

Nucleotide Polymorphisms in the α_2 Gene Define Multiple Alleles That Are Associated With Differences in Platelet $\alpha_2\beta_1$ Density

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Three allelic differences in the α_2 gene are associated with expression levels of the $\alpha_2\beta_1$ integrin on the platelet surface. We have previously defined two linked silent polymorphisms in the α_2 gene coding region at nucleotides 807 (C or T) and 873 (G or A). We have now identified one rarer nucleotide polymorphism in the coding region at nucleotide 837 (T or C) and four additional linked polymorphisms within the introns that flank these coding sequences. Moreover, we have determined that the alloantigenic Br polymorphism, which resides in a distal coding region at nucleotide 1648, is also linked to the 837 polymorphism. Thus, three α_2 gene alleles, defined by eight nucleotide polymorphisms, have

INTEGRINS ARE heterodimeric molecules composed of noncovalently associated α and β subunits that mediate cell-cell and cell-matrix adhesion.¹ The integrin receptor for collagen/laminin, $\alpha_2\beta_1$ (also known as the platelet membrane glycoprotein Ia-IIa complex,² the very late activation antigen-2 [VLA-2],³ and the class II extracellular matrix receptor [EC-MII] ⁴), is expressed on a wide variety of cell types, including megakaryocytes, platelets, fibroblasts, endothelial cells, and epithelial cells.⁵ Although $\alpha_2\beta_1$ serves as a collagen receptor on platelets and fibroblasts,⁶ it functions as both a collagen and laminin receptor on endothelial cells and on many epithelial cell types.⁷

Previous studies in our laboratory have shown that platelet levels of $\alpha_2\beta_1$ vary significantly among normal individuals, whereas the levels of other integrins do not.⁸ Because $\alpha_2\beta_1$ mediates platelet adhesion to collagen in vivo, variation in its expression levels could have a significant impact on platelet function. Significantly, our recent analyses have shown that DNA sequence polymorphisms in the α_2 gene are linked to expression levels of $\alpha_2\beta_1$ on platelets.⁹ The DNA sequence variants identified include two conservative changes in the amino acid coding region of α_2 at nucleotides 807 (TTT/TTC at

now been discovered. Allele 1 (807T/837T/873A/Br^b) is associated with increased levels of $\alpha_2\beta_1$; allele 2 (807C/837T/873G/Br^b) and allele 3 (807C/837C/873G/Br^a) are each associated with lower levels of $\alpha_2\beta_1$. Finally, we also show here that the rate of platelet attachment to type I collagen in whole blood under conditions of high shear rate (1,500/s) is proportional to the density of $\alpha_2\beta_1$ receptors on the platelet surface. Thus, the density of platelet $\alpha_2\beta_1$ could have an important impact on platelet adhesion to collagen in whole blood and therefore on platelet function in vivo, contributing to an increased risk of thrombosis or to bleeding in relevant disease states.

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codon Phe²²⁴) and 873 (ACA/ACG at codon Thr²⁴⁶) of the cDNA sequence. Although these particular variants do not change the amino acid sequence of the α_2 protein, we have found a significant correlation between these DNA sequence polymorphisms and expression levels of $\alpha_2\beta_1$. We have found that the 807C/873G sequences are associated with lower levels of $\alpha_2\beta_1$, while the 807T/873A sequences are associated with higher levels of this integrin. In addition, familial studies confirm that regulation of $\alpha_2\beta_1$ levels is an inherited trait linked to these two silent polymorphisms. These alleles were referred to as 807C (for the 807C/873G pair) and 807T (for the 807T/873A pair).

In this report, we have extended our analysis of the α_2 gene to include the introns surrounding the nucleotide polymorphisms at bp 807 and 873. As a result of this work, we are now able to define three α_2 gene alleles; two of these alleles are associated with lower levels of $\alpha_2\beta_1$, whereas one is associated with higher levels of this integrin. These allelic differences in $\alpha_2\beta_1$ levels could modulate platelet function in vivo by influencing the critical initial phase of stabilized platelet adhesion to collagen, which is mediated by $\alpha_2\beta_1$. Indeed, we show here that differences in $\alpha_2\beta_1$ receptor levels are reflected in the rate of platelet attachment to type I collagen under conditions of high shear.

MATERIALS AND METHODS

Identification of the polymorphism at bp 837. The polymorphism at bp 837 was identified after analysis of mRNA or DNA from 85 individuals. The mRNA was amplified and sequenced, whereas the DNA was analyzed by Southern dot blot hybridization for determination of the base at position 837. Procedures were performed as previously described.⁹ Oligonucleotides used for dot blot analysis of the 837 sequence were: 837 C: GCTGAATAGGCATATTT; 837 T: GCTGATAAGCATATTT.

Sequence analysis. DNA was either sequenced manually by the dideoxy termination method using Sequenase 2.0 (US Biochemical Corp, Cleveland, OH) or with an automated sequencer at The Scripps Research Institute DNA Core Laboratory.

