G protein-coupled receptor kinase 5 regulates thrombin signaling in platelets via PAR-1

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Abstract:
The interindividual variation in the functional response of platelets to activation by agonists is heritable. Genome-wide association studies (GWAS) of quantitative measures of platelet function have thus far identified fewer than 20 distinctly associated variants, some with unknown mechanisms. Here, we report GWAS of pathway specific functional responses to agonism by ADP, a glycoprotein VI-specific collagen mimetic and thrombin receptor-agonist peptides, each specific to one of the G protein-coupled receptors PAR-1 and PAR-4, in subsets of 1,562 individuals. We identified an association (P=2.75x10^-40) between a common intronic variant, rs10886430 in the G protein-coupled receptor kinase 5 gene (GRK5), and the sensitivity of platelets to activate through PAR-1. The variant resides in a megakaryocyte-specific enhancer bound by the transcription factors GATA1 and MEIS1. The minor allele (G) is associated with fewer GRK5 transcripts in platelets and greater sensitivity of platelets to activate through PAR-1. We show that thrombin mediated activation of human platelets causes binding of GRK5 to PAR-1 and that deletion of the mouse homologue Grk5 enhances thrombin induced platelet activation sensitivity and increases platelet accumulation at the site of vascular injury. This corroborates evidence that the human G-allele of rs10886430 associates with greater risks of cardiovascular diseases. In summary, by combining the results of pathway specific GWAS and eQTL studies in humans with the results of platelet function studies in Grk5-/- mice, we obtain evidence that GRK5 regulates the human platelet response to thrombin via the PAR-1 pathway.

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Title

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Key Points

- Platelet reactivity via the PAR-1 thrombin receptor is mediated by G protein-coupled receptor kinase 5.
- Platelet GRK5 is associated with thrombus formation in humans and mice.

Short title: GRK5 regulates thrombin signaling in platelets
Abstract

The interindividual variation in the functional response of platelets to activation by agonists is heritable. Genome-wide association studies (GWAS) of quantitative measures of platelet function have thus far identified fewer than 20 distinctly associated variants, some with unknown mechanisms. Here, we report GWAS of pathway specific functional responses to agonism by ADP, a glycoprotein VI-specific collagen mimetic and thrombin receptor-agonist peptides, each specific to one of the G protein-coupled receptors PAR-1 and PAR-4, in subsets of 1,562 individuals. We identified an association ($P=2.75\times10^{-40}$) between a common intronic variant, rs10886430 in the G protein-coupled receptor kinase 5 gene (GRK5), and the sensitivity of platelets to activate through PAR-1. The variant resides in a megakaryocyte-specific enhancer bound by the transcription factors GATA1 and MEIS1. The minor allele (G) is associated with fewer GRK5 transcripts in platelets and greater sensitivity of platelets to activate through PAR-1. We show that thrombin mediated activation of human platelets causes binding of GRK5 to PAR-1 and that deletion of the mouse homologue Grk5 enhances thrombin induced platelet activation sensitivity and increases platelet accumulation at the site of vascular injury. This corroborates evidence that the human G-allele of rs10886430 associates with greater risks of cardiovascular diseases. In summary, by combining the results of pathway specific GWAS and eQTL studies in humans with the results of platelet function studies in Grk5−/− mice, we obtain regulates the human platelet response to thrombin via the PAR-1 pathway.
Introduction

Platelets are essential to prevent bleeding. By continuously surveying vessel walls and activating in response to damage, they initiate the formation of thrombi to close any breaches. If hemostasis is well balanced, thrombus formation is followed by thrombolysis and vessel wall repair. Unfortunately, platelet activation may initiate inappropriate thrombus formation in individuals with vascular disease, leading to vessel occlusion and ischemia. Consequently, platelet inhibitors are a standard treatment for the secondary prevention and treatment of ischemic cardiovascular diseases.

Measures of the activation sensitivity of platelets, henceforth ‘reactivity phenotypes’, have heritable interindividual components of variation\textsuperscript{1,2}. However, this heritable variation is less well studied than that of resting platelet phenotypes such as platelet count (PLT#) and mean platelet volume (MPV). GWAS of platelet phenotypes measured by complete blood counts (CBC) in over half a million participants have identified over 1,800 distinctly associated genetic variants, explaining much of the heritable variation in the European ancestry population\textsuperscript{3,4}. In contrast, studies designed to identify variants associated with platelet reactivity by agonists have each relied on fewer than 4,000 participants and have, in aggregate, identified fewer than 20 distinctly associated alleles\textsuperscript{2,5-9}. The principal reason for relatively low sample sizes is the intricate nature of platelet reactivity phenotyping. The activation of platelets in response to vascular damage is caused by the binding of a variety of agonists to cell surface receptors. In particular, the receptors for collagen and thrombin, which are important initiators of activation, and the receptors for ADP and Thromboxane A2, which are important for amplification. To quantify reactivity \textit{in vitro}, these receptors must be stimulated and the platelet response measured either by light transmission aggregometry (LTA) or by flow cytometry of surface markers of activation.

