The Arg-Gly-Asp (RGD) Recognition Site of Platelet Glycoprotein IIb-IIIa on Nonactivated Platelets Is Accessible to High-Affinity Macromolecules

By Yoshiaki Tomiyama, Tadahiro Tsubakio, Randolph S. Piotrowicz, Yoshiyuki Kurata, Joseph C. Loftus, and Thomas J. Kunicki

We have characterized a murine IgG monoclonal antibody, OP-G2, specific for platelet glycoprotein (GP) IIb-IIIa (αIIbβ3). OP-G2 Fab fragments inhibit fibrinogen-mediated platelet aggregation and competitively inhibit adenosine diphosphate-induced binding of 125I-fibrinogen to washed platelets. OP-G2 binding to GPIIb-IIIa is specifically inhibited by RGD-containing peptides but not the fibrinogen γ-chain carboxy-terminal peptide, and OP-G2 Fab fragments, like RGD-containing peptides, alter the conformation of GPIIb-IIIa resulting in the expression of a ligand-induced binding site (LIBS) recognized by PMI-1. OP-G2 Fab fragments bind more slowly to the recombinant Cam variant of GPIIb-IIIa (αIIbβ3Cam) wherein an Asp199 to Tyr199 substitution in GPIIa abolishes the ability to recognize RGD. These data indicate that OP-G2 recognizes an epitope at or in very close proximity to the RGD recognition site of GPIIb-IIIa and that, in every aspect tested, OP-G2 behaves like a macromolecular RGD ligand. Interestingly, two-color flow cytometry shows that OP-G2 IgG can bind to nonactivated platelets. Quantitative binding assays indicate that nonactivated platelets bind approximately 50,000 125I-OP-G2 molecules/platelet. Furthermore, the affinity of OP-G2 for platelets activated with thrombin is roughly fivefold higher (nonactivated, kd = 24.8 nmol/L; activated, kd = 4.9 nmol/L). These results suggest that the RGD recognition site of GPIIb-IIIa is available to macromolecules that contain RGD even on nonactivated platelets, provided that the affinity of the ligand is adequate.

© 1992 by The American Society of Hematology.
platelet are bound by nonactivated platelets, and that the affinity of OP-G2 for activated platelets is significantly increased. Our findings suggest that the RGD recognition site of GPIIb-IIIa is available even on nonactivated platelets, and that an IgG molecule such as OP-G2 can bind to this RGD recognition site.

MATERIALS AND METHODS

MoAbs. The murine MoAbs OP-G2, AP2 (anti–GPIIb-IIIa complex), and AP3 (anti–GPIIIa) were developed as described. PMI-1 (anti–GPIIb heavy chain) was a gift from Dr M. Ginsberg (La Jolla, CA). MoAb 62 is a monoclonal anti–GPIIb-IIIa that activates GPIIb-IIIa.25 S12,26 is a murine MoAb against the platelet α-granule membrane GP, GMP-140, was a gift from Dr R. McEver (Oklahoma City, OK). Monoclonal IgG was purified from ascites fluid by affinity chromatography on Protein A-Sepharose CL-4B (Pharmacia, Piscataway, NJ).

Purified IgG was labeled with 125I using the chloramine T method. Free 125I was separated from the sample by filtration through a Bio gel P2 column (Bio-Rad, Richmond, CA). A specific activity of 400 to 800 cpm/ng IgG was routinely obtained.

For the preparation of OP-G2 Fab fragments, monoclonal IgG was dialyzed against 0.01 mol/L phosphate-buffered saline (PBS) (pH 7.4) and adjusted to a concentration of 4 mg/mL. After adding 10 mmol/L cysteine and 2 mmol/L EDTA, OP-G2 was digested with mercaptopein (Sigma Chemical Company, St Louis, MO) at a 1:59 ratio of papain to protein for 4 hours at 37°C. The reaction was terminated by adding iodoacetamide to a final concentration of 10 mmol/L. The Fab fragments were separated from Fc fragments and undigested IgG by chromatography on Protein A-Sepharose CL-4B.