Isolation of introns F, G, and H from genomic DNA. The primers used for amplification possessed either an *Xba* I site or an *Xho* I site to facilitate subcloning into pGEM (Promega, Madison, WI). Intron G was amplified and isolated as previously described.⁹ Intron F was amplified using the following primer pair: 5' primer (cDNA bp 634-665): GAAC-TCGAGGTACAAGGCCCTTGATATAGGCC; 3' primer (cDNA bp

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764-786): AGGTCTAGACCATATTGGGATGTCTGGGATG. Intron H was amplified using the following primer pair: 5' primer (intron G bp 3506-3532): AATCTCGAGCGAATACTGGGATAAATACATGCAC; 3' primer (cDNA bp 1090-1114): TCCTCTAGACCCAGCCTTTTCTAG-TAGAGCTGC.

The polymerase chain reaction (PCR) products were ligated into pGEM (Promega) after digestion with *Xba* I and *Xho* I. Intron F was found to possess an internal *Xba* I site. Therefore, each segment of intron F was subcloned into pGEM separately.

Br polymorphism analysis. Determination of Br genotype was performed as described.¹⁰ Sixty-three individuals were typed for this polymorphism; of these, 13 were heterozygous for this polymorphism, whereas the remainder were homozygous for the Br^b polymorphism.

Determination of α_2 genotype using the *Bgl* II/*Nde* I restriction fragment length polymorphism (RFLP) assays. The ~600-bp segment of intron G encompassing the *Bgl* II and *Nde* I sites was amplified from genomic DNA using the following primer pair: 5' primer (intron G bp 2789-2812): GATTTAACCTTCCCGACTGCCTTC; 3' primer (intron G bp 3346-3369): CATAGGTTTTGGGGAACAGGTGG.

The PCR product (5 μ L) was digested in a 15 μ L reaction volume using 0.5 μ L of *Bgl* II or *Nde* I (NEB) in the recommended reaction buffer at 37°C overnight. Reaction products were analyzed on a 1.4% agarose gel.

Preparation of collagen-coated cover slips. Acid soluble type I collagen from human placenta (Sigma, St Louis, MO) was diluted to a concentration of 200 mg/mL in phosphate-buffered saline (PBS), pH 7.4, and applied evenly over a horizontal glass cover slip (Corning, Inc, Corning, NY; 24 mm \times 50 mm), covering all but the first 10 mm, which remained uncoated to facilitate handling. Coated cover slips were then placed in a humid environment at room temperature for 60 minutes. Excess collagen was removed by four sequential rinses with PBS, pH 7.4, and assembled in the flow chamber. Blocking the cover slips with bovine serum albumin (0.1 mg/mL) did not affect initial platelet adhesion or subsequent thrombus formation, and uncoated cover slips did not support platelet adhesion.^{11,12}

Perfusion chamber and epifluorescence video microscopy. Platelet interaction with immobilized collagen was studied using a modification of a Hele-Shaw flow chamber described elsewhere.^{11,12} Collagen-coated cover slips formed the lower surface of the chamber with a flow path height of 254 μ m (determined by a silicone rubber gasket). The flow chamber was assembled and filled with PBS, pH 7.4. A syringe pump (Harvard Apparatus Inc, Holliston, MA) was used to aspirate blood through the flow chamber. A flow rate of 1.94 mL/min produced a wall shear rate of 1,500/s at the inlet of the flow chamber. All measurements of platelet adhesion and thrombus formation were made at a position adjacent to the inlet of the chamber so as to avoid prior exposure of flowing platelets to the thrombogenic surface and preformed thrombi. Platelets were labeled in whole blood by direct incubation with the fluorescent dye mepacrine (quinacrine dihydrochloride; 10 mmol/L, final concentration). Although this dye also labels leukocytes, these cells could be readily distinguished from platelets by their relatively large size and scarcity; moreover, permanent leukocyte attachment to collagen was negligible at wall shear rates above 500/s. Red cells were not visualized due to fluorescence quenching by hemoglobin. Mepacrine concentrates in the dense granules of platelets and has no effect on normal platelet function at the concentration used.¹³ Platelet secretion after adhesion does not prevent their visualization. Furthermore previous studies have shown that mepacrine does not interfere with platelet adhesion.¹² The flow chamber, mounted on an epifluorescence microscope (Axiovert 135M inverted microscope, Carl Zeiss Inc, New York, NY), allowed direct visualization in real time of the platelet adhesion process, which was recorded on a video cassette recorder.

Platelet surface coverage measurements. The total area occupied by adherent platelets in an area of 16,384 mm² was measured by capturing images from the video tape using a frame grabber (Matrox

Image LC; Matrox Electronics System Ltd, Dorval, Quebec, Canada) and processing using the Metamorph software package (Universal Imaging Corporation, Westchester, PA). A threshold was applied to the image to distinguish platelets from the background. The microscope settings (including contrast, brightness, and magnification settings) were maintained at constant values to facilitate valid comparisons between different experiments.

