An Amino Acid Polymorphism within the RGD Binding Domain of Platelet Membrane Glycoprotein IIIa Is Responsible for the Formation of the Pen^a/Pen^b Alloantigen System

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Abstract

The human Pen^a/Pen^b alloantigen system represents a naturally occurring polymorphism of human platelet membrane glycoprotein (GP) IIIa, and has previously been implicated in the onset of two important clinical syndromes, neonatal alloimmune thrombocytopenic purpura and posttransfusion purpura. To investigate the molecular basis of the polymorphism underlying the Pen alloantigen system, we used the polymerase chain reaction to amplify platelet-derived GPIIIa mRNA transcripts. DNA sequence analysis of amplified GPIIIa cDNAs from nucleotides 161 to 1341 (encompassing amino acid residues 22–414) revealed a $G_{526} \leftrightarrow A_{526}$ polymorphism that segregated precisely with Pen phenotype in twelve other individuals examined. This nucleotide substitution results in an Arg (CGA) to Gln (CAA) polymorphism at amino acid 143 of GPIIIa. Interestingly, this polymorphic residue is located within the putative RGD binding site (residues 109–171) of GPIIIa. Platelet aggregation patterns of a Pen$^{b/b}$ individual, however, were nearly normal in response to all physiological agonists tested, indicating that this polymorphism does not grossly affect integrin function. Short synthetic peptides encompassing residue 143 were unable to mimic either the Pen^a or Pen^b antigenic determinants, suggesting that the Pen epitopes are dependent upon proper folding of the polypeptide chain. Finally, we constructed allele-specific recombinant forms of GPIIIa that differed only at amino acid residues 143. Whereas anti-Pen^a alloantibodies were able to recognize the Arg_{143} recombinant form of GPIIIa, anti-Pen^b antibodies were not. Conversely, anti-Pen^b alloantibodies were reactive only with the Gln_{143} isoform of the GPIIIa molecule. It thus appears that amino acid 143 of GPIIIa is not only associated with Pen phenotype, but specifically controls the formation and expression of the Pen alloantigenic determinants. (*J. Clin. Invest.* 1992. 90:2038–2043.) Key words: platelet antigens • neonatal alloimmune thrombocytopenic purpura • polymerase chain reaction • integrins • platelet RNA

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Introduction

The human platelet membrane glycoprotein (GP)[1] IIb-IIIa complex is a member of the integrin family that is involved in adhesive interactions. In stimulated platelets, GPIIb-IIIa mediates platelet aggregation by serving as the receptor for fibrinogen and von Willebrand factor. The binding of these adhesive proteins to GPIIIa partly depends on their Arg-Gly-Asp (RGD) sequence which interacts with residues 109–171 of GPIIIa (1).

In addition to its physiological role, both GPIIb and GPIIIa are known to bear a number of clinically important alloantigenic determinants that can induce an alloimmune response in two immunopathologic syndromes, posttransfusion purpura (PTP) and neonatal alloimmune thrombocytopenic purpura (NATP) (2). To date, three different alloantigen systems have been localized to the GPIIb-IIIa complex, including Pl^A, Bak, and Pen. The molecular basis of the Pl^A and Bak system has been previously solved, an isoleucine to serine substitution at amino acid 843 of GPIIb that is responsible for Bak, and a leucine to proline dimorphism at amino acid 33 of GPIIIa for Pl^A (for a review, see reference 3).

The Pl^A-associated polymorphism is most often implicated in causing NATP and PTP in the Caucasian population. However, the Pen alloantigen system is a more frequent cause of both of these alloimmune diseases in Asian individuals. In 1985, Friedman and Aster (4) reported the first description of the Pen alloantigen system in two cases of NATP, and showed that it was likely to be localized to either GPIIb or GPIIIa. Subsequently, Shibata et al. (5) reported an alloantigen system, designated Yuk, responsible for two cases of NATP in Japan. A case of PTP associated with the Pen alloantigen system was later reported by Simon et al. (6). The localization of the Yuk and Pen alloantigens to GPIIIa was demonstrated by Shibata et al. (7) and by Furihata et al. (8), respectively, and together with serological data, it has been widely supposed that these two alloantigen systems may be identical.

