Calcium Ions and the Conformation of Glycoprotein IIIa That is Essential for Fibrinogen Binding to Platelets: Analysis by a New Monoclonal Anti-GP IIIa Antibody, TM83

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Using a newly developed murine monoclonal antibody (MoAb), TM83, against glycoprotein IIIa (GPIIIa) of human platelets, we have analyzed the relationship between platelet fibrinogen binding and conformational changes in GPIIIa under EDTA treatment. Crossed radioimmunoelectrophoresis demonstrated that TM83 reacted with only the GPIIb/IIIa complex but also with GPIIIa alone. TM83 dose-dependently inhibited both thrombin-induced aggregation and fibrinogen binding to activated platelets. 

Using 2 mmol/L EDTA-containing medium, pH 7.4, at 22°C for 30 minutes, TM83 binding to the platelets markedly decreased to 70% of the control level. The decreased binding was fully recovered to the control level when the platelets were resuspended in Ca²⁺-containing medium. These platelets retained their aggregability. In contrast, when platelets were incubated in 2 mmol/L EDTA-containing medium, pH 7.4, at 37°C for 30 minutes, TM83 binding to the platelets markedly decreased to 7% of the control, which could only be recovered to 40% of the control by replacing the medium with calcium-containing medium; these platelets lacked thrombin-induced aggregability. These findings suggest that the epitope for TM83 may be located near the fibrinogen binding site on GPIIIa and that its conformation is dependent on Ca²⁺ ions.

PLATELET MEMBRANE glycoprotein Ib (GPIIb) and IIIa (GPIIIa) form a calcium-dependent heterodimer complex in which receptors for adhesive proteins such as fibrinogen, von Willebrand factor (vWF), and fibronecetin are exposed upon platelet activation. When platelets are stimulated, fibrinogen binds on the exposed receptor predominantly, and the bridge formations by the bound fibrinogen between platelet membranes consequently result in platelet aggregation. It is postulated that conformational changes in the GPIIIa/IIIa complex on activated platelet membranes are required for the expression of fibrinogen binding sites. In this reaction the effect of EDTA is well known. Zucker and Grant first reported that platelets irreversibly lost their ability to aggregate when they were incubated with 2 to 4 mmol/L EDTA or EGTA for five to seven minutes at 37°C at a pH under 7.8. The mechanism for this effect was precisely analyzed by Pidard et al. Although sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of EDTA-treated platelets indicated normal amounts of GPIIb and GPIIIa, they found by crossed-immunoelectrophoresis with intermediate gels containing anti-GPIIb or GPIIIa monoclonal antibody (MoAb) that EDTA induces an irreversible change in the GPIIIa/IIIa complex at 37°C. A reduction in the size and probable dissociation of the GP IIb/IIIa precipitate was accompanied by the appearance of precipitates corresponding to free GPIIb, free GPIIIa, and a major new cathodal precipitate, which bound to either anti-GPIb or anti-GPIIIa MoAbs but could not bind to a MoAb against the GPIIb/IIIa complex.

Molecular cloning and sequencing of GPIIb showed that it contains four calcium-binding domains, similar to other calcium-binding proteins such as calmodulin and troponin C. As expected by the sequence of GPIIb, calcium ions seem to regulate surface orientation of GPIIb. In contrast, GPIIIa appeared not to have a typical calcium-binding domain. Meanwhile, chemical crosslinking of adhesive proteins and their RGD-containing peptides to activated platelets have suggested that a site on or proximal to GPIIIa may be a component of the functional adhesive receptor on platelets. Conformational changes in GPIIIa appear to be important for maintaining the structure and function of the adhesive protein receptor and appear to be requisite for the exposure of the receptor upon platelet activation. Calcium ions might be important in these processes, but there have been no reports on calcium-dependent conformational changes in GPIIIa on the platelet membrane. Recently we have developed a new monoclonal anti-GPIIIa antibody, and we have used it to clarify the relationship between the fibrinogen binding activity of platelets and conformational changes in GPIIIa under EDTA treatment of platelets.

