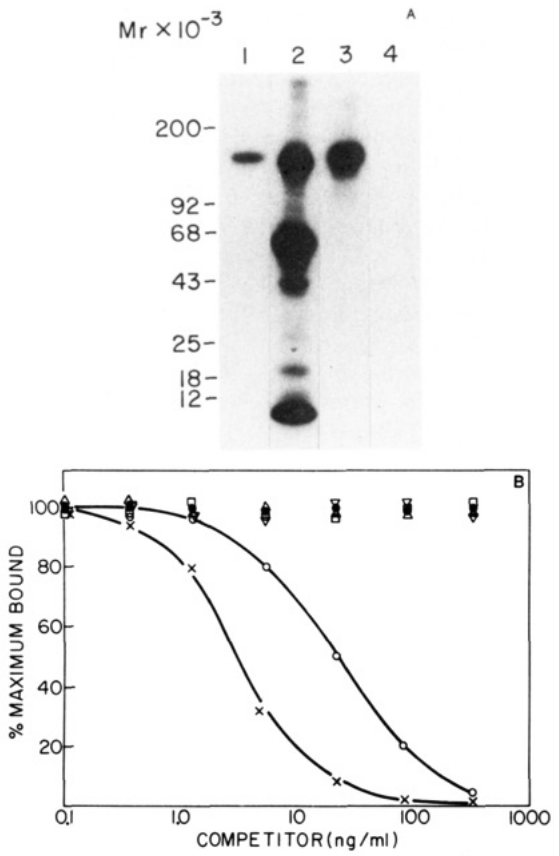

FIGURE 1: Specificity of Mab A2.5. (A) Iodinated proteins were immunoprecipitated with Mab A2.5. Lane 1, immunoprecipitate of iodinated purified TSP; lane 2, iodinated supernatant from thrombin-activated platelets; lane 3, immunoprecipitate of the supernatant shown in lane 2; lane 4, immunoprecipitate of the supernatant with a control Mab. (B) Radioimmunoassay in which Mab A2.5 was immobilized on PVC wells and the binding of a constant amount of ^{125}I-TSP was challenged with the following: ×, purified TSP; ○, supernatant from thrombin-activated platelets; ●, same platelet supernatant after adsorption with heparin–Sepharose to remove TSP; □, fibronectin; ▽, von Willebrand factor; △, fibrinogen.

platelet supernatant and purified TSP compete. Thus it is unlikely that Mab A2.5 recognizes a protein other than TSP.

Effect of Mab A2.5 on Hemagglutinating Activity of TSP. As shown in Figure 2 (top row), TSP is a potent agglutinin of trypsinized, glutaraldehyde-fixed human red cells with an end point in this assay of 2.5 μg/mL (well 5). To test the effect of the Mabs on the hemagglutination activity of TSP, the lectin was present in all the remaining wells (wells 1–10) at a concentration of 5 μg/mL (that in well 4, top row). The Mabs were serially diluted in these remaining rows. Both Mab A2.5 and Mab C6.7 were inhibitors of the lectin activity having minimum inhibitory concentrations of 15 and 30 μg/mL, respectively. As seen in wells 11, the Mabs alone had no lectin activity, and in wells 12, the erythrocytes in the absence of TSP remain unagglutinated. Row 4 in Figure 2 shows that a control Mab against human FN has no anti-lectin activity toward TSP. Also tested were 10 other Mabs against TSP, all of which were without effect (not shown).

Effect of Mabs on Agglutination of Fixed, Activated Platelets. Figure 3 (top row) illustrates the standard agglutination assay for TSP using, instead of fixed erythrocytes, fixed, thrombin-activated platelets (Haverstick et al., 1985). The top row is a serial dilution of TSP that is active in this assay through well 7, which contains 1.25 μg/mL TSP. To assess the effects of the Mabs on this activity of TSP, the amount in well 6 (2.5 μg/mL) was used in all the remaining rows, wells 1–10. Hence, in the absence of any inhibitor all these wells should be positive. As can be seen, only Mab A2.5 has any inhibitory effect in this assay, and its minimum inhibitory concentration is 60 μg/mL, about 4-fold higher than that required to inhibit the hemagglutination activity of TSP (Figure 3). The panel of 10 other anti-TSP Mabs was also tested and found to be without effect in this assay.