Here, we present the results of GWAS of platelet reactivity phenotypes in subsets of 1,562 healthy adults enrolled in the Cambridge Platelet Function Cohort (PFC). Using flow cytometry, we measured responses to agonism by adenosine 5’-diphosphate (ADP), by the GPVI-specific ligand collagen-related peptide (CRP-XL) and by thrombin receptor-agonist peptides, each specific to PAR-1 or PAR-4. Analyses of these phenotypes replicated several known pathway-specific associations. Additionally, we identified an association on chromosome 10q26, between a variant in a platelet expression quantitative trait locus (eQTL) for \textit{GRK5} and reactivity to thrombin, replicating a recently published association in which reactivity was measured by LTA\textsuperscript{10}. By analyzing reactivity to PAR-1 and PAR-4 specific peptides, we provide compelling evidence that the association signal in \textit{GRK5} is mediated through the PAR-1 receptor. Furthermore, by deleting the mouse homologue \textit{Grk5}, we show that mouse platelets without Grk5 exhibit greater \textit{ex vivo} platelet reactivity to thrombin, but not to other agonists. \textit{Grk5\textsuperscript{-/-}} mice showed greater platelet accumulation upon laser-induced vascular injury and increased thrombin-induced pulmonary thromboembolism compared to wild-type (WT) controls. Collectively, the results of our genetic and functional studies in humans and mice provide robust evidence that G protein-coupled receptor (GPCR) kinase 5 encoded by \textit{GRK5}, is a key regulator of platelet activation by thrombin via the GPCR PAR-1.
Methods

Detailed descriptions of our materials and methods are provided in the Supplemental Information.

Human Study Participants

Donors registered with NHS Blood and Transplant (NHSBT) were invited to join the NIHR BioResource, a cohort of volunteers who have consented to be recalled for studies by genotype. Subsequently, these NIHR BioResource members were invited to participate in the Cambridge PFC. Blood for the protein binding experiments was given by healthy donors at Thomas Jefferson University. Written informed consent was obtained of participants prior to blood collection. Information about the study participants and the permissions of the institutional review board and research ethics committee are given in the Supplemental Information.

Platelet Function Association Study

DNA extracted from EDTA treated blood from Cambridge PFC participants was genotyped using the UK Biobank Axiom™ Array (Thermo Fisher, Santa Clara, CA). A description of the QC and imputation methods is within the Supplemental Information.

Platelet activation was measured by whole blood flow cytometry assays as previously described (Supplemental Information). Platelets were agonized with ADP (Sigma-Aldrich), CRP-XL (crosslinked monomeric sequence GCO[GPO]10GCG, Department of Biochemistry, University of Cambridge, UK), PAR-1, PAR-4 and TRAP-6 (Tocris) agonist peptides. The binding of a PE-labelled anti-P-selectin monoclonal antibody (CD62P, NHSBT, Bristol, UK) and a FITC-labelled polyclonal anti-fibrinogen (Agilent Dako) was quantified using Beckman Coulter flow cytometers. Individual level summaries of platelet reactivity were derived by logit transforming the percentage of cells with a signal greater than a threshold determined from background, measured in corresponding aliquots of un-agonized blood.

PLT# and MPV were measured from EDTA treated blood using Beckman Coulter and Sysmex CBC analyzers. We regressed the variation explained by sex, age, PLT# and MPV and reagent batch from the individual level summaries of reactivity (Supplemental Information).

Except for one subset of 450 individuals, in which the activation response to agonism by ADP was measured in duplicate only by fibrinogen binding, each activation response was measured four times, twice using P-selectin expression and twice using fibrinogen binding. Outliers and discordant repeated measurements were discarded and the average of the repeats taken. The effect of PLT#, MPV, sample batch, sex and age were regressed out of each average. Outliers and samples for which the P-selectin and fibrinogen binding averages were discordant were discarded. The P-selectin expression and fibrinogen binding averages were highly correlated (Supplemental Figure 1), therefore, to increase the power to detect novel genetic associations, we performed a GWAS for each agonist using the score vector corresponding to the first...
principal component of the averaged P-selectin and averaged fibrinogen measurements as the phenotype, after further adjustment for PLT#, MPV, sample batch, sex, age and time elapsed between the start of the study and the time of measurement. The GWAS association tests were performed using linear mixed models to control for relatedness. For response to agonism by ADP, separate GWAS were performed in the subset of individuals for which reactivity was measured by fibrinogen binding only and in the subset for which reactivity was measured by both fibrinogen binding and P-selectin expression. The evidence for association was combined using inverse variance weighted meta-analysis after scaling the statistics from the fibrinogen binding GWAS to place them on the same scale of measurement as the statistics from the other GWAS.

For replication, platelet activation was measured in an independent cohort of donors, using a plate-based aggregation end-point assay as described\textsuperscript{13}. The aggregation of platelets in response to increasing doses of TRAP-6 was measured using absorbance and curves were generated to calculate EC50 values.

A detailed description of the genetic association analysis is provided in the Supplemental Information.

**GRK5 and PAR-1 immunoprecipitation**

Washed human platelets were activated using thrombin and then lysed (Supplemental Information). Proteins were precipitated with an anti-PAR-1 (ATAP-2) antibody or inert control immunoglobulin (Ig) and then probed for GRK5 before reprobing with an anti-PAR-1 antibody.

