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# The disulfide-rich region of platelet glycoprotein (GP) IIIa contains hydrophilic peptide sequences that bind anti-GPIIIa autoantibodies from patients with immune thrombocytopenic purpura (ITP)

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**Abstract**

Immune thrombocytopenic purpura (ITP) is an autoimmune blood disease caused by autoantibody-mediated destruction of blood platelets. Platelet glycoprotein (GP) IIb/IIIa is a common target for antiplatelet autoantibodies. The present studies were undertaken (1) to confirm whether the disulfide rich repeat region of GPIIIa contains target epitopes for antiplatelet antibodies in patients with ITP; (2) to determine whether these antigens were defined by peptide sequences in the absence of post-translational modification; and (3) to correlate observed immunologic reactivity with the recently solved X-ray crystallographic structure of an analogous integrin complex, the vitronectin receptor, $\alpha_V\beta_3$. Recombinant fusion proteins of four GPIIIa extracellular sequences were prepared and purified. Immunoblotting results with purified recombinant peptides showed potent reactivity of 16 of 24 ITP patient serum anti-GPIIb/IIIa antibodies with the fusion protein containing the GPIIIa sequence of residues from 468 to 691. These results are consistent with a report by Kekomaki et al. that a 50 kDa chymotryptic digestion product of GPIIIa isolated from blood platelets contains target epitopes for serum antiplatelet antibodies in 16 of 33 ITP patients. Smaller peptides including residues 446–501 and residues 593–691 each reacted with only 5 of the 24 patient sera; furthermore all but 3 of these interactions were very weak. Visualization of the conformation of the extracellular portion of $\alpha_V\beta_3$ reveals the location of the 222-residue antigenic GPIIIa ($\beta_3$) peptide 'B' at the immediately extracellular region of the protein that includes a β-tail domain and several integrin-EGF domains. In summary, predictions of hydrophilicity, surface accessibility and antigenicity and the three dimensional structure of the $\beta_3$ integrin correlate with autoantibody binding to a recombinant GPIIIa peptide 'B' containing residues 468–691.


*Keywords:* Platelet autoantigen; Immune thrombocytopenia; Integrin structure-function correlation; Recombinant fusion protein; GPIIIa autoepitopes

*Abbreviations:* ITP, Immune thrombocytopenic purpura; GPIIb/IIIa, Glycoprotein IIb/IIIa; MAIPA, Monoclonal antibody immobilization of platelet antigen; GST, Glutathione-*S*-transferase; EGF domains, Epidermal growth factor-like domains

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## 1. Introduction

Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disease characterized by an isolated decrease in the concentration of blood platelets in the circulation. The pathogenic mechanism involves destruction of platelets mediated by autoantibodies with clearance of the opsonized platelets in the reticuloendothelial system, particularly in the spleen. Affected patients are at an increased risk for hemorrhage. Current understanding of the precise molecular details of platelet autoantigens is limited, and most therapeutic modalities for ITP have been identified empirically. Development of novel specific treatments for ITP would be greatly facilitated by an improved molecular understanding of the precise targets of the causative antiplatelet antibodies.

A number of studies have determined that several platelet surface proteins can be the targets of antiplatelet antibodies and that antibodies directed against more than one target protein may occur [1–4]. However, autoantibodies against the $\alpha II\beta_3$ integrin complex, glycoprotein IIb/IIIa (GPIIb/GPIIIa), have been implicated most frequently as mediators of platelet destruction in ITP [1–7]. Based upon results of antigen capture experiments, platelet GPIIb/IIIa target antigens have been hypothesized to be 'conformational epitopes' that are dependent upon the tertiary conformation of the target protein [8]. Furthermore, some investigators have reported that GPIIb/IIIa autoantigens depend upon maintaining the quaternary structure of this bimolecular glycoprotein complex [9]. Specific antibodies directed against each individual component of this glycoprotein complex (GPIIb and GPIIIa) have been detected in ITP patients' sera, but the precise submolecular portions of GPIIb or GPIIIa that define the target autoepitopes for most of these antibodies are not well characterized.

Immunoblotting studies in our laboratory found that anti-GPIIIa antibodies were common in the sera of patients with ITP [10–13]. A soluble recombinant form of GPIIb/IIIa was produced by Peterson et al. and shown to bind three of the autoantibodies that were tested [14]. Only a few studies have addressed the question of which portion of the GPIIIa molecule is involved in the target epitopes for anti-GPIIIa antibodies. Antibodies against the cytoplasmic portion of GPIIIa were reported in blood samples obtained from patients with a clinical diagnosis of ITP [8,15]. Since these cytosolic portions of the glycoprotein are not displayed on the intact platelet surface, the antibodies directed against them are probably secondary to, rather than causative of, ITP. Kekomaki, Kunicki and colleagues reported that 14 of 31 ITP sera bound to a 50 kD GPIIIa chymotrypsin digestion product containing much of the disulfide rich extracellular portion of GPIIIa [16]. Their studies used an immunoblotting assay against protein fragments isolated by immunoabsorption after proteolytic digestion of GPIIIa from blood platelets; multiple peptide bands resulted after reduction of the disulfide bonds. Chymotrypsin digestion experiments would not differentiate between antigens defined by the peptide sequence and those that depended upon carbohydrate.

The present experiments were performed to determine whether recombinant peptides including sequences within the disulfide-rich region of GPIIIa define target epitopes for antiplatelet autoantibodies in sera from patients with ITP. Portions of this transmembrane protein that were candidate targets of clinically important autoantibodies would be expected to reside in the extracellular portion of GPIIIa where they would be accessible to the extracellular plasma in which destructive antiplatelet antibodies circulate. Hydrophobicity/hydrophilicity, surface accessibility, and antigenicity prediction techniques were used to identify peptide sequences of GPIIIa for immunologic study. Synthetic fusion proteins containing these peptide sequences were produced and assayed for immune reactivity with sera from patients with a clinical diagnosis of primary ITP.