Localization of the Epitope for Mab A2.5. It has been shown that TSP can be digested with a variety of proteases to yield relatively stable domains of protein structure (Dixit et al., 1984a; Coligan & Slayter, 1984; Dixit et al., 1984b; Mumby et al., 1984). To localize the epitope with which Mab A2.5 interacts, TSP was radioiodinated by both the Bolton–Hunter and Iodobead methods, and this TSP was subjected to chymotryptic digestion (0.5% w/w chymotrypsin, 30–60 min at 25 °C). The digest was immunoprecipitated with Mab A2.5 and the precipitated material solubilized and subjected to SDS–PAGE, followed by autoradiography. Figure 4A (reducing conditions) shows the chymotryptic digest of labeled TSP (lanes 1 and 2, two different exposures), the immunoprecipitate with Mab A2.5 (Figure 4A, lane 3), and an immunoprecipitate with a control Mab (Figure 4A, lane 4). Mab A2.5 cleanly precipitates a 25-kDa species from the chymotryptic digest. We have shown, using affinity chromatography of the chymotryptic digest on heparin–Sepharose (Dixit et al., 1985a), that this fragment represents the heparin binding domain of TSP. Since the heparin binding domain is not linked to larger fragments of TSP by disulfide bonds (Dixit et al., 1984a; Coligan & Slayter, 1984), it should run on SDS–PAGE as a 25-kDa species, even in the absence of reductant. This is shown in Figure 4B. In Figure 4A, it can

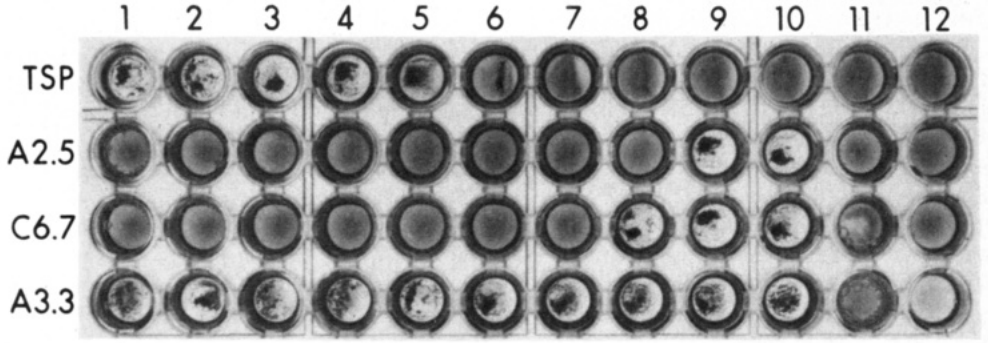

FIGURE 2: Inhibition of hemagglutinating activity of TSP by Mabs. In the top row, TSP was serially diluted and assayed for activity against fixed, trypsinized human erythrocytes as described under Materials and Methods in the absence of any additions to the standard assay. Well 12 had no added TSP (negative control). For the remaining three rows, TSP was held constant at 5 μg/mL (that amount in well 4 of the top row) in wells 1–10, and the indicated monoclonal antibodies were serially diluted. Well 11 was with antibody alone (no TSP); well 12 was with erythrocytes alone. The concentration of all antibodies in wells 1 was 2 mg/mL. The minimum inhibitory concentrations are 15 μg/mL for Mab A2.5, 30 μg/mL for Mab C6.7, and no effect for Mab A3.3.