**Mouse models**

All mouse protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania or Thomas Jefferson University.

Mice protocols are described within the Supplemental Information. Grk5 knockout mice were generated with a C57BL/6 background using CRISPR-Cas9 genome-editing\textsuperscript{14} and fetal liver chimeras were generated using transplantation from WT control or Grk5\textsuperscript{-/-} mice.

**Agonist-mediated platelet activation in vitro**

Mouse platelet activation experiments were performed as described previously using flow cytometry and LTA\textsuperscript{15} (Supplemental Information). For flow cytometry, platelets were stimulated with PAR-4 agonist peptide (PAR-4-AP), ADP, Thromboxane A2 mimetic (U46619), or convulxin (CVX) in diluted heparinized blood. Binding of fluorochrome conjugated antibodies against CD41, P-selectin (CD62P, BD Bioscience) and activated integrin were used to measure platelet activation with a BD LSRII (BD Biosciences). For LTA assays, platelets were collected from platelet rich plasma and counts were adjusted to 2x10\textsuperscript{8}/ml. Aggregation was observed in a PAP-8E Platelet Aggregation Profiler (BIO/DATA Cooperation). Single endpoint luminescence assays were used to quantify agonist-induced platelet ATP release as described\textsuperscript{15}.
Vascular injury and pulmonary thromboembolism models

Thrombus formation upon vascular injury was measured in the cremaster muscle microcirculation of male mice aged 8 to 12 weeks as described\textsuperscript{16}. Pulmonary thromboembolism assay was performed as previously described with some modifications\textsuperscript{17}. Further details of each method are provided in the Supplementary Information. Data sets were compared using the Student $t$ test or the Mann-Whitney test. $P \leq 0.05$ was considered statistically significant.

Results

Genome Wide Association Study for Platelet Activation

We measured platelet reactivity to agonists in subsets of 1,562 healthy participants in the Cambridge PFC by pathway specific \textit{ex vivo} stimulation. We agonized the platelet GPCR-signaling pathways, activating the receptors P2Y\textsubscript{1}, P2Y\textsubscript{12} with ADP and PAR-1 and PAR-4 (the thrombin receptors) with receptor specific peptides (PAR-1 and PAR-4). Additionally, we agonized the GPVI-FcεRI/ITAM signaling pathway with CRP-XL. We quantified platelet reactivity at the individual level by summarizing cell level measurements of surface P-selectin expression and bound fibrinogen.

GWAS identified five genome-wide significant association signals ($P < 5 \times 10^{-8}$, Table 1, Figure 1). These association signals recapitulate previous reports, including reports by us based on analyses of subsets of the present dataset. They include associations with reactivity to ADP in \textit{PEAR1} (rs12566888) and \textit{ARHGEF3} (rs7624918) and associations with reactivity to CRP-XL in \textit{GP6} (rs1613662)\textsuperscript{2,9,11}. The analyses of reactivity to the two thrombin receptor agonists relied on entirely new data and identified two associations. Firstly, we replicated an association in \textit{F2RL3} (rs773902), the gene encoding the PAR-4 receptor, identified with reactivity to PAR-4 agonism measured by LTA\textsuperscript{18}. Secondly, we identified a newly discovered association in \textit{GRK5} (rs10886430, chr10:121010256A>G, GRCh37) with reactivity to PAR-1 agonism\textsuperscript{10}.

The PAR-1 association signal in the \textit{GRK5} locus was explained completely by conditioning on the imputed minor allele (G) count of rs10886430, the variant exhibiting the strongest evidence for association with platelet reactivity ($n = 546$, $\hat{\beta} = 1.09$ Standard Deviations (SD), Standard Error (SE) = $8.21 \times 10^{-2}$, $P = 2.75 \times 10^{-40}$, Table 1, Figure 2A). Our analyses showed that rs10886430 is not associated with platelet activation by ADP or by CRP-XL. However, an association was identified with reactivity to the PAR-4 specific peptide, although with a smaller estimated effect size ($\hat{\beta} = 0.44$ SD, SE = 0.092, $P = 2.20 \times 10^{-6}$)(Supplemental Figure 3).

We replicated the association with rs10886430, in the same study participants, by replacing the PAR-1 agonising peptide with TRAP-6, another PAR-1 receptor specific agonist peptide ($\hat{\beta}$ = $1.18$ SD, SE = 0.0801, $P = 8.91 \times 10^{-49}$). Additionally, we performed an out of sample replication experiment using the TRAP-6 agonist and a microplate-based platelet aggregation assay\textsuperscript{13},...
which provided evidence for an association with the same direction of effect (n=253, \( \hat{\beta} = -0.103 \) SD, SE=0.042, \( P = 0.014 \); Supplemental Figure 4).

Genomic characterization of PAR-1 platelet activation association at \textit{GRK5}

To explore the molecular mechanism underlying the platelet reactivity association at \textit{GRK5}, we interrogated eQTL data for platelets aggregated from two independent studies (Supplemental Information). The minor allele (G) of rs10886430, corresponding to greater platelet reactivity, was associated with a lower abundance of \textit{GRK5} mRNA in platelets (n=388, \( \hat{\beta} = -0.50 \) SD, SE 0.0473, \( P = 4.79 \times 10^{-23} \)) (Figure 2B).

