

Published in final edited form as:

Atherosclerosis. 2008 June ; 198(2): 280–286. doi:10.1016/j.atherosclerosis.2007.10.005.

β_3 Integrin Haplotype Influences Gene Regulation and Plasma von Willebrand Factor Activity

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Abstract

The Leu33Pro polymorphism of the gene encoding β_3 integrin (*ITGB3*) is associated with acute coronary syndromes and influences platelet aggregation. Three common promoter polymorphisms have also been identified. The aims of this study were to (1) investigate the influence of the *ITGB3* –400C/A, –425A/C and –468G/A promoter polymorphisms on reporter gene expression and nuclear protein binding and (2) determine genotype and haplotype associations with platelet $\alpha_{IIb}\beta_3$ receptor density. Promoter haplotypes were introduced into an *ITGB3* promoter-pGL3 construct by site directed mutagenesis and luciferase reporter gene expression analysed in HEL and HMEC-1 cells. Binding of nuclear proteins was assessed by electrophoretic mobility shift assay. The association of *ITGB3* haplotype with platelet $\alpha_{IIb}\beta_3$ receptor density was determined in 223 subjects. Species conserved motifs were identified in the *ITGB3* promoter in the vicinity of the 3 polymorphisms. The GAA, GCC, AAC, AAA and ACC constructs induced ~50% increased luciferase expression relative to the GAC construct in both cell types. Haplotype analysis including Leu33Pro indicated 5 common haplotypes; no associations between *ITGB3* haplotypes and receptor density were found. However, the GCC-Pro33 haplotype was associated with significantly higher vWF activity (128.6 [112.1–145.1]%) compared with all other haplotypes (107.1 [101.2–113.0]%, $p=0.02$). In conclusion, the GCC-Pro33 haplotype was associated with increased vWF activity but not with platelet $\alpha_{IIb}\beta_3$ receptor density, which may indicate *ITGB3* haplotype influences endothelial function.

Keywords

β_3 integrins; polymorphisms; gene regulation

Introduction

Receptor-mediated interactions between leukocytes, endothelial cells and platelets and coagulation factors and extracellular matrix proteins at the sites of vascular injury are central to the pathogenesis of atherosclerosis and thrombosis [1]. β_3 integrin is a component of the platelet-specific $\alpha_{IIb}\beta_3$ receptor and the more widely distributed $\alpha_v\beta_3$ receptor. Platelets play a key role in arterial thrombosis and the $\alpha_{IIb}\beta_3$ receptor is central to these processes as the

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mediator of platelet aggregation at the site of vascular injury. The $\alpha_v\beta_3$ receptor is variably expressed by a number of cell types including endothelial cells and vascular smooth muscle cells [2]. Increased endothelial cell and vascular smooth muscle cell expression of $\alpha_v\beta_3$ in coronary artery atherosclerotic plaques has been reported [3]. Consequently variants of the gene encoding β_3 integrin (*ITGB3*) may influence the development of cardiovascular disease through modification of the function of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$.

Several polymorphisms in the *ITGB3* gene have been identified including the Leu33Pro (P1^A) variant and the -400C/A, -425A/C and -468G/A promoter polymorphisms [4]. Studies have demonstrated associations between the Pro³³ allele (P1^{A2}) and premature myocardial infarction (MI), ischemic stroke and restenosis following coronary angioplasty [5,6]. Pro33 is associated with a lower threshold for $\alpha_{IIb}\beta_3$ activation in response to ADP, and enhanced α -granule release, clot retraction and adhesion to fibrinogen [7,8]. Limited information is available regarding the functional effects of the -400C/A, -425A/C and -468G/A promoter polymorphisms. The aims of this study were to (i) investigate the influence of the -400C/A, -425A/C and -468G/A promoter polymorphisms on reporter gene expression and nuclear protein binding and (ii) determine genotype and haplotype associations with platelet $\alpha_{IIb}\beta_3$ receptor density.

Materials and Methods

Bioinformatics

Bioinformatics analyses were carried out to compare the 5' region of the human (accession number **AY706100**), chimp (**AY413572**), mouse (**AY413573**) and chicken (**X75348**) *ITGB3* genes to determine regions of conservation. The first 500 bp 5' to the ATG of each species were analysed by FootPrinter (<http://abstract.cs.washington.edu/~blanchem/FootPrinterWeb/FootPrinterInput2.pl>) against the default phylogenetic tree to indicate potential regulatory elements [9]. The software produced short regions of sequences (up to 10 bp in length) that were highly conserved (maximum of 2 bp difference) between species.