Crossed immunoelectrophoresis (CIE). CIE was performed as described. Briefly, 100 μg of Triton X-100 (BioRad Laboratories, Richmond, CA) solubilized platelet protein was electrophoresed at 10 V/cm at 16°C for 75 minutes in a first-dimension gel consisting of 1% agarose dissolved in 38 mmol/L Tris, 0.1 mol/L glycine, 10 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L KCl, pH 7.4. Second-dimension electrophoresis was performed at 2 V/cm for 18 hours against an intermediate gel containing 1 × 10^6 cpm of 125I-monoclonal IgG followed by an upper gel containing rabbit antiplatelet antibody. Precipitin arcs containing 125I-monoclonal IgG were shown by autoradiography of the CIE plate.

Platelet isolation. Six volumes of blood were mixed with 1 vol of acid-citrate-dextrose solution (ACD, National Institutes of Health [NIH] formula A) and centrifuged at 250 g for 10 minutes to obtain platelet-rich plasma (PRP). Prostaglandin E, (PGE,; Sigma) was maintained at 2.5 mmol/L. Various concentrations of 125I-OP-G2 were added to the platelet suspension and after addition of OP-G2 to yield specific binding.

Flow cytometry. For two-color analysis, nonactivated or thrombin-activated platelets (2.5 × 10^5/mL) were first incubated with biotinylated-S12 (4 μg/mL) for 60 minutes. The platelets were then pelleted, resuspended in HEPES/NaCl-PGE, and incubated with a 1:40 dilution of phycoerythrin (PE)-conjugated streptavidin (Becton Dickinson, Mountain View, CA) and a saturating concentration of fluorescein isothiocyanate (FITC)-conjugated OP-G2 (60 μg/mL) for 60 minutes. FITC-conjugated anti-Leu 11a MoAb (IgG1 isotype; Becton Dickinson) was used as negative control. The platelet suspension was then fixed with 1% paraformaldehyde and analyzed in a flow cytometer (Becton Dickinson FACS StarPLUS).

Stably transfected cell lines expressing wild type GPIIIa and/or GPIIIa were used wherein a point mutation in GPIIIa abrogates RGD-binding function, as described previously. Cells were harvested with 3.5 mmol/L EDTA and an aliquot of 5 × 10^5 cells was pelleted, resuspended in Tyrode’s buffer (1% BSA, 2 mmol/L MgCl2, 137.5 mmol/L NaCl, 12 mmol/L NaHCO3, 2.6 mmol/L KCl, pH 7.4) containing 10 μmol/L MoAb 62 and incubated on ice for 30 minutes. A saturating concentration of biotinylated OP-G2 (100 μg/mL) was added and the cells were incubated for an additional 30 minutes on ice. Cells were pelleted, washed once, and incubated with FITC-avidin (Cappel, Durham, NC) for 30 minutes. Cells were pelleted, resuspended in 0.5 mL Tyrode’s buffer, and the binding of OP-G2 to cells was analyzed by flow cytometry.

For analysis of the ligand-induced binding site (LIBS) recognized by PMI-1, flow cytometry was performed according to the method of Ginsberg et al30 with slight modifications. Washed platelets were resuspended in Tyrode’s buffer and adjusted to 3 × 10^9/mL. Five-microliter aliquots of platelet suspension were added to tubes containing FITC-conjugated PMI-1 (20 μg/mL) in Tyrode’s buffer. Platelets were incubated in a total volume of 50 μL for 60 minutes at ambient temperature with (+)RGDW peptide or Fab fragments of OP-G2 or AP2. Platelets were then diluted to 0.5 mL with Tyrode’s buffer and analyzed.

Platelet aggreometry. Aggregation was monitored using a model PAT-4 NKK platelet aggregation tracer (Nikou Bioscience Inc, Tokyo, Japan) at 37°C and a stirring rate of 1,000 rpm. To prepare PRP, blood (9 vol) was anticoagulated with 1 vol 3.8% trisodium citrate, centrifuged at 250 g for 10 minutes, and adjusted to 3 × 10^8 platelets/mL by addition of platelet-free plasma. To prepare washed platelets, PRP was obtained from blood (9 vol) anticoagulated with 1 vol ACD-A and washed twice with RCD containing 20 mmol/L of PGE, pH 6.5. The platelets were resuspended in 5 mmol/L HEPES, 0.3 mmol/L NaH2PO4, 12 mmol/L NaHCO3, 5.5 mmol/L glucose, 1 mmol/L MgCl2, 2 mmol/L CaCl2, 2 mmol/L KCl, 137 mmol/L NaCl, pH 7.4.