MATERIALS AND METHODS

Materials. Collagen was obtained from Horm (Munich, West Germany). Hirudin (1,000 U/mL), bovine serum albumin (BSA), human fibrinogen (fraction I), galactose oxidase (Dactylidiomerdixides, Type V: 200 to 600 U/mg), potato apyrase (grade I) and bovine thrombin (specific activity of 2,000 U/mg) were obtained from Sigma Chemical Co (St Louis). Protein A Sepharose CL 4B, Sephadex G-25M packed in Columns PD-10 and DEAE-Sephacel were from Pharmacia Fine Chemicals (Uppsala, Sweden). Neuraminidase (Streptococcus sp) and Cellulofine 2000 m were from Seikagaku Fine Chemicals Co (Tokyo, Japan). Radioactive sodium iodide (¹²⁵I) and sodium borohydride (¹H) were purchased from New England Nuclear (Boston). Mouse normal IgG was from Cappel Laboratories (Cochranville, PA). Immunoplates were from Costar (Cambridge, MA). Enzymebeads and Affi-Gel 10 were from Bio-
Rad Laboratories (Richmond, CA). The other reagents were of analytic grade.

Preparation of a MoAb against GPIIb/IIIa. The murine MoAb TM83 (IgG3) was obtained by the method of Koehler and Milstein. TM83 is an antibody from a clone with inhibitory activity against collagen-induced aggregation. The cloning was carried out twice by a limiting dilution, resulting in a statistical probability of monoclonality greater than 99%. TM83 was purified from cultured medium by affinity chromatography on Protein A according to the manufacturer's manual. Antibody concentration was determined spectrophotometrically at 280 nm by assuming A = 14.0 at 1% concentration. Purified MoAb was radiiodinated by the chloramine T method; the specific radioactivity of iodinated TM83 was 139,400 dpm/μg (2261 bq/μg).

Preparations of washed platelets. Venous blood from healthy volunteers was drawn into a syringe containing one-ninth volume of 3.8% trisodium citrate. Platelets were washed by the method of Mustard et al with several modifications. Platelet-rich plasma (PRP) was prepared by centrifugation of the whole blood at 120 g for 15 minutes and then acidified to pH 6.5 by adding 1/1,000 volume of 1 mol/L citric acid. Platelets were sedimented by centrifugation at 1,200 g for 15 minutes and suspended in HEPES-Mg2+ buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 4.2 mmol/L NaH2PO4, 2 mmol/L MgCl2, 5.5 mmol/L dextrose, 0.35% BSA, and 3.8 mmol/L HEPES, pH 6.5, containing 25 U/mL heparin and 3 U/mL of apyrase). After incubation at 37°C for ten minutes, platelets were sedimented by centrifugation at 1,200 g for 15 minutes and resuspended in the HEPES-Mg2+ buffer containing 3 U/mL of apyrase. After incubation at 37°C for ten minutes, platelets were sedimented and suspended into the final buffer as described below.

Immunoprecipitation. Platelets (7.0 x 1010) washed as above and finally suspended in HEPES buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 4.2 mmol/L NaH2PO4, 1 mmol/L MgCl2, 2 mmol/L CaCl2, 5.5 mmol/L dextrose, 0.35% BSA, and 3.8 mmol/L HEPES, pH 7.4) were surface labeled with tritium by the method of Phillips and Agin. The labeled platelets were washed once by the method described above. The labeled platelets were solubilized in 1% Triton X-100 Tris-glycine buffer, pH 7.4 (38 mmol/L Tris, 150 mmol/L glycine, 1 mmol/L EDTA, 4 mmol/L NaNEM (N-ethylmaleimide), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 4 mmol/L iodoacetamide, and 2 mmol/L benzamidase), and then they were sonicated by a Sonifer Cell Disruptor 185 (Branson, West Germany) at 30 s three times at a power reading of 10. The solubilized platelet solution was centrifuged at 100,000 g for 90 minutes. The supernatant was immunoprecipitated with TM83-coupled Affi Gel-10 (Bio-Rad Laboratories) by our previously described method at room temperature, and the precipitated antigens were analyzed by nonreduced and reduced SDS-PAGE. Following by fluorography.