Generation of Variant *ITGB3*Reporter Constructs

The *ITGB3* promoter region was cut from a previously reported *ITGB3*-pGL2 vector [10] and ligated into pGL3 by digestion with 5 units of *Kpn I* and *Nhe I*, and ligation with T4 ligase. The wild type *ITGB3* 5' region construct will be referred to as *ITGB3*-pGL3-GAC in relation to the more common alleles located at positions -468, -425 and -400. Haplotypes were introduced into the *ITGB3*-pGL3-GAC construct by site directed mutagenesis using the GeneEditorTM *in-vitro* Site Directed Mutagenesis system (Promega). Mutagenic oligonucleotides were designed with the relevant mismatch in the middle:

-468A: 5'-GGCATTCAACAGATGTTTG-3';

-425C: 5'-GTGTGAATGAAT GACACTCGAGGTAGTGG-3';

-400A: 5'-GTGAATGTGTACCAAGAATC-3'

The -400A and -468A variants were introduced using the manufacturer's standard protocol. A modified protocol was utilised to generate the -425C variant; the mutagenic oligonucleotide was added at the alkaline denaturation stage, and the initial temperature for oligonucleotide annealing was increased to 83°C. Twenty colonies were picked for each mutagenesis reaction and successful mutagenesis confirmed by genotyping and sequencing. The sequence of the

ITGB3 promoter (from the ATG codon to -2070) has been submitted to GenBank, accession number [AY706100](#).

Cell Culture and Transient Transfections

Human erythroleukemic (HEL 92.1.7) cells ($\alpha_{IIb}\beta_3$ expressing representative of megakaryocytic cells) were grown in RPMI 1640 medium (Sigma), supplemented with 10% foetal calf serum (FCS) (First Link Ltd), 2 mM glutamine and antibiotic mix (10,000 U penicillin, 10 mg streptomycin and 25 μ g amphotericin B per ml). Cells were transiently co-transfected by electroporation with the *ITGB3*-pGL3 constructs and Renilla Luciferase-CMV (pRL-CMV) vector (Promega), as the internal control, at a ratio of 50:1. For transfection, 1×10^7 cells per sample were pelleted and resuspended in 0.8 ml PBS, 10 μ g of DNA was added and cells were electroporated at 500 μ F, 0.4 kV (BioRad Gene Pulser). The electroporated cells were seeded in 25 ml of pre-warmed media and incubated at 37°C and 5% CO₂ for 48 hours prior to reporter gene analysis.

Human Microvascular Endothelial cells (HMEC-1, $\alpha_V\beta_3$ expressing, representative of endothelial cells) were grown in MCDB 131 media (Invitrogen), supplemented with 10% FCS, antibiotic mix, 2mM glutamine, 0.001% epidermal growth factor and 1 μ g/ml hydrocortisone. HMEC-1 cells were transiently transfected with Lipofectin (Invitrogen): 5×10^5 cells were plated on 60 mm plates 24 hours prior to transfection with 3 μ g DNA (100:1 ratio of *ITGB3*-pGL3 constructs to pRL-CMV) and 20 μ l Lipofectin, according to manufacturer's instructions, followed by incubation at 37°C and 5% CO₂ for 24 hours prior to reporter gene analysis.

Luciferase Reporter Gene Expression Analysis

Luciferase reporter gene analysis was carried out using the Dual-Luciferase Reporter Assay system (Promega). Transfected HEL cells were pelleted by centrifugation, washed, and 1×10^7 cells lysed with 250 μ l passive lysis buffer (PLB). Transfected HMEC-1 cells were washed, and lysed with 1 ml PLB. Renilla and firefly luciferase activities were determined in 20 μ l of centrifuged cell lysate according to manufacturer's instructions using a PHL luminometer (Mediators). Firefly luciferase values were normalised to Renilla luciferase activity. Eight independent sets of transfections were carried out, and results are expressed as the activity of each construct relative to the *ITGB3*-pGL3-GAC construct.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins were extracted from 5×10^6 HEL and HMEC-1 cells according to the method described by Andrews & Faller [11]. Fluorescein-labelled sense oligonucleotides were synthesised with the polymorphic variant in the centre, as follows:

-468G/A: forward 5'- AGAGAAGGCATTCAG/ACAGATGTTTGCCAG -3'

reverse 5'- CTGGCAAACATCTGC/TTGAATGCCTTCTCT-3'

-425 A/C: forward 5'- GTGTGAATGAATGAA/CACTCGAGGTAGTGG-3'

reverse 5'- CCACTACCTCGAGTT/GTCATTCATTCACAC-3'

-400C/A: forward 5'- GTGGGTGAATGTGTC/ACAAGAATCCAGCG-3'

reverse 5'- CGCTGGATTCTTGG/TACACATTCACCCAC-3'

Sense and antisense oligonucleotides were annealed and gel purified, and 1.5 pmol used in each binding reaction. EMSA reactions were performed in binding buffer (20% glycerol, 20mM Tris, pH7.5, 100mM KCl, 1mM DTT) with 0.1 μ g/ μ l poly dI/dC and 35 μ g of nuclear

protein extract. For competition assays, 1-, 10- and 50-fold molar excess of unlabelled specific or non-specific competitor (SP1: forward 5'- ATTCGATCGGGGCGGGGCGAGC-3' and reverse 5'-GCTCGCCCCGCCCCGATCGAA-3') were added. Reaction mixes were incubated at RT⁰ for 30 min and then electrophoresed on an 8% polyacrylamide gel with 5% stacking gel in 0.25x TBE (22.5 mM Tris-borate and 0.5 mM EDTA, pH 8.3) at 200V for 6 hours at 15°C. The gel was visualised using a FX Molecular Imager (BioRad).

Genotyping of *ITGB3* polymorphisms

The -468G/A polymorphism was genotyped using an Assays-by-DesignSM SNP genotyping assay (Applied Biosystems):

forward primer 5'- GGCAAGAAAAAATTAGTGAATAATAAAGGACTGA-3'

reverse primer 5'- ACTCGAGGTAGTGGGTGAATGT-3'

probes 5'-AAGGCATTCAG/ACAGATG-3'

Probes were labelled with VICTM for detecting the -468G allele and 6-FAMTM for detecting the -468A allele, and following PCR amplification, fluorescence detection was carried out using an ABI PRISM 7700 SDS (Applied Biosystems). The -425 A/C polymorphism was genotyped by PCR-RFLP with primers: forward 5'-CGAGGCTCTTCATGGACCTA-3' and reverse 5'-CACATTCACCCACTACCTCGAAT-3' and digestion with *Apo I* (the underlined base is a mismatch to introduce an *Apo I* site in the presence of the -425A allele). The -400 C/A polymorphism was genotyped by PCR-RFLP with primers: forward 5'-AGTGAATAATAAAGGACTGAACCG-3' and reverse 5'-GCGCTCGCATCTCGTC-3' and digestion with *Rsa I*. The Leu33Pro polymorphism was genotyped as previously described [12].

Subject recruitment and analysis of $\alpha_{IIb}\beta_3$ receptor expression

Subjects (n=233) were recruited by the Baylor College of Medicine Thrombosis Research Group for analysis of the relationship between *ITGB3* polymorphisms and $\alpha_{IIb}\beta_3$ receptor expression. The study population comprised healthy subjects, recruited through local advertising, who were free from a history of cardiovascular disease and were not taking aspirin (n=138), as previously described [13] and subjects who had previously (>3 months) undergone coronary artery bypass surgery (CABG, n=85) who were taking aspirin. All subjects gave informed consent according to a protocol approved by the Baylor College of Medicine institutional review board. Platelet $\alpha_{IIb}\beta_3$ receptor expression was measured by flow cytometry using phycoerythrin-conjugated monoclonal antibody HIP8 (anti-CD41a; BD-Pharmingen) and expressed as mean fluorescent intensity, as previously described [14] Fibrinogen concentration was determined by the Clauss method and vWF activity was determined using a ristocetin cofactor assay, as previously described [14].

Statistical Analysis—Non-normally distributed variables were log-transformed to enable parametric analyses. Comparisons between two groups of continuous variables were carried out using Student's Independent t-test and more than two groups by ANOVA. Data are presented as mean (geometric mean) with either SD (log SD) or 95% confidence intervals (CI). Age did not conform to normal distribution and is presented as median with 25th and 75th percentiles in descriptive statistics. Differences between categorical variables were ascertained using chi-square analysis. All statistical analyses were performed using SPSS for Windows version 12 (SPSS Inc).