At the time these experiments were undertaken the X-ray crystallographic structure for GPIIb/IIIa or the related integrin, $\alpha_V\beta_3$, was not available for identification of candidate epitopes. Based upon the proteolytic digestion studies of Kekomaki et al. [16], the present studies focused on the disulfide-rich region of GPIIIa that is predicted to be extracellular. Within this region, candidate linear sequences were selected by prediction of sequences

with high hydrophilicity or sequences with high probability to occupy surface regions of the folded molecule [17–21]. The recombinant fusion proteins containing GPIIIa peptides that were synthesized express platelet autoantigens. Since these antigenic peptides can be expressed in a prokaryotic system, carbohydrate modification of the peptides is not required for these antigens.

## 2. Experimental methods

### 2.1. Reagents

The cDNA clone for GPIlIa was supplied by Dr Paul Bray (Houston, TX). Primers used for the polymerase chain reaction (PCR) were synthesized at the Pathology Department DNA Synthesis Laboratory at Yale University School of Medicine using an Applied Biosystem 392 DNA/RNA synthesizer. The pGEX-2T expression vector was purchased from Pharmacia LKB Biotechnology Inc. (Piscatway, NJ). All enzymes and oligonucleotides were purchased from New England Biolabs (Beverly, MA). All other reagents were of analytic grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

### 2.2. Prediction of antigenic peptide regions

Assessment of hydrophobicity/hydrophilicity in GPIIIa was done by two methods, Kyte–Doolittle [18] and Hopp–Woods [17], using the Protean module of Lasergene Software (DNAStar, Inc., Madison, WI). Both methods predict regions of hydrophobicity/hydrophilicity by summing hydrophobicity/hydrophilicity over a range of amino acids. The number of residues averaged was nine in the Kyte–Doolittle method and seven in the Hopp–Woods calculation. For prediction of regions that might contain autoepitopes, the results were graphed with positive hydrophilicity above the abscissa; hydrophobic peptide sequences would appear below the horizontal axis.

Antigenic index was calculated by the method of Jameson and Wolf [22] which produces an index of antigenicity, by combining values for hydrophilicity (Hopp–Woods, $H[\langle 2\rangle 0.5\langle 1\rangle 0\langle -1\rangle -0.5\langle -2\rangle])$, surface probability (Emini, $S[\langle 1\rangle 1.0\langle 0\rangle])$ [21], flexibility (Karplus–Schultz, $F[\langle 1\rangle 1.0\langle 0\rangle])$ [23], secondary structure predictions of Chou–Fasman (CF[$T=2$, $t=1$, 0]) [19] and Garnier–Robson (GR[$T=2$, $t=1$, 0]) [20] by the following formula: $A=0.3\mathrm{H}+0.15\mathrm{S}+0.15\mathrm{F}+0.2\mathrm{CF}+0.2\mathrm{GR}$.

Surface accessibility prediction was performed according to the published method of Emini [21]. This surface probability plot method predicts the surface regions by forming the product of the residue specific surface propensities over a range of 5 amino acids. Probability threshold: (0.6): 1 000 000.

### 2.3. Construction of the expression vector containing truncated GPIIIa

The cDNA coding for GPIIIa was used in PCR amplification of partial sequences coding for the desired peptide fragments. The primers used in the amplification reactions were: peptide A-forward: 5′-GCG AGA TCT GGG CCC AAC ATC TGT ACC-3′, peptide A reverse: 5′-CGC GAA TTC ACT TGG GAC ACT CTG GCT C-3′; peptide B-forward: 5′-GCG AGA TCT GGG CCT GGC TGG CTG GGA T-3′, peptide B-reverse: 5′-CGC GAA TTC ACT TGG GAC ACT CTG GCT C-3′; peptide C-forward: 5′-GCG AGA TCT CAT CGC TGC AAC AAT GCC AAT-3′, peptide C-reverse: 5′-CGC GAA TTC ACT CGC CCC GCT GGC TGC AGA-3′; peptide D-forward: 5′-GCA GGA TCC TAT GGG GAC ACC TGT GAG AAG-3′, peptide D-reverse: 5′-CGC GAA TTC ACT TGG GAC ACT CTG GCT C-3′ with appropriate restriction enzyme recognition sites for subcloning into *Bam H1/Eco R1* site of pGEX-2T indicated. The polymerase chain reaction was carried out in 100 μl of reaction volume containing 20 mM Tris–HCl, pH 8.8, 10 mM KC1, 10 mM $(NH4)2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 2.5 units of pfu DNA polymerase, 50 pmol of primer and 10 ng of template DNA. The PCR reaction was carried out for 30 cycles with each cycle consisting of denaturation at 94 °C for 1.5 min, annealing at 55 °C for 2 min and extension at 72 °C for 3 min. The amplified PCR products B, C and D were purified using QIA quick-spin PCR purification kit (Qiagen Inc., Valencia, CA).

These DNA fragments were then digested with appropriate restriction enzymes to generate cohesive ends for ligation to the *Bam H1/Eco R1* digested pGEX-2T vector. The digestion products were subjected to agarose gel electrophoresis followed by the QIAEX DNA gel extraction protocol (Qiagen Inc., Valencia, CA). Purified digests were cloned into plasmid pGEX-2T at the *Bam H1/Eco R1* site. The recombinant plasmid was transformed into competent *E. coli* $DH5_{\alpha}$ cells and the clones were screened by restriction digestion analysis and subsequent sequencing. In addition, the fragment obtained by *Bam H1* and *Eco R1* digestion of the amplified product I, coding for residue 468–691, was also subcloned into the pGEX-2T vector.

