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A regulatory node involving Gα_q, PLCβ, and RGS proteins modulates platelet reactivity to critical agonists

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Abstract

Background: Most platelet agonists work through G protein-coupled receptors (GPCRs), activating pathways that involve members of the G_q, G_i, and G_{12}/G_{13} families of heterotrimeric G proteins. G_q signaling has been shown to be critical for efficient platelet activation. Growing evidence suggests that regulatory mechanisms converge on GPCRs and G_q to prevent overly robust platelet reactivity.

Objectives: To identify and characterize mechanisms by which G_q signaling is regulated in platelets.

Methods: Based on our prior experience with a Gα_{i2} variant that escapes regulation by RGS (regulator of G protein signaling) proteins, a Gα_q variant was designed with glycine 188 replaced with serine (G188S) and then incorporated into a mouse line so that its effects on platelet activation and thrombus formation could be studied *in vitro* and *in vivo*.

Results and Conclusions: As predicted, the G188S substitution in the Gα_q disrupted its interaction with RGS18. Unexpectedly, it also uncoupled PLCβ3 from activation by platelet agonists as evidenced by a loss rather than a gain of platelet function *in vitro* and *in vivo*. Binding studies showed that in addition to preventing the binding of RGS18 to Gα_q, the G188S substitution also prevented the binding of PLCβ3 to Gα_q. Structural analysis revealed that G188 resides in the region that is also important for Gα_q binding to PLCβ3 in platelets. We conclude that the Gα_q signaling node is more complex than has been previously understood, and suggest that there is cross-talk between RGS proteins and PLCβ3 in the context of Gα_q signaling.

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Declaration of Competing Interests
The authors have no relevant conflicts of interest to disclose.

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Keywords

hemostasis; platelets; G protein-coupled receptors; signaling networks

1. Introduction

Binding of an agonist to its GPCR enables the receptor to activate heterotrimeric G proteins (Gα and Gβγ) by promoting the exchange of GTP for GDP on the α-subunit. $G\alpha_q$-mediated downstream signaling is critical for functional responses during platelet activation. $G\alpha_q$ stimulation leads to activation of the β-isoforms of phospholipase C (PLCβ) and subsequent increases in intracellular Ca^{2+}. A defect in $G\alpha_q$ expression in patients leads to impaired platelet aggregation and granule secretion, resulting in bleeding diathesis [1]. Similarly, platelets from $G\alpha_q$-deficient mice have a diminished activation response to nearly all the GPCR signaling agonists [2], highlighting the significance of $G\alpha_q$ signaling to achieve efficient hemostasis. On the other hand, signaling through $G\alpha_{i2}$, the predominant G_i family member expressed in platelets, is important for platelet activation because it inhibits the accumulation of cAMP via activation of ADP $P2Y_{12}$ receptor [3] and promotes Rap1 activation [4, 5]. The differences in $G\alpha_q$- and $G\alpha_i$-dependent signaling events during platelet activation suggest distinct downstream signaling effectors, further suggesting that both G proteins could be differentially regulated.

Although much is known about $G\alpha_q$- and $G\alpha_i$-mediated signaling pathways during platelet activation, how activated $G\alpha_q$ is regulated in order to achieve an optimal platelet response to injury has yet to be deciphered. RGS proteins suppress G protein signaling by accelerating the hydrolysis of GTP bound to activated Gα [6–8]. The predominant RGS proteins expressed in human and mouse platelets are RGS10 and RGS18 [9]. Recent studies from our laboratory and others have shown that RGS10 and RGS18 provide negative feedback to both $G\alpha_q$- and $G\alpha_i$-dependent signaling during platelet activation [6, 10–16]. Part of the evidence for this conclusion comes from studies in which RGS10 and/or RGS18 were knocked out in mice [12, 14–16]. Another part comes from studies in which a single amino acid substitution in $G\alpha_{i2}$(G184S) known to block the interaction between $G\alpha_{i2}$ and RGS proteins was incorporated into a mouse line and shown to result in an increase in platelet reactivity to agonists [10]. The gain-of-function observed in platelets from $G\alpha_{i2}$(G184S) mice was limited only to $G\alpha_{i2}$-dependent signaling events. At the proteomics level, RGS proteins have significantly lower expression than $G\alpha_q$ or $G\alpha_i$ proteins [9], suggesting that $G\alpha_q$ or $G\alpha_i$ may be regulated by other factors in platelets.

