Expression and Purification of Functional Recombinant Epitopes for the Platelet Antigens, PIA¹ and PI²

By Emily A. Barron-Casella, Thomas S. Kickler, Ophelia C. Rogers, and James F. Casella

The platelet antigens, PIA¹ and PI², are responsible for most cases of posttransfusion purpura (PTP) and neonatal alloimmune thrombocytopenia (NAIT) in the causative population and are determined by two allelic forms of the platelet glycoprotein GPIIIa gene. To study the interaction between these antigens and their respective antibodies, we inserted a sequence that encodes the signal peptide and the N-terminal 66 amino acids of the PIA¹ form of GPIIIa into the expression vector pGEX1. To express the PI² antigen, nucleotide 196 of the PIA¹ coding sequence was mutated to the PI² allelic form. When transformed and induced in Escherichia coli, the two constructs produce glutathione S-transferase (GST)/N-terminal GPIIIa fusion proteins, one containing leucine at position 33 (PI²¹), the other proline (PI²²). These proteins are easily purified in milligram quantities using glutathione-Sepharose and react specifically with their respective antibodies by immunoblot and enzyme-linked immunosorbent assay. Antigenicity of the PI²¹ fusion protein in reduced glutathione increases with time; moreover, the addition of oxidized glutathione accelerates this process, presumably because of formation of the native disulfide bonds. Neutralization assays indicate that the PI²¹ fusion protein competes for all of the anti-PI²¹ antibody in the serum of patients with PTP and NAIT that is capable of interacting with the surface of intact platelets. This study shows that the GST/N-terminal GPIIIa fusion proteins contain conformational epitopes that mimic those involved in alloimmunization, and that regions other than the amino terminal 66 amino acids of GPIIIa are not likely to contain or be required for the development of functional PI²¹ epitopes. Furthermore, these recombinant proteins can be used for the affinity-purification of clinical anti-PI²¹ antibodies and specific antibody identification by western blotting, making them useful in the diagnosis of patients alloimmunized to PI²¹ alloantigens.

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From the Division of Hematology of the Department of Pediatrics, and Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD.

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Address reprint requests to James F. Casella, MD, Division of Hematology, Department of Pediatrics, Johns Hopkins University School of Medicine, Ross Bldg 1125, 720 Rutland Ave, Baltimore, MD 21205.

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of anti-PI\textsuperscript{A} antibodies by western blots. Using purified GST-PI\textsuperscript{A} fusion protein, we also have affinity-purified several examples of anti-PI\textsuperscript{A} antibodies from human serum, thus allowing the development of affinity-purified typing reagents. In addition, we have determined that the antigenicity of GST-PI\textsuperscript{A} and GST-PI\textsuperscript{B} fusion proteins is reduction sensitive, as are the native PI\textsuperscript{A} and PI\textsuperscript{B} epitopes. Methods for optimizing the antigenicity of the GST-PI\textsuperscript{A} fusion protein are described. We also show that the GST-PI\textsuperscript{A} fusion protein is capable of neutralizing essentially all of the IgG in the serum of patients with NAIT and PTP that is capable of interacting with the platelet surface. These findings suggest that the amino terminal 66 amino acids of GPII\textalpha retain all of the secondary structures required for recognition of the PI\textsuperscript{A} epitope(s) by clinical anti-PI\textsuperscript{A} antibodies used in our studies. It appears that additional regions of the protein are unlikely to be required for expression of the authentic PI\textsuperscript{A} epitope(s).

MATERIALS AND METHODS

Antibodies and Platelets

Anti-PI\textsuperscript{A} antibody was obtained from well-characterized antisera from patients with PTP\textsuperscript{1} and five patients who were typed as PI\textsuperscript{B} and delivered infants with NAIT.\textsuperscript{9} Human PI\textsuperscript{B} antisera was obtained from Dr E. Taaning (Glostrup Hospital, University of Copenhagen, Glostrup, Denmark). PI\textsuperscript{A} and PI\textsuperscript{B} immunoetry was performed using a radiolabeled antiglobulin test as previously described.\textsuperscript{8} Platelet gel samples were prepared as previously described.\textsuperscript{3} Anti-PI\textsuperscript{A1} antibody was obtained from well-characterized antisera (Amrad, Kew, Victoria, Australia). The basic chain reaction (PCR) fragment of GPII\textalpha was cloned into the pGEX\textsuperscript{I} expression vector (Amrad, Kew, Victoria, Australia). The fragment was inserted in-frame, maintaining the correct codons for the initiating methionine and second amino acid. The opposite described. Platelet gel samples were prepared as previously described.\textsuperscript{33} was amplified by PCR.\textsuperscript{*} The forward PCR primer (5'TAGAGC-