RESULTS

Additional nucleotide polymorphisms linked to expression levels of $\alpha_2\beta_1$ define three alleles of the α_2 gene. We have previously described the variation in $\alpha_2\beta_1$ levels that exists among individuals. In studying this variability in expression levels, our analysis of mRNA from six individuals expressing different levels of $\alpha_2\beta_1$ revealed two linked nucleotide polymorphisms, separated by almost seventy nucleotides, at bp 807 and 873 in the α_2 coding region. These were the only two nucleotide polymorphisms identified within the ~3.5-kb α_2 coding region that consistently varied among the samples studied. Based on this work, subsequent analyses of 30 individuals showed that the 807/873 nucleotide polymorphisms were associated with differences in expression levels of $\alpha_2\beta_1$; the 807C/873G pair was found to be associated with lower levels of $\alpha_2\beta_1$, the 807T/873A pair with higher levels of this integrin.

In the present study, we have continued our analysis of the α_2 gene to determine if additional sequence polymorphisms exist that are associated with the 807 and 873 polymorphisms and with expression levels of $\alpha_2\beta_1$. Analysis of a larger group of individuals has now led to the identification of an additional, rarer nucleotide polymorphism (C or T) in the coding region of the α_2 gene, at bp 837. As with the polymorphisms at bp 807 and 873, the polymorphism at bp 837 does not change the α_2 coding sequence. In this variant, a C rather than a T is found at bp 837 in a small subset of individuals (Fig 1). In our study group, 13 out of 85 individuals screened were heterozygous C/T at this position; all 13 of these individuals were either heterozygous or homozygous for 807C/873G, indicating strong linkage with the 837C polymorphism.

This nucleotide variation therefore defines a third allele of the α_2 gene, which is present at a frequency of approximately 8% in the population. It is striking that the three nucleotide polymorphisms we have identified are clustered in one segment of the α_2 coding region. For this reason, we extended our analysis of the α_2 gene to include the three introns flanking bp 807, 837, and 873 (introns F, G, and H). We have cloned these introns, and the sequences of this region have been deposited with GenBank (Accession No. AF035968).

We have identified several sequence variations in the ~4 kb intron that separates bp 807 and 873 (intron G). Each of these additional nucleotide polymorphisms is always found associated with the particular α_2 allele indicated in Fig 1, thereby establishing their linkage to the 807 and 873 polymorphisms. Of particular interest for screening purposes (see below) is a *Bgl* II restriction site created by the nucleotide sequence present in intron G of allele 1. As depicted in Table 1, this *Bgl* II site is found associated only with allele 1. Seventy nucleotides upstream of the *Bgl* II site, we have also identified a *Nde* I site, present in alleles 1 and 2, but are absent in allele 3. Interestingly, this nucleotide variation is linked to the polymorphism at bp 837, where only individuals carrying a C at bp 837 were found

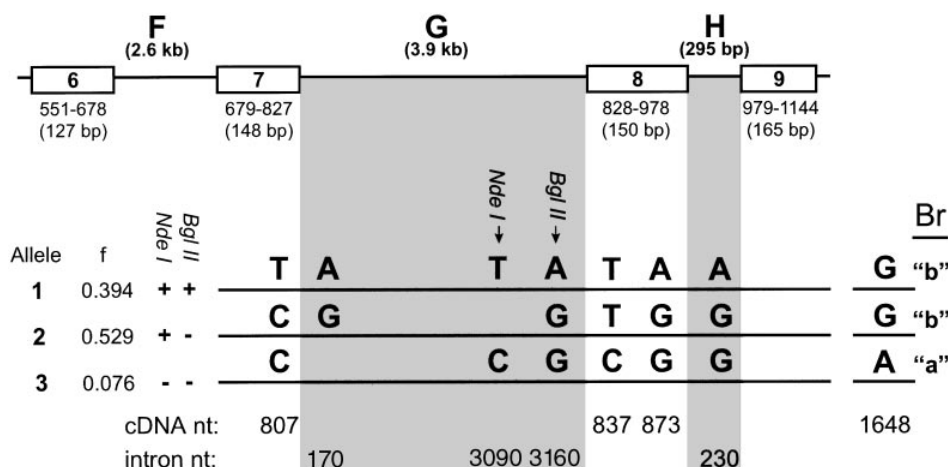


Fig 1. Structure of the α_2 gene surrounding the 807 and 873 polymorphisms. Three α_2 gene alleles, defined by eight nucleotide polymorphisms, are indicated in the figure. Exons 6 through 9 are shown in boxes; positions of the exons in the cDNA sequence and the length of each exon are indicated beneath each exon box. The polymorphisms at bp 807, 837, and 873 are shown in bold. The Br polymorphism at bp 1648 is also shown in bold to the right. Introns F, G, and H are indicated. The length of each intron is shown in parentheses. The frequency (f) of each allele was determined from a random pool of 85 individuals. cDNA and intron positions of the polymorphisms are indicated. The ability of each allele to be cleaved by *Nde* I or *Bgl* II (+ or -) is indicated in tabular form next to each allele. The precise bp differences that change susceptibility to cleavage by *Nde* I or *Bgl* II within intron G are indicated.

to lack the *Nde* I site. The previously defined Br polymorphism at nucleotide 1648¹⁰ also appears to be linked to these polymorphisms; only individuals carrying a C at bp 837 were found to carry the Br^a polymorphism.

