A Point Mutation Leads to an Unpaired Cysteine Residue and a Molecular Weight Polymorphism of a Functional Platelet $\beta_3$ Integrin Subunit

THE Sr$^a$ ALLOANTIGEN SYSTEM OF GPIIIa

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Recently, we described a low frequency platelet alloantigen on human platelet membrane glycoprotein (GP) IIIa, termed Sr$, that was involved in neonatal alloimmune thrombocytopenia. To identify the molecular nature of the Sr$^a$ alloantigen, we analyzed the nucleotide sequence of polymerase chain reaction-amplified GPIIIa mRNA, and found a C to T substitution in seven Sr$^a$ positive individuals which results in an Arg$^{636} \rightarrow$ Cys polymorphism within the cysteine-rich region of GPIIIa. Analysis of allele-specific recombinant forms of GPIIIa that differed only at amino acid residue 636 showed that anti-Sr$^a$ alloantibodies reacted with the Cys$^{636}$, but not the Arg$^{636}$, recombinant form of GPIIIa. Interestingly, under reducing conditions, the Cys$^{636}$ form of GPIIIa migrated with a slightly increased apparent molecular weight compared with the Arg$^{636}$ form. Following treatment with Endoglycosidase F, both allelic forms of GPIIIa exhibited the same mobility, however the Sr$^a$ epitope was lost. Sr$^a$ positive platelets express the same number of GPIIb-IIIa complexes on their surface as wild-type Sr$^a$ negative platelets, and also aggregate normally in response to a variety of platelet agonists. Based upon these results, we conclude that 1) GPIIIa residue 636 specifically controls the formation and expression of the Sr$^a$ alloantigenic determinant, and 2) an unpaired cysteine residue alters the N-linked glycosylation pattern of the extracellular domain of GPIIIa, but affects neither the degree of surface expression nor the adhesive function of the GPIIb-IIIa complex.

Integrins constitute a large family of cell surface $\alpha\beta$ heterodimers that are widely distributed on many cells and are involved in cell-matrix or cell-cell interactions. Human platelet glycoprotein IIIa (GPIIIa) is the common $\beta$ subunit of the $\beta_3$ subfamily of integrins and associates with either $\alpha_{IIb}$ (GPIIb) to form the major platelet fibrinogen receptor, GPIIb-IIIa (integrin $\alpha_{IIb}\beta_3$) on human platelets (1), or with $\alpha_\gamma$, to form the vitronectin receptor, $\alpha_\gamma\beta_3$, which is more widely distributed. Mature GPIIIa protein consists of 762 amino acids, including six potential N-glycosylation sites in the extracellular domain that have been shown to be post-translationally modified with high-mannose carbohydrate residues (2-4). GPIIIa also contains 56 cysteine residues in highly conserved locations within the extracellular domain of the molecule, all of which are normally disulfide-linked. Thirty-one of these cysteine residues comprise a cysteine-rich, protease-resistant core of the molecule and are clustered between residues 433 and 655 into four tandemly repeated segments of about 40 amino acids each (4). Another 7 cysteines are concentrated within the first 50 amino-terminal amino acids (4, 5). The cysteine pairing pattern of GPIIIa has been largely established by Calvete et al. (6), who showed that a number of long range disulfide bonds form within the molecule, bringing together at least two linearly distant polypeptide segments. Overall, these cysteine residues likely serve an important role in preserving the overall threedimensional structure of the integrin complex, as perturbation or absence of 1 or more of these residues has been shown to severely affect stability and/or ligand binding function (7).

In addition to its role in mediating cell adhesive interactions, GPIIIa is the most polymorphic of the integrin subunits, with six currently recognized alleles known to exist in the human gene pool. Differences in these allelic isofoms have been shown to be responsible for eliciting an alloimmune response leading to platelet destruction in two clinically significant pathologic syndromes, post-transfusion purpura and neonatal alloimmune thrombocytopenia (for recent reviews, see Refs. 5, 8, 9). The advent of platelet mRNA PCR technology (10) has made it possible to elucidate the molecular basis for a number of these platelet membrane glycoprotein polymorphisms. To date, all of these have been found to result from single nucleotide, and consequently single amino acid substitutions (11-17), which are in turn thought to subtly affect the conformation of the protein, leading to expression of the offending antigenic determinant.