Results

Bioinformatics Analysis of the *ITGB3* Promoter

FootPrinter analysis (Fig. 1) indicated conserved motifs in the *ITGB3* 5' region located in the region up to 500 bp from the ATG codon. The -400C/A polymorphism is within a motif conserved between human and chimp (TGTGTCCCAA) and the -425A/C and -468G/A polymorphisms are between conserved motifs. The evolutionary conservation of regions within the 5' gene regulatory region indicated that these sequences might be functionally relevant. To determine if the promoter polymorphisms were functional, the luciferase reporter gene system was utilised together with analysis of nuclear protein binding.

Influence of *ITGB3* -468/-425/-400 Haplotypes on Luciferase Reporter Gene Expression and Nuclear Protein Binding Profile

Luciferase reporter gene analyses indicated significant differences in reporter gene expression according to *ITGB3* promoter haplotype in both HEL (p=0.035) and HMEC-1 cells (p=0.048), as shown in Figure 2. Similar luciferase expression patterns were obtained from the HEL and HMEC-1 cells. The GAA, GCC, AAC, AAA and ACC constructs induced ~50% increased luciferase expression relative to the GAC construct whereas the GCA and ACA constructs showed similar expression to GAC in both cell types. These results indicated that the three polymorphisms in the *ITGB3* promoter may be functional and lead to a difference in nuclear protein binding. Each polymorphic variant was, therefore, analysed for differences in nuclear protein binding with HEL and HMEC-1 nuclear protein extracts. No difference in the nuclear protein binding profile was observed for the -400C/A, -425A/C and -468G/A allelic variants (data not shown).

Association of *ITGB3* polymorphisms and haplotype with platelet $\alpha_{IIb}\beta_3$ receptor density

The relationship between *ITGB3* polymorphisms and $\alpha_{IIb}\beta_3$ receptor density was investigated in 223 subjects. The characteristics of the study population, including ethnicity, are presented in table 1. The mean receptor density (expressed as mean fluorescence intensity, MFI) was 136 (range: 81 to 192). There was no significant difference in mean receptor density between the different ethnic groups studied (Caucasian: 137 [SD 18]; African American: 134 [SD 19]; Mexican American: 135 [SD 16]; Asian: 143 [SD 27]; Other: 131 [SD 22], p=0.59). $\alpha_{IIb}\beta_3$ receptor density was significantly inversely correlated with BMI (r=-0.264, p<0.001) and fibrinogen (r=-0.155, p=0.02) but there was no correlation between $\alpha_{IIb}\beta_3$ receptor density and vWF activity (r=-0.019, p=0.78). There was no significant correlation between receptor density and age (r=-0.096, p=0.16) and no significant difference in $\alpha_{IIb}\beta_3$ receptor density between men and women, or between smokers and non-smokers (data not shown). There was no significant difference in receptor density between CABG patients taking aspirin and healthy subjects not taking aspirin, nor was there a significant difference in receptor density between those with and without a history of MI, or hypertension (data not shown).

The genotype distributions of the -400C/A, -425A/C, -468G/A and Leu33Pro polymorphisms conformed to Hardy Weinberg equilibrium. The -400C/A polymorphism was in significant linkage disequilibrium with the -425A/C (D'=-1.0, p=0.006) and -468G/A polymorphisms (D'=0.93, p<0.0001) but not with the Leu33Pro polymorphism. The -425A/C polymorphism was in significant linkage disequilibrium with the -468G/A (D'=-0.66, p=0.04) and the Leu33Pro (D'=0.67, p<0.0001) polymorphisms. There was no significant linkage disequilibrium between the -468G/A and Leu33Pro polymorphisms. Six promoter haplotypes were identified, as shown in table 2 (denoted A to F), with the 4 most frequent haplotypes accounting for 98% of the 446 chromosomes analysed. Inclusion of the Leu33Pro polymorphism in haplotype analysis gave rise to 11 different haplotypes, as shown in table 2

(denoted 1 to 11), with the 5 most common haplotypes accounting for 94% of the 446 chromosomes analysed.

Analysis of the relationship of individual SNPs with receptor density did not indicate any association of genotype with receptor density, as shown in table 3. The relationship of receptor density with promoter haplotype and haplotype including Leu33Pro was also analysed; there was no significant difference in receptor density according to *ITGB3* haplotypes (data not shown).