The linkage associations between the polymorphisms at bp 807/873/837, the *Nde* I site, and the Br polymorphisms are given in Table 2. Two additional nucleotide polymorphisms linked to bp 807/873 were also identified, one near the 5' end of intron G and one in the 300 bp intron that lies downstream of bp 873 (intron H), although our analysis is limited to only a few individuals at the present time. Finally, our preliminary data indicate that nine additional base variations in intron F, which lies upstream of bp 807, may be linked to the already defined alleles. However, these particular base changes (determined for just one 807T allele and one 807C allele) need to be confirmed among a larger pool of 807C and 807T individuals.

Thus, three α_2 gene alleles, defined by eight nucleotide polymorphisms, have now been defined. We had previously shown that alleles 1 and 2 of the α_2 gene, defined only by the polymorphisms at bp 807 and 873, were associated with high or low $\alpha_2\beta_1$ levels, respectively. The third allele we now define carries the 807C/873G polymorphisms and would have therefore been associated with lower levels of $\alpha_2\beta_1$ in our previous studies. Individuals homozygous for allele 3 have not yet been identified by us, precluding a direct analysis of $\alpha_2\beta_1$ levels

Table 1. Association Between the Presence of the *Bgl* II Site and Expression of α_2 Allele 1

Allele 1 Copy Number	<i>Bgl</i> II Digestion Pattern*		
	Complete Digest	Partial Digest	Undigested
2	4	0	0
1	0	17	0
0	0	0	17

*The number of donors whose DNA amplicon gave the indicated digestion pattern is noted, as per the examples depicted in Fig 2A.

associated with that genotype. Nonetheless, the clustering of nucleotide polymorphisms identified so far suggests that this region of the α_2 gene might harbor elements important in the regulation of α_2 expression.

*α_2 allele genotyping using *Bgl* II/*Nde* I restriction analysis.* We have developed a strategy to type individuals for each of the three alleles using the polymorphic *Bgl* II and *Nde* I restriction sites described above. The region of intron G encompassing these sites was amplified from genomic DNA as described in Materials and Methods, and the resulting PCR products were digested with *Bgl* II or *Nde* I. Analysis by agarose gel electrophoresis resulted in clearly resolved patterns from which the genotype of individuals could be readily determined (Fig 2). As seen in panel A, the 600-bp PCR product amplified from individuals expressing alleles 2 and/or 3 (lanes 5 and 6) or from the control sequence (lane 7) remained undigested by *Bgl* II. The PCR product produced from individuals homozygous for

Table 2. Association Between the Presence of the *Nde* I Site and α_2 Genotype

Genotype α_2 Alleles	<i>Nde</i> I Site			Br Type		
	Complete Digest	Partial Digest	Undigested	a/a	a/b	b/b
1/1	4	0	0	0	0	12
2/2	5	0	0	0	0	17
1/2	7	0	0	0	0	21
1/3	0	2	0	0	4	0
2/3	0	5	0	0	9	0
3/3	—	—	—	—	—	—

Allelic genotype combinations are depicted in the first column. Individuals homozygous for allele 3 have not yet been detected in this study. The patterns of *Nde* I digestion were determined, as per the examples depicted in Fig 2B. Br typing was performed as described in Materials and Methods. The number of individuals whose DNA amplicon gave the representative digestion pattern with *Nde* I is depicted for each allelic genotype.