Expression and Purification

PGEXII\textalpha-PI\textsuperscript{A} and pGEXII\textalpha-PI\textsuperscript{B} were transformed into competent DH\textsubscript{5}\textalpha bacterial cells (GIBCO-BRL, Gaithersburg, MD). Single colonies were used to inoculate 100 mL ofuria broth (LB) medium containing 50 g/mL ampicillin and the cultures were grown overnight. The next day, the 100-mL cultures were added to 1 L of fresh LB with ampicillin and grown 2 hours. The expression of the fusion protein was induced by the addition of 0.1 mmol/L isopropl p-D-thiogalactopyranoside (IPTG). The cells were allowed to grow another 2 hours then harvested and resuspended in 10-mL ice-cold phosphate-buffered saline (PBS) with 10 mmol/L EDTA and 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF) to prevent protease activity. Cells were lysed by sonication with a microtip probe (Heat System Ultrasonics, Inc) for 1 minute. One hundred microliters of Triton X-100 was added to the lysate. After centrifugation (10 minutes at 5,000g), the supernatant was collected. Five milliliters of 50% slurry of glutathione-Sepharose 4B beads (Pharmacia, Piscataway, NJ) in PBS with 1% Triton X-100, pH 8.0, were added and incubated overnight at 4°C with gentle mixing. The beads with the captured GST-fusion proteins were poured into a 1 x 5 cm column and the nonspecifically bound material was washed away with 10 to 15 bed volumes of PBS containing protease inhibitors. The GST-GPIII\textalpha fusion proteins were eluted (1 mL/5 min) with 20 mmol/L reduced glutathione (GSH) and 50 mmol/L TRIS-HCl, pH 8.0. Protein concentrations were determined by the Bradford assay (BioRad Laboratories, Richmond, VA).\textsuperscript{13}

Affinity Purification of Anti-PI\textsuperscript{A} Antibodies

Anti-PI\textsuperscript{A1} antibody was purified from the plasma of a patient with PTP and from five patients with NAIT. A 1:1 x 3.5-cm Sepharose 4B-PI\textsuperscript{A} fusion protein affinity column was prepared by dialyzing 0.5 mg of the PI\textsuperscript{A} fusion protein against 500 mmol/L KCl, 10 mmol/L potassium phosphate, pH 8.0, after which the coupling reaction was performed according to manufacturer's instructions for CnBr-activated Sepharose 4B (Pharmacia). Five to twenty milliliters of diluted antiserum (1:1 antiserum to 10 mmol/L TRIS-HCl, pH 7.5) was passed over the column. The column was washed with 20 mL of 0.5 mmol/L NaCl, 0.2% Triton X-100, and 10 mmol/L TRIS-HCl, pH 8.0 followed by 0.1 mol/L sodium acetate, pH 5.0 until the absorbance at 280 nm of the eluate reached zero. The bound antibody was eluted with 1 mol/L acetic acid in 0.9-mL fractions into tubes containing 0.1 mL of 1 mol/L TRIS base. The peak fractions con-
Proteins were separated on 0.15-× 12- × 18-cm SDS-polyacrylamide gels (37:1 acrylamide to bis-acrylamide) with 5% stacking gels, pH 6.8, and 10% resolving gels, pH 8.8. Care was taken to heat the samples for precisely 1 minute at 100°C. Gels were run at a constant current of 30 to 35 milliamperes for 3 hours according to the method of Laemmli. After completion, the proteins were either stained with Coomassie Blue or transferred to a nitrocellulose membrane (Bio-Rad; 0.45 μm) for 2 hours at 0.7 amperes using 0.096 mol/L glycine, 0.01% SDS, 12.5 mmol/L TRIS-HCl, pH 8.3, as transfer buffer. The nitrocellulose filters were blocked by incubation in 5% to 10% dried milk, 150 mmol/L NaCl, 0.05% Tween-20, 10 mmol/L TRIS-HCl, pH 8.0, for 30 minutes at room temperature. To fresh blocking buffer, the specific antibody was added at a dilution of 1:1000 for affinity-purified anti-PIA1, 1:10 for PI A2 antisemum, and 1:5 for the anti-PIA2 antibodies from patients with NAIT. The filters were incubated at 4°C overnight and washed briefly several times with PBS containing 0.05% Tween-20, followed by two 15-minute and two 5-minute washes with the same buffer. Blots were incubated with a 1:10,000 dilution of recombinant protein A/G conjugated with horseradish peroxidase (Pierce, Rockford, IL), and again washed with PBS-Tween-20 as described above. Using enhanced chemiluminescence reagents (ECL; Amersham, Arlington Heights, IL), proteins recognized by the antibodies were detected and visualized on Hyperfilm-MP (Amersham).