Purification of GPIIb/IIIa complex. Crude GPIIb/IIIa complex from platelets was obtained by Triton X-114 extraction according to the method of Bordier. Briefly, washed platelets (4.5 x 1010), prepared as described above, were solubilized in 1 mL of Tris-saline buffer (10 mmol/L Tris, 145 mmol/L NaCl, 1 mmol/L CaCl2, pH 7.4) containing 1% Triton X-114 at 4°C. Triton X-114-insoluble material was removed by centrifugation at 20,000 g for 30 minutes at 4°C. The supernatant was warmed at 25°C for 10 minutes, and the Triton X-114 phase was collected by centrifugation at 300 g for 10 minutes. To wash the Triton X-114 phase, 1 mL of cold Tris-saline buffer (10 mmol/L Tris, 145 mmol/L NaCl, 1 mmol/L CaCl2, pH 7.4) was mixed with the Triton X-114 phase, the mixture warmed to 25°C, and then centrifuged under the above conditions. These washing procedures were repeated twice. Finally, 1 mL of Tris-saline buffer, pH 7.4, containing 10 mmol/L CHAPS (3-(3-cholamidopropyl)dimethylammonio-1-propane sulfonate) was added to the Triton X-114 phase to maintain a single phase at room temperature. Crossed-radioimmunoelctrophoresis (CRIE). CRIE was performed by the method of Hagen et al. The crude GPIIb/IIIa complex fraction was run on a 1% agarose for the first dimension at 90 V for 45 minutes at 22°C. For the second dimension, 100 μL of radiiodinated TM83 (1.4 x 106 cpm) was placed in the intermediate gel, and electrophoresis in the second dimension was performed in a gel containing antiwhole platelet antiserum (14 μL/cm2) at a constant voltage of 60 V for 20 h at 22°C. The antiwhole platelet antiserum was prepared according to Hagen et al. After staining with Coomassie brilliant blue (CBB), the plate was subjected to autoradiography on x-ray film (X-Omat AR film, Eastman Kodak Co, Rochester, NY) in a cassette for three days at -80°C.

Fibrinogen binding to platelets. Human fibrinogen was purified from Kabi fibrinogen and radiiodinated by the lactoperoxidase-glucose oxidase method. Binding of the radiolabeled fibrinogen to thrombin-activated platelets was measured by the method of Plow et al. Briefly, 50 μL of platelet suspension (1012/μL) suspended in HEPES buffer containing 2 mmol/L CaCl2, pH 7.4, were stimulated with 0.1 U/mL of thrombin at 22°C without stirring. After five minutes hirudin was added to final concentration of 1 U/mL. Radiolabeled fibrinogen was added at a final concentration of 1 μL/μL. After a 60-minute incubation, platelet associated radioactivity was recovered by centrifugation at 10,000 g for five minutes at 22°C through a silicon oil mixture (Tory silicone, Tokyo, Japan) consisting of five parts light silicone oil (gravity, SH:550) and one part heavy silicone oil (gravity, SH:200), which allowed all the platelets to be recovered in the oil phase.

Binding of radiolabeled TM83 to the platelet surface. Fifty microliters of washed platelet suspension (4.0 x 1011 cells/μL) in HEPES buffer, pH 7.4, containing 2 mmol/L CaCl2, pH 7.4, was placed in each well of a millipititer plate (type HA; Millipore Corp, Bedford, MA) with various concentrations of 125I-TM83 (0 to 200 mCi/μL) and incubated for 120 minutes at 22°C. After incubation, the medium was filtrated out by using an aspirator. Platelets retained on the membrane of the millipititer plate were washed three times with 200 μL of the HEPES buffer by filtration. The filter membranes were cut out by a puncher (Millipore, Corp, Bedford, MA). The radioactivity of 125I-TM83 on the filters associated with platelets and the free 125I-TM83 in the filtrates were counted by an Autogammacounter (Alola, Tokyo, Japan). Nonspecific binding was determined in the presence of a 100-fold excess of cold TM83.

Effect of divalent cations on the binding of TM83 to platelets. The following buffers were used in this series of studies: HEPES buffer (137 mmol/L NaCl, 2.6 mmol/L KCl, 4.2 mmol/L NaH2PO4, 5.5 mmol/L dextrose, 0.35% BSA, 3.8 mmol/L HEPES, pH 7.4); HEPES-EDTA buffer (HEPES buffer containing 2 mmol/L CaCl2, pH 7.4); HEPES-EDTA buffer (HEPES buffer containing 2 mmol/L MgCl2, 0.5 mmol/L EGTA); HEPES-Mg2+ buffer (HEPES buffer containing 2 mmol/L MgCl2); and HEPES-Ca2+ buffer (HEPES buffer containing 2 mmol/L CaCl2 and 1 mmol/L MgCl2). Each buffer was adjusted to pH 7.4 by adding 0.1 mol/L NaOH. The HEPES-EDTA buffer used for the calcium deprivation studies was estimated to have a Ca2+ concentration of less than 10 mmol/L by using Quin 2 as previously reported by us. HEPES-Mg2+ buffer without any addition of Ca2+ contained 10 to 20 mmol/L calcium as determined by the Quin 2 method in this study, a value of which is similar to that reported by Harfenist et al.