The effect of OP-G2 Fab fragments or synthetic peptides on platelet aggregation was measured by preincubating the PRP with antibody or peptides for 3 minutes at 37°C with stirring before the addition of aggregation-inducing agents. ADP, epinephrine, collagen, and ristocetin were used as aggregating agents. Thrombin, 0.1
U/mL was added to washed platelet suspension without added fibrinogen.

**Fibrinogen binding to ADP-stimulated platelets.** Fibrinogen binding to washed platelets was measured as previously described. To initiate fibrinogen binding, 10 μmol/L of ADP was added to the suspension. After 5 minutes without stirring at ambient temperature, the platelets were sedimented through 30% sucrose dissolved in resuspension buffer as described above for binding of MoAbs. Nonspecific binding was determined in parallel tubes that contained 10 mmol/L EDTA. The effect of OP-G2 Fab fragments on ADP-stimulated fibrinogen binding was determined by preincubating platelets with OP-G2 Fab at a concentration of 40 μg/mL for 5 minutes before initiating the fibrinogen binding assay.

**Enzyme-linked immunosorbent assay (ELISA).** ELISA was performed as previously described. Microtiter wells coated with purified GPIb-IIIa were incubated with 50 μL of a solution containing peptide for 60 minutes at ambient temperature. Fifty microliters of OP-G2 or AP2 was then added to the wells, and the plates were incubated an additional 60 minutes. The wells were washed six times with PBS-0.05% Tween 20 (PBS-Tween), 50 μL of alkaline phosphatase-conjugated goat antimouse IgG (Zymed, Inc, South San Francisco, CA; 1:1,000 dilution in PBS-Tween) was added to each well, and the plates were incubated for 60 minutes at ambient temperature. The wells were washed six times, the substrate (p-nitrophenylphosphate in diethanolamine buffer, pH 9.8) was added, and absorbance at 405 nm was recorded.

**Peptide synthesis.** The peptides were synthesized using a Milligen/BioResearch Labs 9050 Automated Pepsynthesizer (San Rafael, CA) employing PepSyn KA resin and Fmoc-amino acids, as previously described. Peptides were cleaved from the resin with trifluoroacetic acid and purified by reverse-phase high-performance liquid chromatography (Beckman System Gold; Beckman Instruments, Inc, Alex Division, San Ramon, CA) using Vyadec C18 preparative columns (The Sep/a/ra/tions Group, Hesperia, CA). All peptides were greater than 90% homogenous.

**RESULTS**

**Characterization of murine MoAb OP-G2.** The isotype of OP-G2 is IgG1-κ, as determined by Ouchterlony immunodiffusion analysis. To define the epitope recognized by OP-G2, indirect immunoprecipitation was performed. OP-G2 immunoprecipitated two radiolabeled proteins having apparent molecular weights of 140 and 92 Kd under nonreduced electrophoretic conditions (data not shown). These proteins correspond to GPIIb and GPIIIa. To further characterize the OP-G2 epitope on GPIIb-IIIa, Triton X-100-soluble platelet protein prepared in the presence or absence of 5 mmol/L EDTA was analyzed by CIE using radiolabeled OP-G2, PMI-1, or AP3 in the intermediate gel (Fig 1). Autoradiograms of the gels indicate that all of these antibodies bind, as expected, to the GPIIb-IIIa complex. When platelets were solubilized in the presence of 5 mmol/L EDTA, most of the GPIIb-IIIa complex dissociates into free GPIIb and GPIIIa. Autoradiograms of the gels in which EDTA-treated proteins were analyzed indicate that 125I-PMI-1 bound to the residual GPIIb-IIIa complex and dissociated GPIIb (Fig 1C), while 125I-AP3 bound to the residual GPIIb-IIIa complex and dissociated GPIIIa (Fig 1D). In contrast, 125I-OP-G2 bound only to the residual GPIIb-IIIa complex (Fig 1B).