### 2.4. Expression and purification of recombinant GPIIIa fusion protein

Recombinant GPIIIa proteins were expressed and purified from *E. coli* cells as described [24]. Overnight cultures of *E. coli* $DH_{5\alpha}$ transformed with pGEX-GP IIIa constructs were diluted 1:10 with Luria broth medium and grown for 1 h at 37 °C. Isopropylthiogalactopyranoside (0.25 mM) was then added. After 3–4 h of growth, bacteria were pelleted and resuspended at 4 °C in 1:50 culture volume of a sonication buffer containing 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA and 0.15 mM PMSF. Induction was maximal at 3–4 h and fusion proteins comprised 20–80% of total bacterial proteins as determined by densitometry of Coomassie blue-stained SDS-gel (data not shown). All subsequent steps were performed at 4 °C. Bacteria were treated with 1 mg/ml lysozyme for 30 min and then 10 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM PMSF, 1 mM DTT, 1 mM EDTA and 1 mM benzamidine were added. The suspension was frozen at −80 °C, thawed at 30 °C and incubated with Triton X-100 (1%), deoxyribonuclease I (0.1 mg/ml) and $MgCl_2$ (10 mM) for 30 min and then sonicated for 30 s. The lysate was centrifuged at 48 000×*g* for 40 min and the supernatant was applied to a reduced glutathione–agarose column. The column was washed with 10 mM sodium phosphate buffer, pH 7.5, containing 130 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 1 mM EDTA (PBSI) and 0.5% Triton X-100, followed by 100 ml of PBSI and 25 ml of sonication buffer. Fusion proteins bound to the column were eluted with sonication buffer containing 10 mM reduced glutathione, and subsequently concentrated with Centricon microconcentrators (Amicon Corp., Danvers, MA).

### 2.5. Immunoblotting analysis

Immunoblotting assays were performed as described [12,13]. Briefly, 2 μg of purified GPIIIa fusion proteins were resolved by electrophoresis on 10% SDS-polyacrylamide gel and then electrotransferred to a nitrocellulose membrane using a Bio-Rad electroblotting apparatus. The membrane was blocked at 25 °C for 1 h with 5% (w/v) non-fat dry milk in TBST buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl and 0.05% Tween-20) and subsequently incubated at 25 °C for 3 h with 1:100 diluted ITP patient serum in the same buffer. After washing thoroughly with 5% non-fat dry milk in TBST buffer, the membrane was incubated for 1 h with 1:5000 dilution of rabbit anti-human IgG-HRP conjugate (Bio-Rad, Hercules, CA), and then washed with TBST buffer. Immune complexes were revealed with the enhanced chemiluminescence detection method by immersing the blot for 1 min in a 1:1 mixture of chemiluminescence reagents A and B (Amersham, Amersham, UK) and then exposed to Kodak XCL film.

### 2.6. Platelets

Samples of 30 ml of blood from blood group O donors were collected in syringes containing 5 ml acid citrate dextrose (ACD) with 10 mM EDTA. Platelet rich plasma was obtained by centrifugation twice at 230×*g* for 20 min at room temperature. Contamination with white blood cells was <1 cell/1000 platelets. Platelets were collected by centrifugation at 800×*g* for 20 min at room temperature. They were subsequently washed 3 times in a modified TES Tyrodes buffer containing 136 mM NaCl, 10 mM TES (*N*-tris) (hydroxymethyl)-2-aminoethanesulfonic acid (Calbiochem, Los Angeles, CA), 2.6 mM KCl, 0.5 mM $NaH_2PO_4$, 2 mM EDTA, 2 mM $MgCl_2$, 5.5 mM

glucose pH 7.4. For adsorption and elution studies, platelets were suspended in 1 mg/ml BSA in PBS with 2 mM EDTA at a density of $2\times10^9$ platelets/ml. For polyacrylamide gel electrophoresis, platelets were suspended in TES Tyrodes buffer at a density of $2\times10^9$ platelets/ml and solubilized by addition of an equal volume of 60 mM Tris HCl, pH 6.8, with or without 2% β-mercaptoethanol and then heated at 100 °C for 5 min.

### *2.7. Patient sera*

Serum specimens from 24 patients with a clinical diagnosis of ITP were studied. All 24 patients had circulating anti-GPIIb/IIIa IgG antibodies as demonstrated by monoclonal antibody immobilization of platelet antigens ('MAIPA') antigen capture assay [25]. A diagnosis of ITP was based upon documentation of isolated thrombocytopenia in the absence of splenomegaly, microangiopathy, or systemic autoimmune disease. The investigations were approved by the Human Investigation Committee of Yale University.

### *2.8. Separation of platelet proteins and transfer to nitrocellulose*

Polyacrylamide gel electrophoresis was performed with Laemmli discontinuous gels using 3% polycrylamide stacking and 5, 7 or 12% separating slab gels. Samples of $10^8$ platelets or 2 μg of purified recombinant fusion protein were loaded on each lane. Gels were run overnight at 15 V constant voltage until a bromphenol blue tracking dye reached the bottom of the gel. The separated proteins were then electrophoretically transferred to 0.45 μm pore sized nitrocellulose paper (Schleicher and Schuell, Keene, NH) using an E-C Corp 'Electroblot apparatus'. Transfer was complete in 2 h at 5 W as assayed by staining the remaining gel with Coomassie blue.