Anti-Pen^a alloantibodies completely inhibit ADP-induced platelet aggregation (8), suggesting that the region of GPIIIa containing the Pen epitope may lie within a functionally important region of this membrane glycoprotein complex. Analysis of proteolytic fragments of GPIIIa have shown that the Pen^a determinant is lost from GPIIIa coincident with the removal of a 30-kDa fragment of GPIIIa by chymotrypsin (9). Subsequent

1. *Abbreviations used in this paper:* ASOH, allele-specific oligonucleotide hybridization; GP, glycoprotein; NATP, neonatal alloimmune thrombocytopenic purpura; PCR, polymerase chain reaction; PTP, posttransfusion purpura.

studies performed by Niewiarowski et al. (10) and Beer and Coller (11) have demonstrated the presence of a large disulfide-bonded loop in GPIIIa that is cleaved by chymotrypsin at residues 121 and 348. This region likely comprises the 30-kDa fragment removed from GPIIIa by chymotrypsin. Taken together, we have previously hypothesized that the Pen epitopes may be localized to the region bounded by GPIIIa residues 121–348 (3). In this report, we provide evidence that an amino acid polymorphism within the RGD binding domain of GPIIIa is responsible for the expression of the Pen^a/Pen^b alloantigen system.

Methods

Amplification of platelet mRNA. Platelet mRNA was prepared from whole blood of individuals of known Pen serological phenotypes according to methods described previously (12, 13). Two sets of primers were designed to amplify the region of GPIIIa mRNA from bases 161–1341 (encoding amino acid residues 22–414). The first set of primers consisted of a forward primer 161–183 (5′-CATGTGTGCCTGGTGCTCTGATG-3′) and a reverse primer 698–674 (5′-CACCTGGTCAGTTAGCGTCAGCACG-3′), while the second primer pair was made up of a forward primer from bases 633–658 (5′-GATATGAAGACCACCTGCTTGCCCAT-3′) and a reverse primer from nucleotides 1341–1315 (5′-TAAAGGACTTCTCCTTCTCCTGGGGAC-3′). PCR amplification was carried out in a DNA Programmable Thermal Controller (MJ Research, Inc., Watertown, MA) programmed to denature at 94°C for 1.5 min, anneal at 50°C for 1.5 min, and chain extend at 72°C for 3 min. The reaction was allowed to proceed for 30 cycles followed by a final chain extension at 72°C for 7 min to allow completion of strand synthesis. In some cases, two 25-bp primers flanking exon III (nucleotides 13622 to 14134) of the GPIIIa gene were used to amplify this region from genomic DNA that was isolated from peripheral whole blood.

Analysis of PCR products. The resulting PCR products were subcloned into plasmid vector pGEM-5Zf (Promega Corp., Madison, WI) and the inserts sequenced by the dideoxy chain termination method using Sequenase T7 DNA polymerase (US Biochemical Corp., Cleveland, OH). In addition, PCR products were directly sequenced using the modified method of Bachmann et al. (14). Briefly, glass-milk purified PCR products (Bio 101, Inc., La Jolla, CA) were resuspended in 5× Sequenase buffer, 16–20 pmol of sequencing primer, and 0.4 μl 10% Nonidet P-40 in a final vol of 10 μl. DNA was denatured by boiling for 5 min, followed by immediate cooling in a dry ice bath. Sequencing reactions were performed by using diluted dGTP labeling mix in a 5-min labeling reaction at room temperature followed by a 1-min chain termination reaction at 45°C. Amplified PCR products from both genomic DNA and cDNAs were also subjected to allele-specific hybridization analysis using two 13-base allele-specific oligonucleotide probes (13). Probe A (5′-AGATGCGAAAGCT-3′) corresponds to the published sequence of $GPIIIa_{520-532}$, while probe B is nearly identical (5′-AGATGCAAAAGCT-3′) differing only in the middle base that distinguishes the Pen^a versus Pen^b alleles of GPIIIa (see Results).