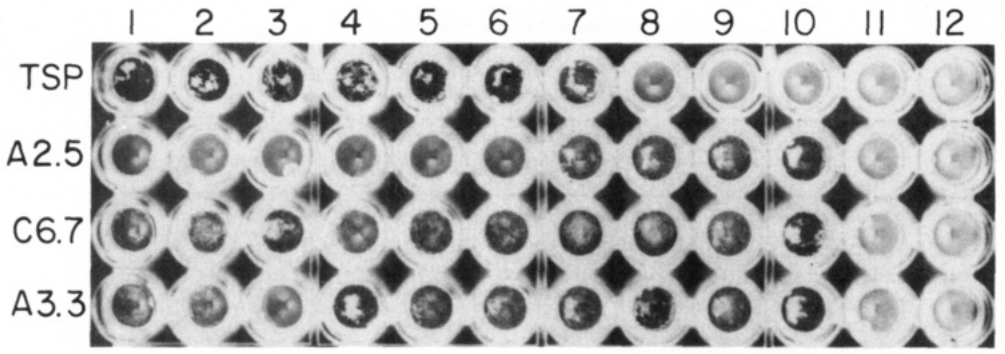

FIGURE 3: Inhibition of TSP-mediated agglutination of fixed, activated platelets by Mabs. In the top row, TSP was serially diluted into the wells, fixed, activated platelets were added, the plate was agitated for 20 min, and the plate was read and photographed against a black background. In well 1, TSP is present at 75 μg/mL, and the lowest active concentration (the end point) is in well 7 at 1.25 μg/mL. Well 12 has no added TSP (negative control). For the remaining three rows, the amount of TSP in each well was held constant at 2.3 μg/mL (that amount in well 6, top row), and Mabs were serially diluted, beginning with a concentration of 2 mg/mL in wells 1. Only Mab A2.5 is inhibitory in this assay, and its minimum inhibitory concentration in well 6 is 60 μg/mL.

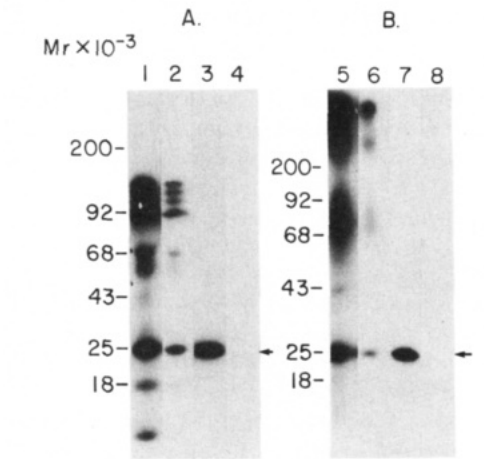

FIGURE 4: Immunoprecipitation of the chymotryptic digest of TSP with Mab A2.5. Intact TSP was iodinated by both the Bolton–Hunter reagent and the Iodobead procedure with $Na^{125}I$ and digested with chymotrypsin (0.5% w/w, 30–60 min at 25 °C). The digest was terminated with PMSF, samples were immunoprecipitated with Mab A2.5 as described under Materials and Methods, and material bound to the Mab was analyzed by SDS–PAGE, followed by autoradiography. Samples run in lanes 1–4 were reduced with 2-mercaptoethanol, and those in lanes 5–8 were run in the absence of reducing agent. Lanes 1 and 5, chymotryptic digest of doubly iodinated TSP (overexposed); lanes 2 and 6, same digest, shorter exposure; lanes 3 and 7, immunoprecipitates of the digest with Mab A2.5; lanes 4 and 8, immunoprecipitation of the digest with a control Mab. The arrow indicates the position of the specifically immunoprecipitated fragment of TSP with an M_r near 25000.

