**Extracellular Epitopes of Platelet Glycoprotein Ib\(\alpha\) Reactive With Serum Antibodies From Patients With Chronic Idiopathic Thrombocytopenic Purpura**

By Ruyi He, Diane M. Reid, Charles E. Jones, and N. Raphael Shulman

Glycoproteins (GPs) IIb/IIIa and Ib/IX are principal targets of autoantibodies (autoAbs) in idiopathic thrombocytopenic purpura (ITP). Platelet-associated Abs against GP\(\text{IIb/IIIa}\) primarily recognize discontinuous or nonlinear epitopes (Fujisawa et al, *Blood* 81:1284, 1993). This study focused on whether Abs against the extracellular domain of GP\(\text{IIb/IX}\) might react with short linear amino acid (aa) sequences of GP\(\text{Ib/\alpha}\). Complementary DNAs (cDNAs) coding for two overlapping fragments of GP\(\text{Ib/\alpha}\) were amplified, cloned into pFLAG.2 plasmids, and expressed in *Escherichia coli* DH5\(\alpha\) competent cells as FLAG fusion proteins, which were purified by anti-FLAG immunaffinity chromatography. Of 16 selected ITP sera containing anti-GP\(\text{IIb/\alpha}\), 6 reacted in microtiter radioimmunoassays (RIAs) with recombinant protein fragment 2 (aa 240 to 485); 1 also with fragment 1 (aa 1 to 247). When synthetic peptides corresponding to 4 segments of fragment 2 with high antigenic indices (P1 to P4) were used as targets in RIAs, all 6 sera reacted with P2 (aa 326 to 346); 1 also reacted with P4 (aa 389 to 412). P2 was shown to be present on the surface of intact platelets by adsorption studies, and anti-P2 was detected in direct eluates of platelets from ITP patients. Glycocalcin in solution effectively competed with immobilized P2 for anti-P2; P2 in solution was a less effective competitor. Epitope scanning with a panel of synthetic 15-mer peptides localized the P2 epitope to the sequence, TKEQTTFP. Epitope definition may offer insight into the pathophysiology of and more specific treatments for ITP.

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**MATERIALS AND METHODS**

**Study sera.** Sera from 16 patients with chronic ITP were selected for study based on their reactivity against extracellular determinants of GP\(\text{IIb/\alpha}\) by standard antigen capture immunobead assays and adsorption studies, as described previously. Controls included 10 ITP sera not containing anti-GP\(\text{IIb/\alpha}\) and 20 sera from normal individuals. Patients with ITP met the diagnostic criteria of having thrombocytopenia with no underlying illness or abnormality that might account for it and of having normal or increased numbers of marrow megakaryocytes. All were adults with ITP for more than 2 years; 5 patients were male and 11 were females. A few sera were used fresh; the remainder had been stored up to 10 years in small aliquots at −20°C and were centrifuged at 11,000g for 10 minutes before use. None was subjected to more than one freeze-thaw.

**Platelet preparation.** Blood was drawn using a double syringe technique with a 19-gauge needle. EDTA was the anticoagulant. Prostaglandin E\(\text{\textsubscript{2}}\) (3 \(\mu\text{mol/L}\)) was added. Platelet-rich plasma obtained by centrifugation at 180g for 8 minutes was gel-filtered on Sepharose 2B (Pharmacia, Uppsala, Sweden) using Tyrode’s buffer (138 \(\text{mmol/L NaCl}, 2.9 \text{mmol/L KCl}, 12 \text{mmol/L NaHCO\textsubscript{3}}, 0.4 \text{mmol/L NaHPO\textsubscript{4}}, 0.1\% \text{glucose}, \text{pH 7.4}) with 0.35\% bovine serum albumin (BSA). Platelets were used the same day for Ab adsorption studies, flow cytometry, or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Platelet for purified membrane fragment. Adsorption and competitive inhibition studies were performed to determine whether the peptide epitopes were present in native GP\(\text{Ib/\alpha}\) on the platelet surface.
and GP preparation were obtained by apheresis of normal volunteers at the Department of Transfusion Medicine, Clinical Center, National Institutes of Health (Bethesda, MD).