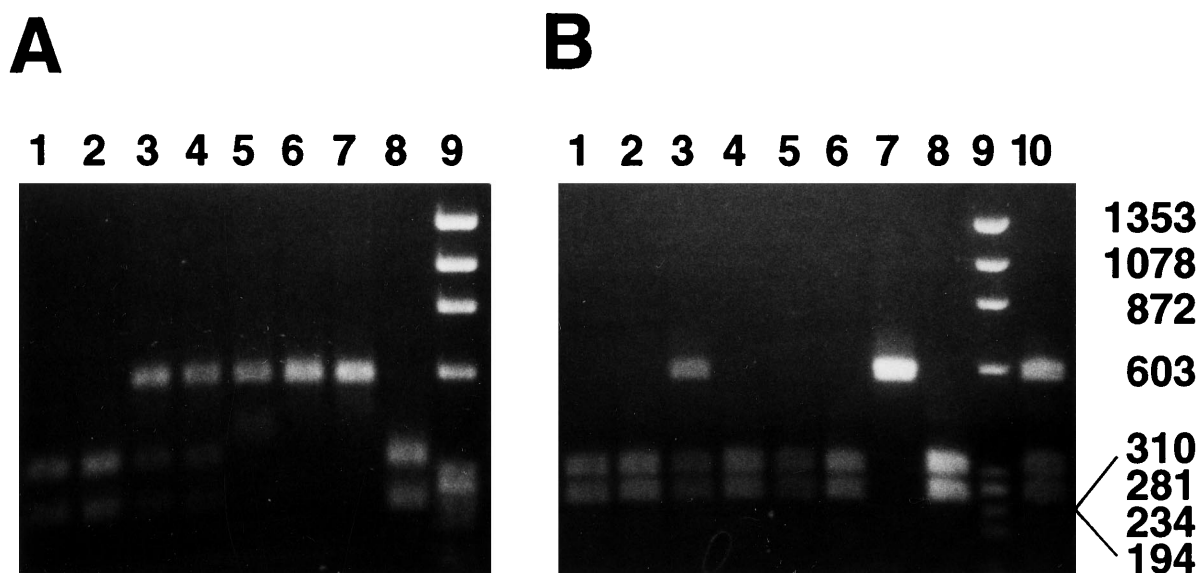


Fig 2. α_2 allele genotyping using *Bgl* II/*Nde* I digestion. The region of the α_2 gene encompassing the *Bgl* II and *Nde* I sites was amplified from genomic DNA as described in Materials and Methods. The 600-bp PCR product was digested with *Bgl* II (A) or *Nde* I (B), and the resulting products were analyzed by agarose gel electrophoresis. Lanes 1 and 2, homozygous (allele 1); lane 3, heterozygous (allele 1, allele 3); lane 4, heterozygous (allele 1, allele 2); lanes 5 and 6, homozygous (allele 2); lane 7, control sequence 807C/837C/873G; lane 8, control sequence 807T/837T/873A; lane 9, molecular weight (MW) λ Hind III/ ϕ X174Hae III; lane 10 (B only), heterozygous (allele 2, allele 3). The 807, 837, and 873 designations refer to the genotype as determined by Southern dot blot analysis of genomic DNA.⁹ Size of MW markers (in bp) is indicated to the right of the figure.

allele 1 (lanes 1 and 2) or from the control sequence (lane 8) was completely digested by *Bgl* II to produce fragments of 200 bp and 400 bp, whereas that produced from individuals expressing allele 1 and either allele 2 or 3 yielded fragments of 200 bp, 400 bp, and 600 bp (lanes 3 and 4).

The same PCR products were digested with *Nde* I to permit determination of allele 3. As seen in panel B, only those products generated from individuals carrying allele 3 or from the control sequence remained undigested by *Nde* I (lanes 3, 7, and 10). As none of the individuals screened were homozygous for allele 3, *Nde* I in each case cuts some product (excluding the control sequence in lane 7). Thus, we describe here a novel RFLP method for rapidly determining the α_2 genotype at positions 807, 837, and 873 from genomic DNA. This technique therefore provides a simple method for discriminating among the three α_2 gene alleles described above.

Sequence analysis of intron G. Sequence analysis of intron G has further revealed the presence of a unique inverted repeat in this region (Fig 1). Separated by ~ 2.5 kb, the inverted repeat sequences, which do not have significant homology with sequences currently in the database, are 275 bp long, have the potential to form a stem loop structure based on sequence analysis, and are present in all of the gene alleles described above.

The rate of platelet adhesion to collagen at high shear rate increases with increasing $\alpha_2\beta_1$ receptor density. We compared the behavior of platelets from individuals homozygous for allele 1 with those expressing only alleles 2 or 3 to assess the influence of $\alpha_2\beta_1$ receptor density on adhesion to type I collagen. Whole blood was perfused over immobilized type I collagen under high shear rate conditions (1,500/s). The results from one representative comparison between an individual homozygous for allele 1 (807T) and an individual homozygous

for allele 2 (807C) are depicted in Fig 3. Coverage of the collagen-coated surface increased over time with platelets from both individuals, and by 3 minutes there was essentially no difference in the total surface coverage between the two samples. However, the rate at which platelet deposition occurred differed significantly between paired donor samples. The results from a comparison of three 807T and three 807C donors are summarized in Fig 4. In the case of the 807T donors, the rate of platelet attachment was significantly faster, particularly within the first 2 minutes of adhesion (Fig 4). Thus, at high shear rates in whole blood, the rate of platelet attachment to type I collagen increases with increasing density of $\alpha_2\beta_1$.

DISCUSSION

We have previously defined two linked, silent polymorphisms in the α_2 gene that are associated with variable expression levels of $\alpha_2\beta_1$ on platelets and that define two alleles of the α_2 gene.⁹ In this report, we describe the identification of five additional nucleotide polymorphisms in the region surrounding bp 807 and 873. These polymorphisms are linked to those previously described and to expression levels of α_2 . Indeed, three α_2 gene alleles, defined by eight nucleotide polymorphisms, have now been confirmed.

It is not clear whether these sequence changes are themselves involved in differential α_2 expression or if they are merely physically linked to the region responsible for modulating α_2 levels. Studies are currently underway to investigate this question. In the discussion that follows, we describe several possible mechanisms through which these exonic and/or intronic polymorphisms might be linked to expression levels of the $\alpha_2\beta_1$ integrin; other mechanisms are certainly possible.