To understand whether the mechanism of $G\alpha_q$ inactivation in platelets is similar to $G\alpha_i$ or if unique regulatory dynamics exist in $G\alpha_q$-dependent signaling pathways, we generated mice with a single amino acid substitution of glycine to serine at G188 in the RGS binding domain of $G\alpha_q$, analogous to the G184S mutation in $G\alpha_i$. This substitution has been shown to lead to a gain of $G\alpha_q$ signaling function in CHO cells transfected with serotonin (5-HT) 5-HT_{2c} [17]. Here we have validated the predicted effects of the G188S substitution on the interaction of $G\alpha_q$ with RGS18 and then, after incorporating the substitution into the germline of mice, tested its effects on platelet reactivity *in vitro* and *in vivo*.

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2. Materials and Methods:

2.1. Mice and antibodies

$G\alpha_q$(G188S) knock-in mice were generated using CRISPR-Cas9 genome-editing [18]. The G188S missense mutation, encoded by a GGG→TCG change, also adds a diagnostic RsaI restriction digestion site. The CCCC, upstream of TCG change, was replaced with ACCA and introduced a silent mutation to prevent re-cutting by Cas9 after editing. The full-length cDNA of $G\alpha_q$ was sequenced, and no other mutations were found.

Anti-G_{i2} and anti-PLCβ3 were from Santa Cruz (St. Louis, MO). Anti-$G\alpha_q$ was from EMD Millipore (Burlington, MA). Anti-RGS18 was from Abcam (Cambridge, United Kingdom). Anti-actin and anti-Flag were from Cell Signaling (Danvers, MA). Jon/A-PE was from Emfret Analytics (Wuerzburg, Germany). Anti-mouse CD62P was from BD Biosciences (Franklin Lakes, NJ). Antibodies were used at a dilution of 1:1000 for Western Blot. 2μg of anti-Flag antibody was used for immunoprecipitation.

2.2. Platelet function studies

Immunoblotting, immunoprecipitation, flow cytometry, platelet aggregation, ATP release, intracellular calcium, and vascular injury experiments were performed as described [15, 19–21].

2.3. Structural and computational alanine scanning analysis

The interaction interface of $G\alpha_q$ and its known *in vivo* binding partners was predicted using the Robetta Computational Interface Alanine Scanning Server [22] and existing structures available from the RCSB Protein Data Bank (www.rcsb.org) [23]. Structures were viewed and the interfaces were manually mapped using PyMOL.

2.4. Statistical analysis.

Results are presented as mean ± SEM. Data were analyzed using the Student's t-test or two-way ANOVA test. $p<0.05$ was considered statistically significant.

3. Results and Discussion

3.1. G188S mutation on $G\alpha_q$ disrupts its interaction with RGS18 in mouse platelets

Mice bearing the G188S substitution in exon 4 of $G\alpha_q$ were generated using CRISPR-Cas9 genome-editing (Figure 1A). Mice heterozygous for the substitution (denoted +/G188S) were born in expected Mendelian ratios and developed normally. However, only 7.2% of homozygous mice (G188S/G188S) survived to weaning age. Those that did survive were smaller in size, and exhibited markedly reduced growth independent of sex. The homozygous mice are referred to as G_q^{G188S} hereafter. Complete blood counts, including platelet counts, were normal in G_q^{G188S} mice, as was the expression of $G\alpha_q$ protein. Notably, the $G\alpha_q$(G188S) substitution prevented the interaction of RGS18 with activated $G\alpha_q$ in the presence of GDP+AlF_4^-, which constrains $G\alpha_q$ to its GTP bound (transition) state (Figure 1Bi&ii).

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3.2. Diminished GPCR signaling in G_q^{G188S} platelets

To assess the functional consequences of the $G\alpha_q$(G188S) substitution, integrin activation and α-granule secretion were compared in platelets from WT and G_q^{G188S} littermates using flow cytometry with antibodies specific to activated integrin $\alpha_{IIb}\beta_3$ and P-selectin. The assays were performed using diluted platelet suspensions to minimize signaling induced by secreted mediators such as ADP and thromboxane A_2 (TXA_2). The results in Figure 1C&D&E show a normal response to convulxin, which is a ligand for the platelet collagen receptor glycoprotein (GP) VI, but a greatly reduced response to a PAR4 agonist peptide (PAR4AP; AYPGKF), ADP, and the stable TXA_2 analog U46619 in platelets from the G_q^{G188S} mice. The surface expression of integrin $\alpha_{IIb}\beta_3$ was also found to be normal on G_q^{G188S} platelets (Figure 1F). Platelet aggregation studies (Figure 2A) also showed a normal response when platelets from G_q^{G188S} mice were stimulated with convulxin, but a substantial decreased response when stimulated with PAR4AP, ADP or U46619. In each case, raising the agonist concentration partially restored aggregation. Dense granule secretion (measured with ATP release) was reduced in response to PAR4AP and U46619, but not convulxin in G_q^{G188S} platelets (Figure 2B).