Association of *ITGB3* polymorphisms and haplotype with plasma fibrinogen and vWF activity

There was no significant difference in fibrinogen according to *ITGB3* genotype, as shown in table 3, or haplotype (data not shown). There was no significant association of the -400C/A and -468G/A polymorphisms with vWF activity, however there was a significant association of the -425A/C polymorphism and a borderline association of Leu33Pro with vWF activity, as shown in table 3. Analysis of the relationship of promoter haplotypes and haplotypes including Leu33Pro with vWF activity did not indicate any significant associations (data not shown), however, the data was suggestive of potential differences in vWF activity, particularly in haplotype combinations including haplotype 3 (GCC-Pro33). To investigate this further we created a dummy variable including all subjects with haplotype combinations including haplotype 3 (n=43) compared with subjects homozygous for haplotype 1 (GACLeu33) and compared vWF activity: vWF was significantly higher in subjects possessing haplotype 3 (128.6 [112.1, 145.1]%) compared to subjects homozygous for haplotype 1 (106.3 [98.1, 114.5]%, p=0.02). vWF was also significantly higher in subjects possessing haplotype 3 when compared with all other subjects (107.1 [101.2, 113.0]%, p=0.02). vWF activity differed significantly by ethnicity, with African American subjects having significantly lower vWF (96.6 [SD 37.8]%) than Caucasian subjects (120.4 (SD 44.1)%, p=0.02 after Sheffe post hoc analysis). Furthermore, although *ITGB3* haplotype did not differ significantly by ethnicity, haplotype 3 was less prevalent in African Americans (10.4%) than Caucasians (21.4%, p=0.08). Therefore we analysed the relationship of haplotype 3 with vWF activity accounting for ethnicity and haplotype 3 remained significantly higher in those possessing haplotype 3 (p=0.03).

Discussion

Regions of a gene that are conserved between species are likely to be functionally significant and bioinformatics analysis of the region of *ITGB3* promoter containing the -400C/A, -425A/C and -468G/A polymorphisms indicated a number of regions conserved between species, suggesting they may be important in regulation of *ITGB3* expression. Luciferase reporter gene analyses in cells representative of megakaryocyte and endothelial cells indicated modest (~50%) increases in reporter gene expression for the GAA, GCC, AAC, AAA and ACC constructs relative to the GAC construct, supporting a potential role for the polymorphisms in modulating gene expression. In contrast, EMSA analyses did not indicate any clear difference in nuclear protein binding pattern between the alleles of each polymorphism. However, luciferase reporter gene and EMSA analyses do not take into account the native chromatin structure of the human *ITGB3* gene and therefore the inconsistency of results between the 2 methods does not in itself negate an effect of the polymorphisms on *ITGB3* expression *in vivo*. Therefore, to determine whether the differences in reporter gene expression translated into differences in $\alpha_{IIb}\beta_3$ expression in platelets, genotype and haplotype associations with receptor expression were determined.

The extent of linkage disequilibrium between the promoter polymorphisms and the Leu33Pro polymorphism were similar to those reported by O'Haloran *et al.* [15]. Six of the 8 potential promoter haplotypes were identified in the present study and inclusion of the Leu33Pro

polymorphism resulted in the detection of 11 haplotypes, in keeping with those of O'Halloran *et al.* [15]. The prevalence of the 3 most common haplotypes (GAC-Leu33, AAA-Leu33, GCC-Pro33) were approximately equivalent in the two study groups, with the majority of Pro33 alleles associated with the GCC promoter haplotype and the Leu33 allele associated predominantly with the GAC promoter haplotype. There was some discrepancy in the occurrence of the less frequent haplotypes, which likely reflects ethnic heterogeneity in the present study.

We found a wide range in platelet $\alpha_{IIb}\beta_3$ expression which was not explained by *ITGB3* promoter and Leu33Pro genotype or haplotype. Previous reports of the association of Leu33Pro with receptor density have been inconsistent [7]; however, the results of the present study lend further support for a lack of association of *ITGB3* promoter polymorphisms and Leu33Pro with receptor density in unstimulated platelets. These results are inconsistent with the luciferase reporter gene results in which the common GCC and AAA promoter haplotypes were associated with a 50% increase in luciferase reporter gene expression compared with the GAC haplotype. The influence of the promoter polymorphisms on $\alpha_V\beta_3$ expression in human endothelial cells or smooth muscle cells cannot be inferred from the results of our study regarding $\alpha_{IIb}\beta_3$ receptor expression due to cell specific regulation of $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ receptor expression. Increased $\alpha_{IIb}\beta_3$ receptor expression is reported to arise from increased steady-state mRNA levels of both α_{IIb} and β_3 mRNA resulting from enhanced transcription [16]. In contrast, the expression of $\alpha_V\beta_3$ appears to be largely dependent upon regulation of β_3 expression, both at the transcriptional and post-transcriptional level [17,18]. Therefore it cannot be excluded that the influence of the promoter polymorphisms on *ITGB3* gene expression may have more of an influence on the ultimate expression of $\alpha_V\beta_3$ than on the expression of $\alpha_{IIb}\beta_3$.