Inspection of our previously published erythroblast and MK ATAC-seq data showed that rs10886430 lies in an MK-specific nucleosome depleted element\(^9\). Chromatin immunoprecipitation and sequencing (ChIP-Seq) of MKs showed the element to have the H3K27Ac profile characteristic of an enhancer and identified strong evidence for binding by GATA1 and MEIS1, two of the key MK lineage transcription factors\(^9\). However, we saw no evidence for binding by the MK factors FLI1, GATA2, RUNX1 and TAL1\(^{19,20}\) (Figure 2C). A difference of affinity between the two alleles of rs10886430 to bind to one or both of GATA1 and MEIS1 is the most likely explanation for the \textit{GRK5} eQTL. This hypothesis is supported by Rodriguez \textit{et al.} who showed that the transcriptional activity of constructs containing the enhancer region is reduced when the G allele of rs10886430 is introduced into K562 and HUVEC cell lines\(^{10}\).

Together, these findings indicate that the molecular mechanism explaining the association of rs10886430 with platelet reactivity to agonism through PAR-1 is mediated by the allele-specific differences in expression of \textit{GRK5} mRNA.

Binding of \textit{GRK5} to PAR-1 during platelet activation

The regulation of PAR-1 signaling by \textit{GRK5} suggests that \textit{GRK5} binds to PAR-1 in response to thrombin stimulation. To evaluate this interaction we precipitated these proteins in washed human platelets and then probed using antibodies either against \textit{GRK5} or PAR-1 (Figure 3). During platelet activation using thrombin (1 U/ml), immunoprecipitation of PAR-1 co-precipitated \textit{GRK5} (Figure 3A) and conversely immunoprecipitation of \textit{GRK5} pulled down PAR-1 (Figure 3B). Furthermore, when platelets were activated with a lower concentration of thrombin (0.1 U/ml), immunoprecipitation of \textit{GRK5} also pulled down PAR-1 (Supplemental Figure 5). When we applied the same experimental procedure to resting platelets we detected little or no binding of \textit{GRK5} to PAR-1. \textit{GRK5} and \textit{GRK6} are 72\% homologous at the amino acid level\(^{21,22}\), therefore we excluded the possibility that our results were a consequence of cross reactivity between anti-\textit{GRK5} and \textit{GRK6} (Supplemental Figure 6).

Generation and characterization of \textit{Grk5}\(^{-/-}\) mice
We studied the consequences of the inhibition of Grk5 for thrombin mediated activation signaling using Grk5 knockout mice. Grk5<sup>-/-</sup> mice were generated by introducing a premature stop codon using CRISPR-Cas9, resulting in loss of Grk5 expression (Supplemental Figure 7). The Grk5<sup>-/-</sup> mice that were generated by crossing were born in the expected Mendelian inheritance ratios and were viable and grossly normal in appearance. The average values of CBC measured hematological variables did not differ significantly between the knockouts and their WT littermates (Supplemental Table 1). Furthermore, deletion of Grk5 did not affect the expression of the Grk2 and Grk6 proteins in the mouse brain (Supplemental Figure 7D).

**Grk5<sup>-/-</sup> platelets display increased agonist-mediated platelet activation ex vivo**

We compared the reactivity of platelets from Grk5<sup>-/-</sup> mice to those from WT mice, using flow cytometry to measure surface bound fibrinogen and anti-P-selectin as markers of α<sub>IIbβ<sub>3</sub> activation and α-granule secretion respectively<sup>15,23</sup>. We agonized platelets using a mouse Par-4 thrombin receptor agonist peptide (AYPGKF), as Par-4 is the mouse homologue of human PAR-1. Mean integrin activation (P=0.04) and P-selectin expression (P=0.01) were both greater in platelets generated by Grk5<sup>-/-</sup> than WT mice in response to a low-dose of the Par-4 agonist peptide. However, there was no significant difference when high-doses of Par-4 agonist peptide were used (Figure 4A). There was no evidence for differences in activation response to agonism by ADP, by the Thromboxane-A2 analog U46619, or by convulxin, which is an alternative ligand to CRP-XL for GpVI (Supplemental Figure 8). Phenotyping by LTA of reactivity to the Par-4 agonist corroborated the flow cytometry data (Figure 4B). Furthermore, when platelets were incubated with thrombin, an increased response was also observed in Grk5<sup>-/-</sup> platelets (Figure 4C and 4D). LTA measured responses to other agonists indicated a slight initial increase in Grk5<sup>-/-</sup> platelet response to ADP, but there was no difference in responses to U46619 or CVX (Supplemental Figure 9).

We used single endpoint luminescence assays<sup>15</sup> to measure the ATP released from platelets in response to agonism by Par-4 agonist peptide and ADP. Increased ATP release was observed in platelets of Grk5<sup>-/-</sup> mice, compared to WT mice, on activation using a low dose of Par-4 agonist peptide (P=0.04). However, there was no evidence for a difference in response to agonism by ADP (Supplemental Figure 10). These results are compatible with the notion that platelets without Grk5 secrete a greater number of δ-granules when activated via the thrombin receptor Par-4 compared to normal WT platelets (Figure 4E).