### *2.9. Elution of platelet-associated immunoglobulins from platelets*

Platelet-associated antibodies were eluted by diethyl ether. Briefly, 200 μl of platelet suspension ($10^9$/ml in PBS) from the ITP patient and from normal donors were mixed with 200 μl of diethyl ether by vigorous vortexing for 1 min. The mixture was placed in a 37 °C water-bath for 15 min to evaporate ether. The mixture was then centrifuged at 2600 rpm for 10 min at 20 °C, and the bottom layer was obtained as a platelet eluate. The eluates were stored at −80 °C until use.

## 3. Results

### *3.1. Prediction of hydrophilicity, surface accessibility and antigenicity*

At the time the potentially antigenic platelet GPIIIa peptides were designed, the three dimensional conformation for GPIIb/IIIa or related integrins was unknown. Prediction of sequences of low hydrophobicity (high hydrophilicity) were calculated by published methods [17,18]. Results of these calculations are shown in Fig. 1. Qualitative analysis of the hydrophilicity profiles revealed that the disulfide-rich region and the β-tail domain of Peptide 'B' are predicted to be hydrophilic for the majority of their sequence, features consistent with location at the protein surface as expected for potential antigenic targets. The calculated antigenic profile using the method of Jameson and Wolf [22] predicted that much of the sequence comprising Peptide 'B' could form antigenic conformations. Estimation of surface accessibility by the method of Emini [21] predicted surface localization for the entire β-tail domain as well as smaller peaks centered about residues 460 and 500, all contained within the sequence of Peptide 'B.'

Location of the recombinant GPIIIa peptides in the tertiary conformation of the $\beta_3$ integrin was assessed by comparison with the conformation of the extracellular portion of $\alpha_V\beta_3$ as recently determined to 3.1 Å resolution by X-ray crystallography [26]. Figs. 2 and 3 illustrate the structure of the portions of the integrin complex with well defined locations (i.e. excluding residues 1–54 and residues 435–531 for which the temperature factors indicate uncertainty in the location of this portion of the $\beta_3$ structure.) (In both Fig. 2 and Fig. 3 the platelet membrane would be positioned at the bottom of the figure). The alpha chain is demonstrated in grey, while the $\beta_3$ protein is shown in

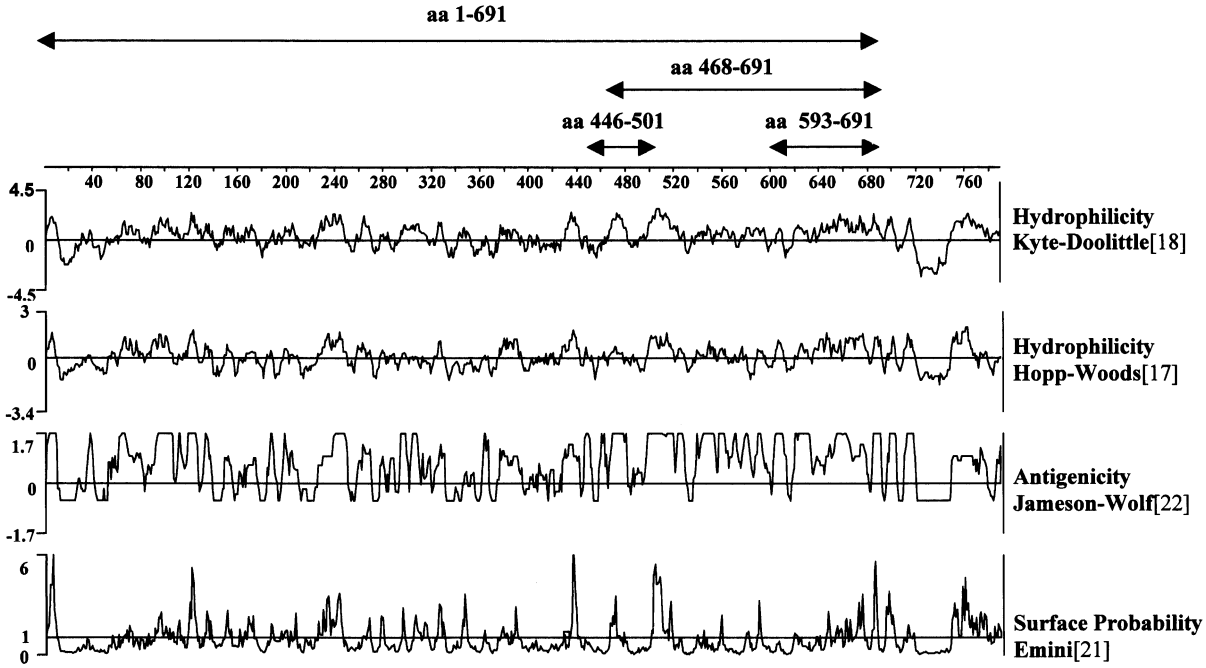


Fig. 1. Results of predictions that relate to likely antigenic regions of GPIIIa. Fig. 1 shows hydrophilicity profiles as calculated according to Kyte and Doolittle [18] and Hopp and Woods [17]. Note that the hydrophilicity index by both methods is the reciprocal of the hydrophobicity index. The antigenicity index was calculated by the method of Jameson and Wolf [22], and surface accessibility was determined according to the method of Emini [21].

orange (residues 55–434) and blue (532–690); the blue area indicates the structure included in our synthetic peptide region 'B.' Positioning the $\alpha_V\beta_3$ crystallographic model as in Fig. 2 reveals the large region of unresolved structure for the $\beta_3$ integrin (435–531). The orientation in the stereo pair diagrams of Fig. 3 illustrates the $\alpha_V\beta_3$ structure rotated to position recombinant Peptide 'B' to the front of the stereo view. In Fig. 3 the sequence of $\beta_3$ that comprises Peptide 'D' is highlighted in a lighter blue color.