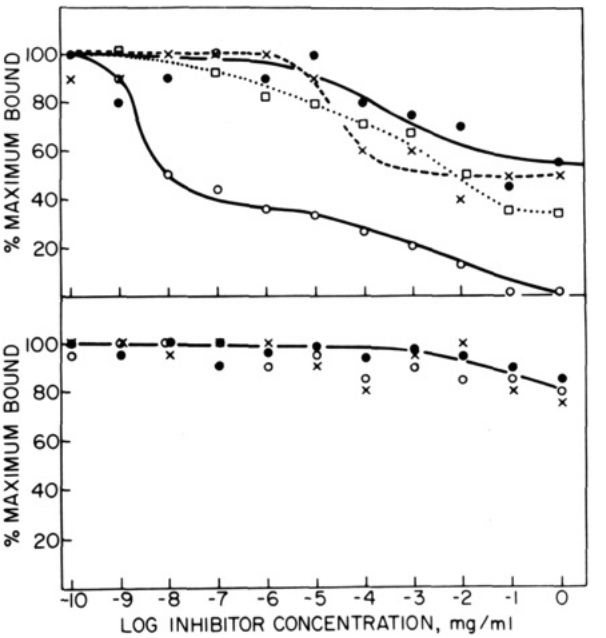

FIGURE 5: Effect of glycosaminoglycans on the binding of Mabs to TSP. The Mabs were immobilized on PVC wells, and the binding of a constant amount of radiolabeled TSP (100000 cpm) was challenged with the indicated concentrations of glycosaminoglycan. Top panel: ○, heparin and Mab A2.5; □, heparin and Mab C6.7; ●, hyaluronic acid and Mab A2.5; ×, chondroitin sulfate and Mab A2.5. Bottom panel: Binding of TSP to Mab A6.1 challenged with the following: ●, hyaluronic acid; ×, chondroitin sulfate, ○, heparin.

be seen that Mab A2.5 does not recognize the 18-kDa species present in the chymotryptic digests (lane 1). This fragment contains the epitope recognized by Mab C6.7 (Dixit et al., 1985a).

Effect of Glycosaminoglycans on the Binding of Mab A2.5 to TSP. As shown above, Mab A2.5 specifically immunoprecipitates the 25-kDa heparin binding domain of TSP; thus it was of interest to determine if heparin could inhibit the binding of Mab A2.5 to TSP. To do this, the Mab was immobilized on PVC wells and the binding of radiolabeled TSP was challenged with a concentration range of several glycosaminoglycans. Figure 5 (top panel) shows that heparin is an extremely potent inhibitor of the binding of TSP to Mab A2.5, with 50% inhibition occurring at 10 pg/mL heparin. However, total inhibition of binding is only achieved at much higher concentrations of heparin approaching 1 mg/mL. Hyaluronic acid and chondroitin sulfate (Figure 5, top panel) show no inhibition at low concentrations and only partially inhibit over the same concentration range (10 μg/mL to 1 mg/mL) at which heparin abolishes the binding. We also tested the effect of heparin on the binding of labeled TSP to immobilized Mab C6.7 (Figure 5, top panel, open squares). In this case, the concentration dependence of the inhibition corresponds to that found with hyaluronic acid and chondroitin sulfate on the binding of TSP to Mab A2.5. That is, there is no effect at low concentrations of heparin and only partial inhibition even at 1 mg/mL. Figure 5, bottom panel, shows that the binding of TSP to a Mab raised against denatured TSP (Mab A6.1) was virtually unaffected by the glycosaminoglycans at any concentration.

Discussion

In this paper we have characterized a Mab specific for human platelet TSP that can inhibit the agglutination by purified TSP of both fixed, trypsinized human erythrocytes and fixed, activated platelets. This Mab, designated A2.5, was raised against native, calcium-replete TSP and does not bind to reduced and alkylated TSP or TSP transferred to nitro-

cellulose blots after SDS gel electrophoresis. Mab A2.5 specifically immunoprecipitates the heparin binding domain of TSP that is found as a 25-kDa species in chymotryptic digests of TSP in the presence or absence of Ca^{2+} (Dixit et al., 1984a). The binding of Mab A2.5 to TSP is inhibited by heparin at very low concentrations of the glycosaminoglycan. Another Mab, designated C6.7, also inhibits the hemagglutinating activity of purified TSP but has no effect on the agglutination of fixed, activated platelets. This Mab recognizes a distinct region of TSP structure, an 18-kDa fragment derived from chymotryptic digests in the presence of Ca^{2+}. We have previously shown that Mab C6.7 is able to inhibit the thrombin- or A23187-induced aggregation of live platelets, while Mab A2.5 is inactive in this assay (Dixit et al., 1985b). These findings raise the probability that these three assays for TSP function are inherently different or reveal different aspects of TSP function.