Platelet membrane preparation. Platelets were washed in phosphate-buffered saline (PBS), pH 7.4, containing 5 mmol/L EDTA (PBS-EDTA), loaded with glyceral, hypotonically lysed, and sedimented through a sucrose-density step gradient as previously described. Membranes collected from the step interface were washed twice in PBS and used the same day for preparation of indirect eluates.

Eluate preparation. Platelet membranes were used instead of whole platelets to reduce nonspecific IgG and other proteins in indirect eluates. Forty milligrams of platelet membranes was incubated for 16 hours at 4°C with 40 mL of ITP or normal plasma anticoagulated with EDTA. Membranes were pelleted at 62,000 g for 30 minutes, washed twice with PBS-EDTA, suspended in 5 mL of normal saline, acidified to pH 2.8 with 0.1 N HCl, rocked gently for 10 minutes, and repelleted. The supernatant was removed, neutralized to pH 6.5 with 0.01 N NaOH, and clarified by centrifugation at 10,000 g for 15 minutes. Direct eluates were prepared similarly, starting with washed patient’s platelets suspended in normal saline.

Flow cytometry. Fresh, unfixed, gel-filtered platelets were incubated with ITP or control eluates for 1 hour at 20°C, washed twice in flow buffer (PBS containing 0.5% BSA and 2.5 mmol/L EDTA), and incubated for 1 hour with a 1/500 dilution of fluorescein isothiocyanate (FITC)-labeled goat γ-specific antihuman IgG (Kirkegaard-Perry, Gaithersburg, MD). Ten thousand cells were analyzed per test on a FACSscan instrument (Becton Dickinson, San Jose, CA).

Glycocalcin preparation. Following a published method,13 approximately 20 μL of washed platelets was incubated in 3 mol/L KCl at 37°C for 30 minutes, and the platelet supernatant, after dialysis, was applied to a column of wheat germ lectin Sepharose 6-MB (Pharmacia). The eluate, obtained with 5% N-acetylglucosamine in PBS-0.5% EDTA, was subjected to molecular sizing on a column of Sepharose CL-4B (Pharmacia). Protein collected in fractions representing the molecular weight range for GC appeared as a single band at approximately 110 kDa on Coomassie blue-stained SDS-PAGE and on an immunoblot developed with murine monoclonal anti-GPlb (clone S22; Immunotech, Westbrook, ME). The protein concentration of GC was measured by a bicinchoninic acid assay (Pierce, Rockford, IL).

SDS-PAGE and protein immunoblotting. SDS-PAGE and protein immunoblotting were performed according to the methods of Laemmli and Bumette, respectively, using 7.5% gels (BioRad, Hercules, CA). Membranes were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Amplification of GPlba DNA fragments. Full-length 2,420-bp GPlba cDNA constructs, generated in vector pVEGT was a gift from Dr Gerald J. Roth (University of Washington, Seattle Veteran’s Administration Medical Center, Seattle, WA). Two overlapping fragments of GPlba cDNA were amplified by polymerase chain reaction (PCR) on an OmniGene thermocycler (Hybaid, Middlesex, UK). Fragment 1 spanned bps 91 to 831, and fragment 2 spanned bps 808 to 1545. In a total volume of 100 μL, approximately 1 ng of cDNA was incubated with 50 pmol of each oligonucleotide primer (A and B or C and D), 2.5 U Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT), and 0.2 mmol of each dNTP (Promega, Madison, WI) in the appropriate PCR buffer. Primers A, spanning nucleotides 91-110 (5'-GGTTCAGTGCTTCCGACCGCCAAGGTTGC-3'), and B, spanning nucleotides complementary to 812-831 (5'-CGCTCCAGGTCAGTTTGTTGTAG-3'), were used to amplify fragment 1.14 Primers C, spanning nucleotides 808-826 (5'-CGGAGTGTGTCAGTGAACGCGGGTTGGAA-3'), were used to amplify fragment 2. EcoRI restriction sites in primers A and C and Xho I sites in primers B and D are underlined. Thirty-five cycles were performed as follows: 1 minute at 95°C, 1 minute at 52°C, and 2 minutes at 72°C. Afterward, a single final elongation step was performed at 72°C for 5 minutes. Samples were analyzed on 0.75% agarose gels stained with ethidium bromide and visualized under UV light.