**Inhibition of platelet aggregation by OP-G2 Fab fragments.** OP-G2 Fab fragments inhibit ADP (5 μmol/L)-, epinephrine (10 μmol/L)-, or collagen (1 μg/mL)-induced platelet aggregation in a dose-dependent manner (Fig 2). OP-G2 Fab fragments also inhibit thrombin-induced platelet aggregation (data not shown). A slight inhibitory effect of OP-G2 Fab fragments on ristocetin-induced aggregation was also observed, probably reflecting inhibition of secondary, secretion-dependent aggregation (Fig 2). However, when PRP was preincubated with 5 mmol/L EDTA for 2 minutes, OP-G2 Fab had no effect on ristocetin-induced agglutination (data not shown). OP-G2 Fab fragments did not affect shape change of platelets and did not themselves induce platelet aggregation or agglutination.

**Inhibition of fibrinogen binding by OP-G2 Fab fragments.** To examine the effect of OP-G2 Fab fragments on fibrinogen binding to ADP-stimulated platelets, 40 μg/mL of OP-G2 Fab was added to washed platelet suspension without added fibrinogen. The platelets were preincubated with 5 μmol/L ADP for 2 minutes, followed by incubation with 5 μg/mL of OP-G2 Fab for 5 minutes. Fibrinogen binding was then measured by ELISA.

![Fig 1. Crossed immunoelectrophoresis of Triton X-100-solubilized platelet protein. One hundred micrograms of platelet protein solubilized in 1% Triton X-100 in the absence (−) or presence (+) of 5 mmol/L EDTA were analyzed. Depicted are a Coomassie blue-stained gel (A), and autoradiographs of identical gels wherein the second dimension contained: 125I-OP-G2 (B); 125I-PMI-1 (C); or 125I-AP3 (D) in the intermediate gel.](image-url)
oxygen binding to ADP-stimulated platelets, suspensions of washed platelets were incubated with OP-G2 Fab at a concentration of 40 μg/mL at ambient temperature for 5 minutes. Various concentrations of $^{125}$I-fibrinogen and 10 μmol/L ADP were then added to the suspensions. After an additional 5-minute incubation at ambient temperature, the bound fibrinogen was measured. As shown in Fig 3A, prior incubation of platelets with OP-G2 Fab resulted in specific inhibition of fibrinogen binding. Examination of the binding data by using double-reciprocal plots showed that OP-G2 Fab fragments are a competitive inhibitor of fibrinogen binding (Fig 3B). The mean Ki from two experiments using platelets from different normal donors is 68 nmol/L. These findings suggest that the epitope recognized by OP-G2 is in close proximity to the fibrinogen binding site on platelets.

**Effect of synthetic peptides on OP-G2 binding to GPIIb-IIIa.** Because peptides containing the RGD sequence and peptides containing the carboxy-terminal amino acid sequence of the fibrinogen γ-chain can inhibit fibrinogen binding to activated platelets in a competitive manner,$^{5,30,31}$ we examined the effect of these peptides on OP-G2 binding to purified GPIIb-IIIa. The RGD-containing peptides (dextrorotatory-Arg)-Gly-Asp-Trp [(+RGDW], CG (+RGDWG], [(+)RGD-8], and YAVTGRGDS-PASSK [Fn14], and the dodecapeptide, HHLG-GAKQAGDV [H12], corresponding to the carboxy-terminal sequence of the γ-chain of fibrinogen, were tested. RGEW and an irrelevant sequence from GPIIIa, ALPLGS, were used as negative controls. Table 1 shows the inhibitory effect of these peptides on ADP-induced platelet aggregation. The order of potency with respect to inhibition of aggregation was: (+)RGDW > (+)RGD-8 > Fn14 = H12.