In addition to the results reported here, other findings have implicated the heparin binding domain of TSP in its interaction with red cells. Roberts et al. (1985) have shown that TSP binds with high affinity to a sulfated glycolipid or sulfatide found on red cell and platelet membranes and that heparin is a very potent inhibitor of this binding. The same Mab, Mab A2.5, that specifically precipitates the heparin binding domain also blocks the binding of TSP to sulfatides at very low concentrations. However, Mab C6.7 also inhibits the binding to sulfatide, but higher concentrations of the Mab are required. Gartner et al. (1984) have also suggested that bovine TSP can bind to red cells via the heparin binding domain. On the other hand, we have reported that TSP trimers composed of 140-kDa chains obtained by digestion with thermolysin in Ca^{2+} (Haverstick et al., 1984) retain the ability to bind to red cells, even though they are lacking the heparin binding domain (Dixit et al., 1984a). These data suggest that both the heparin binding domain and the 140-kDa peptide can bind to red cells, probably via distinct receptors. The fact that both Mab A2.5 and Mab C6.7 can block the hemagglutination activity of TSP may indicate that two different binding sites on TSP are involved in cross-linking adjacent red cell membranes; that is, the mechanism of agglutination is asymmetric. Additionally, the possibility must be considered that the effect of one of the two Mabs is due to a conformational change propagated from its epitope to some other region of the TSP molecule. The immunoprecipitation of the heparin binding domain by Mab A2.5 and of the 18-kDa chymotryptic fragment by Mab C6.7 (Dixit et al., 1985a) establishes where these Mabs bind but does not establish that a particular effect is mediated at that site on TSP. Evidence in support of such a conformational communication between the epitopes for these two Mabs is found in results of experiments in which each Mab was labeled with [^{75}Se]selenomethionine and the other Mab tested for inhibition of binding of the labeled Mab to TSP. These experiments revealed that Mab C6.7 could inhibit the binding of Mab A2.5 to TSP, but Mab A2.5 had no effect on the binding of Mab C6.7 (not shown). The trivial explanation for these results of greatly different affinities of the two Mabs was ruled out by direct measurement of their affinity for TSP. Mab C6.7 bound with a K_d of 3×10^{-9} M and Mab A2.5 with a K_d of 8×10^{-9} M, less than 3-fold lower affinity (not shown).

To more precisely define the interactions that we suggest may occur between the 18-kDa fragment which contains the epitope for Mab C6.7 and the 25-kDa heparin binding domain with which Mab A2.5 reacts, much more structural data on TSP are needed. However, the results reported here indicate that one should interpret with caution the effects of antibodies on the agglutinating activities of TSP and on platelet aggregation. If, as we propose, some conformational change may be induced in TSP by the binding of antibody, a region of TSP remote from the actual epitope might be responsible for the inferred activity. For example, Gartner et al. (1984) reported that polyclonal antiserum raised against the heparin binding domain of bovine TSP could partially inhibit the aggregation of bovine platelets and suggested that this result indicated a direct role of the heparin binding domain in platelet aggregation. We, on the other hand, have reported here that Mab A2.5, directed against the heparin binding domain, has no effect on platelet aggregation while blocking hemagglutination and fixed platelet agglutination by TSP. Furthermore, Mab C6.7, which effectively inhibits platelet aggregation and also blocks hemagglutination, recognizes a site on TSP that is structurally distinct from the heparin binding domain. Clearly, a much higher resolution mapping of epitopes for these Mabs is needed to determine the precise mechanism by which they exert their effects on TSP function.

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REFERENCES

Baenziger, N. L., Brodie, G. N., & Majerus, P. W. (1972) *J. Biol. Chem. 247*, 2723–2731.