Platelets washed twice as described above were finally resuspended in HEPES-EDTA A buffer (2 x 1010/μL) or HEPES-Ca2+ buffer to serve as the control. The HEPES-EDTA–suspended platelets were equally divided into two tubes. One was incubated at 22°C and the other at 37°C for 30 minutes. After the incubation, from each tube, one fourth of the platelets was taken for the binding
experiments, and the remaining platelets were equally divided into two tubes, which were then centrifuged at 1200 g for ten minutes to pellet the cells. One pellet was resuspended in HEPES-Ca²⁺ buffer and the other in HEPES-Mg²⁺ buffer; these resuspended pellets were then incubated at 37°C for 30 minutes and then adjusted to a platelet count of 4 x 10⁴ cells/µL by adding the appropriate medium. The bindings of TM83 to platelet samples were measured as described above. Aggregations of platelets suspended in each of the above buffers in response to thrombin (0.1 to 1.0 U/mL) or collagen (3 to 10 µg/mL) were monitored by an aggregometer (Model DP274E, Sienco, Morrison, CO).

RESULTS

Determination of the epitope of MoAb TM83. TM83 immunoprecipitated two proteins with mol wt of 145 Kd and 92 Kd on nonreduced and 135 Kd and 110 Kd on reduced SDS-PAGE fluorography, corresponding to GPIIb/IIIa (Fig 1). Several trials to identify the epitope by Western blots were unsuccessful (data not shown).

To define the epitope of TM83 exactly, the crude extract of GPIIb/IIIa was subjected to CRIE using an antiplatelet polyclonal antibody with the intermediate gel containing radioiodinated TM83. When the extract was subjected to CRIE after an addition of 1 mmol/L Ca²⁺ to the extract, an immunoprecipitin arc was stained with CBB (Fig 2A), and autoradiography indicated that iodinated TM83 was incorporated into this immunoprecipitin arc (Fig 2C). This precipitate was cut out and subjected to reduced SDS-PAGE, which showed two protein bands with mol wt of 135 Kd and 110 Kd, corresponding to GPIIb and GPIIIa, respectively, and rabbit IgG subunits with mol wt of less than 66 Kd (Fig 3, lane 1). In contrast, when CRIE was carried out after adding 25 mmol/L EDTA to the extract, three immunoprecipitin arcs were stained with CBB on the CRIE gel (Fig 2B), where the most cathodal immunoprecipitate (Fig 2, arc b) fused with the lower anodal arc (arc a), and the iodinated TM83 was incorporated into both the lower anodal (arc a) and the most cathodal (arc b) immunoprecipitates (Fig 2D). Analyzing each of these arcs by reduced SDS-PAGE (Fig 3) revealed the following: arc b (lane 3) yielded a single 110 Kd protein band, identifying it as GPIIIa; arc a (lane 2) gave 2 protein bands with mol wt of 135 Kd (GPIIb) and 110 Kd (GPIIIa), indicating that this precipitate contained the GPIIb/GPIIIa complex; and arc c (lane 4) yielded a single 135 Kd band, corresponding to GPIIb.

To further clarify the epitope of TM83 and to avoid any damage to the structure of GPIIb/IIIa that might occur during the separation procedure, we carried out CRIE on 1%
Fig 3. SDS-PAGE analysis of precipitin arcs shown in Fig 2. Precipitin arcs in Fig 2. A and B, were cut out and subjected to reduced SDS-PAGE. Lane 1, the single arc on Fig 2A; lanes 2, 3 and 4, precipitin arc a, b and c, respectively, on Fig 2B; lane 5, precipitin-free gel on Fig 2B, as a background. Bands with mol wt of less than 66 Kd are fragments of rabbit IgG.