Agonists whose receptors are coupled to G_q cause activation of PLCβ leading to the hydrolysis of phosphotidylinositol-4,5-bisphosphate (PIP_2) and the production of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). The subsequent increase in $[Ca^{2+}]_i$ is due to the release of Ca^{2+} from intracellular stores followed by the influx of Ca^{2+} from the extracellular milieu. To determine whether the $G\alpha_q$(G188S) substitution in platelets affects PLCβ activation and Ca^{2+} mobilization, we measured changes in $[Ca^{2+}]_i$ in response to each of the GPCR agonists. The response to U46619 and a lower dose of PAR4AP (100 μM) was blunted in G_q^{G188S} platelets, and recovered to only 30-40% in response to a higher dose of PAR4AP (Figure 2C&D). There was a trend of decreased Ca^{2+} mobilization in G_q^{G188S} platelets upon ADP stimulation (Figure 2D).

3.3. G_q^{G188S} mice display diminished rather than increased platelet accumulation in response to hemostatic injury

To understand the impact of the $G\alpha_q$(G188S) substitution on hemostasis, G_q^{G188S} platelet function was assessed *in vivo* using real-time confocal fluorescence microscopy in the cremaster muscle microcirculation after producing a penetrating injury with a laser, an injury that evokes a hemostatic response [24]. The hemostatic thrombi formed in this type of injury are not occlusive, containing a small core of P-selectin$^+$ degranulated platelets that are localized close to the injury site and are covered by a distal shell of P-selectin$^-$ platelets. In WT mice, CD41$^+$ platelets accumulated rapidly, reaching a plateau approximately 2 minutes after injury (Figure 3A). We have shown previously that mice bearing the RGS-insensitive $G\alpha_{i2}$(G184S) substitution have an enhanced response to injury in this model [10]. Here we found that the rate of G_q^{G188S} platelet accumulation following injury was decreased, rather than increased. Total platelet accumulation was reduced by 80% at the end of the observation period (Figure 3A). The size of the core region in the G_q^{G188S} mice were significantly decreased as measured by the area of P-selectin$^+$ platelets (Figure 3B). Fibrin accumulation was normal, indicating that tissue factor exposed at the injury site is sufficient for thrombin generation (Figure 3C).

3.4. G188S mutation on $G\alpha_q$ disrupts its interaction with PLCβ-3 upon platelet activation

Based on the unexpected observation that the $G\alpha_q$(G188S) substitution reduced rather than increased platelet accumulation at the site of injury, we examined the effects of the substitution on the interaction of $G\alpha_q$ with its principal effector, PLCβ. No difference in PLCβ-3 protein expression was observed in G_q^{G188S} mutant platelets (Figure 4A). Agonist binding to a GPCR leads to the exchange of $G\alpha_q$–bound GDP for GTP, resulting in $G\alpha_q$ signaling activation via its binding to PLCβ. An *ex vivo* co-immunoprecipitation assay was used to determine if the G188S mutation in $G\alpha_q$ impairs the activation-dependent association between $G\alpha_q$ and PLCβ-3. Flag-tagged PLCβ-3 was used to pull-down either $G\alpha_q$ or G_q^{G188S} from WT or G_q^{G188S} platelets respectively in the presence of GDP+AlF_4^-. A robust interaction was observed in platelet lysates from WT platelets following incubation with Flag-tagged PLCβ-3 under activation conditions. However, this interaction was completely abolished in G_q^{G188S} platelets (Figure 4B). As anticipated, the interaction between PLCβ-3 and G protein was specific to $G\alpha_q$, but not to $G\alpha_{i2}$. The above observation was further confirmed via co-immunoprecipitation in HEK293 cells that were transfected with Flag-tagged PLCβ-3 along with either WT $G\alpha_q$ or Gq^{G188S}. As observed in Gq^{G188S} platelets, the G188S mutation caused a significant reduction in the G_q^{G188S}/PLCβ-3 interaction when compared to the $G\alpha_q$/PLCβ-3 interaction in the control cells expressing WT $G\alpha_q$ and PLCβ-3 (Figure 4C). These results demonstrate that the G188 residue of $G\alpha_q$ is not only critical for its association with RGS proteins but is also critical for $G\alpha_q$/PLCβ3 interactions upon GPCR activation. Lastly, the surface regions of $G\alpha_q$ expected to be involved in binding interactions with its effectors RGS18 and PLCβ were mapped based on computational alanine scanning. An overlapping interface of these complexes was found, with the G188S mutation residing in the RGS and PLC interfaces (Figure 4D).