Amplificates were subcloned into IBI Escherichia coli pFLAG.2 expression vectors (Eastman Chemical Co, New Haven, CT) that were transfected into E coli DH5α competent cells (GIBCO BRL, Gaithersburg, MD) by heat shock per the manufacturer’s instructions. Successful cloning was confirmed by extensive restriction analysis. FLAG-fusion proteins were purified by immunoadfinity chromatography using monoclonal anti-FLAG M2 affinity gel (Eastman Chemical Co). Recombinant protein fragment 1 consisted of aas 1 to 247 and fragment 2 consisted of aas 240-485, each tagged at their N-terminal ends with the FLAG octapeptide. They were stored in buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4, at −20°C until used in radioimmunoassays (RIAs).

Pepitides. Pepitides were prepared by BioSynthesis, Inc (Lewisville, TX). The amino acid sequence of GPlbo was determined, and its antigenicity index plot,22,23 was accessed through GenBank (Los Alamos, NM) using MacVector software (Eastman Kodak, New Haven, CT), used as a basis for producing four synthetic peptides, designated P1 to P4 within fragment 2 (Fig 1). The decapetide, TKEQTTFPRR, contained the 9 aas that further localized the P2 (21mer) epitope (see Results and Fig 6); one extra aa, arginine, was added to simplify the synthetic process, which is difficult for pepitides with C-terminal prolines. The irrelevant pepitide used as a negative control in this study had the following sequence: YGCKNFWKFTFTSC. All pepitides were readily soluble in PBS, pH 7.4, and were stored at −20°C.

Radio labeling Igs. Murine monoclonal antihuman IgG (clone 8A4; Immunotech, Westbrook, ME) was radiolabeled with 125I using iodogen (Pierce, Rockford, IL) according to the manufacturer’s directions. In a typical experiment, 0.2 mg of Ab was labeled. Ninety-five percent of cpn was precipitable with 10% trichloroacetic acid, and the specific activity of the 125I-protein was 1.0 to 2.0 μCi/μg.

RIA. Immun 4 polystyrene removawell strips (Dynatech, Chantilly, VA) were incubated overnight at 4°C with 100 μL per well of purified protein or peptide that had been diluted to a concentration of 10 μg/mL in coating buffer, pH 9.5 (Kirkegaard-Perry). Wells were washed three times with PBS containing 0.05% Tween-20, pH 7.4 (PBS-T), blocked for 1 hour at 20°C with 2% BSA in PBS-T, washed three times, incubated for 1 hour with sera diluted 1/10 or with direct eluates from 5 × 109 platelets, washed three times, incubated for 1 hour with 50 ng 125I-labeled monoclonal antihuman IgG per well, washed three times, and counted. Samples were always tested in triplicate. Tests were considered positive if the average cpn exceeded by 3 standard deviations (SDs) the average cpn of 3 or more control samples tested on the same plate. Results with wells coated with an irrelevant pepitide did not differ significantly for ITP sera and normal control sera.

Adsorption of anti-P2 by intact platelets. Fresh, gel-filtered platelets were aliquoted into 1.5 mL microfuge tubes in total quantities ranging from 2.5 × 108 to 1 × 109 and pelleted by centrifugation at 1,000g for 10 minutes. Supernatants were removed and platelets were suspended in 100 μL of a 1/10 dilution of test serum in PBS-T. After incubation for 60 minutes at 20°C, platelets were removed and sera were tested for reactivity with P2 by RIA. Results were compared with anti-P2 reactions of unadsorbed sera. Control adsorptions consisted of 1 × 109 O-negative washed erythrocytes suspended in 100 μL of a 1/10 dilution of each test serum in PBS-T.