We evaluated the effect of these peptides on OP-G2 and AP2 binding to purified GPIIb-IIIa (Fig 4). In this assay, to ensure that antibody was present in limiting amounts, we used OP-G2 and AP2 at concentrations of 2 μg/mL and 20 ng/mL, respectively. These were the antibody concentrations that resulted in roughly 50% of maximum binding to GPIIb-IIIa in the same ELISA. None of these synthetic peptides had an effect on AP2 binding to GPIIb-IIIa (Fig 4B). In contrast, OP-G2 binding to GPIIb-IIIa was inhibited by the RGD-containing peptides, but not by RGEW or ALPLGS (Fig 4A). The order of potency with respect to inhibition of OP-G2 binding was exactly the same as the order of potency with respect to inhibition of ADP-induced aggregation. It is noteworthy that H12 did not inhibit OP-G2 binding to GPIIb-IIIa, although the inhibitory effects of H12 and Fn14 on ADP-induced aggregation were of the same order of magnitude.

Next, we examined the effect of (+)RGDW on OP-G2 binding to GPIIb-IIIa using nonactivated or activated platelets. The platelet suspensions were incubated with various concentrations of (+)RGDW for 60 minutes. $^{125}$I-OP-G2 at a final concentration of 1 μg/mL was then added to the platelet suspensions (2.5 × 10⁹/mL). After incubation for 60 minutes, platelet-bound OP-G2 was separated from free OP-G2 by sedimentation of the platelets through 30% sucrose. Figure 5 shows that (+)RGDW can inhibit OP-G2 binding to GPIIb-IIIa on nonactivated as well as activated platelets. Again, 1 mmol/L of RGEW did not inhibit $^{125}$I-OP-G2 binding to GPIIb-IIIa; neither did 1 mmol/L (+)RGDW inhibit $^{125}$I-AP-2 binding to GPIIb-IIIa (data not shown). These findings are consistent with those using ELISA, and demonstrate that (+)RGDW can bind to GPIIb-IIIa even on nonactivated platelets.

**Binding of OP-G2 to nonactivated or activated platelets.** To determine whether OP-G2 binds to GPIIb-IIIa on nonactivated platelets, we analyzed the platelets for binding of both biotinylated-S12 (with PE-streptavidin) and FITC-OP-G2, using two-color flow cytometry. S12 is an IgG MoAb specific for a 140-Kd α-granule membrane GP (GMP-140) that becomes associated with the platelet surface during secretion.$^{21}$ A saturating concentration of FITC-OP-G2 (60 μg/mL for 2.5 × 10⁹ platelets/mL) was used in this analysis (see Fig 7). Figure 6 shows that OP-G2 can bind to nonactivated platelets that are S12-negative. In
Fig 3. Inhibition of fibrinogen binding to ADP-activated platelets by OP-G2 Fab fragments. (A) Suspensions of washed platelets were preincubated with buffer (C) or OP-G2 Fab fragments at a concentration of 40 μg/mL (Φ) for 5 minutes. Various concentrations of [Fibrinogen] (abscissa) and 10 μmol/L ADP were then added to the suspensions. After an additional 5-minute incubation, bound fibrinogen (ordinate) was measured. (B) Double-reciprocal plots of the data from (A).

Table 1. Inhibitory Effect of Synthetic Peptides on ADP-Induced Platelet Aggregation

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Concentration for 50% Inhibition (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+) RGDW</td>
<td>~0.8</td>
</tr>
<tr>
<td>(+) RGD-8</td>
<td>~20</td>
</tr>
<tr>
<td>Fn14</td>
<td>~225</td>
</tr>
<tr>
<td>H12</td>
<td>~780</td>
</tr>
<tr>
<td>RGEW</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>ALPLGS</td>
<td>&gt;1,000</td>
</tr>
</tbody>
</table>

Citrated PRP was preincubated with various amounts of peptides for 3 minutes at 37°C with stirring before the addition of 5 μmol/L ADP. Abbreviations: (+) RGD-8, CG(+)RGDWGY; Fn14, YAVTRGRD-3PASSK; H12, HHLGGAKOAGDV.