Triton X-100 solubilized washed whole platelets using anti-platelet antibody with the intermediate gel containing the radioiodinated TM83. When the solubilized platelet proteins were subjected to CRIE in the presence of 0.5 mmol/L Ca²⁺, several typical precipitation lines, including the arc which represents the GPIIb/IIIa complex, were observed (Fig 4A and C). We have confirmed that this arc was absent in the CRIE patterns of solubilized proteins of platelets collected from patients with Granzmann's thrombasthenia. Autoradiography indicated that iodinated TM83 was incorporated into the arc corresponding to the GPIIb/IIIa complex (Fig 4C). In contrast, when CRIE was carried out in the presence of 10 mmol/L EDTA, the typical arc representing the GPIIb/IIIa complex became weak, and two new arcs having characteristics suggesting that they were precipitin lines of free GPIIb and GPIIIa appeared (Fig 4B). The arc representing GPIIIa was more cathodal than that of the complex, and autoradiography demonstrated that iodinated TM83 was incorporated into this arc (Fig 4D). The amount of iodinated TM83 was remarkable in the presence of 10 mmol/L EDTA (Fig 4D) as well as that in the presence of 0.5 mmol/L Ca²⁺ (Fig 4C). Each arc was cut out and subjected to reduced SDS-PAGE and verified by mol wt to contain GPIIb, GPIIIa, or GPIIb/GPIIIa (data not shown).

According to these results, the iodinated TM83 was incorporated into GPIIIa as well as the GPIIb/GPIIIa complex but not incorporated into GPIIb. We concluded that the epitope of TM83 was located on GPIIIa, and the antibody could also recognize the GPIIb/GPIIIa complex.

**Effects of TM83 on platelet functions.** TM83 dose-dependently inhibited platelet aggregations induced by 3 μmol/L ADP, 1 μg/mL of collagen, and 15 μmol/L A23187 (at 37°C, pH 7.4). However, it inhibited neither the shape change induced by thrombin nor adenosine diphosphate (ADP) nor ristocetin-induced platelet agglutination (data not shown). As shown in Fig 5, aggregation of washed platelets induced by 0.1 U/mL of thrombin was also dose-dependently inhibited by TM83. This inhibition, however, was not complete even in the presence of 600 nmol/L or more of TM83. This is in contrast to aggregations induced by other agonists such as ADP, A23187, and collagen, which were completely abolished by 240 nmol/L or more of TM83. The inhibitory effects of TM83 on platelet aggregation studies were not changed when platelet aggregation studies were carried out 22°C or 37°C.

Fibrinogen binding was measured at 22°C. Binding of radioiodinated fibrinogen to thrombin-activated platelets was saturated at a concentration of 1 μmol/L fibrinogen (data not shown). At this concentration of fibrinogen, 450 nmol/L or more of TM83 almost abolished fibrinogen binding to the platelets suspended in HEPES-Ca²⁺ buffer that were incubated with 0.1 U/mL of thrombin at 22°C pH 7.4 (Fig 5). Under these experimental conditions, the platelets did not aggregate because stirring was not applied.

**Binding of TM83 to platelet surface.** When the binding studies were done with washed platelets resuspended into...
The binding of radioactive TM83 to platelets was carried out at a concentration of 30 nmol/L, because this was the saturating concentration for the number of platelets contained in 50 μL of 4 × 10^7/μL suspension. The incubation temperature or pH of the medium containing 2 mmol/L Ca^2+ (Fig 7, open circles) had little effect on the binding of TM83 to the platelets. In contrast, the bindings decreased with increasing temperature at each pH and decreased with increasing pH at 22°C and 37°C in the presence of 2 mmol/L EDTA (Fig 7B, closed circles). Even in the presence of 2 mmol/L EDTA, increasing pH at 4°C had almost no effect on the binding (Fig 7A, closed circles).

Effect of divalent cation-chelators on binding of TM83. When platelets were incubated in HEPES-EDTA buffer at 22°C for 30 minutes and further incubated with radioactive TM83 for another 120 minutes, the TM83 binding to the platelets (Fig 8A, closed circles) was decreased to 70% that of the control platelets that were incubated in HEPES-Ca^2+ buffer (Fig 8A, open circles). In contrast, when the platelets were incubated in HEPES-EDTA buffer at 37°C, the binding of TM83 to the platelets (Fig 8A, triangles) was decreased to 7% of the control level.