### *3.2. Synthesis of recombinant GPIIIa peptide-GST fusion proteins*

Fragment A was chosen to eliminate the transmembrane residues and the intracellular carboxy terminus. Peptide 'B' encompasses residues 468–690 (Table 1). This region includes most of the extracellular portion of the 50 kDa chymotrypsin resistant, disulfide-rich region. Kekomaki, Kunicki and their colleagues determined that 15 of 31 ITP patients' serum antibodies reacted with that region and that 16 of 17 patients with demonstrable anti-GPIIb/IIIa antibodies showed binding to this proteolytic fragment [16]. Peptide 'C,' a 56 residue peptide, contains the first of three disulfide-rich motifs. Peptide 'D' includes 98 extracellular residues immediately outside of the transmembrane region. Peptides 'C' and 'D' were determined to be likely candidates for epitopes because they are highly hydrophilic regions as demonstrated in the hydrophilicity profiles of GPIIIa shown in Fig. 1.

### *3.3. Recombinant GPIIIa fusion proteins*

To probe for the reactivity of ITP antibodies with the polypeptide structure comprising the extracellular portion of GPIIIa, the fusion protein containing residues 1–691 and smaller peptides ('B', 'C' and 'D') were expressed, purified and tested for antigenicity by immunoblotting. Fig. 4

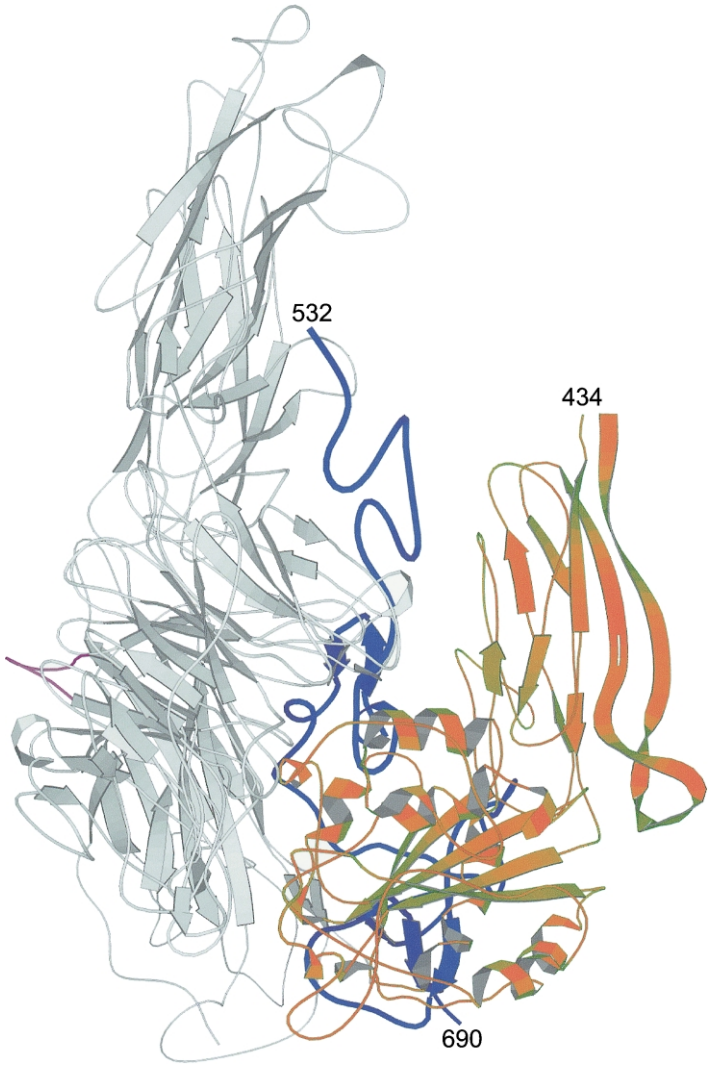


Fig. 2. Structure of the extracellular portion of $\alpha_V\beta_3$ as determined by X-ray crystallography [26]. The protein complex is oriented as if it were rising upward from the transmembrane portion (not included in this structure.) The $\alpha_V$ chain is shown in grey. The $\beta_3$ structure was uncertain at the N-terminus (residues 1–54) and in the region of EGF1 and EGF2 (residues 435–531); these regions are not included in the figure. The sequence from reside 55 to residue 434 is shown in orange, while the sequence from 532 to residue 690 is shown in blue. The orientation in Fig. 2 illustrates the gap in the sequence between 434 and 532 at the top of the figure.

shows Coomassie blue stained SDS-PAGE results indicating that a single band was purified for each fusion protein. The apparent MW of each band was identical to the expected migration of each of the four fusion proteins (peptide MW in addition to 26 kDa for the GST component).

### *3.4. Immune reactivity of sera from patients with ITP with the GPIIIa peptide*

#### *3.4.1. Fusion proteins*

Sera from ITP patients were tested for reactivity with the fusion proteins ‘A’, ‘B’, ‘C’ and ‘D’ in

immunoblotting assays. The sera used in these experiments had been demonstrated to react with the GPIIb/IIIa complex by the monoclonal antibody immobilization of platelet antigens ('MAIPA') antigen capture assay [25]. Immunoblotting studies utilizing the fusion protein containing Peptide 'A' (the entire extracellular portion of GPIIIa) did not bind specifically any of the serum specimens assayed (results not shown). However, when Peptide 'B' reactivity was tested with the same 24 sera by immunoblotting, 16 of 24 were positive as illustrated in Fig. 5 (lane B.) Since proteins produced by *E. coli* are not glycosylated, these results indicate that the antibodies detected were bound to GPIIIa peptide (rather than glycopeptide) antigens. Controls for these experiments were sera from individuals without ITP utilized in immunoblotting assays with autologous platelets; the controls had no binding to any of the GPIIIa peptide bands (results not shown.)