Competition between GC in solution and immobilized P2 for anti-P2. One-hundred microliter aliquots of a 1/10 dilution of ITP se-
run were incubated with various quantities of purified GC for 60 minutes at 20°C, after which the mixtures were tested for anti-P2 activity by RIA. Results were compared with those obtained using untreated serum.

**Inhibition of RIA measurement of anti-GC and anti-P2 by P2 in solution.** One hundred microliter aliquots of a 1/10 dilution of ITP serum in PBS-T were incubated with various amounts of P2 for 60 minutes at 20°C, after which anti-GC or anti-P2 activity was measured by RIA and compared with values obtained with untreated serum.

**Adsorption of anti-GC by immobilized P2.** One hundred microliters of a 1/20 dilution of serum in PBS-T were incubated for 1 hour at 20°C in a well precoated with P2, as described above, and then transferred to another identical well for a second 1 hour of adsorption. Reactivity of adsorbed serum with GC was then tested by RIA and compared with that of unadsorbed serum.

**Detection of anti-P2 and platelet-bindable IgG in platelet eluates.** Eluates were checked for anti-P2 activity by RIA. Flow cytometric measurements of surface-bound IgG were made on normal platelets after their exposure to ITP or control eluates.

**Epitope scanning using a panel of overlapping peptides.** A panel of 40 biotinylated 15-mer peptides spanning fragment 2 were synthesized© by Chiron Mimotopes, Inc (Victoria, Australia), according to the format, biotin-SGSG-NH₂-peptide-COOH. Peptide 1 begins at aa 237 and peptide 40 ends at aa 485. Sequences of peptides 14 through 19, bracketing P2, are listed in Table 1. Each peptide overlapped the preceding one by 9 aa residues, with an offset of 6 aas. Most peptides were easily solubilized in 40% acetonitrile/water solution and stored at -70°C until used. Peptides not soluble in this manner (nos. 1, 2, 8, 13, 14, 19, 20, 28, 30, 31, 32, and 39) were dried down and solubilized in 100% dimethyl sulfoxide (DMSO). Microtiter plates were coated with 100 µL per well of a 5 µg/mL solution of streptavidin (affinity-purified, salt-free; Sigma, St Louis, MO) and evaporated to dryness overnight at 37°C. A standard enzyme-linked immunosorbent assay (ELISA) protocol supplied by Chiron was used. One hundred microliters of peptide at approximately 12 µg/mL was coated per well. All peptides were coated in duplicate on wells of a single microtiter plate that was then exposed to one serum. Sixteen ITP and 11 normal sera were tested, each at 1/100 dilution. The amount of 2 ITP and 5 control eluates tested were derived from approximately 5 × 10⁶ platelets. Peroxidase-labeled goat antihuman IgG was used at 1/2,000 dilution, and absorbance was measured at 410 nm with a Dynatech MR 650 plate reader after addition of ABTS substrate (Kirkegaard-Perry).

**RESULTS**

**Reactions of sera with immobilized GC.** Of 16 ITP sera containing anti-GPIb/IX by antigen capture assays, 6 (no. 1 through 6) reacted with purified GC in RIAs. Cpsm of positive samples exceeded by 3 SDs the mean of normal sera run in triplicate concurrently. For normal sera, the ratio of the mean + 3 SDs divided by the mean (control ratio) was 1.5. A total of 16 normal sera were tested to calculate this ratio. Ratios for positive sera (mean patient’s cpm divided by the normal mean) ranged from 2 to 5.2.

None of the six positive sera, used at 1/20 dilution, elicited reactions in immunoblots with GPIb from solubilized platelets or with purified GC.