Peptide competition and direct binding assays. Two 7-amino acid peptides encompassing amino acid 143 of GPIIIa, TQMRKLT specific for Pen^a and TQMQKLT specific for Pen^b, were synthesized at the Blood Research Institute Peptide Synthesis Core Facility. For the competition assay, 0.5 μg/well of the anti-GPIIIa monoclonal antibody, AP3, was plated overnight at 4°C in microtiter wells, and used to capture GPIIIa from Triton X-100 detergent lysates prepared from the platelets of a serologically defined $Pen^{a/b}$ individual. Preliminary studies using different dilutions of anti-Pen^a and anti-Pen^b alloantisera established that a 1:12 dilution gave 80–90% maximal binding to the AP3-captured GPIIIa. Therefore, this dilution was used in all future studies. 50 μl of either anti-Pen^a or anti-Pen^b alloantisera, diluted 1:12, was preincubated in the presence or absence of these two peptides at concentrations ranging from 0.05 to 5.0 mM at room temperature for 1 h, and then added to the wells containing immobilized GPIIIa. After a 1-h incubation at room temperature, the binding of alloantibodies to the GPIIIa was detected using biotinylated HB43 (mouse anti-human IgG antibody) and avidin/biotinylated alkaline phosphatase complex (ABC reagent; Vector Laboratories, Inc. Burlingame, CA), according to manufacturer's directions. Bound HB43 was quantitated by measuring the OD at wavelength of 405 nm in an ELISA reader.

For the direct binding assay, 0.05–0.2 μg of these two peptides dissolved in 100 μl of carbonate buffer (pH 9.6) were plated directly in microtiter wells according to the method described by Flug et al. (15). After blocking unbound sites with PBS containing 0.05% Tween 20 and 1% gelatin, alloantibodies (50 μl of a 1:12 dilution of serum) were added to the wells and their binding was detected using biotinylated HB43 as described above. Several different peptides to which we had available specific anti-peptide antibodies were also plated under identical conditions and used as positive controls. Each of these control reactions yielded ODs of > 1.0 (not shown).

Construction of allele-specific recombinant forms of GPIIIa. Allele-specific recombinant forms of GPIIIa were produced using a full length GPIIIa cDNA (Arg_{143} form), the internal EcoRI restriction site of which had been removed by site-directed mutagenesis (kindly provided by Dr. Gilbert C. White II, The University of North Carolina, Chapel Hill, NC). A 261-bp fragment encompassing polymorphic base 526 was produced by digestion of amplified platelet cDNA derived from a $Pen^{b/b}$ individual using Xmn I and Bbv II. This fragment was then shuttled into the Bluescript vector containing $GPIIIa/Arg_{143}$ cDNA which had been digested with the same enzymes. Substitution of A for G at base 526, as well as junctional sequences that had been modified to produce recombinant GPIIIa isoforms, were confirmed by nucleotide sequence analysis. Finally, both the Arg_{143} and Gln_{143} cDNA constructs were subcloned into the EcoRI cloning site of the mammalian expression vector EMC-3 (generously provided by Dr. Glenn Larsen, Genetics Institute, Cambridge, MA). Plasmids used for subsequent transfection studies were purified by banding twice through CsCl gradients.

Expression and analysis of recombinant GPIIIa. Transfection of COS-7 cells with allele-specific recombinant forms of GPIIIa was performed using the DEAE-dextran method as described previously (16). Proteins synthesized by the transfected COS cells were metabolically labeled with [^{35}S]methionine overnight. ^{125}I surfaced-labeled Triton X-100 platelet extracts derived from $Pl^{A1/A1}$ and $Pen^{a/a}$ individual were used as positive controls. Recombinant GPIIIa molecules synthesized by COS cells were immunoprecipitated using a monoclonal anti-GPIIIa antibody, AP3 (not shown), as well as anti-Pen^a, anti-Pen^b, and anti-Pl^{A1} human alloantisera. The samples were analyzed on 7%–9% SDS-PAGE gel under reducing conditions.

Platelet and COS cell lysates. The Triton-treated platelet lysates were prepared as described previously in the presence of EDTA and used in the ELISA (8). ^{125}I-surface-labeled platelets were lysed in a buffer containing 20 mM Tris, pH 7.4, 100 mM NaCl, 1% Triton X-100, 10 mM *N*-ethyleneimide, 2 mM phenylmethylsulfonyl fluoride. (PMSF), and 100 μg/ml leupeptin. COS cells were lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 2 mM PMSF.