There are a number of ways in which DNA sequence alterations could influence gene expression. For example,

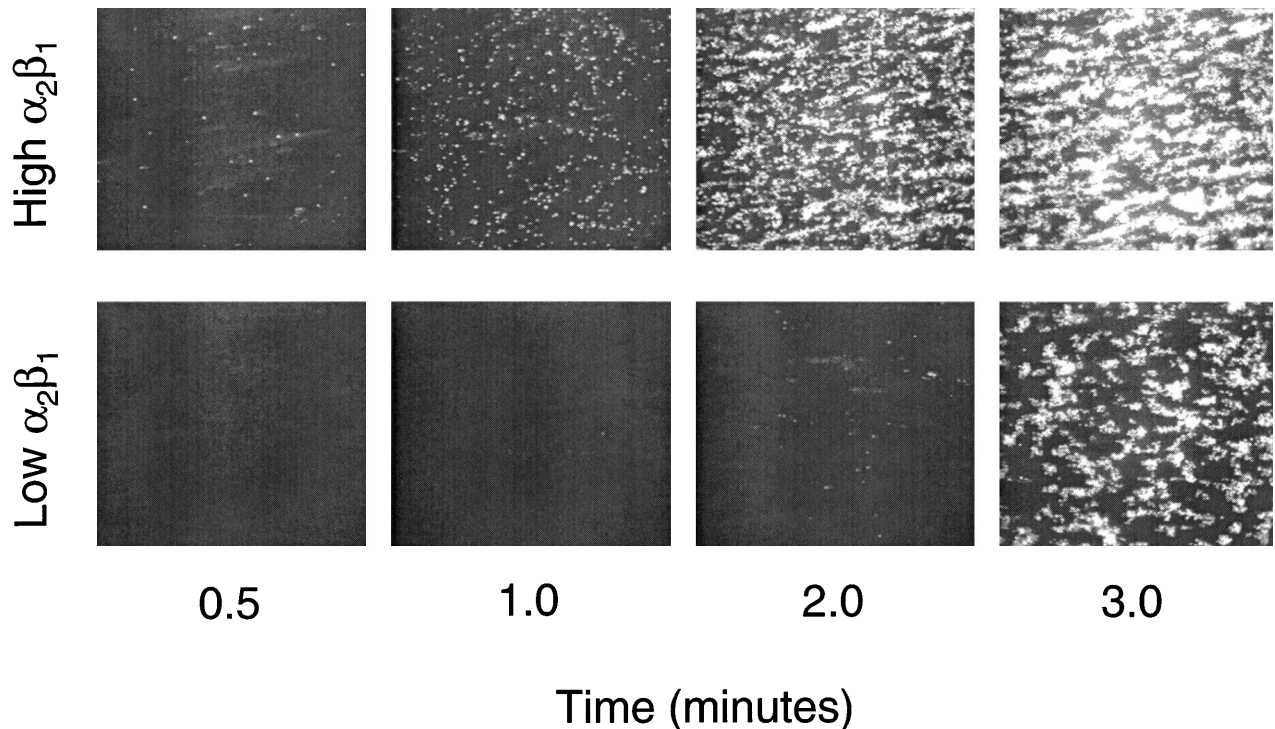


Fig 3. Video microscopy of platelet attachment to human type I collagen at high shear (1,500/s). Real-time epifluorescence video microscopy showing the time courses of platelet adhesion in whole blood to surface-bound solubilized human type I collagen at 1,500/s. Single-frame images obtained during a typical comparison are depicted. These data, derived from one donor pairwise analysis, are representative of results obtained from six donors. Upper row: normal donor, homozygous allele 1 (807T) genotype, High platelet $\alpha_2\beta_1$ density; bottom row: normal donor, homozygous allele 2 (807C) genotype, Low platelet $\alpha_2\beta_1$ density. Time after initiation of whole blood flow is shown above each column. Platelet $\alpha_2\beta_1$ density was determined by flow cytometry and is expressed as a normalized mean fluorescence intensity (nMFI).⁹ The range of nMFI for 32 normal subjects is 1.0 to 9.4. In the experiment depicted here, the nMFI values for each donor are: High, 9.2; Low, 2.2.

changes in specific promoter elements could influence promoter activity, resulting in increased or decreased mRNA levels. Expression of $\alpha_2\beta_1$ in hematopoietic cells is restricted to megakaryocytes and platelets.¹⁴ Zutter et al¹⁵ have shown that induced expression of $\alpha_2\beta_1$ during megakaryocytic differentiation is due to transcriptional activation of the α_2 gene; no changes in the level of β_1 mRNA expression were detected during megakaryocytic differentiation. Approximately 5 kb of the 5' sequence flanking the α_2 transcriptional start site has been cloned, and regions critical for α_2 expression have been identified.¹⁶ Although a strong core promoter region located in the first ~100 bp upstream of the transcriptional start site was found to be necessary for gene activity, it was not found to be cell-type specific. A silencer element located upstream of this region (between -92 and -351) was found to function in cells of hematopoietic lineage, and enhancer elements identified further upstream (between -1426 and -2592) were found to be megakaryocyte-specific and were required for high-level expression of the α_2 gene in megakaryocytic cells. In addition, a number of sites for transcription factors and other regulatory molecules have been identified in the region upstream of the transcriptional start site. Sequence variations in these or other regulatory elements could have a profound impact on expression levels of α_2 .