After the incubation of platelets in HEPES-EDTA buffer at 22°C or 37°C, these platelets were centrifuged, resuspended into HEPES-Ca^2+ buffer (Fig 8B), and incubated further at 37°C for 30 minutes. The reduced TM83 binding to the platelets preincubated with 2 mmol/L EDTA at 22°C was completely recovered to the control level (Fig 8B, closed circles). The platelet aggregability in response to 3 μg/mL of collagen or 0.01 U/mL of thrombin was preserved (data not shown). In contrast, the reduced TM83 binding (7% of

### Table 1. Binding of TM83 to Resting and Activated Platelets

<table>
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<th>Experimental Conditions</th>
<th>Binding of TM83 (pmol/10^9 Platelets)</th>
<th>Ratio (%)</th>
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<tr>
<td>Control*</td>
<td>24.3 ± 0.6</td>
<td>100</td>
</tr>
<tr>
<td>PGE1 treatment†</td>
<td>19.8 ± 0.8</td>
<td>81</td>
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<tr>
<td>Thrombin activation‡</td>
<td>29.9 ± 1.3</td>
<td>123</td>
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*Platelets were resuspended in HEPES buffer, pH 7.4 at 22°C.
†Platelets were treated with 1 μmol/L PGE1.
‡Platelets were activated with 0.1 U/mL of thrombin.
control) to the platelets preincubated with EDTA at 37°C was recovered to only 40% of the control level (Fig 8B, triangles). These platelets remained unable to aggregate even when 10 μg/mL of collagen or 0.1 U/mL of thrombin was used as the stimulant.

When platelets that had been preincubated in HEPES-EDTA buffer containing 2 mmol/L EDTA at 22°C (Fig 8A, closed circles) were resuspended into HEPES-Mg²⁺ buffer, pH 7.4, which contained 2 mmol/L Mg²⁺ (Ca²⁺ concentration was 10 to 20 μmol/L), the binding of TM83 to the platelets increased from 70% to 80%, with complete recovery of aggregability (Fig 8C, closed circles). In contrast, when the platelets preincubated in HEPES-EDTA buffer at 37°C (Fig 8A, triangles) were resuspended in HEPES-Mg²⁺ buffer, the binding of TM83 to the platelets remained low, showing no improvement (Fig 8C, triangles), with irreversible loss of aggregability.

To observe the specific effect of calcium on the binding of TM83 to platelets, we observed the binding of TM83 to platelets that had been incubated in HEPES-EGTA buffer. Binding experiments were performed at saturating concentration of TM83 (30 to 40 nmol/L). TM83 binding to the platelets in the presence of 0.5 mmol/L EGTA and 2 mmol/L Mg²⁺ (HEPES-EGTA buffer) at 22°C was not reduced (Table 2). These platelets retained their ability to aggregate in response to 0.1 U/mL of thrombin. When platelets were incubated at 37°C for 30 minutes in the same buffer, the binding of TM83 decreased to 57% of the control. The decreased binding of TM83 could not be recovered to the control level by subsequent incubation with 2 mmol/L Ca²⁺.

**DISCUSSION**

We have developed a new MoAb against GPIIIa, designated as TM83, that is able to immunoprecipitate both the GPIIb and GPIIIa of solubilized platelet membranes in the presence of 1 mmol/L EDTA (at 22°C, pH 7.4), as verified by SDS-PAGE (Fig 1). However, the Western blotting technique could not determine in which of the GPs (IIb or IIIa) the epitope is present, although the results suggest that the epitope of TM83 is relatively sensitive to a conformational change in GPIIb and GPIIIa that would occur under SDS-PAGE, since TM83 could not bind to the blots. To determine the epitope of TM83, we performed CRIE on the crude extract of GPIIb and GPIIIa as well as whole platelet proteins, where the milder detergents Triton X 114 and 100 were employed instead of SDS, using the antiplatelet antibody with an intermediate gel containing the radioiodinated TM83. In the presence of EDTA, the iodinated TM83 was incorporated into the arcs representing the GPIIb/GPIIIa complex and the separate GPIIIa molecules as indicated by the autoradiograms. The amount of incorporated TM83 was markedly less in the GPIIIa arc obtained by CRIE of crude extract as compared to that from CRIE of whole platelet proteins. This suggested that the epitope on GPIIIa may...
Table 2. Effect of Calcium and Temperature on Binding of TM83 to Platelets