Results

Amplification and analysis of nucleotides 161–1341 of GPIIIa. Based upon our prediction that the region between residues 121 and 348 of GPIIIa might carry the Pen epitopes, we used two sets of primers to amplify platelet GPIIIa cDNA from nucleotides 161–1341 (encoding amino acid 22–414). The first 537 nucleotides in this region was PCR-amplified from platelet mRNA of known Pen phenotypes using primers that bounded bases 161–698, and as predicated, the major band obtained was 537 bp long (Fig. 1). Nucleotide sequence analysis of the 537-bp fragment derived from known $Pen^{a/a}$ and $Pen^{b/b}$ platelets

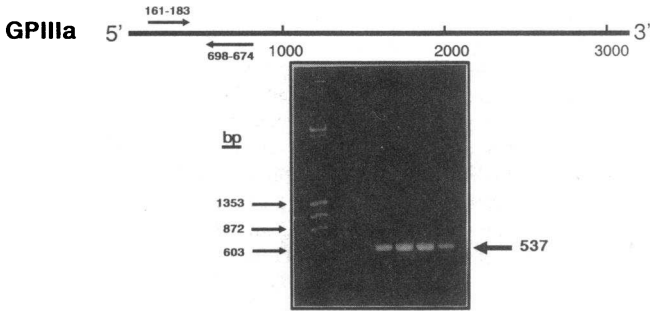

Figure 1. Amplification of nucleotides 161–698 of human platelet membrane GPIIIa mRNA. The locations of the two oligonucleotide primers used for PCR-amplification of the GPIIIa mRNA transcript are illustrated above. cDNA synthesized from platelet mRNA derived from a known $Pen^{b/b}$ individual was amplified by PCR and analyzed in 4 lanes of a preparative 1.4% agarose gel stained with ethidium bromide. The resulting 537-bp-long product (*arrow*) was subsequently eluted from the gel and used for nucleotide sequence analysis (*Fig. 2, below*). DNA size standards (Drigest III; Pharmacia Diagnostics, Inc., Silver Spring, MD) are shown at the left.

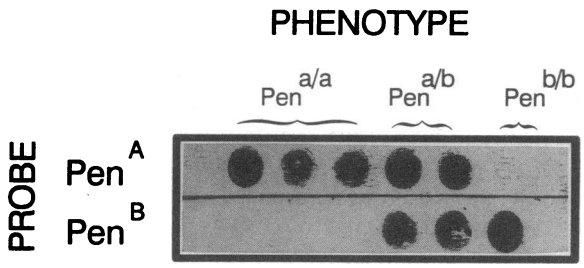

Figure 3. Dot-blot hybridization analysis of the Pen^a and Pen^b allelic forms of GPIIIa. Platelet RNA was converted into cDNA and PCR-amplified as described in Methods. 10 μl of each PCR reaction product was then spotted equally onto two nylon membranes which were then probed with either of two 13-base allele-specific oligonucleotide probes that differed only in the middle base, as detailed in Methods. Platelet mRNA derived from $Pen^{a/a}$ homozygous individuals hybridized to only the Pen^a-specific DNA probe, while RNA from a $Pen^{b/b}$ individual bound only the Pen^b probe. Platelet RNA from two serologically heterozygous individuals reacted with both probes equally, as predicted.

revealed a single nucleotide substitution from G ↔ A at base 526 of GPIIIa (Fig. 2). A second set of primers were used to amplify a 708-bp fragment bounded by nucleotides 633–1341, but no further nucleotide differences between Pen^a and Pen^b individuals were observed (data not shown).

To examine whether the polymorphism at base 526 segregated with the phenotypes of known serologically determined individuals, we performed direct PCR sequencing as well as allele-specific oligonucleotide hybridization (ASOH) analysis using 13 base oligonucleotide probes that differed only in the central nucleotide (13). PCR amplification products used in these experiments were derived from both amplified genomic DNA and amplified cDNAs from 10 $Pen^{a/a}$, 2 $Pen^{a/b}$, and 1 $Pen^{b/b}$ individuals. ASOH analysis of 6 representative amplified cDNA samples is shown in Fig. 3, and further confirms that the single base substitution at base 526 of GPIIIa segregates precisely with Pen phenotype.