In addition to promoter variations influencing gene expression, the 3' untranslated region (3'UTR) of α_2 , which is

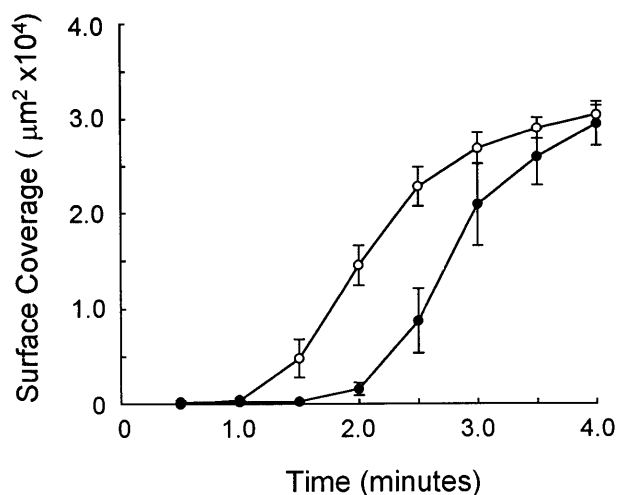


Fig 4. Time course of platelet adhesion to human type I collagen at 1,500/s. Time course of platelet attachment to surface-bound solubilized human type I collagen. The area (square microns) covered by platelets is depicted on the ordinate as a function of time after initiation of blood flow, in minutes (abscissa). The results of three paired comparisons are shown ($n = 3$; mean \pm SEM). (○) High-density donors (mean nMFI = 6.5); (●) low-density donors (mean nMFI = 2.1). The levels of expression of $\alpha_2\beta_1$ in high-density donors was threefold that in low-density donors. Differences at 1.5, 2.0, and 2.5 minutes are statistically significant ($P < 0.01$).

approximately 5 kb, might modulate protein expression; 3'-untranslated regions have been shown to influence mRNA stability as well as message translation.¹⁷ It is also possible that the polymorphisms at bp 807 and 873 are directly related to allelic differences in α_2 expression. Modulation of expression levels by sequences within the coding region of RNA has been described previously.¹⁸⁻²⁰ Differences in intragenic sequences could also influence α_2 gene expression. There are numerous reports of enhancer or repressor elements located in intronic sequences.²¹⁻²³ Indeed, the fact that the polymorphisms so far identified are clustered in one region of the α_2 gene suggests that this region might harbor critical control elements involved in the regulation of α_2 expression.

In whole blood, under a broad range of shear rates (50 to 1,500/s), $\alpha_2\beta_1$ mediates the initial phase of stabilized platelet adhesion to collagen that will lead to accelerated prothrombin conversion on the platelet surface and thrombus formation supported by the integrin $\alpha_{IIb}\beta_3$.²⁴⁻²⁶ However, even when the function of $\alpha_{IIb}\beta_3$ is completely inhibited and thrombus formation is blocked, $\alpha_2\beta_1$ -mediated adhesion itself still induces the cellular changes that catalyze prothrombin conversion to the same extent as one would otherwise see in the absence of inhibitors.²⁶ Consequently, $\alpha_2\beta_1$ has the potential to play a major role in platelet function in vivo both as a primary mediator of adhesion to collagen and as a primary stimulus for prothrombin conversion at the platelet surface.

As shown here, the density of platelet $\alpha_2\beta_1$ has an important impact on platelet adhesion to collagen in whole blood even under conditions of high shear rate (1,500/s), such that the rate of adhesion to type I collagen increases with increasing receptor density. This stage of adhesion, occurring between 30 seconds and 3 minutes, is likely to be a critical period of thrombus formation in vivo, because there are many compensatory antithrombotic mechanisms that would come into play to inhibit thrombus expansion or to dissociate the nascent thrombus. Within 3 to 5 minutes, a sufficient number of stimuli have been received, regardless of $\alpha_2\beta_1$ density, and mature thrombi begin to cover the collagen surface. The expansion of the initial platelet clusters to form larger thrombi is certainly mediated by the binding of integrin $\alpha_{IIb}\beta_3$, because macroaggregate formation (but not surface attachment) is inhibited by monoclonal antibodies specific for that integrin.

The expression of $\alpha_2\beta_1$ by platelets is critical in promoting platelet adhesion to the subendothelium; adhesion of platelets to collagen is critical for normal platelet activity, in hemostasis, and in wound repair. Hereditary variation in platelet levels of $\alpha_2\beta_1$, defined by the existence of multiple alleles of the α_2 gene that are associated with variable $\alpha_2\beta_1$ expression levels, could therefore have a significant impact on platelet function, contributing to an increased risk of thrombosis or bleeding in relevant disease states.