Table 1
Composition of the recombinant GPIIIa-GST fusion proteins synthesized and assayed for antigenicity

| Peptide | Residues | Size (residues) | Size (kDa-incl. GST) |
|---|---|---|---|
| A | 1–691 | 691 | 100 |
| B | 468–691 | 222 | 65 |
| C | 446–501 | 56 | 35 |
| D | 593–691 | 98 | 40 |

Five of the 24 sera were positive when studied with each of the affinity purified smaller fusion

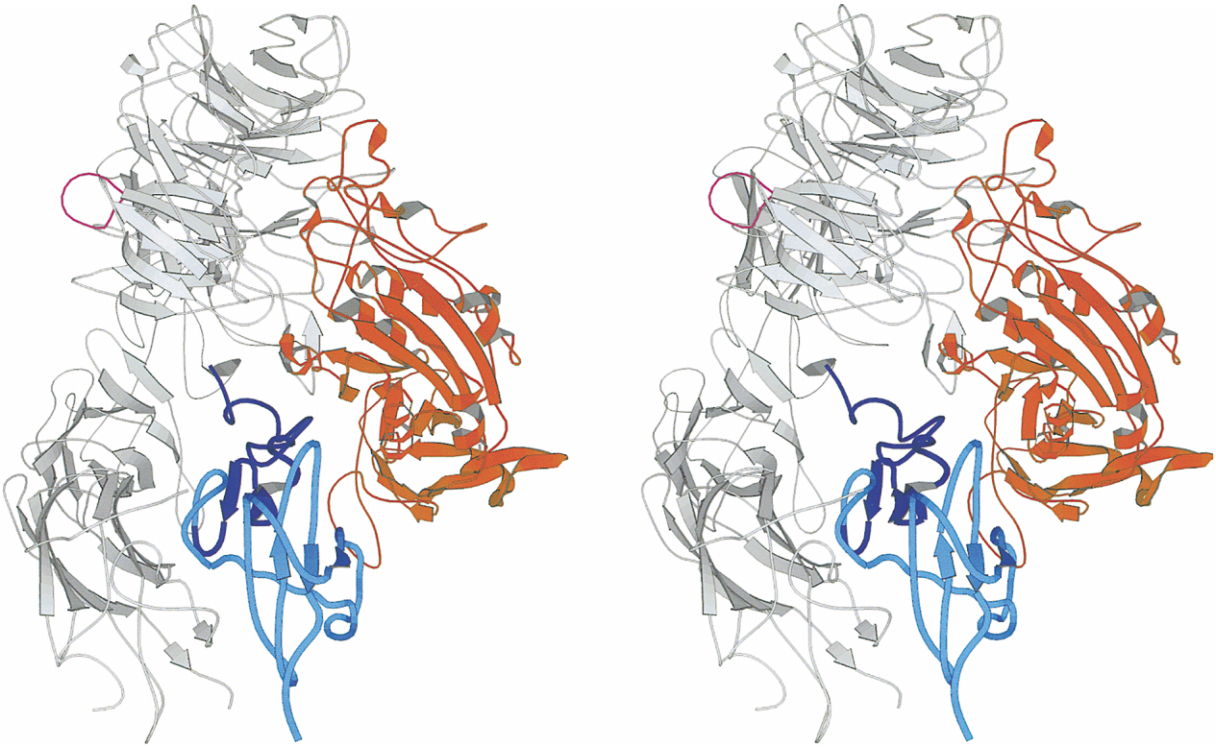

Fig. 3. Stereo pair diagrams of the tertiary conformation of $\beta_3$ integrin component of the portions of the $\alpha_V\beta_3$ integrin structure solved to a resolution of 3.1 Å [26]. As in Fig. 2, the $\alpha_V$ sequence is shown in grey. The $\beta_3$ integrin structure from reside 55 to residue 434 is shown in orange; the sequence from 532 to residue 690 is shown in blue. The lighter blue chain highlights recombinant Peptide 'D' studied by immunoblotting. The orientation of the heterodimer in Fig. 3 shows the immediate extracellular region (in blue) moved to the front in this view. (This sequence is included within the recombinant Peptide 'B' that was a focus of experimental immunologic analysis.)

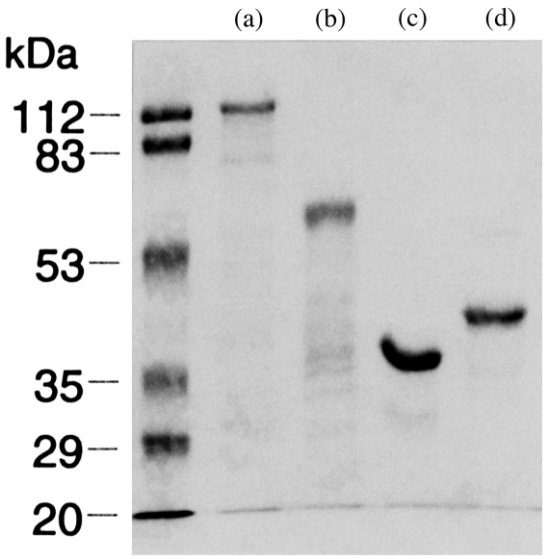


Fig. 4. The SDS-PAGE gel analysis of expressed and purified GPIIIa fusion proteins. Overnight cultures of *E. coli* DH5α transformed with pGEX-GPIIIa constructs were grown and induced as described under 'Methods.' Lane A: GST-GPIIIa-peptide 'A' (entire extracellular portion, residues 1-691), Lane B: GST-GPIIIa-peptide 'B', including GPIIIa residues 468-691. This region includes most of the 50 kDa chymotrypsin-resistant, disulfide-rich region. Lane 'C' and Lane 'D': GST-GPIIIa-peptide 'C' (residues 446–501) and peptide 'D' (residues 593–691). The position and size (kDa) of molecular weight markers are indicated.