ADP activates platelets by binding to G<sub>q</sub>-coupled P<sub>2</sub>Y<sub>1</sub> and G<sub>i2</sub>-coupled P<sub>2</sub>Y<sub>12</sub> receptors<sup>24</sup>. ADP P<sub>2</sub>Y<sub>12</sub> signaling triggers activation/phosphorylation of the G<sub>i2</sub>-mediated serine/threonine kinase Akt<sup>25</sup>. To determine if deletion of Grk5 affects P<sub>2</sub>Y<sub>12</sub>-dependent signaling, we compared phosphorylation of Akt between WT and Grk5<sup>-/-</sup> platelets in response to ADP stimulation in the presence or absence of the P2Y<sub>1</sub> antagonist, MRS2500, or the P2Y<sub>12</sub> antagonist, cangrelor (Supplemental Information). Upon ADP stimulation, Grk5<sup>-/-</sup> platelets exhibited greater phosphorylation of Akt than WT platelets, but the increase was not statistically significant (Supplemental Figure 11). Greater phosphorylation of Akt from Grk5<sup>-/-</sup> platelets than from WT platelets was still observed upon blocking P2Y<sub>1</sub> with MRS2500 (Supplemental Figure 11C). However, in the presence of the P2Y<sub>12</sub> antagonist Cangrelor, the phosphorylation of Akt in
response to stimulation by ADP was abolished in both WT and Grk5<sup>−/−</sup> platelets (Supplemental Figure 11A).

**Grk5 regulates the hemostatic response to injury in vivo**

To investigate if enhanced *in vitro* platelet responsiveness to Par-4 receptor activation in Grk5<sup>−/−</sup> mice had an effect on thrombus formation following vascular damage, we used the laser-induced injury model in cremaster muscle arterioles<sup>16,23</sup>. In this model, the core of the hemostatic plug consists of P-selectin positive platelets bound by fibrin which is surrounded by a loosely packed shell of less-activated P-selectin negative platelets. We measured platelet accumulation for 180 seconds following injury using fluorescently labeled anti-CD41 (Figure 5). Initially, platelets accumulated at similar rates in Grk5<sup>−/−</sup> and WT mice (Figure 5A). However, we observed greater accumulation at the late stage in Grk5<sup>−/−</sup> mice compared to WT mice, as demonstrated by the increase in the mean area under the curve (*P*=0.03) and mean total platelet accumulation at 180 second post injury (*P*=0.016) (Figure 5B, D and E, Supplemental Figure 12). Since there was no difference in mean P-selectin deposition between the two groups (Supplemental Figure 13), the increased thrombus formation was due to an expansion of the P-selectin negative shell region rather than the P-selectin positive core region. Deleting Grk5 did not affect fibrin accumulation, suggesting that thrombin generation at the site of injury is normal in Grk5<sup>−/−</sup> mice (Supplemental Figure 13). To exclude possible effects of the deletion of Grk5 on cells within the vascular wall, we have performed additional laser injury studies in irradiated wild-type mice reconstituted with hematopoietic cells harvested from wild-type or Grk5<sup>−/−</sup> fetal livers. The result showed that there was a similar increase in platelet accumulation at the site of vascular injury in reconstituted Grk5<sup>−/−</sup> mice as seen in whole-body Grk5<sup>−/−</sup> mice (Supplemental Figure 14).

**Absence of Grk5 increases thrombin-induced pulmonary thromboembolism**

Finally, we evaluated the role of Grk5 in thrombin signaling using a thrombosis model. Thrombin-induced pulmonary thromboembolism is a model of systemic occlusive pulmonary microthrombi, which are composed of platelet aggregates and fibrin<sup>26,27</sup>. To determine whether deletion of Grk5 might increase the incidence of formation of thromboembolism induced by platelet activation, Grk5<sup>−/−</sup> mice and their WT control littermates were injected intravenously with thrombin. In the absence of Grk5, there is a 1.6-fold increase in thrombus formation in the lung compared to WT control littermates (Figure 5F and Figure 5G).

**Discussion**

We have performed GWAS analyses of platelet reactivity phenotypes in subsets of 1,562 healthy blood donors, recapitulating genetic association signals previously reported by us and by others (in *ARHGEF3* and *PEAR1* for ADP, in *GP6* for CRP-XL and in *F2RL3* for PAR-4
specific agonist peptide). Additionally, we identified a new association between the variant rs10886430 in *GRK5* and platelet reactivity to a PAR-1 agonist peptide, corroborating the recent report of association with platelet reactivity to thrombin measured by LTA. Our analyses have clarified the molecular mechanism distal to the allelic variation in *GRK5*. The direct consequence of the polymorphism almost certainly generates differential binding affinity of the transcription factors GATA1 and MEIS1 to an MK specific enhancer element, causing allelic differences in the abundance of *GRK5* transcript in platelets.

Interestingly, the association of rs10886430 with platelet reactivity colocalizes with an association with MPV reported by Astle et al. The G allele, which is associated with lower platelet *GRK5* transcript levels and greater platelet reactivity via PAR-1 is also associated with greater MPV ($P=6.6 \times 10^{-18}$). To exclude the possibility that the platelet reactivity effect is mediated entirely through variation in platelet volume, we regressed MPV from the phenotype prior to performing our GWAS.