Neutralization Assay

Increasing concentrations of recombinant GST-PIA1 or GST-PI A2 fusion protein (0.5 to 13 μg/ml) were added to PIA1 antiserum diluted to a titer of 1:100. After incubation at room temperature for 30 minutes, the mixtures were added to 2 × 107 PI A1 platelets. The amount of free-PI A1 antibody capable of binding to the platelets was determined by a radiolabeled antigenblin technique as previously described. The experiments for the patient with PTP were performed in solution, those for the patients with NAIT were performed in microtiter plates.

Results

Recombinant Fusion Proteins Contain Epitopes Recognized by Anti-PI A1 and Anti-PI A2 Antibodies

By SDS gel electrophoresis (Fig 1, left panels), the purified GST-PI A1 and GST-PI A2 fusion proteins migrate similarly with an approximate molecular mass of 37 kD. A
contaminating protein that comigrates with glutathione S-transferase (26 kD) and is recognized by anti-GST antibody copurifies with the fusion protein. When the sample is not exposed to a reducing agent, the fusion protein band is diffuse, presumably because of the multiple conformations created by disulfide bonds. Addition of a reducing agent results in a predominant, slower migrating band. This behavior appears to be similar to that of the native GPIIIa protein, where reduction results in loss of intramolecular sulfhydryl bonds and a higher apparent molecular weight on gels.

An immunoblot of the same samples (Fig 1, center panels) shows that affinity-purified anti-PIA1 antibodies from a patient with PTP recognize the 37-kD GST-PIA1 fusion protein and a band of the same size as GPIIIa (100 kD) from PIA1-positive platelet lysates. Reactivity is lost if the samples are exposed to a reducing agent. GPIIIa in the platelet lysate from a PIA2 individual and the GST-PIA2 fusion protein were recognized by the anti-PIA2 antibody (Fig 1, right panels).

The ELISA (Fig 2) confirms the results of the immunoblot. The affinity-purified anti-PIA1 antibody binds to the GST-PIA1 fusion protein, but reacts only minimally with the GST-PIA2 fusion protein. The affinity-purified antibody can be diluted greater than 1:1,000 (0.2 μg/mL) and still maintain good reactivity, as shown in Fig 2.

Immunoblots and ELISAs were performed with PIA1 antibody in both assays. Backgrounds were much lower with the affinity-purified antibody in both assays.

Factors Affecting Antigenicity

Effect of time and oxidant (GSSG). Although we expected that the reducing environment (20 mmol/L GSH) used to elute the protein would have an adverse effect on antigenicity, we noted that antigenicity spontaneously increased with time under these conditions (results not shown).

We hypothesized that some of the reduced glutathione might be oxidizing, allowing disulfides present in the oxidized glutathione to exchange with accessible sulfhydryls in the fusion protein. The combination of both oxidized and reduced glutathione has been shown to promote disulfide formation in other disulfide-bonded proteins, presumably after the formation of mixed disulfide bonds.