**Reactions of sera with recombinant GP Ibα protein fragments.** When recombinant GPIbα protein fragment 1 (aa 1 to 247) and fragment 2 (aa 240 to 485) were used as target antigens (Ags) in RIAs for the 16 ITP study sera, the same 6 that reacted with GC reacted with fragment 2. One (no. 6) also reacted with fragment 1. The control ratio was 1.4, based on determinations with 18 normal sera, and the ratios for positive sera ranged from 2 to 5.3. On the basis of these results, attention was focused on epitope mapping fragment 2.

**Reactions of sera and direct eluates with synthetic GPIbα peptides (P1 to P4).** When the four synthetic peptides P1 to P4 shown in Fig 1 were plated as target Ags in RIAs, sera no. 1 through 6 reacted with P2; serum 3 also reacted with P4. Results are summarized in Table 2. There were no positive reactions to P1 or P3 among the 16 ITP study sera reactive with GPIb/IX, and no positive reactions to peptides P1 to P4 among the 10 ITP sera nonreactive with GPIb/IX. Direct eluates from patients no. 1 and 2 reacted positively

### Table 1. Overlapping 15-mer Peptides in the P2 Region of Fragment 2

<table>
<thead>
<tr>
<th>Peptide No.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>TASLDQMPSSLHHT</td>
</tr>
<tr>
<td>15</td>
<td>QMPSSLHTPQESTKE</td>
</tr>
<tr>
<td>16</td>
<td>HPQESTKEOTTFFP</td>
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<td>18</td>
<td>FPFPRTNPFTHLHES</td>
</tr>
<tr>
<td>19</td>
<td>PNFTHLHMESITFSKT</td>
</tr>
</tbody>
</table>

![Fig 1. Peptides in fragment 2 of GPIbα selected for study. Constituent aa and antigenicity profiles are shown for GPIbα peptides (P1 to P4).](image-url)
Table 2. Reactions of ITP Sera and Eluates With GPIbα Oligopeptides (P1 to P4)

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sera</th>
<th>Eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>262</td>
<td>993</td>
</tr>
<tr>
<td>2</td>
<td>392</td>
<td>1,104</td>
</tr>
<tr>
<td>3</td>
<td>370</td>
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<td>1,143</td>
</tr>
<tr>
<td>5</td>
<td>484</td>
<td>1,159</td>
</tr>
<tr>
<td>6</td>
<td>375</td>
<td>1,499</td>
</tr>
<tr>
<td>Control</td>
<td>300</td>
<td>290</td>
</tr>
</tbody>
</table>

*Elute experiments were performed with a different radiolabeled antiliglobin preparation than that used for serum assays. The amount of direct eluate used per well was derived from 5 × 10⁶ platelets.
† Mean control + 3 SDs.

with P2 compared with 5 direct eluates of normal platelets (Table 2). To further substantiate that the specificity of reactions with P2 was the same as reactions with intact platelets and GC, adsorption and competitive inhibition studies were performed.

Adsorption of anti-P2 by intact platelets. Figure 2 shows the adsorption of anti-P2 from sera no. 1, 2, and 3 by intact platelets. Anti-P2 was greater than 80% adsorbed from 10 μL of serum by 5 × 10⁸ to 10⁹ platelets; anti-P2 was not adsorbed in detectable amounts from these sera by 10⁶ O-negative erythrocytes.

Inhibition of anti-P2 by purified GC in solution. Figure 3 shows results of adding purified GC to sera no. 1, 2, and 3 before reacting them with immobilized P2. Anti-P2 was decreased 60% to 85% by the presence of 9 × 10⁻⁶ mol/L GC (10 μg/10 μL). The amount of anti-P2 competitively inactivated by GC was less than the amount of anti-P2 removed by adsorption with platelets containing equivalent amounts of GC (see Fig 2: 2 × 10⁶ platelets contain approximately 1 μg GC).