Recently, we described a new low frequency platelet alloantigen on GPIIIa, termed Sr$, that was responsible for a case of neonatal alloimmune thrombocytopenia (18). Since that time several other private platelet alloantigens (CA, Va, Mo) that reside on GPIIIa have also been reported (16, 19-21). In this study, we have further characterized the biochemical and molecular properties that underlie the polymorphism of human platelet GPIIIa that is responsible for the immunogenicity of the Sr$^a$ alloantigen, as well as their effects on expression and function of this unique integrin isoform.
MATERIALS AND METHODS

Seraological Studies—Typing of human platelets for the presence of P1 anti-P1′ antibodies was performed by the glycoprotein-specific immunoassay, MAIPA, using anti-P1′ and anti-SR-specific alloantiserum and the monoclonal antibody Gi5 directed against GPIIIa/IIb as capture antibody (22). The platelet-specific alloantibodies P1′ and SR′ were obtained from mothers of children with neonatal alloimmune thrombocytopenia, as previously described (18). Monoclonal antibodies in precooled PBS were allowed to react with 2×10^5 washed platelets in PBS buffer, pH 7.4, containing 10 mM EDTA and 10 mM benzamidohydrochloride (PBS/EDTA) incubated with 30 μCi of tritiated N-ethylmaleimide (NEM) as described by Kalmarics and Coller (26) with minor modifications. Briefly, aliquots of 5×10^8 washed platelets in PBS buffer, pH 7.4, containing 10 mM EDTA and 10 mM benzamidohydrochloride (PBS/EDTA) were incubated with human sera, and analyzed by SDS-PAGE under reduced conditions. Gels containing biotinylated proteins were transferred to nitrocellulose by immunoblotting and visualized using streptavidin-horseradish peroxidase and chemiluminescent substrate protein A-Sepharose mixture (Amersham Buchler, Braunschweig, Germany) was added at 85°C and “hot-start” PCR was performed in a DNA thermal cycler (Biometra, Göttingen, Germany). Amplification proceeded for 30 cycles, with denaturation for 1.5 min at 94°C, annealing of primers for 1.5 min at 50°C, and extension for 3 min at 72°C.

Isolation and Amplification of Genomic DNA—Genomic DNA was isolated from peripheral blood leukocytes using the proteinase/salting out procedure of Miller et al. (27). A 335-bp region of the GPIIIa gene encompassing the SR polymorphic nucleotide was amplified using the PCR primer pair shown in Table I. PCR was performed using 1-2 μg of DNA and 5.5 μL of each primer using 2.5 units of Taq polymerase in PCR buffer in a total volume of 100 μL as described above. Thirty cycles of 1 min at 96°C, 1.5 min at 57°C, and 3 min at 72°C were performed. Analysis of PCR Products—Five μL of PCR-amplified products were analyzed on 1.4% agarose gel containing ethidium bromide (Dianova, Hamburg, Germany). Selected amplified forms of cDNA were purified using a GeneClean (Hana, Iowa, USA) and sequenced by the dideoxy termination method using the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Construction and Expression of Allele-specific Recombinant Forms of GPIIIa—Allele-specific recombinant forms of GPIIIa were created using cartilage metaplasia. A full-length GPIIIa cDNA, the internal EcoRI restriction site of which had been removed by site-directed mutagenesis (kindly provided by Dr. Gilbert C. White II, University of North Carolina, Chapel Hill, NC) was used as a host vector for construction of allele-specific GPIIIa isoforms. Plasmids (pGEM-S2Z, Promega Biotech, Madison, WI) from two clones containing GPIIIa nucleotides 1666–2237 of platelet GPIIIa, and having either a T or a C at position 2004 were digested with MluI and AflII (New England BioLabs, Inc., Beverly, MA) and were ligated into the EcoRI site of the mammalian expression vector pcDNA3 (TCl, Heidelberg, Germany). Purified plasmids used for subsequent transfection were validated by nucleotide sequence analysis.