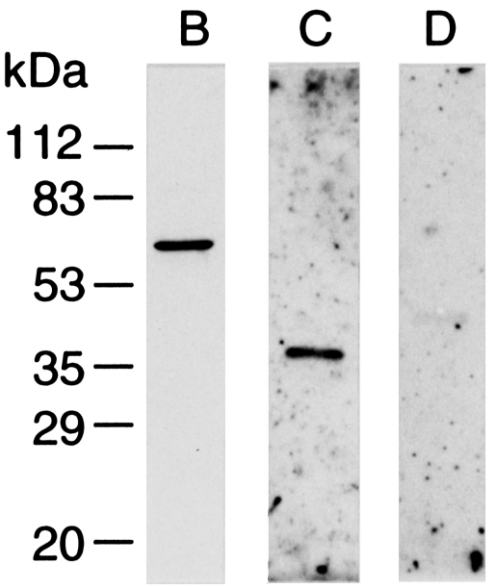


Fig. 5. Detection of antibody binding by immunoblotting performed as described under 'Methods.' In each lane, 2 μg of purified GPIIIa fusion proteins 'B', 'C' and 'D' can be detected by sera from ITP patients. Sera were selected for study on the basis of positive reactivity with the GPIIb/IIIa complex by the MAIPA antigen capture assay.

proteins (Peptide 'C' with residues 446–501 and Peptide 'D' with residues 593–691). Fig. 5 (lane 'C') illustrates immunoblotting results with one of the three sera that showed strong reactivity with Peptide 'C.' The other two antibodies that bound to Peptide'C' produced faint bands indicating very weak antibody binding. Fig. 6 lane 'D' shows one of five antibodies weakly reactive with Peptide 'D'. None of these antibodies reacted strongly with the most carboxy-terminal sequence that was studied. Fig. 6 summarizes the findings for the 24 sera tested. The smaller (56 and 98 residues) submolecular regions (Peptides 'C' and 'D') that were predicted to contain hydrophilic, surface-accessible sequences were found to comprise autoepitopes

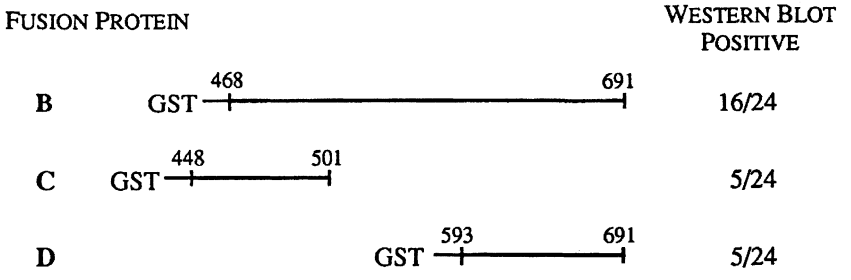


Fig. 6. Summary of immunoblotting studies using the fusion proteins containing GPIIIa peptides B, C and D.

for only a minority of antisera studied (5 of 24 against each.) With the exception of three potent antibody reactions with Peptide 'C,' all of the reactions of anti-GPIIIa autoantibodies with the smaller fusion proteins produced very weak bands on immunoblotting. All of the sera with antibodies that bound to Peptides 'C' or 'D' also had positive reactions with Peptide 'B.' Only one patient had antibodies that reacted with both fusion proteins 'C' and 'D,' suggesting that this patient's anti-GPIIIa autoantibodies were of broad specificity and likely to be polyclonal. The weak intensity of these bands on immunoblotting precluded reciprocal elution and rebinding experiments.

### *3.5. Platelets contain immunoglobulin within their alpha granules*

To implicate an antiplatelet antibody as causative of immune thrombocytopenia, the antibody would need to be directed against the surface of autologous platelets. Elution studies could only be performed if at least $10^9$ fresh platelets could be obtained. One patient who presented with mild thrombocytopenia and a positive direct MAIPA assay could be evaluated in this manner. As shown in Fig. 7, there was strong reactivity with the recombinant fusion protein 'B' that reacted more weakly with Peptides 'C' and 'D' indicating the presence of platelet surface bound anti-GPIIIa and suggesting a broad specificity for these anti-GPIIIa antibodies.

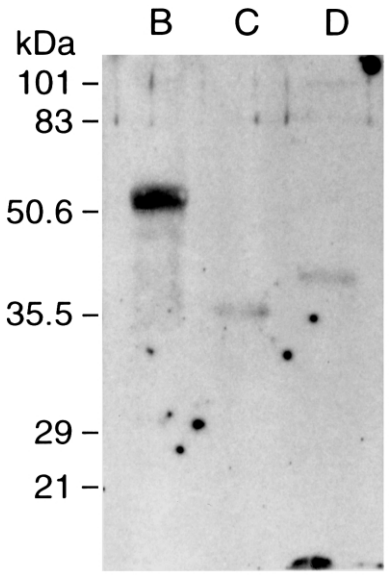


Fig. 7. Immunoblotting results of ether elution of autoantibodies from platelets obtained from a patient with ITP. Platelet preparation and ether elution of ITP antibody from platelet were performed as described under 'Methods.' This patient's platelet bound immunoglobulin was reactive with Peptides 'B', 'C' and 'D', although the strength of the reaction was strongest against the largest recombinant protein containing Peptide 'B'.