Rodriquez et al identified the same genetic variant, rs10886430, associated with platelet reactivity to thrombin and by generating knockdown GRK5 platelets derived from megakaryocyte progenitor cell lines and using a GRK5 inhibitor in human platelets showed that GRK5 regulates signaling primarily via PAR-4. In contrast, our study provides compelling evidence suggesting that GRK5 also regulates thrombin signaling via PAR-1. The platelet reactivity flow cytometry measurement in our study differs from the standard LTA because amplification of the main subsidiary activation pathways is inhibited by aspirin, hirudin and apyrase, when activating platelets with PAR-specific peptides. Hence we can examine the genome-wide association results in a pathway specific manner excluding effects brought about by subsidiary activation. Furthermore the association of PAR-1 is supported by co-immunoprecipitation of GRK5 with PAR-1 upon platelet activation by thrombin. While not at genomewide significance, our results also suggest that rs10886430 is associated with platelet reactivity via PAR-4. Together these results likely indicate that in human platelets, thrombin signaling via PAR-1 and PAR-4 are both regulated by GRK5. The current challenges for studying this pathway are due to the lack of specific anti-PAR4 antibodies. Future work will look to elucidate the contribution of GRK5 regulation in human platelet thrombin signaling once specific reagents are available.

In humans, the protease-activated GPCRs PAR-1 and PAR-4 are the receptors for thrombin. Cleavage of their exodomains by thrombin releases tethered ligands, which bind in a cognate manner to the receptor. The synthetic PAR-1 and PAR-4 agonist peptides used for the present study mimic this specific mode of receptor activation. Low concentrations of thrombin initiate platelet activation via PAR-1 which is quickly terminated. PAR-4 requires higher thrombin concentrations, resulting in a later activation response, but a response which is sustained. Phosphorylation of the thrombin PAR receptors by GPCR kinases, including *GRK5*, induces the GRK-β-arrestin cascade, resulting in the termination of signaling by the internalization and destruction of the active receptor in the lysosomes. Studies indicate that the signaling kinetics of PAR-1 and PAR-4 differ at least in part due to the rate or extent of signaling dependent phosphorylation. Our results suggest that *GRK5* levels, and thus receptor phosphorylation, in
platelets may have a lesser effect on PAR-4 than on PAR-1 receptor signaling, supporting the conclusions of these previous studies of receptor kinetics in human platelets.

In contrast to human platelets, mouse platelets express Par-3 and Par-4, but not Par-1. Thrombin signaling in mouse platelets is mediated by Par-3-facilitated cleavage of Par-4, which responds quickly to thrombin stimulation. Thus, in the context of thrombin receptor signaling, mouse Par-4 has similar kinetic properties to human PAR-1. Our study shows that mice unable to express Grk5 exhibit greater platelet activation sensitivity, principally downstream of Par-4-dependent signaling pathways. This observation is consistent with the association of the human G allele of rs10886430 with reduced levels of GRK5 transcript and increased platelet reactivity to a PAR-1 specific agonist peptide. Recent data generated by authors of the present manuscript show that like GRK5, GRK6 is also involved in PAR-1-mediated signaling in human platelets. In keeping with this observation, deletion of Grk6 in mice enhances Par-4-dependent signaling. Thus, mouse Par-4 signaling is regulated by Grk6 in a similar manner to human PAR-1. The change in platelet reactivity, as measured by flow cytometry and ATP release, caused by knockout of Grk5, is less than that caused by knockout of Grk6. This is probably due to the lower abundance of Grk5 than Grk6 in mouse platelets (Supplemental Figure 15). Inhibition of platelet activation is known to reduce the risk of secondary thrombotic ischemia in patients with vascular disease. It therefore seems reasonable to hypothesize that variation in the sensitivity of platelets to activation by thrombin generates variation in the population risks of thrombotic diseases. Indeed, the G allele of rs10886430 has been associated with greater risks of thrombotic disease in the 0.5 million participants in the UK Biobank, most notably with risk of pulmonary embolism (PE) (OR=1.28, \( P=8.05 \times 10^{-13} \)). Associations have also been identified with risk of venous thromboembolism in the INVENT Consortium (OR=1.12, \( P=2.2 \times 10^{-12} \)) and in the Million Veteran Program (\( P=6.7 \times 10^{-11} \)). In addition, mendelian randomisation analysis in UK Biobank individuals suggests a causal role for thrombin induced platelet reactivity in thrombotic disease, most notably with PE. The observation that Grk5−/− mice exhibit more rapid platelet accumulation during in vivo thrombus formation upon vascular damage than WT mice, supports the hypothesis that these population associations are mediated through variation in platelet reactivity. This suggests that associations with platelet reactivity as measured by our in vitro assays can act as a good model for associations of platelet mediated thrombotic pathologies of the venous circulation.