Platelet Aggregation—Platelet-rich plasma was obtained as previously described (29). For surface expression experiments, COS cells were cotransfected with full-length GPIIIa/pDNA NEO (kindly provided by Dr. Ronggang Wang, Blood Research Institute, The Blood Center of Southeastern Wisconsin, Milwaukee, WI). Recombinant proteins synthesized by the transfected COS cells were metabolically labeled with [35S]methionine and immunoprecipitated as described above and analyzed on SDS-PAGE under reduced or non-reduced conditions. Site-directed mutagenesis of COS cells was performed using the Muta-gene Kit (Promega, Madison, WI) using the oligonucleotide-directed mutagenesis protocol as described previously (18). The resulting plasmids were analyzed on a 1.4% agarose gel containing ethidium bromide (Dianova, Hamburg, Germany). Purified plasmids used for subsequent transfection were validated by nucleotide sequence analysis.

COS cells were transfected with both allele-specific recombinant forms of GPIIIa using the DEAE-dextran method, as previously described (29). For surface expression experiments, COS cells were cotransfected with full-length GPIIIa/pDNA NEO (kindly provided by Dr. Ronggang Wang, Blood Research Institute, The Blood Center of Southeastern Wisconsin, Milwaukee, WI). Recombinant proteins synthesized by the transfected COS cells were metabolically labeled with [35S]methionine and immunoprecipitated as described above and analyzed on SDS-PAGE under reduced conditions. Site-directed mutagenesis of COS cells was performed using the Muta-gene Kit (Promega, Madison, WI) using the oligonucleotide-directed mutagenesis protocol as described previously (18). The resulting plasmids were analyzed on a 1.4% agarose gel containing ethidium bromide (Dianova, Hamburg, Germany). Purified plasmids used for subsequent transfection were validated by nucleotide sequence analysis.

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codes for cysteine at amino acid 636 of the mature GPIIIa polypeptide chain. The presence of an additional cysteine within the cysteine-rich region of GPIIIa is likely to be responsible for the formation of the Sr^a epitope.

Biochemical Properties of Recombinant GPIIIa Allelic Forms—To examine directly whether the Arg^636 → Cys polymorphism actually controls the formation of the Sr^a antigenic determinant, we constructed an allele-specific recombinant form of GPIIIa that differs only by the presence of T at nucleotide 2004 and analyzed the Cys^636 protein product in a mammalian cell transfection system. Whereas PI\(^{A1}\) alloantibody bound equally well to either the Arg^636 or Cys^636 isoforms of the GPIIIa molecule (Fig. 3B, compare lanes 2 and 3 with lanes 4 and 5), anti-Sr^a reacted only with the Cys^636 form (Fig. 3A). These results demonstrate that amino acid 636 is directly involved in the expression of the Sr epitopes. Interestingly, the recombinant Cys^636 isofrom (lane 4) migrated slightly slower in SDS-PAGE than the recombinant wild-type Arg^636 form (lane 3), even under reducing conditions. Identical results were obtained using two independently constructed clones (lanes 2 and 5).

It is possible that the presence of an additional cysteine residue in the Sr^a allelic form of GPIIIa could have altered the conformation of the molecule such that the glycosylation pattern would also be affected. To examine whether the altered mobility of the Cys^636 isofrom was unique only to the recombinant COS-7 cell product, or might be intrinsically present in Sr^a positive versus Sr^a negative platelets, biotin surface-labeled platelets were subjected to immunoprecipitation analysis. As shown in Fig. 4, GPIIIa molecules derived from the platelets of an Sr^a positive (heterozygous) individual migrated as a doublet (lane 1), corresponding to the wild-type (lower band) and Cys^636 Sr^a (upper band) allelic forms. As predicted, only the upper GPIIIa band was reactive with anti-Sr^a alloantisera (lane 2), confirming the results obtained above using recombinant isoforms produced in COS cells (Fig. 3). GPIIIa derived from wild-type Sr^a negative platelets (lane 3), was present as a single band, as expected, and was not reactive with anti-Sr^a antibodies (lane 4). After deglycosylation with Endoglycosidase H, however, both GPIIIa allelic forms migrated with the same mobility (lane 5), indicating that the molecular size polymorphism observed in the Sr^a positive allele of GPIIIa is due to a variable degree of N-linked glycosylation, most likely the presence of an additional single high mannose moiety. In contrast to continued reactivity with anti-PI\(^{A1}\) alloantibody (lane 5, and Ref. 34), however, deglycosylated GPIIIa failed to react with anti-Sr^a alloantibodies (lane 6). These data suggest that the Sr^a antigenic determinant is, at least in part, dependent on the presence of one or more of the high-mannose carbohydrate residues that are known to comprise approximately 15% of the molecular mass of the GPIIIa molecule. Whether or not the additional carbohydrate residue added as a result of the Arg^636 → Cys amino acid substitution forms part of the alloantibody-combining site remains to be determined.