## 4. Discussion

The studies reported here indicate that the 'B' fusion protein containing residues in the disulfide rich portion of GPIIIa (468–691) immediately external to the proposed membrane spanning domain (residues 693–721) contains peptide target antigens for platelet autoantibodies from ITP patients. Moreover, when anti-GPIIb/IIIa antibody was eluted from platelets, the antibodies were reactive against the fusion protein containing Peptide 'B,' indicating that the platelet bound antibodies are extracellular and would be capable of opsonizing platelets in vivo. Unlike antibodies to the cytoplasmic portion of GPIIIa, autoantibodies to the surface portion of the molecule could be implicated as the cause of autoimmune platelet destruction in ITP.

We could not demonstrate antibody binding to the fusion protein containing the entire extracellular portion of GPIIIa (Peptide 'A'), suggesting that the recombinant fusion protein may have a different conformation than native GPIIIa contained in the platelet surface. Study of the conformation of $\alpha_V\beta_3$, a related integrin complex that includes the same β component (Figs. 2 and 3) reveals that the heterodimer component proteins are in contact over a large area. Studies by Beglova et al. [27] using solution NMR of the $\beta_2$ integrin indicate that the cysteine-rich repeats of the EGF2 and EGF3 are a region that undergoes a change in conformation upon activation of the integrin complex. It is possible that the isolated GPIIIa Peptide 'A' fusion protein folded in a conformation in which autoepitopes are not accessible. Peterson et al. produced a soluble form of recombinant GPIIb/

IIIa in a baculovirus expression system that reacted with each of the three ITP sera that were tested [14] suggesting that their recombinant heterodimer glycoprotein complex does form epitopes for anti-GPIIb/IIIa autoantibodies.

The GPIIIa fusion protein containing Peptide 'B' with residues 468–691 showed reactivity with 16 of 24 anti-GPIIb/IIIa autoantibodies that were studied. This peptide contains much of the disulfide-rich region of GPIIIa including EGF-like domains as well as the β tail domain that is situated at the platelet membrane. The 50 kDa chymotrypsin GPIIIa digestion fragment that was found to contain autoepitopes by Kekomaki et al. [16] consists of residues 427–654 that would include our Peptide 'B' except for 37 residues at the carboxy-terminal end of the β tail domain.

Initial attempts to localize platelet GPIIIa autoepitopes further were approached by creation of recombinant Peptides 'C' and 'D' selected because they consisted of sequences with hydrophilic profiles as shown in Fig. 1. Only 5 of 24 patient sera bound to each of these peptides, and reactivity was weaker as estimated from the faint bands on immunoblotting. In the X-ray structure of $\alpha_V\beta_3$ the sequence of GPIIIa Peptide 'C' occurs in the stretch with poorly defined structure from residue 435 to 531 [26]. Peptide 'D' includes much of the β tail domain of the $\alpha_V\beta_3$ conformation. This portion of the integrin complex is situated between the amino-terminal portion of $\beta_3$ and the α chain of the heterodimer and would be largely obscured from the surface as shown in the Fig. 3 stereo diagram.

McMillan et al. [15] have also prepared recombinant fusion proteins containing GPIIIa peptide sequences. They reported that only one of 33 ITP sera reacted with only one of the extracellular GPIIIa peptides (containing residues 350–550). It is not clear why their experiments gave so few positive results while we found frequent reactivity with Peptide 'B.' Possible explanations for the differences between McMillan's results and our own include the different sequences synthesized, the different sensitivities of the assay systems used (microtiter well-vs.-immunoblot), the different patient specimens studied, and the different expression vectors chosen. Both studies evaluated sera already shown to be reactive with the GPIIb/IIIa complex.

The current studies did not address the possible antigenicity of residues 501–593, the section between Peptides 'C' and 'D.' This region comprises the EGF3 and EGF4 domains of $\beta_3$ integrin. Based upon the more frequent and more potent antibody reactivity we observed with Peptide 'B' than with Peptide 'C' or 'D,' the EGF3 and EGF4 domains could contain epitopes for platelet autoantibodies. However, this region is not hydrophilic and has low predicted surface probability (see Fig. 1); therefore, it was not selected for this study.

## 5. Conclusions

Recombinant fusion proteins of the extracellular disulfide-rich region of GPIIIa were shown to react with 16 of 24 ITP patients' serum anti-GPIIb/IIIa antibodies. Specific sequences of GPIIIa containing residues 468–691, residues 446–501 and residues 593–691 were selected for study from predictions of their hydrophilic character, surface accessibility, and antigenicity index. Visualization of the conformation of the extracellular portion of $\alpha_V\beta_3$ reveals the location of these GPIIIa ($\beta_3$) peptide sequences in the three dimensional structure of the protein. Antiplatelet antibodies from 16 of 24 patients with ITP reacted with the 222 residue antigenic peptide 'B' (468–691) includes the β-tail domain and a series of four EGF-like domains. These results support the findings of Kekomaki et al. that a 50 kDa chymotryptic digestion product from this region of blood platelet GPIIIa contains target epitopes for serum antiplatelet antibodies in 16 of 33 ITP patients. We conclude that predictions of hydrophilicity, surface accessibility, and antigenicity correlate with observed autoantigen localization. Furthermore, the experimental finding of autoantibody binding to recombinant GPIIIa peptide 'B' containing residues 468–691 correlates with the three dimensional structure of the $\alpha_V\beta_3$ integrin that contains the same $\beta_3$ component as GPIIb/IIIa.

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