REFERENCES

- Hynes RO: Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* 69:11, 1992
- Pischel KD, Bluestein HG, Woods VL: Platelet glycoprotein Ia, Ic, and IIa are physicochemically indistinguishable from the very late activation antigens adhesion-related proteins of lymphocytes and other cell types. *J Clin Invest* 81:505, 1988
- Takada Y, Wayner EA, Carter WG, Hemler ME: Extracellular matrix receptors, ECMRII and ECMRI, for collagen and fibronectin correspond to VLA-2 and VLA-3 in the VLA family of heterodimers. *J Cell Biochem* 37:385, 1988
- Kunicki TJ, Nugent DJ, Staats SJ, Orzechowski RP, Wayner EA, Carter WG: The human fibroblast class II extracellular matrix receptor mediates platelet adhesion to collagen and is identical to the platelet glycoprotein Ia-IIa complex. *J Biol Chem* 263:4516, 1988
- Zutter MM, Santoro SA: Widespread histologic distribution of the $\alpha_2\beta_1$ integrin cell-surface collagen receptor. *Am J Pathol* 137:113, 1990
- Elices MJ, Hemler ME: The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others. *Proc Natl Acad Sci USA* 86:9906, 1990
- Kirchhofer D, Languino LR, Ruoslahti E, Pierschbacher MD: $\alpha_2\beta_1$ integrins from different cell types show different binding specificities. *J Biol Chem* 265:615, 1990
- Kunicki TJ, Orzechowski R, Annis D, Honda Y: Variability of integrin alpha 2 beta 1 activity on human platelets. *Blood* 82:2693, 1993
- Kunicki TJ, Kritzik M, Annis DS, Nugent DJ: Hereditary variation in platelet integrin $\alpha_2\beta_1$ copy number is associated with two silent polymorphisms in the α_2 gene coding sequence. *Blood* 89:1939, 1997
- Kalb R, Santoso S, Unkelbach K, Kiefel V, Mueller-Eckhardt C: Localization of the Br polymorphism on a 144 bp Exon of the GPIa gene and its application in platelet DNA typing. *Thromb Haemost* 71:651, 1994
- Usami S, Chen HH, Zhao Y, Chien S, Skalak R: Design and construction of a linear shear stress flow chamber. *Ann Biomed Eng* 21:77, 1993
- Savage B, Saldivar E, Ruggeri ZM: Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell* 84:289, 1996
- Dise CA, Burch JW, Goodman DBP: Direct interaction of mepacrine with erythrocyte and platelet membrane phospholipid. *J Biol Chem* 257:4701, 1982
- Santoro SA, Zutter MM: The $\alpha_2\beta_1$ integrin: A collagen receptor on platelets and other cells. *Thromb Haemost* 74:813, 1995
- Zutter MM, Fong AM, Krigman HR, Santoro SA: Differential regulation of the $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ integrin genes during megakaryocytic differentiation of pluripotential K562 cells. *J Biol Chem* 267:20233, 1992
- Zutter MM, Painter AA, Staatz WD, Tsung YL: Regulation of α_2 integrin gene expression in cells with megakaryocytic features: A common theme of three necessary elements. *Blood* 86:3006, 1995
- Jackson RJ: Cytoplasmic regulation of mRNA function: The importance of the 3' untranslated region. *Cell* 74:9, 1993
- Burstein KL, Jewell CM, Sar M, Cidlowski JA: Intragenic sequences of the human glucocorticoid receptor complementary DNA mediate hormone-inducible receptor messenger RNA down-regulation through multiple mechanisms. *Mol Endocrinol* 8:1764, 1994
- Kaludov NK, Pabon-Pena L, Hurt MM: Identification of a second conserved element within the coding sequence of a mouse H3 histone gene that interacts with nuclear factors and is necessary for normal expression. *Nucleic Acids Res* 24:523, 1996
- Beattie DT, Mahan MJ, Mekalanos JJ: Repressor binding to a regulatory site in the DNA coding sequence is sufficient to confer transcriptional regulation of the vir-repressed genes (vrg genes) in *Bordetella pertussis*. *J Bacteriol* 175:519, 1993
- Ottavio L, Chang C-D, Rizzo M-G, Travali S, Casadevall C, Baerga R: Importance of introns in the growth regulation of mRNA levels of the proliferating cell nuclear antigen gene. *Mol Cell Biol* 10:303, 1990
- Frenkel B, Montecino M, Stein JL, Lian JB, Stein GS: A composite intragenic silencer domain exhibits negative and positive transcriptional control of the bone-specific osteocalcin gene: Promoter and cell type requirements. *Proc Natl Acad Sci USA* 91:10923, 1994

23. Jeffers M, Pellicer A: Multiple intragenic elements regulate the expression of the murine N-ras gene. *Oncogene* 7:2115, 1992
24. Coller BS, Beer JH, Scudder LE, Steinberg MH: Collagen-platelet interactions: Evidence for a direct interaction of collagen with platelet GPIa/IIa and an indirect interaction with platelet GPIIb/IIIa mediated by adhesive proteins. *Blood* 74:182, 1989
25. Savage B, Marzec UM, Chao BH, Harker LA, Maraganore JM,

Ruggeri ZM: Binding of the snake venom-derived proteins applaggin and echistatin to the arginine-glycine-aspartic acid recognition site(s) on platelet glycoprotein IIb/IIIa complex inhibits receptor function. *J Biol Chem* 265:11766, 1990

26. Kirchhofer D, Tschopp TB, Steiner B, Baumgartner HR: Role of collagen-adherent platelets in mediating fibrin formation in flowing whole blood. *Blood* 86:3815, 1995