The consequence of complete loss of expression of $G\alpha_q$ in mouse platelets has been well-characterized, with diminished platelet activation and aggregation observed in response to all GPCR agonists [2]. However, the $G\alpha_q$ knock-out model does not provide insight into the underlying mechanisms of regulation of GPCR signaling in platelets. In this study, G_q^{G188S} serves as a useful tool to identify novel patterns of regulator binding to $G\alpha_q$. We show for the first time that the $G\alpha_q$/RGS interaction interface is also critical for maintaining $G\alpha_q$/PLCβ-3 associations upon platelet activation. We suggest the possibility that negative regulation by RGS proteins and signal propagation by PLCβ overlap and alter each other's effectiveness. Activation of PLCβ triggers inositol signaling cascades, leading to intracellular calcium mobilization and PKC activation [25]. However, PLCβ has been observed to negatively regulate GPCR signaling via GTPase-activating protein (GAP) activity [25, 26]. The magnitude of the stimulation of the GTP hydrolysis effect of PLCβ is similar to that of RGS proteins [27].

There is growing structural evidence that many effectors are coupled with $G\alpha_q$ [25]. While the action of some of these effectors in regulating $G\alpha_q$ signaling have been well characterized in an isolated context, any potential interactions between effectors at the $G\alpha_q$ signaling node and the functional consequences of such interactions on platelet function have not been characterized. We have shown here that PLCβ-3 and RGS18 share a portion

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of their binding region on $G\alpha_q$. Results of this study and others suggest that the $G\alpha_{i2}$ and $G\alpha_q$ signaling nodes differ in ways that are significant. Because of the differences between the $G\alpha_q$ and $G\alpha_{i2}$ signaling nodes, the novel G188S mutant mouse line provides an exciting new avenue for exploring the dynamics of regulation by multiple effectors and potential cross-talk between RGS proteins and PLCβ suggested by our results.

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Essentials:

- $G\alpha_q$-mediated signaling is critical for functional responses during platelet activation.
- The feedback mechanism of $G\alpha_q$ inactivation is different from that of $G\alpha_{i2}$ and involves RGS proteins and PLCβ-3.
- A G188S mutation in $G\alpha_q$ prevents RGS proteins and PLCβ-3 from binding to $G\alpha_q$, which results in diminished platelet activation both *in vitro* and *in vivo*.
- This G188S mutant mouse model will facilitate our understanding of the molecular interaction of $G\alpha_q$ with its regulators and effectors in platelets.