To investigate the effect of oxidation on antigenicity of the PIA1 fusion protein, we lowered the GSH concentration and added increasing amounts of the oxidant, GSSG, to freshly made protein preparations. The total glutathione concentration remained 20 mmol/L in each sample. As shown in Fig 3, increasing the GSSG/GSH ratio to 50:50 (10 mmol/L final concentration of GSSG and GSH) resulted in a progressive increase in anti-PIA1 reactivity with the GST-PIA1 fusion protein, to a level approximately three times that of the original material in 20 mmol/L GSH. The reactivity between anti-PIA1 antibodies and the PIA1 fusion protein was small and did not change appreciably with GSSG treatment. When samples were treated with a 40:60 ratio of GSSG:GSH for different lengths of time (Fig 4), a steep increase in reactivity occurred within the first 30 minutes, after which a gradual increase was seen up to 19 to 22 hours. The spontaneous increase in antigenicity in 100% GSH appeared to occur over days.

Other factors affecting antigenicity. In our initial experiments, we observed some variability of antigenicity on immunoblots as compared with ELISAs. An examination of the methods used to prepare samples for electrophoresis disclosed that the recombinant protein showed different requirements for antigenicity on gels than the intact native protein from platelets. For example, antigenicity of the recombinant protein was almost completely destroyed by boiling for greater than 2 minutes in 1% SDS. Alternatively, the same protein had excellent reactivity on nondenaturing polyacrylamide gels or on SDS-polyacrylamide gels after heating to
Antibodies From Patients With NAIT Can Be Distinguished by Their Interactions With GST-PIA1 and GST-PIA2

To further determine the potential usefulness of the recombinant proteins in the diagnosis of NAIT, affinity-purified antibodies from five patients with NAIT were examined using Western blots. As shown in Fig 5C, antibodies from all five patients showed reactivity with GST-PIA1 and not with GST-PIA2.

DISCUSSION

We have expressed and purified two GST fusion proteins containing the 26 amino acid signal sequence and 66 amino acid N-terminus of the GPIIIa protein. One of the fusion proteins is recognized by anti-PIA1 antibodies, the other reacts with anti-PIA2 antibodies. The only difference between the two proteins is the amino acid at position 33; leu33 is present in the GST-PIA1 fusion protein and pro33 in the GST-PIA2 fusion protein. The similarity of the PIA1 epitope to that of native GPIIIa on the surface of platelets is established by the ability of the recombinant protein to compete for essentially all of the antiplatelet antibodies in antisera from patients with both PTP and NAIT. This also suggests that all of the structural elements required for recognition of the PIA1 epitope or epitopes are contained in the amino terminal 66 amino acids of GPIIIa.

Other expression systems have been used to produce PI\textsuperscript{A} alloantigenic epitopes. Bowditch et al.\textsuperscript{7} restricted the PIA1 epitope in \textalpha\textsuperscript{GTP}22 to the 26 amino acid signal sequence and 66 amino acid N-terminus of GPIIIa. Anti-PIA1 antibodies recognized the resulting \textbeta-galactosidase-GPIIIa fusion protein containing leu33, but not pro33. It was not determined whether the same domain expressing pro33 could be recognized by anti-PIA2 antibodies and thereby define the PIA2 epitope. This expression system was designed to identify PIA1 epitope(s), but does not produce the recombinant proteins in large quantities. A mammalian system has also been used to express PIA\textsuperscript{A} and PIA\textsuperscript{B} forms of GPIIIa in COS-1 cells and these recombinant proteins can be immunoprecipitated by their respective alloantibodies.\textsuperscript{19} However, because other alloantibodies and autoantibodies recognize GPIIIa, these intact proteins are limited in their ability to characterize the specificity of clinical antibodies.

Attempts to make synthetic peptides that are recognized by anti-PIA\textsuperscript{A} antibodies from patients with NAIT and PTP have been unsuccessful. A 13-amino acid peptide that included the leu33 site did not bind anti-PIA\textsuperscript{A} antibodies.\textsuperscript{7} In another study, monoclonal antibodies made against synthetic peptides spanning the polymorphism recognized GPIIIa-PIA\textsuperscript{A} and GPIIIa-PIA\textsuperscript{B} after reduction, yet failed to react with the native epitopes.\textsuperscript{8} These observations suggest that the clinical antibodies recognize different conformations of GPIIIa, and not simply the primary structure. This is also supported by the fact that both epitopes are reduction sensitive.