Effect of a Free Thiol Group on GPIIIa Expression and Function in Sr^a Positive Platelets—In order to verify biochemically the presence of an additional, unpaired cysteine residue in GPIIIa derived from Sr^a positive individuals, intact platelets were incubated with tritiated NEM to derivatize accessible free thiol groups, solubilized in Triton X-100, and then immunoprecipitated with the anti-GPIIIa murine monoclonal antibody, Gi5. Since the β-subunit of the GPIb complex has previously been shown to contain a free sulfhydryl group (26), aliquots of these same lysates were subjected to immunoprecipitation analysis using the anti-GPIb\(_{β}\)-specific monoclonal antibody Gi22, which served to validate the specificity of the labeling reaction. As shown in Fig. 5, GPIb\(_{β}\) was visualized by auto-

![Fig. 1. DNA sequence analysis of amplified GPIIIa cDNA derived from an Sr^a positive individual.](image-url)

**RESULTS**

Amplification and Analysis of the NH\(_{2}\) and COOH-terminal Regions of GPIIIa Platelet mRNA—Previous immunohistochemical studies have shown that anti-Sr^a alloantibodies react with the 68-kDa fragment of GPIIIa generated by chymotryptic treatment of intact platelets (18). Since this fragment has been shown to lack residues 121–348 of the large disulfide-bonded loop formed by disulfide bonding of Cys^6 in with Cys^435 (6, 31–33), we were able to predict that the remaining region formed by amino acid residues 1–120 and 349–762 (encompassing nucleotides 99–470 and 1143–2384, respectively) of GPIIIa is likely to carry the Sr^a epitope. Thus, we amplified this region as two separate segments of 642 (nucleotides 56–698) and 571 (nucleotides 1666–2237) base pairs. Nucleotide sequence analysis of the resulting 571-bp COOH-terminal fragment derived from an Sr^a positive individual revealed a single C → T nucleotide substitution at base 2004 (Fig. 1) in five of seven subclones examined, consistent with the fact that all Sr^a positive individuals examined to date have been serologically heterozygous for this low frequency allelic form of GPIIIa. In contrast, all clones from an Sr^a negative individual encoded a C at this position (not shown). No other nucleotide differences between Sr^a positive versus negative individuals were found.

Correlation of C\(^{2004}\) → T Polymorphism with Sr Allotype—In order to determine whether the C → T polymorphism seen in the one Sr^a positive individual was associated with Sr phenotype, genomic DNA from six Sr^a positive and 10 different Sr^a negative individuals (four family members and six unrelated donors) were amplified using PCR to yield a 335-bp product (not shown). Allele-specific oligonucleotide typing was then performed using 17-base probes containing the putative genotype-specific nucleotide in the middle (Table I, right). As shown in the top panel of Fig. 2, the putative Sr^a-specific probe containing a T in the middle hybridized with the PCR products derived from all six Sr^a positive heterozygous individuals, but was negative with 10 Sr^a negative individuals. In contrast, the wild-type “Sr^a”-specific probe, containing a C in the middle, hybridized with both Sr^a heterozygous and Sr^a negative individuals. Based upon these results, we conclude that the observed base change at nucleotide 2004 segregates directly with the phenotypic presence of the Sr^a alloantigen. Importantly, the C → T mutation changes a CGT codon for arginine into a TGT that
The antisense primer beginning in the intron following exon 11 128 bases from splice junction at base 2034 was used to prime the synthesis of amplified DNA from genomic GPIIIa DNA (39). The second strand was generated by the sense primer beginning in the intron preceding exon 11 102 bases from the splice junction at base 1934 during the first round of PCR. The oligonucleotide probes specific for the Sr(a) and the Sr(b) forms of the GPIIIa gene differed only in the central base (underlined).