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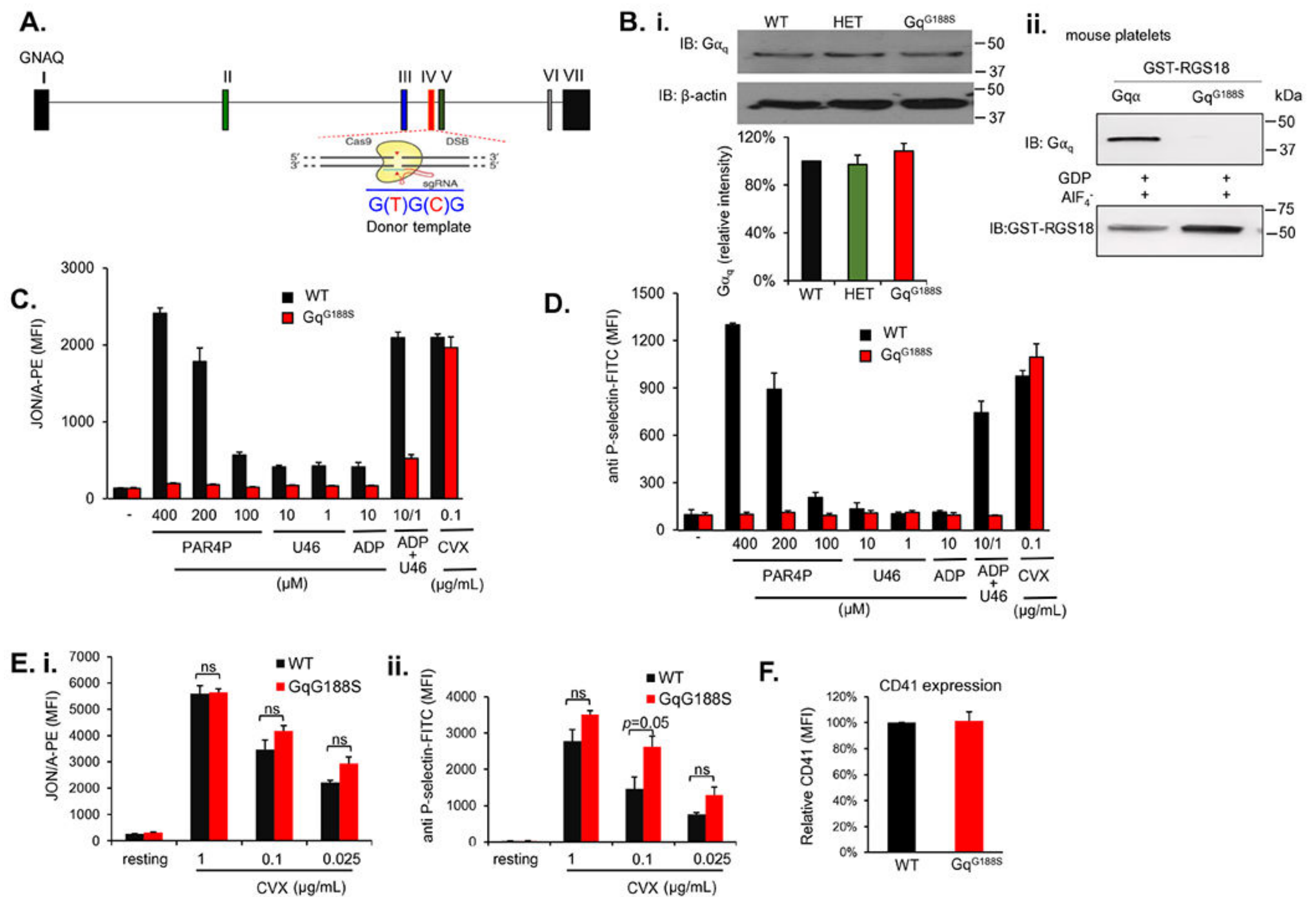

Figure 1. Generation and Characterization of $G_{\alpha q}$(G188S) mice.

(A) Strategy for introducing G188S mutation in exon 4 of mouse GNAQ gene. A synthetic homology-directed repair (HDR) template (GG→TCG) was designed to introduce the G188S mutation in $G\alpha_q$. The sgRNA and HDR donor template were then combined with Cas9 mRNA for subsequent cytoplasmic injection of fertilized mouse eggs. (B) Bi: $G\alpha_q$ protein expression in WT and G188S-expressing mice. N=3. Bii: GST-RGS18 was used to pull down WT $G\alpha_q$ or G_q^{G188S} expressing mouse platelets in the presence of GDP and AlF_4–, which causes $G\alpha_q$ to adopt the transition state recognized by RGS proteins. Bound proteins were subjected to electrophoresis and probed with an anti-RGS18 antibody to detect GST-RGS18 fusion protein. (C&D) Decreased integrin activation and α-granule exocytosis in platelets from G_q^{G188S} mice. Platelets from G_q^{G188S} and littermate control mice (WT) were stained with fluorophore-conjugated antibodies to either activated integrin $\alpha_{IIb}\beta_3$ (Jon/A antibody) (C) or P-selectin (D) after incubation with a PAR4 agonist peptide (PAR4AP), a TXA_2 mimetic (U46619), ADP, U46619/ADP, or convulxin (CVX) at the concentrations indicated (N=4). (E) Platelets from G_q^{G188S} and littermate control mice (WT) were stained with fluorophore-conjugated antibodies to either activated integrin $\alpha_{IIb}\beta_3$ (Jon/A antibody) (i) or P-selectin (ii) after incubation with CVX at the concentrations indicated (N=4). (F) The expression of integrin in G_q^{G188S} and littermate WT platelets

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measured by flow cytometry. Platelets were stained with fluorophore-conjugated CD41 antibody specific to integrin α_{IIb}. N=5.