<table>
<thead>
<tr>
<th>Experimental</th>
<th>Binding of TM83 (mol/10^9 Platelets) after incubation for 30 minutes (n = 8)</th>
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<tbody>
<tr>
<td></td>
<td>At 22°C</td>
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<tr>
<td>HEPES-Ca(^{2+}) buffer(\dagger)</td>
<td>24.3 ± 0.5</td>
</tr>
<tr>
<td>HEPES-EGTA buffer(\dagger)</td>
<td>24.4 ± 0.5</td>
</tr>
<tr>
<td>HEPES-EGTA buffer + Ca(^{2+})</td>
<td>25.9 ± 0.6</td>
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\(\dagger\)HEPES-Ca\(^{2+}\) buffer contained 2 mmol/L Ca\(^{2+}\) and 1 mmol/L Mg\(^{2+}\), pH 7.4.

\(\dagger\)HEPES-EGTA buffer contained 2 mmol/L Mg\(^{2+}\) and 0.5 mmol/L EGTA.

\(\dagger\)Platelets were incubated in HEPES-EGTA buffer at 22°C or 37°C and then in 2.5 mmol/L Ca\(^{2+}\) suspension medium.

The complex. Immunoprecipitations of both that the antibody could also recognize the GPIIb/GPIIIa epitope of TM83 was located on the GPIIIa molecule and of iodinated CRIE sample, almost the whole platelet proteins in the to obtain the crude extract of membrane GPs. When we used can be coprecipitated by TM83 even in the presence of EDTA. We concluded that the presence of EDTA by a MoAb to GPIIb alone and one to GPIIIa alone in the previously reported. These changes were lower than those previously reported. However, there are some MoAbs for which less than 40,000 to 45,000 binding sites are expressed on the resting platelet surface (eg, PAC-1 and 7E3 bind 10,000 to 15,000 and 17,900 sites per platelet respectively). However, the amount of these antibodies bound increases on platelet activation. These observations suggest that binding of the MoAbs is dependent on the orientation of the epitope and/or on conformational changes in the molecule which occur on activation. In the case of TM83, binding was increased 23% on activation by thrombin, was decreased 19% by treatment with 1 mmol/L PGE\(_1\) (Table 1). These changes were lower than those previously reported for PAC-1 and 7E5. This relatively small range of change in the binding suggests that the epitope of TM83 may be located on surface GPIIIa but not present on the GPIIb/GPIIIa in alpha granule membrane that becomes exposed upon activation.\(^{24}\) The number of the binding sites of TM83 was the same as the numbers reported independently by DiMinno et al., who used their MoAb, B59.2, against the GPIIb/GPIIIa complex and by Furihata et al., who used an antibody against Pen, which is a platelet-specific alloantigen associated with GPIIIa molecule. Furihata et al suggested that the number of Pen antigen sites (20,000 sites/platelet) could be explained as being half of the total GPIIIa expressed on the platelet surface if there are two populations of GPIIb/GPIIIa molecules. Since we used platelets individually collected from 12 healthy volunteers and performed a fibrinogen binding experiment using platelets from each individual, it is conceivable that TM83 recognizes an alloantigen on GPIIIa. There may not be an allelic variation in the antigen. One explanation is that the binding of TM83 may be inherently bivalent, thereby restricting the number of antibody molecules bound at saturation, as speculated by Furihata et al. Another speculation is that the binding site of TM83 may be located on or near the site where the GPIIb/GPIIIa complex formation occurs, so that half of the TM83 epitope may be covered under the stereochemical structure of the complex.

TM83 dose-dependently inhibited fibrinogen binding to platelets activated by thrombin (Fig 5), and the binding was completely inhibited at 450 nmol/L. The result suggests that the epitope of TM83 is located in the vicinity of the fibrinogen binding site on GPIIIa. However, the epitope of TM83 is not the fibrinogen binding site itself, since the fibrinogen binding site is not exposed on the membrane of the resting platelets.\(^{12}\) To estimate the relative distance between the fibrinogen binding site and the epitope of TM83 in GPIIIa, we studied the binding of RGDSP peptide\(^{22}\) to the thrombin-activated platelet. At saturation levels of TM83, binding of the peptide to platelets was inhibited 60% (data not shown). These results suggested that the fibrinogen binding sites are not far from the epitope of TM83 on GPIIIa. Moreover, all the inhibitory effects of TM83 on platelet aggregation induced by ADP, collagen, A23187, and thrombin are compatible with the assumption that the TM83 epitope is located in the vicinity of the fibrinogen binding site on GPIIIa. However, thrombin-induced aggregation remained at about 20% of the control value even in the presence of a sufficient amount of TM83. We have observed that some aggregation in response to thrombin can occur without the participation of fibrinogen.\(^{37}\) It is suggested that the binding of another adhesive protein, such as the binding of thrombospondin to GPIV,\(^{38}\) might enhance thrombin-induced platelet aggregation.