Table I

| Primer (5' → 3') | Probe (5' → 3') |
|-----------------|----------------|
| Sense           |                |
| Antisense       | AGGCTGCAAAATCTCCCGAGG |
|                 | TGGAGGCTGGACAGCTTTCTG |
| Sense           | Sr (a) GATTCTGCTGGACAGG |
|                 | Sr (b) GATTCTGCCTGGACAGG |

Fig. 2. Allele-specific oligonucleotide probe hybridization analysis of Sr phenotype. PCR products from Sr positive and Sr negative individuals derived by amplification of the GPIIIa gene. PCR products were applied to nylon strips and hybridized with either Sr (corresponding with the Sr sequence) or Sr (corresponding with the wild-type GPIIIa published sequence) oligonucleotide probes (see Table I). All Sr individuals were found to be heterozygous for the polymorphic allele.

Fig. 3. Immunoprecipitation analysis of allele-specific recombinant GPIIIa isoforms. Recombinant forms of GPIIIa/IIla complex were produced in COS-7 cells that had been transfected with cDNAs encoding either ArgGAG or CysGAG (mutant) form of GPIIIa and cotransfected with full-length wild-type GPIIIa. A, the reactivity of anti-Sr alloantibodies with 35S-labeled COS cell lysates transfected with either CysGAG (lane 1) or ArgGAG (lane 2) recombinant forms of GPIIIa cDNAs. B, reactivity of anti-PiA antibodies with the two recombinant forms. Note that the CysGAG form (lanes 4 and 5) has a slightly lower mobility than the wild-type ArgGAG form (lanes 2 and 3).

Fig. 4. Molecular weight polymorphism of the Sr allelic form of GPIIIa. Human platelets from wild-type (lanes 3 and 4) or Sr positive (lanes 1, 2, 5, and 6) were biotin labeled and subjected to immunoprecipitation analysis using either anti-PiA (lanes 1, 3, and 5) or anti-Sr (lanes 2, 4, and 6) alloantisera. Note the presence of a GPIIIa doublet in lane 1, corresponding to the lower ArgGAG and upper CysGAG allelic forms in this heterozygous individual. Whereas both forms express the PiA epitope (lane 1), only the upper GPIIIa band is reactive with anti-Sr antibodies (lane 2). Following deglycosylation with EndoH (lanes 5 and 6), the Sr allelic form co-migrated with the wild-type GPIIIa allele (compare lanes 1 and 5). Deglycosylated GPIIIa continued to express the PiA epitope (lane 5) but became unreactive with Sr alloantisera (lane 6). Immunoprecipitates were analyzed on 7.5% SDS-PAGE under reduced conditions.

Fig. 5. Analysis of GPIIIa immunoprecipitated from Sr negative (lanes 1 and 2) and Sr positive (lanes 3–5) platelets labeled with tritiated N-ethylmaleimide using mAb G56. Lanes 6 and 7 represent GP1b immunoprecipitated from a Sr negative and a Sr positive individual using mAb G22. Immunoprecipitates were analyzed on 7.5% SDS-PAGE under non-reduced (lanes 1–5) or reduced (lanes 6 and 7) conditions.

diography following immunoprecipitation of the GPib complex from either Sr negative (lane 6) or Sr positive (lane 7) platelets. In contrast, only GPIIIa from Sr positive platelets (lanes 3–5) became labeled, whereas GPIIIa derived from Sr negative platelets (lanes 1 and 2) did not. These results demonstrate that a free thiol group is present in the Sr positive allelic form of GPIIIa.