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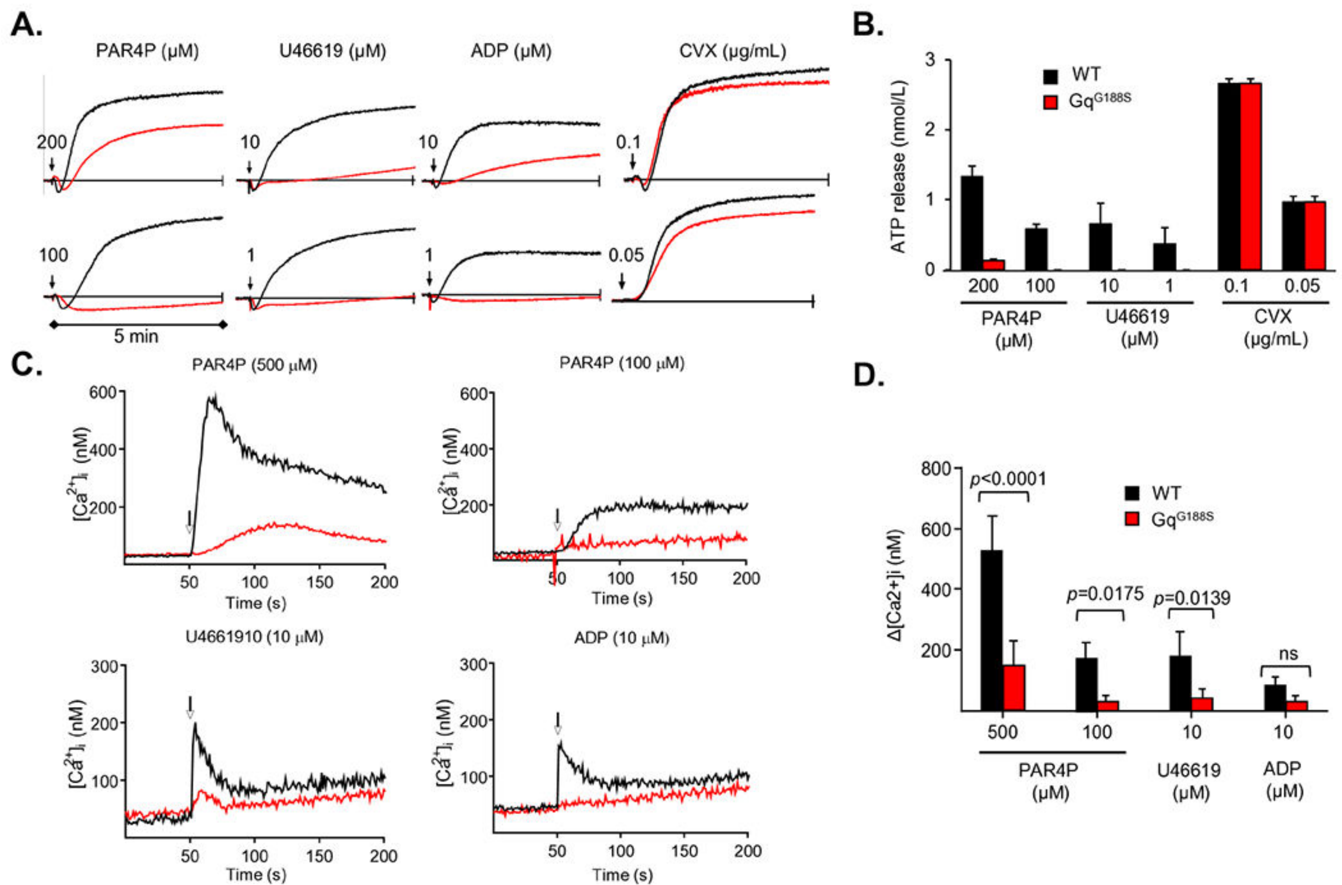

Figure 2. Decreased platelet aggregation/ATP release and diminished Ca^{2+} mobilization in platelets from G_q^{G188S} mice.

(A) Representative aggregation traces for platelets stimulated with PAR4AP, U46619, ADP, or CVX at the concentrations indicated (N=3). (B) ATP release from platelets stimulated with PAR4AP, U46619, or CVX at the concentrations indicated (N=2). (C) Ca^{2+} mobilization. Platelets were stimulated with PAR4AP, ADP or U46619 at the concentrations indicated in the absence of extracellular Ca^{2+}. Representative measurements are shown. (D) The results of 4 experiments (mean ± SEM) are summarized. Data sets were compared using two-way ANOVA test.