Inhibition of anti-P2 and anti-GC by P2 in solution. Figure 4 shows that anti-P2 activities (open symbols) in sera no. 1 and 2 were inhibited 40% to 75% by 2.5 μg P2/100 μL (8.6 × 10⁻⁶ mol/L) of 1/10 diluted serum. P2 in solution at the same concentration inhibited anti-GC activities (solid symbols) by about 20%, and inhibition increased to only 30% by a 10-fold further increase in P2 concentration. In Fig 2, the 10⁸ platelets that adsorbed all anti-P2 from 10 μL of serum contain about 0.1 μg of P2 or 1/500 the maximum amount of soluble P2 used in Fig 4. P2 at concentrations as high as 1.7 × 10⁻⁴ mol/L had no effect on binding of anti-P2 to platelets.

Adsorption of anti-GC by immobilized P2. Figure 5 shows adsorption of anti-GC of from sera no. 1, 2, and 6 by immobilized P2. Anti-GC was fully removed from sera no. 1 and 2 but was only partially removed from serum no. 6, which presumably reflects the presence of residual Ab against fragment 1 and/or other untested epitope(s) on fragment 2 in the sample after Ab against P2 was adsorbed.

Platelet-bindable IgG and anti-P2 in platelet eluates. Normal platelets had increased surface-bound IgG after incubation with indirect and direct ITP eluates from patients no. 1 and 2 as shown by a significant increase in their fluorescence profiles compared with platelets incubated with control eluates (data not shown). Both patients' eluates contained anti-P2 (Table 2).

Epitope mapping of fragment 2 using a panel of overlapping peptides. Figure 6 shows ELISA reactions of sera no. 1 through 6 and of 2 direct eluates from patients no. 1 and 2 with sequential 15-mer peptides of fragment 2. Typical controls are also shown. Each serum and platelet eluate reactive with P2 reacted with peptides no. 16 and 17. The direct eluate of patient no. 1 reacted with peptides no. 26 and 27, which are in the domain of P4, but serum from this patient did not react with P4. Serum from patient no. 3 reacted with P4, but not with any of its smaller constitutive peptides (nos. 25 to 29). As seen in Table 1, the 9 aa sequence, TKEQTTFPP, was common to peptides no. 16 and 17. When synthesized as a decapptide with R at the carboxy-terminal end, it reacted only with sera no. 1 and 4. Ratios for positive sera were 2.2 and 2.3, respectively, compared with the control ratio of 1.5 obtained with 20 normal sera, all used in the same experiment with ITP sera. Preincubation of sera no. 1 through 6 with immobilized P2 completely removed activity against peptides no. 16 and 11.

DISCUSSION

Knowledge of ITP autoAbs has recently been more clearly defined by use of Ag capture techniques to identify specific targeted GPs.1,25 These tests offer increased sensitivity over standard ELISAs and immunoblots, most likely by maintaining native conformations of antigenic GP determinants. From extensive use of these assays,1,7 the patterns of reaction

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Fig 2. Adsorption of anti-P2 with intact platelets. One hundred microliters of 1/10 diluted serum from each of 3 different ITP patients (no. 1, 2, and 3) was incubated for 1 hour with various numbers of fresh gel-filtered platelets, after which anti-P2 activity of the supernatant was measured by RIA. These values were compared with anti-P2 activities in the same sera before adsorption.

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Fig 3. Inhibition of anti-P2 by purified GC in solution. One hundred microliters of 1/10 diluted serum from each of 3 different ITP patients (no. 1, 2, and 3) was incubated with various quantities of GC for 1 hour and the mixtures were then checked for anti-P2 activity by RIA. Values were compared with anti-P2 activities measured in untreated sera. One microgram of GC is approximately equivalent to the amount of GC in $2 \times 10^9$ platelets.

that emerge are as follows. (1) Abs against major platelet membrane GPs, Ib/IIa and Ib/IX, are commonly found on patients' platelets and in their plasma. (2) Sera from individual patients characterizedly contain Abs against more than one target, often on different GPs. (3) Anti-GPs may be of one or more Ig classes and directed against internal and/or external GP epitopes. (4) Not all anti-GP Abs in ITP, such as those against internal GP epitopes, are pathogenic.