Determination of PIA\textsuperscript{A} and PIA\textsuperscript{B} conformations must be caused by the unique properties of the two amino acids associated with position 33. Leucine is a hydrophobic amino acid, whereas proline is noted for its restricted flexibility that often creates bends in proteins. The conformational changes induced by these differing primary structures appear to be stabilized by the formation of sulfhydryl bonds. The observation that the recombinant proteins show significant differences in chemical stability with heating suggests that regions of the protein not included in the fusion protein still have...
**Fig 5.** (A) Inhibition of PI\(^{A1}\) antiserum by GST-PI\(^{A1}\). The ability of the recombinant proteins GST-PI\(^{A1}\) (■) and GST-PI\(^{A2}\) (■) to inhibit binding of antibodies from the antiserum of a patient with PTP to a fixed number of platelets was assayed as described in Materials and Methods. Each data point represents the average of results from two identical experiments performed on different days. (B) The same assay was used to examine the binding of antibodies from the antisera of five patients with NAIT. PI\(^{A1}\) samples are shown with solid lines, PI\(^{A2}\) with dotted lines. Each data point represents the average of three replicate samples. (C) Western blots using affinity-purified antibodies prepared as described in Materials and Methods from the same five patients with NAIT. Lanes containing the GST-PI\(^{A1}\) protein are labeled PI\(^{A1}\), those containing the GST-PI\(^{A2}\) protein are labeled PI\(^{A2}\). Only the region of the 37-kD fusion protein is shown. No signal was seen in the region of the 26-kD protein, indicating lack of reactivity with the GST segment alone.

Important effects on the stability of the native protein. Thus, for example, the sulfhydryl linkage between the cysteines at position 5 and 435 may be biologically important in conferring stability or otherwise inducing subtle conformational changes in the PI\(^{A1}\) epitope(s), despite the fact that it appears that the residues in the region of the cysteine at position 435 are not essential for antibody recognition.

The success of our expression system is most likely due to mild purification conditions and favorable conditions for reconstituting normal sulfhydryl bonds. No denaturant is necessary for solubilizing the recombinant proteins. The GST portion of the proteins enables one to easily capture the material on glutathione-Sepharose and purify the fusion protein away from most bacterial proteins. After elution with buffer containing GSH, GSSG can be added to the protein preparation to rapidly increase its antigenicity. We assume that the mixture of GSH/GSSG helps reshuffle incorrectly formed disulfide bonds, resulting in structure similar to the native PI\(^{A1}\) epitope(s).

The potential diagnostic usefulness of the recombinant PI\(^{A}\) antigens is shown by the specificity of the immunoblotting experiments and the neutralization assays described herein. The availability of both PI\(^{A1}\) and PI\(^{A2}\) recombinant proteins could obviate the need for acquisition of both PI\(^{A1}\) and PI\(^{A2}\) platelets to insure specificity of reactions in clinical assays.

Especially important to improved clinical diagnosis is the ability to purify anti-PI\(^{A1}\) typing reagents. For example, the fusion proteins exclude the region of the Pen (HPA-4) alloantigen system, which is also located on GPIIIa. The removal of contaminating HLA antibodies, a frequent occurrence, and absence of other specificities should help to avoid erroneous immunophenotyping results. We have not yet developed the ELISA to the point that it is as sensitive and specific as western blots for clinical use; however, this may be possible with further purification of the recombinant antigens.

As described above, we have used the GST-PI\(^{A1}\) fusion protein to purify several examples of anti-PI\(^{A1}\) antibodies from the sera of patients with PTP and NAIT. Similar purification and characterization of anti-PI\(^{A2}\) antibodies as well as further restriction of the domains required for anti-PI\(^{A}\) recognition should be feasible. Use of the recombinant proteins as immunogens is also possible. These reagents should prove to be useful in future studies of the pathogenesis, diagnosis, and treatment of NAIT and PTP.

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REFERENCES


Expression and purification of functional recombinant epitopes for the platelet antigens, PlA1 and PlA2

EA Barron-Casella, TS Kickler, OC Rogers and JF Casella