The presence of an unpaired cysteine residue might be expected to result in a loss of stability and/or function of the GPIIb-IIIa complex. To examine whether this additional cysteine influences the efficiency of expression of GPIIIa on the platelet surface, binding isoformers were generated using human anti-Sr and anti-PiA alloantibodies in a quantitative competitive enzyme-linked immunosorbent assay (30). Table II summarizes the results of quantitative binding studies with platelets from several donors differing in their Sr and PiA phenotypes. As shown, platelets from individuals who were homozygous for PiA and heterozygous for Sr (donor 1) bound 50,800 anti-PiA antibody molecules/cell, in agreement with previously obtained values for the number of GPIIIa molecules present on the platelet surface (35). Moreover, approximately 27,300 Sr antibody molecules bound to these cells, as expected for presumably heterozygous individuals. Taken together, these results are consistent with the conclusion that the CysGAG allelic form of GPIIIa is expressed as efficiently as the wild-type ArgGAG form.

To determine the possible effects of an unpaired cysteine on the ability of GPIIIa to bind fibrinogen and support platelet
aggregation, platelets from a Sr⁺ positive individual were compared with Sr⁺ negative (control) platelets in standard platelet aggregation assay. Fig. 6 shows the platelet aggregation responses obtained using PRP from 2 Sr⁺ positive individuals compared with a single Sr⁺ negative healthy donor. As shown, addition of $10^{-5}$ M ADP resulted in a completely normal aggregation response in platelets derived from both Sr⁺ positive individuals. Similar findings were obtained using collagen (5 µg/ml) or ristocetin (1.5 mg/ml) as agonists (not shown). These results suggest that neither the expression nor function of Sr⁺ positive platelets are adversely affected by the presence of an unpaired cysteine residue on the cell surface.

**DISCUSSION**

Although the importance of integrins in mediating cell-cell and cell-extracellular matrix interactions is well recognized, the fact that a number of integrin α- and β-subunits may be encoded by multiple allelic forms is not well appreciated. GPIIIa (β₃) is the most polymorphic integrin subunit in man, and is most frequently responsible for eliciting an alloimmune response leading to thrombocytopenia and perhaps more widespread damage within the vasculature. To date, six different genetic variants of the GPIIIa molecule have been identified at the serological and molecular biological level, and these are summarized in Table III. The P¹¹ allele is by far the most common, with a gene frequency of nearly 85% within the Caucasian population and differs from the second most common form of α₁, P¹², by a single Leu³³ → Pro amino acid substitution. Other alleles of GPIIIa, including the Sr⁺ isoform of this study, are much less frequently represented, but also appear to have arisen by point mutations of the P¹¹ ancestral allele.

The Sr⁺ alloantigen was originally described serologically in a case of alloimmune thrombocytopenia, and subsequent immunohistochemical studies localized the Sr⁺ antigen to the 68-kDa membrane-bound protease-resistant core of GPIIIa (18), either on the amino-terminal 121 amino acid residues or on the carboxyl-terminal segment bounded by residues 349–692. By sequencing cDNA derived from PCR-amplified platelet mRNA (10), we have localized the polymorphism underlying the Sr⁺ polymorphism to a C⁶³⁰→T substitution, which in turn results in an Arg → Cys polymorphism at amino acid 636, just proximal to the membrane from the cysteine-rich region of the molecule. Using mammalian transfection techniques, we were able to demonstrate that the additional cysteine residue at position 636 of GPIIIα controls the formation of the Sr⁺ alloantigenic epitope. Other three-dimensional structural features of GPIIIa appear to be required as well, however, since both disulfide bond reduction (18) as well as deglycosylation (Fig. 4) abolish the presentation of the Sr⁺ epitope.

Interestingly, the Cys⁶³⁶ (Sr⁺) allelic form of GPIIIa was found to have a higher apparent molecular weight than the wild-type Arg⁶³⁶ form. This molecular weight polymorphism appears to be attributable to differential glycosylation of the altered conformer of GPIIIa induced by the presence of an unpaired cysteine residue, since deglycosylation, but not disulfide bond reduction, allowed the Sr⁺ form of GPIIIa to co-migrate on SDS-PAGE with the wild-type allele. At the present time, we do not know whether the glycosylation differences observed are due to altered trimming of the high-mannose carbohydrate side chains, or to the presence of additional carbohydrate moieties to normally cryptic, unmodified sites.