Internal epitopes of plasma Abs have been mapped to short C-terminal cytoplasmic peptides of GPIIIa<sup>22</sup> and GPIIb<sup>5</sup>, but Abs specific for these epitopes appear to be secondarily stimulated by excessive platelet destruction and are not pathogenically significant.<sup>25</sup> On the other hand, PAAbs that react with external epitopes of GPIIb/IIIa quantitatively decrease or disappear during clinical remissions induced by certain drugs such as steroids and cytotoxic agents, suggesting they do have a pathogenic role.<sup>10,11</sup> Similar studies have not yet been performed with anti-GPIb/IX Abs.

Antibodies against globular proteins typically react with discontinuous (nonlinear or assembled) epitopes that are dependent on protein folding.<sup>26</sup> For instance, reactions of most PAAbs against GPIIb/IIIa are dependent on an intact heterodimeric complex.<sup>12</sup> Linear peptides each of approximately 200 aas, together spanning the full length of GPIIIa, reacted with only 2 of 32 anti-GPIIb/IIIa PAAbs.<sup>13</sup> Similarly, nonlinear (higher order) structure was necessary for antigenicity of a 50-kD chymotryptic fragment of GPIIIa that reacted in the native state, but not after disulfide reduction, with serum Abs in 48% of chronic ITP patients and in some patients with secondary and nonimmune thrombocytopenias.<sup>27</sup>

In our study, 6 (38%) of 16 ITP sera that were known to contain anti-GPIb/IX reacted with GC, which accounts for almost all of the extracellular domain of GPIIb. The other 10 sera presumably had specificities for GPIIb<sub>y</sub>, GPIX, a complex-dependent epitope, or an epitope on the short segment of GPIIb<sub>y</sub> remaining after GC cleavage.

To map glycocalicin epitopes, we first studied Ab reactions with two recombinant fragments of GC that lacked.

Fig 4. Inhibition of serum reactions with immobilized GC or P2 by dissolved P2. One hundred microliters of 1/10 diluted serum from each of 2 patients (no. 1 and 2) was incubated with various concentrations of P2 for 1 hour and the mixtures were then tested for anti-GC or anti-P2 activity by RIAs. Values were compared with the respective Ab activities measured in untreated sera. One microgram of P2 is equivalent to the amount of P2 in approximately $8.8 \times 10^9$ platelets. Soluble P2 up the concentrations shown on the abscissa did not inhibit attachment of anti-P2 to platelets. (These 0% values are not plotted.)

Fig 5. Adsorption of anti-GC activity from ITP sera with immobilized P2. One hundred microliters of 1/20 diluted serum from each of 3 different ITP patients (no. 1, 2, and 6) was adsorbed twice with P2 coated on a microtiter well and then checked for reactivity against GC by RIA. Serum no. 6 reacted with both fragments 1 and 2.
Epitope scanning using a panel of overlapping peptides. Each histogram represents reactions of serum or eluate with 40 15-mer peptides spanning aas 237 to 485 of GPⅠbα. The average absorbance at 410 nm of duplicate wells is plotted versus identification numbers for peptides. Peptides no. 2 to 6 bracket P1; peptides no. 14 to 18 bracket P2; peptides no. 19 to 23 bracket P3; and peptides no. 25 to 29 bracket P4. Control plots are representative of 11 performed with normal sera and 5 performed with direct platelet eluates from normal individuals.