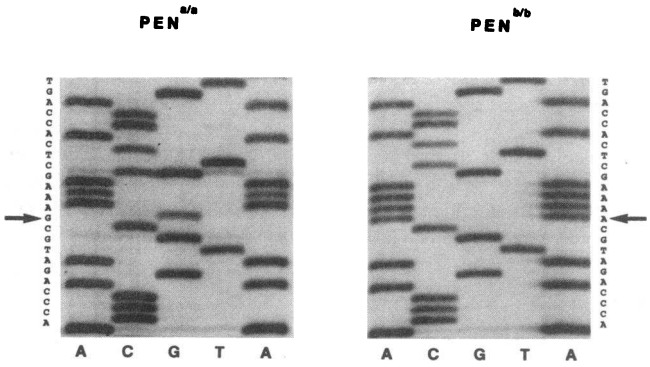

Figure 2. DNA sequence analysis of amplified GPIIIa cDNAs from $Pen^{a/a}$ and $Pen^{b/b}$ individuals. The region from GPIIIa 516–539 is shown. The sequence of the $Pen^{a/a}$ individual is identical to the previously published sequence for GPIIIa, having a G at base 526. The G/C compression seen in this gel following the three consecutive $A^{'s}$ (bases 527–529) was resolved by sequencing the antisense strand (not shown). The sequence derived from the $Pen^{b/b}$ homozygous individual revealed a A instead of G at base 526. This single base change results in a Gln (CAA) for Arg (CGA) substitution at amino acid 143 of GPIIIa. The polymorphic nucleotide is indicated with arrows.

Effect of the Pen dimorphism on platelet function. The G_{526} ↔ A_{526} nucleotide substitution present in $Pen^{b/b}$ individuals manifests itself as an Arg (CGA) ↔ Gln (CAA) polymorphism at amino acid 143 of GPIIIa. Interestingly, residue 143 is located within the putative RGD binding site of GPIIIa molecule (1). Therefore, to examine whether this amino acid substitution might affect the function of GPIIb-IIIa, we performed aggregation studies using platelets derived from both a $Pen^{a/a}$ (Arg_{143}) homozygous and a $Pen^{b/b}$ (Gln_{143}) homozygous individual using ADP, arachidonic acid, thrombin, epinephrine, collagen, and ristocetin as agonists. The platelet aggregation response in the $Pen^{b/b}$ individual was diminished by ~ 20% in response to all agonists tested (not shown). Interpretation of these findings as a direct effect of the polymorphism is complicated by the fact that the $Pen^{b/b}$ individual used in our study has chronic renal failure requiring hemodialysis, and is unfortunately the only homozygote known in North America. Thus, it is possible that the mild decrease in the rate and extent of platelet aggregation in this individual may be caused by the uremia rather than the Pen^b phenotype of GPIIIa.

Interaction of synthetic peptides with anti-Pen^a and anti-Pen^b alloantibodies. Two 7-amino acid peptides that differ only at amino acid 143, TQMRKLT specific for Pen^a and TQMQKLT specific for Pen^b, were examined for their ability to mimic the Pen^a and Pen^b epitopes of GPIIIa. As shown in Fig. 4, neither peptide, at concentrations up to 5 mM, was able to inhibit the binding of anti-Pen^a or anti-Pen^b alloantibodies to immobilized GPIIIa derived from $Pen^{a/b}$ platelets. In addition, as shown in Fig. 5 neither anti-Pen^a nor anti-Pen^b alloantibodies preferentially bound to allele-specific peptides that had been plated directly in the plastic wells. Thus, short synthetic peptides seem unable to mimic the structure of the Pen epitopes.

Immunoprecipitation analysis of recombinant GPIIIa alloforms. To examine the involvement of amino acid 143 in the formation of the Pen^a and Pen^b antigenic determinants, we