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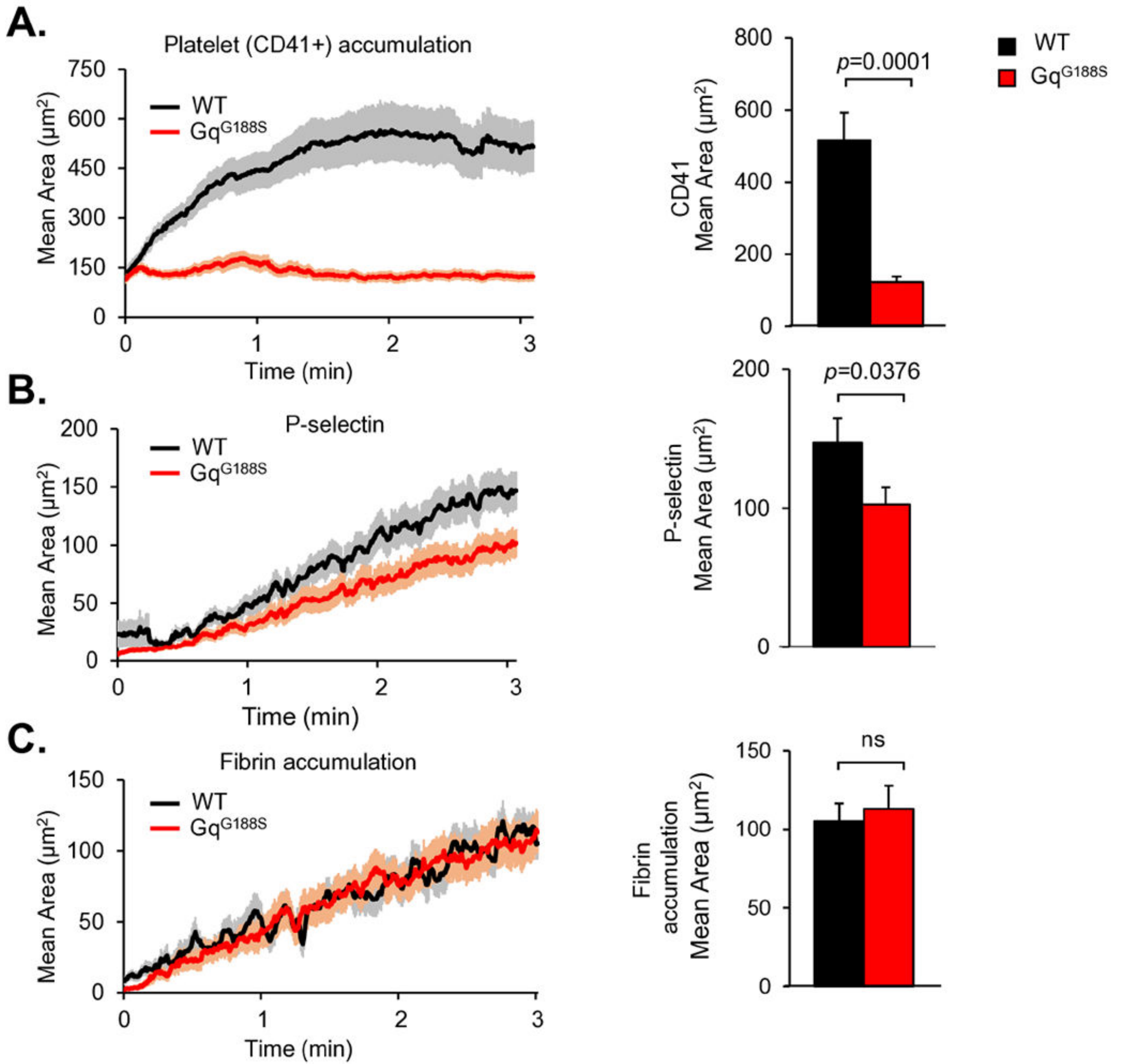

Figure 3. G_q^{G188S} mice display diminished platelet accumulation in response to hemostatic injury.

Various combinations of Anti-CD41 $F(ab)_2$ fragments (0.12 µg/g body weight; clone MWReg30, BD Bioscience, San Jose, CA), anti-P-selectin (0.2 µg/g body weight; clone RB40.34, BD Bioscience), and anti-fibrin (0.2 µg/g body weight; clone 59D8) were infused intravenously via the jugular vein. Antibodies were labeled with Alexa fluor dye monoclonal antibody labeling kits (Alexa-488, Alexa-568 and Alexa-647) according to the manufacturer's instructions (Invitrogen, Waltham, MA). Confocal intravital fluorescence microscopy was performed to follow (A) platelet accumulation, (B) P-selectin expression and (C) fibrin deposition after making small penetrating injuries in cremaster muscle

arterioles with a laser in G_q^{G188S} mice and littermate controls. Bar graphs represent the $CD41^+$ area (A), P-selectin$^+$ areas (B), and fibrin accumulation (C) at the end of the 3-minute observation period. At least 50 injuries were performed in 7-8 mice in each group. Data sets were compared using the unpaired t-test.