We found an association of *ITGB3* -425A/C and Leu33Pro polymorphisms and the GCC-Pro33 haplotype (hap3) with vWF activity. Since vWF is released from endothelial cell Weibel Palade bodies this may reflect an influence on endothelial cell function. Shear stress induces activation of endothelial cell $\alpha_V\beta_3$ [19] and it has been shown that shear stress also induces increased secretion of vWF from endothelial cell Weibel Palade bodies [20]. Therefore it is possible that shear stress elicits differential responses in endothelial cells expressing different *ITGB3* haplotypes. Studies of the Leu33Pro polymorphism indicated differential effects on platelet function, with the Pro33 allele associated with enhanced outside-in signalling in platelets and increased α -granule secretion [21]. In addition, enhanced cell migration on vitronectin and osteopontin was observed in CHO cells expressing $\alpha_V\beta_3$ Pro33 [22], which may lend support for a functional effect of Leu33Pro in endothelial cells.

Conclusions

Bioinformatics and reporter gene analyses suggested that the *ITGB3* -468 G/A, -425 A/C and -400 C/A polymorphisms may be functional. However, promoter haplotypes and promoter haplotypes in combination with the Leu33Pro polymorphism were not significantly associated with $\alpha_{IIb}\beta_3$ receptor density, despite wide inter-individual differences in receptor density. The GCC-Pro33 haplotype was, however, associated with enhanced vWF activity, which may indicate *ITGB3* haplotype influences endothelial function. Further studies are required to determine whether *ITGB3* haplotype is associated with enhanced $\alpha_V\beta_3$ expression or activity.

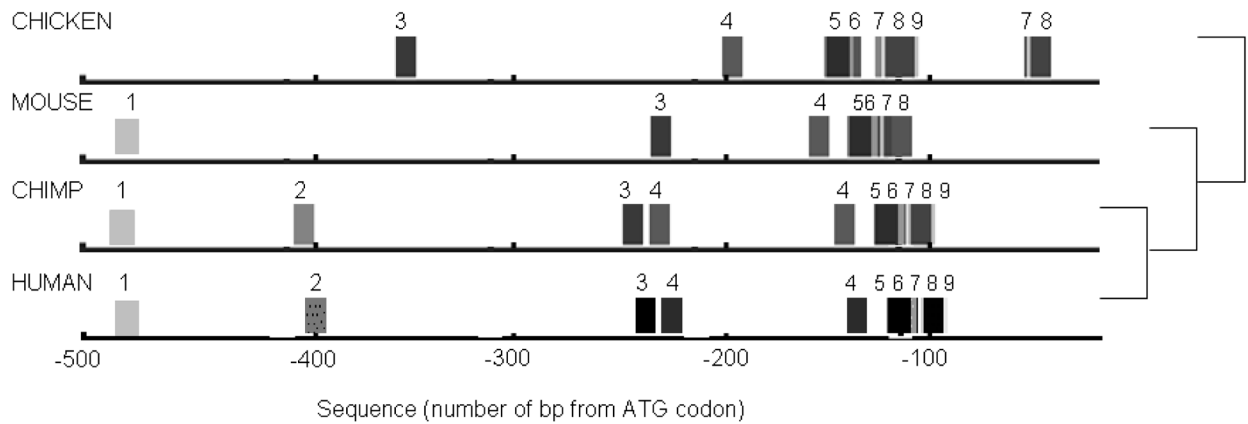
Acknowledgements

This study was funded through grants from the British Heart Foundation (PhD studentship FS/2001012, KEP) and the Heart, Lung and Blood Institute of the National Institutes of Health (HL65229, PB).