Fragment 1 (aa 1 to 247), which reacted with only 1 of the 6 positive sera, approximates the globular N-terminal domain with respect to primary aa sequence. Fragment 2 (aas 240 to 485), which represents the sequence of the native elongated, rod-like domain, reacted with all 6 sera. Next, short peptides within fragment 2 were chosen for synthesis as potential epitopes on the basis of their antigenic indices. Of the 4 promising regions (Fig 1), the 21-aa P2 reacted with all 6 sera and the 24-aa P4 also reacted with one of these sera. P2 and P4 are located within the macroglycopeptide domain of GPⅠbα, where there is minimal folding or tertiary structure because of constraints imposed by numerous proline residues and O-linked glycosylations as well as lack of cysteine residues. The P2 epitope was further defined by Ab reactions with a panel of overlapping 15mer peptides (Fig 6). All positive sera and platelet eluates from 2 of the patients whose sera were positive reacted with peptides no. 16 and 17, which share 9 aas, TKEQTTFPP. Anti-P2 accounted for all anti-GC activity directed at the fragment 2 domain in 2 of 3 sera tested (Fig 5). Purified GC effectively competed for anti-P2 (Fig 3); adsorption of sera with fresh, gel-filtered platelets removed anti-P2 activity (Fig 2); and direct eluates of patients' platelets contained anti-P2. P2 is therefore expressed on native GC and is accessible on the surface of platelets.

Evidence that ITP Abs often have low affinity is the low number of specific anti-GP Abs attached per platelet in the presence of excess Ab despite high-density expression of reactive GPs. Full adsorption of anti-P2 required approximately 25 to 100 times more platelets than necessary to completely adsorb many serum platelet-specific alloAbs, suggesting that anti-P2 Abs may have relatively low affini-
ties. Similarly, large amounts of platelets were also required to remove the plasma ITP factor capable of inducing thrombocytopenia in vivo by passive infusion. In Fig 2, sera no. 1 and 3 appeared to contain a mixture of Abs with distinctly different affinities, with those with higher affinities being adsorbed at the lower concentrations of platelets.

P2 in solution at a concentration as high as $1.7 \times 10^{-4}$ mol/L was ineffective in inhibiting binding of anti-P2 to platelets, was weakly effective in inhibiting reactions with immobilized GC, and was most effective in inhibiting reaction with immobilized P2 (Fig 4). Anti-P2 appears to have the highest affinity when the epitope is on platelets, less when on purified GC, and least for P2 itself. This phenomenon has been described for certain anti-muiglobulin antibodies against continuous determinants and has been attributed to the low probability of short peptides in solution assuming conformations similar to those of the native protein. The decapetide defining the epitope has less affinity for the Ab than P2 in that it reacted weakly with only 2 of the 6 sera that were positive with P2. Thus, P2 or the decapetide would not be effective in vivo as competitors to prevent attachment of ITP Abs to platelets. However, immobilized P2 (21-mer) was effective in removing specific Abs from patient’s plasma. This may be attributable to an increased apparent affinity of antibody for antigen locally concentrated on a solid surface.

The direct eluate of patient no. 1 reacted with peptides no. 26 and 27 in the domain of P4, but her serum did not, possibly because low-titer Abs were present only on platelets. Serum Abs from patient no. 2 reacted with P4 but not with peptides no. 25 through 29 in the domain of P4, perhaps due to lesser affinity for smaller peptides.

Unlike anti-GC autoAbs in one case of acquired Bernard-Soulier syndrome, there is no indication from a clinical standpoint that autoAbs against P2 or P4 cause a functional platelet defect. This is consistent with the fact that the P2 and P4 sequences are distant from the hinge region where von Willebrand factor binds (aa 269 to 287) as well as from a high-affinity thrombin binding site (aa 271-284). P2 and P4 are distinct from the Ko (HPA-2) alloantigen, which is determined by a ThrMet polymorphism at aa 145, and from binding sites of many known antiplatelet antibodies and the factor responsible for thrombocytopenia in patients with chronic immune thrombocytopenic purpura. Blood 83:1024, 1994


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Extracellular epitopes of platelet glycoprotein Ib alpha reactive with serum antibodies from patients with chronic idiopathic thrombocytopenic purpura

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