Coligan, J. E., & Slayter, H. S. (1984) *J. Biol. Chem. 259*, 3944–3948.

Dixit, V. M., Grant, G. A., Santoro, S. A., & Frazier, W. A. (1984a) *J. Biol. Chem. 259*, 10100–10105.

Dixit, V. M., Grant, G. A., Frazier, W. A., & Santoro, S. A. (1984b) *Biochem. Biophys. Res. Commun. 119*, 1075–1081.

Dixit, V. M., Haverstick, D. M., O'Rourke, K. M., Hennessy, S. W., Grant, G. A., Santoro, S. A., & Frazier, W. A. (1985a) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3472–3476.

Dixit, V. M., Haverstick, D. M., O'Rourke, K., Hennessy, S. W., Broekelmann, T. J., McDonald, J. A., Grant, G. A., Santoro, S. A., & Frazier, W. A. (1985b) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3844–3848.

Galfre, G., Howe, S. C., Milstein, C., Butcher, G. W., & Howard, J. C. (1977) *Nature (London) 291*, 421–423.

Gartner, T. K., Williams, D. C., Minion, F. C., & Phillips, D. R. (1978) *Science (Washington, D.C.) 200*, 1281–1283.

Gartner, T. K., Gerrard, J. M., White, J. G., & Williams, D. C. (1981) *Blood 58*, 153–157.

Gartner, T. K., Walz, D. A., Aiken, M., Starr-Spires, L., & Ogilvie, M. L. (1984) *Biochem. Biophys. Res. Commun. 124*, 290–295.

Gerrard, J. M., Phillips, D. R., Rao, G. H. R., Plow, E. F., Walz, D. A., Ross, R., Harker, L. A., & White, J. G. (1980) *J. Clin. Invest. 66*, 102–109.

Haverstick, D. M., Dixit, V. M., Grant, G. A., Frazier, W. A., & Santoro, S. A. (1984) *Biochemistry 23*, 5597–5603.

Haverstick, D. M., Dixit, V. M., Grant, G. A., Frazier, W. A., & Santoro, S. A. (1985) *Biochemistry* (in press).

Jaffe, E. A., Leung, L. L. K., Nachman, R. L., Levin, R. I., & Mosher, D. F. (1982) *Nature (London)* 295, 246–248.

Jaffe, E. A., Ruggiero, J. T., Leung, L. L. K., Doyle, M. J., McKeown-Longo, P. J., & Mosher, D. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 998–1002.

McDonald, J. A., & Kelley, D. G. (1980) *J. Biol. Chem. 255*, 8848–8858.

McPherson, J., Sage, H., & Bornstein, P. (1981) *J. Biol. Chem. 256*, 11330–11336.
Mosher, D. F., Doyle, M. J., & Jaffe, E. A. (1982) *J. Cell Biol. 93*, 343–348.
Mumby, S. M., Raugi, G. J., & Bornstein, P. (1984) *J. Cell Biol. 98*, 646–652.
Phillips, D. R., Jennings, L. K., & Prasanna, H. R. (1980) *J. Biol. Chem. 255*, 11629–11632.
Raugi, G. J., Mumby, S. M., Abbott-Brown, D., & Bornstein, P. (1982) *J. Cell Biol. 95*, 351–354.
Raugi, G. J., Mumby, S. M., Ready, C. A., & Bornstein, P. (1984) *Thromb. Res. 36*, 165–175.
Roberts, D. D., Haverstick, D. M., Dixit, V. M., Frazier, W. A., Santoro, S. A., & Ginsburg, V. (1985) *J. Biol. Chem.* (in press).
Sage, H., Farin, F. M., Striker, G. E., & Fisher, A. B. (1983) *Biochemistry 22*, 2148–2155.
Santoro, S. A., & Cowan, J. F. (1982) *Collagen Res. 2*, 31–43.
Yamada, K. M., Yamada, S. S., & Pastan, I. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3158–3162.