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A**B**

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-500 AAAGGACTGA ACCGGTTCAG AGAAGGCATT CAGCAGATGT TTGCCAGTCA AATGAATTAA AGTGTGAATG AATGMAACTC GAGGTAGTGG GTGAATGTGT
-400 CCCAAGAATC CAGCGAAAACA GGGTCTCCCA GGAGGCGGGA CTGGAAGGGT CCGGAGAGGG GCCACAGGCT CCTGGCCTTT CTAAGCACAC CAAGTGCCCA
-300 GTCGCGGACC CCCGGGACCA GGATGCGCTG ACGACCCGGC TGGCAGGCGG GTCCTCGTGG GCGAGGCGAG GGAGGCGGG AGAGAGGAGC AATAGTTTCC
-200 CACCGCTCCC TCTCAGGCGC AGGGTCTAGA GAAGCGCGAG GGGATCTAGA GAAGCCGGAG GGGAGGAAGC GCGAGTCCGC GGCCCGCCCC GTTGCGTCCC
-100 ACCCACCGCG TCCCCTCCC TCCCCTCCC CTGCGGAAAA GCGGCCGCGG GCGGCGGCG CCCACTGTGG GCGGGGCGGA GCGCCGCGGG GCGGGACGA

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Figure 1. FootPrinter Analysis of Conserved Motifs in the 5' Regions of the Human, Chimp, Mouse and Chicken *ITGB3* Genes

Five hundred base pairs of the 5' region of the human, chimp, mouse and chicken *ITGB3* genes underwent footprinting analysis to determine if there were any regions of conservation between the different species. **Panel A:** Identical motifs detected in each species' sequence have the same number. The phylogenetic tree on the right hand side shows the evolutionary relationship between the different species. **Panel B:** Conserved motifs are underlined, several motifs between -90 and -140 overlapped. The positions of the -400C/A, -425A/C and -468G/A are in bold and underlined.

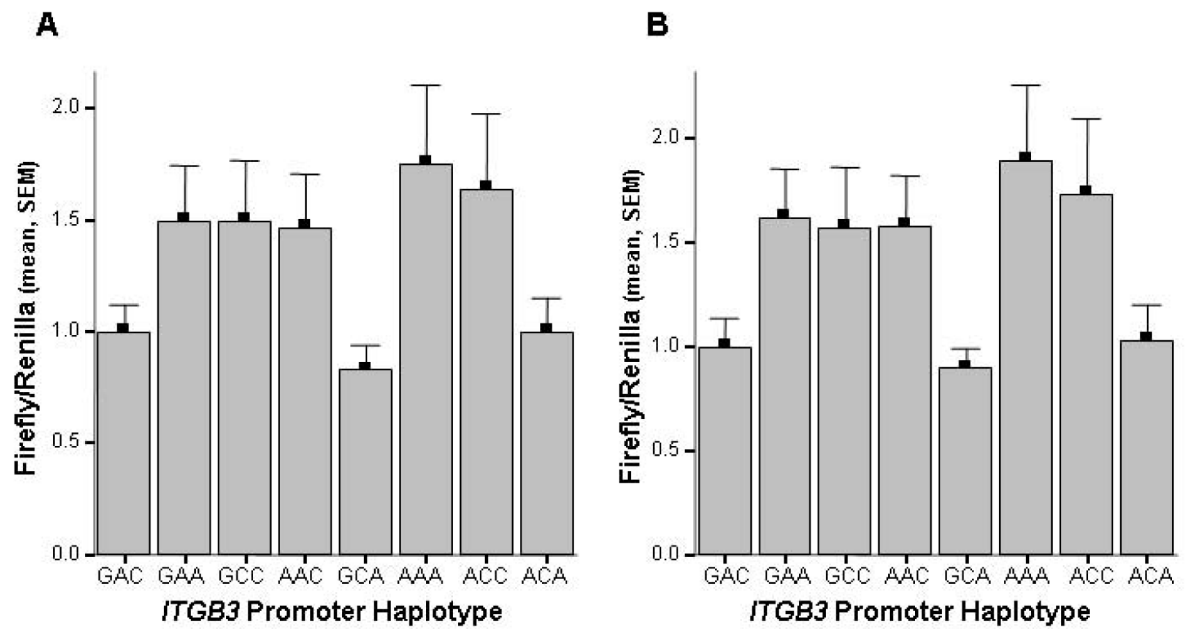


Figure 2. Influence of the -468G/A, -425A/C and -400C/A polymorphisms in the 5' gene regulatory region of *ITGB3* on firefly reporter gene expression in HEL and HMEC-1 cells

Firefly luciferase values were normalised to Renilla luciferase activity and expressed relative to the wild type GAC construct. **Panel A:** firefly/renilla luciferase values for HEL cells transiently transfected with each construct. ANOVA analysis, $p=0.02$. **Panel B:** firefly/renilla luciferase values for HMEC-1 cells transiently transfected with each construct. ANOVA analysis, $p=0.048$.