Following incubation with [³H]NEM, label became incorporated into GPIIIa from Sr⁺ positive platelets, confirming at a biochemical level the presence of a free sulfhydryl group in this rare allelic form of GPIIIa. Parallel experiments using Sr⁺ negative platelets, however, revealed no incorporation of [³H]NEM into platelet GPIIIa, confirming the structural analysis of Calvete et al. (6). The finding of a naturally occurring isof orm of GPIIIa having 57 cysteine residues is remarkable, since both the number and the position of the 57 cysteine residues are absolutely conserved in the integrin β-subunit family ranging from Drosophila to man (36, 37). One might have expected the presence of an additional, unpaired cysteine to have a deleterious effect on either expression of this integrin β-subunit or on platelet function, resembling the effects of similar mutations that have been shown to be responsible for the congenital platelet functional disorder, Glanzmann thrombasthenia (5, 7, 38). However, not only was expression of the Sr⁺ allelic form of GPIIIa quantitatively normal, Sr⁺ positive platelets aggregated to the same extent as wild-type Sr⁺ negative platelets. Moreover, individuals carrying the Sr⁺ allele also show no obvious hemostatic, immunologic, or vascular abnormalities, suggesting that the adhesive functions of cells expressing the vitronectin receptor α₃β₃ are also unaffected. Examination of the effects on expression and function of other molecular variations and alloantigenic forms of platelet surface receptors should continue to provide insights into the structural features of these molecules that influence biosynthesis, trafficking, and ligand binding capacity.
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REFERENCES