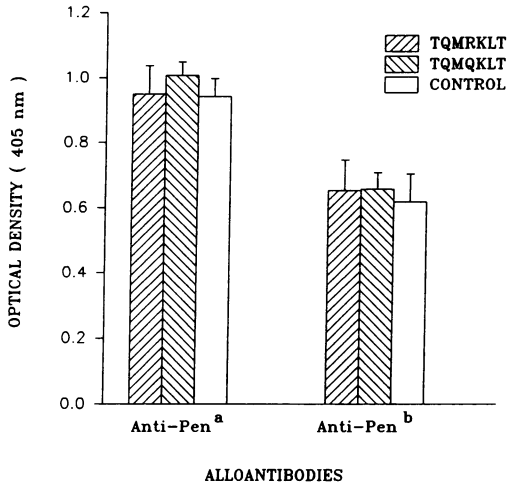

Figure 4. Effect of allele-specific synthetic peptides on alloantibody binding to immobilized GPIIIa. Triton solubilized platelet lysates derived from a $Pen^{a/b}$ individual were incubated in microtiter wells that had been precoated with the anti-GPIIIa monoclonal antibody, AP3. Following antibody capture of GPIIIa, the wells were blocked with PBS/Tween 20/gelatin. Anti-Pen^a and anti-Pen^b alloantibodies were preincubated with either of two allele-specific peptides (TQMRKLT specific for Pen^a, and TQMQKLT specific for Pen^b, 5 mM final concentration) for 1 h at room temperature and then added to the GPIIIa-containing wells. Peptide concentrations of up to 5 mM had no effect on the ability of anti-Pen^a or anti-Pen^b alloantibodies to bind to the immobilized GPIIIa. Control indicates the level of reactivity of alloantibody binding in the absence of peptide. Normal human serum gave OD readings of less than 0.15. Data are expressed as the mean±SD (n = 3).

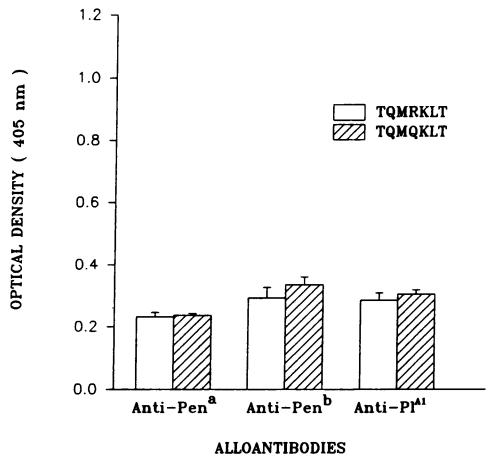

Figure 5. Direct binding of anti-Pen alloantibodies to immobilized allele-specific synthetic peptides. Either TQMRKLT (*open bars*) or TQMQKLT (*hatched bars*) were incubated in microtiter wells at a concentration of up to 2 μg/ml. Unbound sites were blocked with PBS/Tween 20/Gelatin as described in Methods, and then 50 μl of anti-Pen^a, anti-Pen^b, or anti-Pl^{A1} alloantibodies were added to the wells and their binding detected using the biotinylated monoclonal anti-human IgG antibody, HB43. Neither anti-Pen^a nor anti-Pen^b demonstrated specific reactivity with the allele-specific peptides, as compared with the binding of an anti-Pl^{A1} alloantibody control. Normal human serum gave readings between 0.1 and 0.2 OD units. Data are expressed as the mean±SD (n = 3).

constructed allele-specific recombinant forms of GPIIIa that differed only at residue 143, and analyzed them in a mammalian cell transfection system. Whereas anti-Pl^{A1} alloantibodies bound equally well to either the Arg_{143} or Gln_{143} isoforms of the GPIIIa molecule, anti-Pen^a antisera reacted with only the Arg_{143} allelic form (Fig. 6 *A*), while anti-Pen^b immunoprecipitated only the Gln_{143} form (Fig. 6 *B*). These results demonstrate that amino acid 143 directly controls the expression of the Pen epitopes, and that the observed dimorphism is not limited to a simple genetic association.

Discussion

The purpose of the present study was to characterize, at a molecular biological and biochemical level, polymorphisms of human platelet GPIIIa that might be responsible for the immunogenicity of the previously described Pen^a/Pen^b platelet alloantigen system. Although the Pen^b phenotype is rare in the Caucasian population, its frequency among Asian individuals is 5–10-fold higher, and appears to be responsible for a majority of the PTP and NATP cases seen in Japan. We used a combination of platelet RNA PCR techniques, nucleotide sequencing, ASOH, synthetic peptide inhibition analysis, and mammalian transfection techniques to elucidate the molecular etiology of this clinically important platelet immunological disorder.

Nucleotide sequence analysis of GPIIIa mRNA transcripts derived from the platelets of $Pen^{a/a}$ and $Pen^{b/b}$ individuals revealed a single nucleotide substitution at base 526. Subsequent analysis of PCR-amplified genomic DNA using a combination of direct nucleotide sequencing and ASOH analysis demonstrated that this nucleotide substitution segregated perfectly with the serologically determined phenotype of a number of $Pen^{a/a}$, $Pen^{a/b}$, and $Pen^{b/b}$ individuals. The molecular basis for this platelet membrane glycoprotein polymorphism, therefore, is similar to the previously solved Pl^A and Bak alloantigen systems in that it represents a naturally occurring, genetically inherited allelic variant of the GPIIIa molecule, rather than a postsynthetically acquired antigenic determinant.