GRK5 is ubiquitously transcribed. It is expressed across the hematopoietic lineages and in endothelial cells. It is also expressed in most of the tissues surveyed by the GTEx studies. The desensitization of GPCRs is not a mechanism unique to the megakaryocytic lineage, and interactors of GRK5, which include transmembrane, cytosolic and nuclear GPCRs as well as non-GPCR proteins, are involved in multiple signaling pathways including the cell cycle, apoptosis, cell motility and inflammation pathways. This may explain why variants in the GRK5 locus have been associated with many biomedically relevant quantitative traits and disease risks affecting a range of physiological systems. They include height, type II diabetes, lung function and many neurological and behavioral traits, such as the risks of alcohol misuse and post-traumatic depression. This pleiotropy suggests the therapeutic manipulation of GRK5 expression is likely to avoid off target effects only if it can be achieved with cell type specificity.
For example, GRK5 activity has been shown to be increased in heart failure models and is under investigation as a potential target for pharmacological inhibition\textsuperscript{36}. However, our results and those of Rodriguez et al. imply that inhibition of GRK5 in platelets would be prothrombotic and proinflammatory\textsuperscript{10}. On the other hand, further investigation of the platelet specific binding partners of GRK5 as candidate targets for novel antithrombotic drugs may be warranted.

To conclude, by activating platelets through specific signaling pathways, we confirmed previously published associations and identified a new genetic association with platelet reactivity mediated via the thrombin GPCR PAR-1 in GRK5. The platelet eQTL, epigenetic analyses of MKs and functional work in humans platelets and mice evidence the molecular and biological mechanism mediated by the inhibitory effect of GRK5 on PAR-1. This improves our understanding of the signaling pathways associated with thrombin signaling and provides evidence for the distinct and wide-reaching consequences of dysregulation of GPCR-coupled receptor kinases.

Data Sharing

For original data contact kd286@cam.ac.uk.

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Authorship

Contributions: K.D., X.Z., H.M., C.K., J.B., P.L.T., M.C., J.V.M., K.W., K.W., B.V., N.K., performed experiments; K.D., N.G., R.K., H.V., J.L.D., W.J.A., P.M., analysed data; S.A., and K.S., provided research support; S.M., J.M.G., J.Y., provided reagents; K.D., W.H.O, J.M.G., W.J.A. and P.M., designed the study and wrote the manuscript. All authors reviewed the manuscript.

Conflict of Interest


Jing Yang is a full time employee of Bristol Myers Squibb. The remaining authors have no conflicts of interest.

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Figure 1.
Genome-wide associations with platelet reactivity.
A Manhattan plot showing the \( P \)-values of tests for association between genetic variants and each of four phenotypes measuring the reactivity of platelets to agonism by ADP, CRP-XL, PAR-1 and PAR-4 measured in the Cambridge Platelet Function Cohort. Each dot corresponds to a genetic variant in the Haplotype Reference Consortium r1.1 reference panel. The position on the x-axis indicates the physical position of the variant; the position on the y-axis indicates the \(-\log_{10} P\)-value of a Wald test for association from a linear mixed-model (on a log scale). Only variants with an imputation INFO score >0.6 and a \( P \)-value <0.1 are shown. The horizontal dashed line corresponds to the genome-wide significance threshold (5×10^{-8}). The red dots correspond to the variants showing the strongest evidence for association in those loci containing significantly associated variants. The red gene names indicate the gene causally mediating each of these associations.

Figure 2.
Increased platelet reactivity via PAR-1 at rs10886430 is due to decreased expression of GRK5.
Box plots showing the relationships between the genotype of rs10886430 and (A) the reactivity of platelets to PAR-1 (after adjustment for covariates) in 546 participants in the Cambridge Platelet Function Cohort, (B) platelet expression of GRK5 measured using probe ID:3190239 of the Illumina HumanHT-12 v4.0 Expression BeadChip microarray in 388 donors, after adjustment for technical variation. The heavy horizontal bars indicate the median of each conditional distribution; the lower and upper hinges respectively indicate the 25th and 75th percentiles of each distribution; the whiskers extend no further than 1.5 interquartile ranges (IQRs) from the hinges. (C) The genetic association between PAR-1 reactivity and rs10886430, in intron 1 of GRK5, co-localizes with the binding of the transcription factors GATA-1 and MEIS1 in human MKs and an enhancer specific to the human MK blood cell lineage. Top to bottom: the strength of evidence for association between genetic variants in the GRK5 locus and PAR-1 reactivity, measured by \(-\log_{10}(P\text{-value})\); the binding sites of the transcription factors GATA-1, RUNX1, FLI1.
and MEIS1 in human MKs; ATAC-seq read depth indicating regions of open chromatin in human MKs; ChIP-seq read depth, measuring the histone modifications H3K4me1, H3K4me3 and H3K27ac in human MKs; human MK enhancer sites inferred from the ATAC-seq and ChIP-seq data; a model of the GRK5 gene with exons indicated by rectangles.

**Figure 3.**
Activation of platelets with thrombin increases GRK5 and PAR-1 binding in human platelets.
(A) Resting human platelets or thrombin (1 U/ml) activated platelets were prepared and lysed. Proteins were precipitated with an anti-PAR-1 (ATAP-2) antibody or control immunoglobulin (Ig) and then probed with anti-GRK5 antibody and reprobed with an anti-PAR-1 antibody (n=6); (B) Lysates from resting or activated platelets were precipitated with an anti-GRK5 antibody or Ig and probed with anti-PAR-1 before reprobing with anti-GRK5 (n=3).