Fig 4. Effect of synthetic peptides on OP-G2 and AP2 binding to purified GPIIb-IIIa. Microtiter wells coated with purified GPIIb-IIIa were incubated with 50 μL of a solution containing peptide for 60 minutes. The concentration of peptide is indicated on the abscissa. Fifty microliters of OP-G2 (A) or AP2 (B) was then added to the well, the plates were incubated an additional 60 minutes, and the extent of binding of OP-G2 or AP2 was quantitated by addition of alkaline phosphate-conjugated goat antimouse IgG and appropriate color development. Absorbance at 405 nm is plotted on the ordinate.

For personal use only.

From www.bloodjournal.org by guest on October 3, 2017. For personal use only.
sites per platelet, saturation was achieved at about 15 μg OP-G2/mL. Scatchard analysis demonstrates that the apparent affinity of AP2 for nonactivated and thrombin-activated platelets remained the same (nonactivated, $k_d = 0.39 \pm 0.05$ nmol/L; activated, $k_d = 0.42 \pm 0.05$ nmol/L; mean ± SD; n = 3). However, the affinity of OP-G2 for activated platelets was roughly fivefold higher than that for nonactivated platelets (nonactivated, $k_d = 24.8 \pm 1.3$ nmol/L; activated, $k_d = 4.9 \pm 1.6$ nmol/L; mean ± SD; n = 4) (Fig 7B).

Kinetic binding studies were explored to further understand the basis for the apparent increased affinity of OP-G2. After a 5-minute incubation of nonactivated or thrombin-activated platelets with 1 μg/mL of 125I-OP-G2, the dissociation rate constant ($k_2$) was determined by measuring the displacement of 125I-OP-G2 from the platelets at 1 minute after adding a 100-fold excess of unlabeled OP-G2. The value of the association rate constant ($k_1$) was determined by measuring 125I-OP-G2 binding after 1 minute. $k_2$ for thrombin-activated platelets was calculated to be $2.66 \times 10^{-2}$ min$^{-1}$ (mean value from two experiments using platelets from different donors). However, no radioactivity was eluted from nonactivated platelets during a 1-hour follow-up. $K_1$ for nonactivated platelets and thrombin-activated platelets were $1.38 \times 10^6$ moles$^{-1}$min$^{-1}$ and $5.74 \times 10^6$ moles$^{-1}$min$^{-1}$, respectively. Thus, $K_1$ for thrombin-activated platelets was roughly fourfold greater than that for nonactivated platelets. The $k_d$ ($K_2/K_1$) for thrombin-activated platelets determined from kinetic studies was 4.6
Fig 8. Expression of PMI-1-binding site on platelets, as determined by flow cytometry. Washed platelets were reacted with FITC-PMI-1 (20 μg/mL) (A) in the presence (open peak) or absence (solid peak) of 500 pmol/L (+)-RGDW, or (B) in the presence of 200 pg/mL OP-G2 Fab fragments (open peak) or 800 pg/mL AP2 Fab fragments (solid peak). Results are expressed as histograms of cell number (linear scale) on the ordinate versus fluorescence intensity (log scale) on the abscissa.

nmol/L, in excellent agreement with the value determined from the equilibrium binding studies.

Binding of OP-G2 Fab fragments induces LIBS recognized by PMI-1. Because RGD-containing peptides can induce conformational changes in GPIIb-IIIa and expose neoantigenic sites, termed LIBS, as a result of ligand-receptor interaction,25,34 we examined whether OP-G2 Fab fragments induce LIBS in GPIIb-IIIa. As shown in Fig 8, OP-G2 Fab fragments (200 μg/mL) induce PMI-1-binding sites in GPIIb-IIIa. In contrast, AP2 Fab fragments, even at 800 μg/mL, fail to induce PMI-1-binding sites.

Binding of OP-G2 to recombinant α1bβ3 or α1bβ3Cam. To further investigate the epitope recognized by OP-G2, we examined the reactivity of OP-G2 with recombinant α1bβ3 or α1bβ3Cam in the presence or absence of MoAb 62. MoAb 62 stimulates the binding of fibrinogen to the wild-type α1bβ3, which is otherwise in an inactive conformation.20 Figure 9 is a representative of three separate experiments. OP-G2 can bind to resting α1bβ3, and its binding is increased in the presence of MoAb 62. The mean fluorescence intensity (MFI) of OP-G2 binding to resting α1bβ3 is 45 and to stimulated α1bβ3Cam is 18. These data indicate that OP-G2 fails to bind to resting or stimulated α1bβ3Cam.