1. Kieffer, N., and Phillips, D. R. (1990) Annu. Rev. Cell. Biol. 6, 329-357
2. McEver, R. P., Baenziger, J. U., and Majerus, P. W. (1982) Blood 69, 80-85
3. Tagu, T., and Osawa, T. (1986) J. Biochem. (Tokyo) 100, 1387-1398
4. Fitzgerald, L. A., Steinbr, R., Rall, S. C., Jr., Lo, S. S., and Phillips, D. R. (1987) J. Biol. Chem. 262, 3936-3939
5. Newman, P. J. (1991) Thromb. Haemostasis 66, 111-118
6. Calvete, J. J., Herschel, A., and Gonzalez Rodriguez, J. (1991) Biochem. J. 274, 63-71
7. Newman, P. J., Seligsohn, U., Lyman, S., and Coller, B. S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3160-3164
8. Aster, R. H. (1989) in Platelet Immunobiology (Kunicki, T. J., and George, J. N., eds) pp. 387-435, Lippeincott, Philadelphia
9. Mueller-Eckhardt, C., Kiefel, V., and Santoso, S. (1990) Transfus. Med. Rev. 4, 98-109
10. Newman, P. J., Gerski, J., White, G. C., Gidwitz, S., Cretney, C. J., and Aster, R. H. (1988) J. Clin. Invest. 83, 739-743
11. Newman, P. J., Derbes, R. S., and Aster, R. H. (1989) J. Clin. Invest. 83, 1778-1781
12. Lyman, S., Aster, R. H., Visentin, G. P., and Newman, P. J. (1990) Blood 73, 2343-2348
13. Wang, R., Furnhata, K., McFarland, J. G., Friedman, K., Aster, R. H., and Newman, P. J. (1992) J. Clin. Invest. 90, 2028-2043
14. Kuijpers, R. W. A. M., Faber, N. M., Cuypers, H. T. M., Ouwehand, W. H., and Vandenborne, A. E. G. K. (1992) J. Clin. Invest. 89, 381-384
15. Santoso, S., Kalb, R., Walka, M., Kiefel, V., Mueller-Eckhardt, C., and Newman, P. J. (1993) J. Clin. Invest. 92, 2427-2432
16. Wang, R., McFarland, J. G., Kekomaki, R., and Newman, P. J. (1993) Blood 82, 3366-3391
17. Wang, L., Juiji, T., Shihata, Y., Kuwata, S., and Tokunaga, K. (1991) Proc. Jpn. Acad. 67, 102-196
18. Kroll, H., Kiefel, V., Santoso, S., and Mueller-Eckhardt, C. (1990) Blood 76, 2296-2302
19. Kekomaki, R., Raivo, P., and Kero, P. (1992) Trans. Med. 2, 27-33
20. Kekomaki, R., Juhkainen, T., Ollikainen, J., Westman, P., and Laes, M. (1993) Br. J. Haematol. 83, 306-310
21. Kuijpers, R. W. A. M., Simsek, S., Faber, N. M., Goldschmeding, R., van Wermkerken, R. K. V., and Von dem Borne, A. E. G. K. (1993) Blood 81, 70-78
22. Kiefel, V., Santoso, S., Weisheit, M., and Mueller-Eckhardt, C. (1987) Blood 70, 1722-1726
23. Santoso, S., Kiefel, V., and Mueller-Eckhardt, C. (1989) Br. J. Haematol. 72, 191-198
24. Kiefel, V., Santoso, S., Kaufmann, E., and Mueller-Eckhardt, C. (1991) Br. J. Haematol. 78, 295-299
25. Smith, J. W., Hayward, C. P. M., Warkentin, T. E., Horsewood, P., and Kelton, J. G. (1990) J. Immunol. Methods 138, 77-85
26. Kekomaki, R. and Coller, B. S. (1988) Biochemistry 27, 5430-5436
27. Miller, S. A., Dykes, D. D., and Polesky, H. F. (1988) Nucleic Acids Res. 16, 1210-1218
28. McFarland, J. G., Aster, R. H., Bassel, J. B., Gianspoulos, J. G., Derbes, R. S., and Newman, P. J. (1993) Blood 78, 2276-2282
29. Goldberger, A., Kolodziej, M., Poncz, M., Bennett, J. S., and Newman, P. J. (1991) Blood 78, 681-687
30. Kiefel, V., and Mueller-Eckhardt, C. (1999) in Laboratory Methods in Immunology (Zelina, H., ed) pp. 241-248, CRC Press, Boca Raton, FL
31. Niewiarowski, S., Norton, K. J., Eckhardt, A., Lukasiewicz, H., Holt, J. C., and Kornecki, E. (1989) Biochim. Biophys. Acta 985, 91-99
32. Boer, J., and Coller, B. S. (1989) J. Biol. Chem. 264, 17564-17573
33. Kouns, W. C., Newman, P. J., Puckett, K. J., Miller, A. A., Wall, C. D., Fox, C. F., Seyer, J. M., and Jennings, L. K. (1991) Blood 78, 3215-3223
34. Newman, P. J., Martin, L. S., Knipp, M. A., and Kahn, R. A. (1985) Mol. Immunol. 22, 719-729
35. Newman, P. J., Allen, R. W., Kahn, R. A., and Kunicki, T. J. (1985) Blood 65, 227-232
36. Bogaert, T., Brown, N., and Wilcox, M. (1987) Cell 51, 929-940
37. DeSimone, D. W., and Hynes, R. O. (1988) J. Biol. Chem. 263, 5333-5340
38. Burk, C. D., Newman, P. J., Lyman, S., Gill, J., Coller, B. S., and Poncz, M. (1993) J. Clin. Invest. 92, 270-276
39. Zimina, A. B., Gidwitz, S., Schwartz, E., Bennett, J. S., White, G. C., and Poncz, M. (1999) J. Biol. Chem. 265, 8590-8595

Table III

| Allelic form | Gene frequency | Serologic designation |
|-------------|----------------|-----------------------|
| GPIIIa Leu32Arg16Pro407Arg184Asp562 | 0.85 | α |
| GPIIIa Pro23Arg16Pro407Arg184Asp562 | 0.15 | α |
| GPIIIa Leu22Glu114Pro407Arg184Asp562 | <0.01 | α |
| GPIIIa Leu12Arg16Ala204Arg184Asp562 | <0.01 | α |
| GPIIIa Leu12Arg16Ala204Arg184Asp562 | <0.01 | α |
| GPIIIa Leu12Arg16Ala204Arg184Cy562 | <0.01 | α |

* In the Caucasian population. Gene frequencies differ in African and Asian populations.