**Figure 4.**
Increased integrin activation, α-granule exocytosis, aggregation and ATP release in platelets from Grk5−/− mice.
(A) Platelets from Grk5−/− and littermate control (WT) mice were stained with antibodies to either activated αIIbβ3 (Jon/A) or P-selectin and measured by flow cytometry. Platelets were stimulated with Par-4 agonist peptide (Par-4-AP, AYPGKF) (n=3). “ns” - non-significant; (B) Platelet reactivity in response to a Par-4-AP (n=5) and (C) Representative aggregation traces and (D) summarized data for platelets stimulated with thrombin (mean ± SEM, n=3-6 mice at each tested concentration). AUC: area under curve. (E) ATP release for platelets from Grk5−/− and littermate control mice (WT) stimulated with Par-4-AP (AYPGKF). The results of 3 experiments (mean ± SEM) are summarized.

**Figure 5.**
Increased platelet accumulation at the site of vascular injury in Grk5−/− mice.
(A) Platelet accumulation was visualized by CD41 staining after laser injuries in cremaster muscle arterioles and recorded by a confocal intravital fluorescence microscopy; (B) Graph shows the area under CD41 vs time curve (AUC - Area Under Curve); (C) Graph shows the peak of the CD41+ area during 180 seconds after injury; (D) Bar graphs represent CD41 deposition at the end of 180 seconds after injury; (E) Dot plot graphs represent CD41 deposition at the end of 180 seconds after injury. All data are mean ± SEM; 36 injuries in three WT mice and 36 injuries in four Grk5−/− mice; (F) Representative images of anti-GPIX labelled thrombi in lungs harvested from WT and Grk5−/− mice. (G) Thrombosis Score, representing the mean thrombus area and number of thrombi, of 8 WT mice versus 8 Grk5−/− mice are summarized. Group means were compared using the Student’s t-test. P≤0.05 was considered statistically significant.
Table 1
Summary statistics for platelet reactivity genome wide association study (< 5x10^{-8}). Coordinates based on GRCh37. Predicted consequence based on GP6 transcript NM_001083899.2 and PAR-4 transcript NM_003950.4.

| Phenotype | Variant ID   | Chr | Position       | Predicted consequence | Ref. Allele | Alt. Allele | Alt. Allele Frequency | N  | Effect Size (SDs per no. ALT alleles) | Std Err (SDs per no. ALT alleles) | P-value       | Nearest Gene |
|-----------|--------------|-----|----------------|-----------------------|-------------|-------------|-----------------------|----|----------------------------------------|----------------------------------|--------------|--------------|
| ADP       | rs12566888   | 1   | 156,869,047    | intronic variant      | G           | T           | 0.09                  | 1341| -0.391                                 | 0.0706                           | 3.184×10^{-8} | PEAR1        |
| ADP       | rs7624918    | 3   | 56,901,292     | Intron variant        | C           | T           | 0.34                  | 1341| 0.284                                 | 0.0434                           | 5.75×10^{-11} | ARHGEF3      |
| CRP-XL    | rs1613662    | 19  | 55,536,595     | non-synonymous variant c.655C>T, p.(Pro219Ser) | G           | A           | 0.83                  | 1329| 0.923                                 | 0.0432                           | 5.01×10^{-101} | GP6          |
| PAR-4     | rs773902     | 19  | 17,000,632     | non-synonymous variant c.358G>A, p.(Ala120Thr) | G           | A           | 0.20                  | 550 | 0.515                                 | 0.0698                           | 1.66×10^{-13}  | F2RL3        |
| PAR-1     | rs10886430   | 10  | 121,010,256    | intronic variant      | A           | G           | 0.13                  | 546 | 1.09                                  | 0.0821                           | 2.75×10^{-40}  | GRK5         |
Figure 3

(A)

Human platelets:

- IP: PAR-1
- IB: GRK5
- IB: PAR-1

Thrombin (1U/ml)

rest 45s 3min Ig

kDa

50 75

(B)

Human platelets:

- IP: GRK5
- IB: PAR-1
- IB: GRK5

Thrombin (1U/ml)

rest 45s 3min Ig

kDa

50 75

Graphs show the relative intensity of GRK5/PAR-1 and PAR-1/GRK5:

- GRK5/PAR-1:
  - rest: ns
  - 45s: p<0.001
  - 3min: p<0.001

- PAR-1/GRK5:
  - rest: ns
  - 45s: p=0.0224
  - 3min: p<0.001
Figure 4

(A) Aggregation

(B) PAR4-AP (70μM, 75μM, 100μM)

(C) Thrombin (0.086 U/ml, 0.1 U/ml, 0.12 U/ml, 0.13 U/ml)

(D) Aggregation and ADP

(E) PAR4-AP
Figure 5

(A) CD41+ area (μm²)

(B) CD41+ AUC (μm²)

(C) CD41+ leukocyte (μm²)

(D) CD41 (at 180 sec post injury)

(E) CD41 (at 180 sec post injury)
Figure 5

(F) WT+ Thrombin vs. Grk5-/- Thrombin

(G) Pulmonary Thromboembolism

Thrombus Score (Full Charge)

WT vs. Grk5-/-

p=0.0305

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