The $G_{526} \leftrightarrow A_{526}$ polymorphism associated with Pen serologic phenotype results in an $Arg_{143} \leftrightarrow Gln_{143}$ dimorphism that is located within a large loop of GPIIIa formed by disulfide bonding of residues Cys_5 and Cys_{435} (17). D'Souza et al. (1) have identified a 63-amino acid region within this loop (residues 109–171) that interacts with the tripeptide sequence, RGD, that is present in many adhesive protein ligands, including fibrinogen, fibronectin, and von Willebrand factor. The physiological relevance of this region of GPIIIa is underscored by the recent finding that an $Asp_{119} \leftrightarrow Tyr_{119}$ substitution discovered in a Glanzmann thrombasthenia variant (CAM) abolishes the ligand binding properties of GPIIIa (18). In contrast to the $Asp_{119} \leftrightarrow Tyr_{119}$ variation present in the CAM variant of Glanzmann's thrombasthenia, the $Arg_{143} \leftrightarrow Gln_{143}$ Pen polymorphism is located within a less well-conserved region of integrin β subunits. It is notable, however, that anti-Pen^a human alloantibodies are potent inhibitors of platelet aggregation (8), and can result in severe thrombocytopenia, causing in utero intracranial hemorrhage in at least two reported cases of Pen-associated NATP (4). Our localization of the Pen polymorphic site to amino acid 143 of GPIIIa provides further evidence for

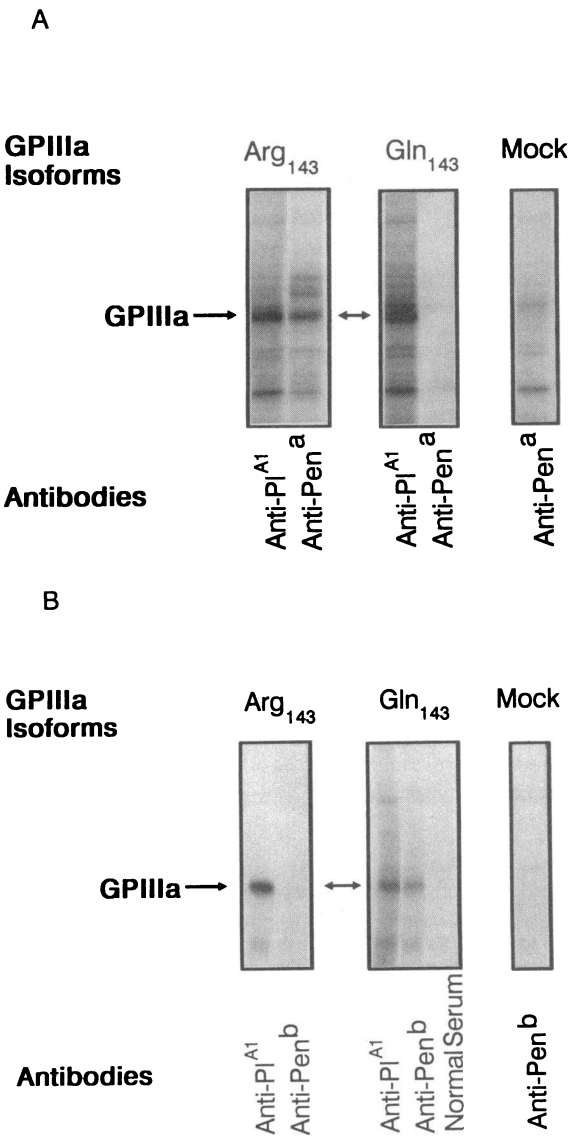

Figure 6. Immunoprecipitation analysis of allele-specific recombinant GPIIIa isoforms. Recombinant forms of GPIIIa produced by COS cells transfected with cDNAs encoding either the Arg_{143} or Gln_{143} form of GPIIIa were analyzed by immunoprecipitation, followed by SDS PAGE gel electrophoresis and autoradiography. Antibodies used for immunoprecipitation are indicated below the lanes, and the recombinant GPIIIa isoforms are shown above. (*A*) The reactivity of anti-Pen^a alloantibodies with ^{35}S-labeled COS cell lysates transfected with either Arg_{143} or Gln_{143} form of GPIII cDNAs. (*B*) Reactivity of anti-Pen^b alloantibodies with the two recombinant GPIIIa isoforms. Anti-Pl^{A1} alloantiserum was used in both sets of experiments as a positive control. The position of GPIIIa is shown with arrows. Anti-Pen^a antisera immunoprecipitated only the Arg_{143} allelic form of GPIIIa, while anti-Pen^b reacted specifically with only the Gln_{143} construct.