The binding of TM83 to platelets was decreased in the presence of 2 mmol/L EDTA. The decrease was not marked at 4°C, but the binding was remarkably depressed when the reaction was performed at 22°C and 37°C, especially at pH values of 7.4 and 8.5. It is known that the chelating activity of EDTA is strongly affected by the pH of reaction mixture. Gogstad et al. suggested that effect of increased pH is due to the increase in chelating capacity of EDTA as well as EGTA (the formation constant of EDTA-metal complexes at pH 9.1 is 27 times that at pH 7.5). TM83 binding lost by incubating platelets with EDTA at 37°C could not be recovered when the cells were resuspended in Ca\(^{2+}\)-containing medium, pH 7.4, at 37°C for 30 minutes. These results coincided with those of Zucker and Grant, who found that platelets irreversibly lost their ability to aggregate when they were incubated with 2 to 4 mmol/L EDTA or EGTA at 37°C at a pH under 7.8. GPIIIa may be degraded or undergoes irreversible structural changes under this condition. On the contrary, decreased binding in platelets that had been incu-
bated with EDTA at 22°C, pH 7.4, was recovered to the control level when the cells were resuspended in the Ca²⁺-containing medium. In this case, a reversible conformational change in GPIIia in the presence of EDTA at 22°C is strongly suggested, especially at an increased pH. When the conformational change in GPIIia occurs after the deprivation of calcium by the chelating agent, the bindings of TM83 and fibrinogen on GPIIia may not occur.

It is postulated that the entire epitope for TM83 is located on the GPIIia molecule, and the epitope may be present on a position that can be modified conformationally in the absence of calcium. The site is located in the vicinity of the fibrinogen binding site. Gogstad et al." reported that GPIIib and GPIIia each contained sites with Ca²⁺ affinity similar to that observed in the GPIIib/GPIIia complex. Therefore, although an EF-hand is not found on the amino acid sequence of the cloned GPIIia, it cannot be excluded that not only GPIIib but also GPIIia may have calcium-binding sites. Several investigators reported that magnesium could not substitute for calcium in maintaining the integrity of the GPIIib/GPIIia complex. We have shown that Mg²⁺ could not substitute for Ca²⁺ to restore the reduction in the binding of GPIIia complex. We have shown that Mg²⁺ could not substitute for Ca²⁺ to restore the reduction in the binding of GPIIia complex. It cannot be excluded that not only GPIIib but also GPIIia may have calcium-binding sites. Several investigators reported that magnesium could not substitute for calcium in maintaining the integrity of the GPIIib/GPIIia complex. We have shown that Mg²⁺ could not substitute for Ca²⁺ to restore the reduction in the binding of GPIIia in the presence of EDTA at 22°C is recovered to the control value (Table 2). These results indicate that Mg²⁺ may partially substitute for Ca²⁺ in maintaining the conformation of the TM83 epitope in GPIIIa than itself when it is present in the heterodimer complex of GPIIib and GPIIia, suggesting that the TM83 epitope conformation may be, at least partially, independent of the domains that form the GPIIib/GPIIia complex.

In conclusion, we have developed a new MoAb, TM83, which reacts with GPIIIa as well as the GPIIib/GPIIia complex. The epitope for TM83 is not the same as the fibrinogen-binding site, but it may be located near the fibrinogen-binding site on GPIIia, and its conformation is dependent on Ca²⁺ ions.

ACKNOWLEDGMENT

We are grateful to Dr GA Jamieson of the Cell Biology Laboratory of the American Red Cross and to Dr SM Jung of Department of Biochemistry of the Jichi Medical School for their critical reviews and valuable suggestions in the preparation of this manuscript.

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Calcium ions and the conformation of glycoprotein IIIa that is essential for fibrinogen binding to platelets: analysis by a new monoclonal anti-GP IIIa antibody, TM83

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