DISCUSSION

The interaction of fibrinogen with the GPIIb-IIIa complex is essential for platelet aggregation, and receptor function becomes apparent only after stimulation with appropriate agonists. In this study we have characterized an IgG MoAb, OP-G2, that reacts specifically with the complex formed by GPIIb and GPIIIa. OP-G2 Fab fragments inhibit fibrinogen-mediated platelet aggregation induced by ADP, epinephrine, collagen, and thrombin in a dose-dependent manner, but do not inhibit ristocetin-induced platelet agglutination that involves the interaction of vWF with GPIb. OP-G2 Fab fragments competitively inhibit ADP-induced binding of 125I-fibrinogen to washed platelets. Furthermore, OP-G2 binding to GPIIb-IIIa is specifically inhibited by RGD-containing peptides.

In this study we elected to use (+)-RGDW rather than RGDS-based peptides because it has been determined that (+)-RGDW has roughly a 200-fold higher affinity for GPIIIa than RGDS as evidenced by data presented here. Having tested three RGD peptides, the order of potency with respect to inhibition of OP-G2 binding is: (+)-RGDW > (+)-RGD-8 > Fn14. This is exactly the same as the order of potency with respect to inhibition of ADP-induced aggregation. These data suggest that OP-G2 recognizes an epitope at or in very close proximity to the RGD recognition site. One might also argue that OP-G2 recognizes an epitope at a distal site that is allosterically modulated by RGD-containing peptides, because RGD-peptides induce conformational changes in GPIIb-IIIa.34-36 However, the latter possibility seems unlikely because OP-G2 failed to bind to recombinant α1bβ3Cam expressing the Cam variant of Glanzmann's thrombasthenia that is due to an Asp119 → Tyr119 substitution in GPIIIa and is characterized by a deficit in fibrinogen recognition by GPIIb-IIIa.24,35 It has also been demonstrated that PAC-1 fails to bind ADP-activated Cam platelets.25 In contrast, four other MoAbs specific for the GPIIb-IIIa complex (A2A9, 7E3, 10E5, and 4F10),11,38-40 all of which bind to GPIIb-IIIa on nonactivated platelets and inhibit fibrinogen binding to platelets, bind to Cam platelets as well as to normal platelets.26 It has been reported that 7E3 exhibits an enhanced on-rate of binding after platelet activation,11 and that the binding of A2A9 is inhibited by the carboxy-terminal decapeptide of the fibrinogen γ-chain.9 However,
because the Cam variant of GPIIb-IIIa fails to recognize fibrinogen or RGD-containing peptides. In addition to the failure to bind to α5β3Cam, OP-G2, like PAC-1, contains a nucleotide sequence that encodes the tripeptide RYD (which mimics RGD) within the complementarity-determining region (CDR) 3 of the heavy chain. Interestingly, OP-G2 Fab fragments, like RGD peptides, induce LIBS recognized by PM1-1 in GPIIb-IIIa. These findings strongly support the hypothesis that OP-G2 recognizes an epitope at or very close proximity to the RGD recognition site and that OP-G2 behaves like a macromolecular RGD ligand.