the importance of this region in binding physiologically important ligands.

To examine whether the $Arg_{143} \leftrightarrow Gln_{143}$ polymorphism might grossly affect the affinity and specificity of GPIIb-IIIa for its naturally occurring ligands, aggregation studies were performed using platelets derived from the only known Pen$^{b/b}$ homozygous individual present in North America. We found that the aggregation response to ADP, arachidonic acid, thrombin, epinephrine, collagen, and ristocetin was diminished by ~ 20%. In light of the fact that this individual has chronic uremia and undergoes dialysis three times/week, it is likely that substitution of Gln for Arg at amino acid 143, in spite of being located within the RGD-binding domain of GPIIIa, has little, if any, additional effect on the ability of this integrin to function. Since Pen$^{b/b}$ individuals are very rare, however, and many extrinsic factors can influence the platelet aggregation response, it may be of future interest to examine more precisely the fibrinogen-binding properties of recombinant allele-specific forms of GPIIb-IIIa in a controlled mammalian transfection system.

We synthesized two short allele-specific synthetic peptides encompassing polymorphic amino acid 143 and examined their ability to competitively inhibit the binding of anti-Pen alloantibodies to immobilized platelet GPIIIa. We also examined the ability of Pen alloantibodies to bind directly to Pen^a and Pen^b synthetic peptides that had been immobilized in plastic microtiter wells. Neither Pen^a nor Pen^b peptides were able to effectively mimic the Pen epitopes in either assay system, suggesting that formation of these two antigenic determinants is dependent upon proper folding of the GPIIIa polypeptide chain.

To overcome the problems inherent in peptide mimicry, we produced full-length recombinant forms of the GPIIIa molecule. Expression of GPIIb and/or GPIIIa in heterologous mammalian cells has previously proven to be an extremely useful means for analyzing the controlling regions of the Pl^A and Bak alloantigen systems at the molecular level (16). Immunoprecipitation analysis of recombinant GPIIIa alloforms containing either an Arg or Gln at amino acid 143 showed that anti-Pen^a alloantibodies recognize only the Arg_{143} (Pen^a) form of GPIIIa, while anti-Pen^b antibodies bind only to the Gln_{143} form of recombinant GPIIIa, and are completely unreactive with the Arg_{143} alloform. These data demonstrate that residue 143 is both necessary and sufficient to control the expression of the Pen^a and Pen^b epitopes. It is not yet known, however, whether other regions might participate structurally in the formation of the actual alloantibody combining site on the GPIIIa molecule.

During the preparation of this paper, Wang et al. published a preliminary report of a $Gln_{143} \leftrightarrow Arg_{143}$ polymorphism that is associated with the Yuk^a/Yuk^b alloantigen system (19). Previous serological studies (8) have suggested that Pen and Yuk are related or identical alloantigen systems (Pen^a = Yuk^b, and Pen^b = Yuk^a). The present work conclusively demonstrates the identity of these alloantigens at the molecular genetic level.

In summary, we have identified a single Arg/Gln amino acid dimorphism at position 143 of GPIIIa that is responsible for the formation of the Pen^a and Pen^b human platelet alloantigens. Further localization of the Pen alloantibody binding sites on the GPIIIa molecule using chimeric integrin constructs in combination with recombinant GPIIIa fragments will likely provide additional clues as to the detailed structure of the Pen alloantigenic determinants. Our elucidation of the molecular genetic basis of the Pen^a and Pen^b allelic forms of the GPIIIa gene can now be exploited for the development of both pre- and post-natal diagnostic DNA typing for Pen phenotype, as has recently been demonstrated for the Pl^{A1}/Pl^{A2} alloantigen system (20).

Acknowledgments

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