Light Activates the Reaction of Bacteriorhodopsin Aspartic Acid-115 with Dicyclohexylcarbodiimide†

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ABSTRACT: Conditions for a light-induced reaction between the carboxyl-modifying reagent *N,N'*-dicyclohexylcarbodiimide (DCCD) and bacteriorhodopsin in Triton X-100 micelles were previously reported [Renthal, R., Dawson, N., & Villarreal, L. (1981) *Biochem. Biophys. Res. Commun. 101*, 653–657]. We have now located the DCCD site in the bacteriorhodopsin amino acid sequence. [^{14}C]DCCD–bacteriorhodopsin (0.67 mol/mol of bacteriorhodopsin) was cleaved with CNBr. The resulting peptides were purified by gel filtration and reverse-phase high-performance liquid chromatography (HPLC). One major ^{14}C peptide (50%) and two minor fractions were obtained. The modified peptides were completely absent in the absence of DCCD, and 10 times less was obtained when the reaction was run in the dark. Amino acid analysis and sequence analysis showed that the major fraction contained residues 69–118. This region includes six carboxyl side chains. Quantitative sequence analysis ruled out significant amounts of DCCD at Glu-74, Asp-85, Asp-96, Asp-102, and Asp-104. The major ^{14}C peptide was also subjected to pepsin hydrolysis. HPLC analysis of the product gave only a single major radioactive subfragment. Amino acid analysis of the peptic peptide showed that it contained residues 110–118. The only carboxyl side chain in this region is Asp-115. Thus, we conclude that Asp-115 is the major DCCD site. The light sensitivity of this reaction suggests that Asp-115 becomes more exposed or that its environment becomes more acidic during proton pumping. The DCCD reaction blue-shifts the retinal chromophore. Such a result would be expected if Asp-115 is the negative point charge predicted to be near the cyclohexene ring of retinal.

Bacteriorhodopsin, the only protein in the purple membrane from *Halobacterium halobium*, is a light-activated proton pump (Stoeckenius & Bogomolini, 1982). The crystal structure (Henderson & Unwin, 1975) and the amino acid sequence (Ovchinnikov et al., 1979; Khorana et al., 1979) are known, providing the opportunity for understanding the molecular mechanism of a simple ion pump. Many proton pumps have been studied by chemical modification methods, and a general phenomenon seems to be inhibition by blocking of carboxyl groups with carbodiimides (Sebald et al., 1980; Esch et al., 1981; Prochaska et al., 1981; Pennington & Fisher, 1981; Phelps & Hatefi, 1981; Beattie & Villalobo, 1982; Sussman & Slayman, 1983). We previously reported that bacteriorhodopsin reacts with water-soluble (Renthal et al., 1979) and water-insoluble (Renthal et al., 1981) carbodiimides, resulting in alteration of H^+ pump function. These studies suggest an important role for carboxyl groups in the bacteriorhodopsin proton pump. In this paper we report the characterization of the product of the light-activated reaction of bacteriorhodopsin with *N,N'*-dicyclohexylcarbodiimide (DCCD)[1] and have identified the major site modified within its primary structure.

Experimental Procedures

Purple Membrane. The method of Oesterhelt & Stoeckenius (1974) was used to obtain purple membrane sheets from *H. halobium* S9. Stock solutions were stored at 10^{-4} M bacteriorhodopsin in 0.025% NaN_3 and washed free of azide prior to use by repeated centrifugation and resuspension in water.

Reaction of Purple Membrane with DCCD. Purple membrane was reacted with [^{14}C]DCCD by the method previously described (Renthal et al., 1981), except that the reaction volume was scaled up. A 12.5-mL suspension of purple membrane sheets (10^{-4} M bacteriorhodopsin) was added to

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[1] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; PTH, phenylthiohydantoin; CNBr 10a, cyanogen bromide fragment of bacteriorhodopsin containing residues 69–118; SPITC, 4-sulfophenyl isothiocyanate; HPLC, high-performance liquid chromatography.

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