Table 1

Characteristics of study subjects

	Subjects (n=223)
Age (years)	42.3 (40.1–44.5)
Male	112 (0.50)
BMI (kg/m ²)	26.6 (5.63)
Current smokers	23 (0.10)
Ethnicity:	
Caucasian	131 (0.59)
African American	49 (0.22)
Mexican American	28 (0.13)
Asian	13 (0.06)
Other	2 (0.01)
Fibrinogen (gL ⁻¹)	3.39 (1.35)
vWF activity (%)	110.6 (42.9)
CABG (%)	85 (0.38)
Previous MI	40 (0.18)
Hypertension	59 (0.27)
Diabetes*	26 (0.12)
Aspirin	85 (0.38)

Age presented as median (25th and 75th percentiles)

Other variables presented as mean or geometric mean (SD or antilog SD) or number (frequency)

CABG: coronary artery bypass graft

*

All subjects taking aspirin were CABG patients

Table 2

ITGB3 haplotype frequencies of study subjects

Promoter haplotype	Number of chromosomes (frequency)	Promoter and Leu33Pro haplotype	Number of chromosomes (frequency)
hapA:	GAC	hap1:	GAC-Leu33
hapB:	AAA	hap7:	GAC-Pro33
hapC:	GCC	hap2:	AAA-Leu33
hapD:	AAC	hap6:	AAA-Pro33
hapE:	GAA	hap5:	GCC-Leu33
hapF:	ACC	hap3:	GCC-Pro33
		hap4:	AAC-Leu33
		hap8:	AAC-Pro33
		hap9:	GAA-Leu33
		hap10:	GAA-Pro33
		hap11:	ACC-Leu33
			ACC-Pro33

Table 3
The relationship of *ITGB3* polymorphisms with platelet $\alpha_{IIb}\beta_3$ receptor density, plasma fibrinogen and plasma vWF activity

	-468 G/A mean (95% CI)	p	-425 A/C mean (95% CI)	p	-400 C/A mean (95% CI)	p	Leu33Pro mean (95% CI)	p
$\alpha_{IIb}\beta_3$ (MFI)	GG: 136 (133, 139) GA: 139 (134, 143) AA: 126 (119, 133)	0.12	AA: 136 (133, 139) AC: 138 (133, 143) CC: 138 (131, 144)	0.85	CC: 137 (134, 140) CA: 237 (132, 142) AA: 128 (120, 137)	0.62	LL: 137 (134, 140) LP: 136 (131, 141) PP: 138 (133, 143)	0.97
Fibrinogen ($g L^{-1}$)	GG: 3.36 (3.21, 3.52) GA: 3.51 (3.24, 3.80) AA: 3.40 (3.06, 3.78)	0.62	AA: 3.36 (3.21, 3.51) AC: 3.60 (3.29, 3.94) CC: 3.35 (2.60, 4.31)	0.33	CC: 3.40 (3.26, 3.55) CA: 3.43 (3.14, 3.74) AA: 3.58 (3.30, 3.90)	0.92	LL: 3.35 (3.20, 3.50) LP: 3.68 (3.36, 4.02) PP: 3.32 (2.67, 4.12)	0.17
vWF activity (%)	GG: 112.7 (105.1, 120.4) GA: 108.3 (99.3, 117.3) AA: 97.1 (76.7, 117.5)	0.47	AA: 105.4 (99.6, 111.2) AC: 124.4 (110.0, 138.8) CC: 139.7 (76.9, 202.5)	0.01	CC: 113.1 (106.0, 120.1) CA: 105.3 (95.2, 115.3) AA: 99.4 (70.2, 128.7)	0.42	LL: 106.8 (100.5, 113.2) LP: 121.8 (109.6, 134.0) PP: 131.6 (73.9, 189.3)	0.06

Data presented as mean or geometric mean (95% confidence intervals)

MFI: Mean fluorescence intensity