Although RGD and the γ-chain fibrinogen peptides appear to interact with the same or mutually exclusive sites on GPIIb-IIIa, recent studies have demonstrated that the binding sites for these peptides are not identical. Using photoactivable derivatives of RGDS and H12, Santoro and Lawing showed that these derivatives were incorporated into different sites on GPIIb-IIIa. RGDS associated more with GPIIIa than with GPIIb, while H12 associated with only GPIIb. More recently, D’Souza et al. localized these peptide recognition sites on GPIIb-IIIa. They observed that the RGD peptide is mainly cross-linked to GPIIIa residues 109 through 171, whereas the fibrinogen γ-chain peptide is cross-linked to GPIIb residues 294 through 314. We also examined the effect of the dodecapeptide of the fibrinogen γ-chain, H12, on OP-G2 binding to purified GPIIb-IIIa. Although the inhibitory effects of H12 and Fn14 on ADP-induced aggregation are of the same order of magnitude (Table 1), H12 did not inhibit OP-G2 binding to GPIIb-IIIa, even at a concentration of 2.5 nmol/L. Our findings are consistent with the results of recent studies that binding sites for RGD peptides and the fibrinogen γ-chain peptide are not identical, and it seems likely that the epitope recognized by OP-G2 is closer to the RGD recognition site than to the γ-chain peptide recognition site. IZ5I-trigramin can bind to nonactivated platelets, and this value is essentially the same as that for OP-G2 and trigramin demonstrate the possibility that the GPIIb-IIIa expression on nonactivated platelets. After thrombin stimulation, the binding sites for OP-G2 and AP2 increased by 67% and 46%, respectively. These increases are consistent with those reported by others. Although the apparent affinity of AP2 remains the same after thrombin stimulation, the equilibrium binding studies demonstrate that the apparent affinity of OP-G2 increases roughly fivefold. Kinetic studies demonstrate that the association rate constant (Kₐ) is roughly fourfold greater than that for nonactivated platelets. Thus, the increase in affinity of OP-G2 for activated platelets can be attributed to an increase in the on-rate of its binding to GPIIb-IIIa. Similar binding characteristics have been observed for trigramin, a naturally occurring peptide (MW: 9 Kd), that is much smaller than the Fab molecule. Trigramin contains an RGD sequence and probably binds at the RGD recognition site. OP-G2 and trigramin bind to nonactivated platelets, yet the affinity of trigramin for ADP-activated platelets is increased by eightfold compared with that for nonactivated platelets (nonactivated, kd = 170 nmol/L; activated, kd = 21 nmol/L). In addition to size selectivity, these findings with OP-G2 and trigramin demonstrate the possibility that the GPIIb-IIIa activation event may result in an increased affinity of the receptor for its ligand, perhaps through an activation-induced conformational change(s) of GPIIb-IIIa.

OP-G2 is the first murine monoclonal IgG antibody to be described that binds so close to the RGD recognition site of GPIIb-IIIa that antibody binding can be completely inhibited by RGD peptides. This antibody has allowed us to study more closely the availability of the RGD recognition site as a function of platelet stimulation. Our results with OP-G2 suggest that the RGD recognition site of GPIIb-IIIa is available even on nonactivated platelets. Our results also provide strong support for the hypothesis that either a conformational change(s) in GPIIb-IIIa itself and/or a change(s) in the microenvironment surrounding GPIIb-IIIa occurs during activation. This change(s) probably results in increased ligand-receptor affinity. In the case of the physiologic ligand, fibrinogen, the affinity for GPIIb-IIIa on nonactivated platelets may be so low as to preclude relevant occupancy. Platelet activation and the resultant changes in GPIIb-IIIa may then result in increased affinity of GPIIb-IIIa for fibrinogen such that threshold levels of bound fibrinogen requisite for platelet cohesion are achieved. Certain macromolecules, such as OP-G2, have a substantially higher affinity for GPIIb-IIIa and can bind to the available RGD recognition site, even on nonactivated platelets.

ACKNOWLEDGMENT

We thank Dr. Mark Ginsberg (La Jolla, CA) for the MoAb, PM1-1; Dr. Rodger McEver (Oklahoma City, OK) for the MoAb, S12; and Dr. Mary Lynn Bajt for performing flow cytometry on recombinant integrins.
OP-G2 AS RGD LIGAND

REFERENCES


From www.bloodjournal.org by guest on October 3, 2017. For personal use only.


42. Tomiyama Y, Kunicki TJ: OP-G2, a murine monoclonal IgG antibody, contains the sequence Arg-Tyr-Asp (RYD) and binds to GP IIb-IIIa on nonactivated platelets. Thromb Haemost 65:1066, 1991 (abstr)


The Arg-Gly-Asp (RGD) recognition site of platelet glycoprotein IIb-IIIa on nonactivated platelets is accessible to high-affinity macromolecules

Y Tomiyama, T Tsubakio, RS Piotrowicz, Y Kurata, JC Loftus and TJ Kunicki