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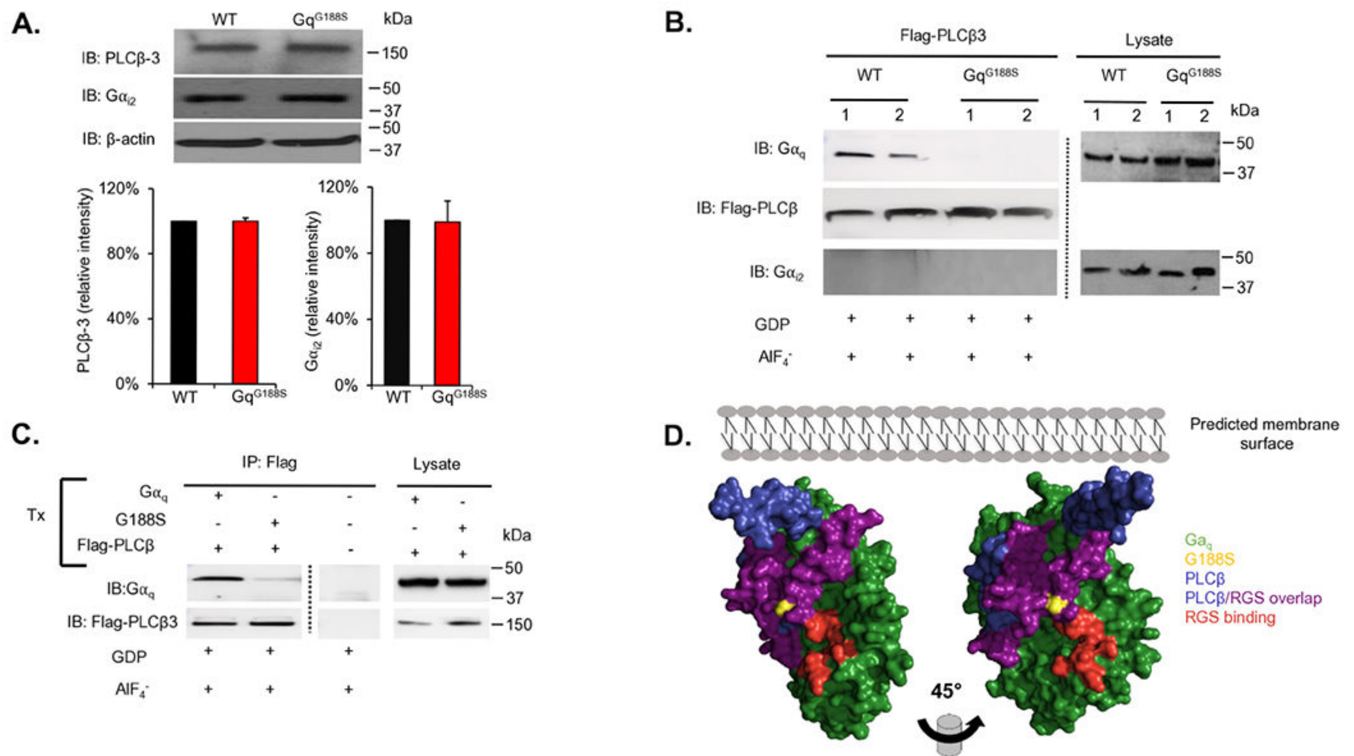

Figure 4. G188S mutation disrupts $G\alpha_q$ interaction with PLCβ-3 upon platelet activation.

The G188S mutation does not affect PLCβ-3 and $G\alpha_{i2}$ protein expression in mice. (N=3). (B) Lysates were prepared from resting WT mouse platelets or G_q^{G188S} expressing platelets. The lysates were then incubated with Flag-tagged PLCβ-3 (Flag-PLCβ-3) coupled to resin beads in the presence of GDP plus AlF_4^-. Bound proteins were subjected to electrophoresis and probed with anti-$G\alpha_q$, -Flag, and -$G\alpha_{i2}$ antibodies to detect $G\alpha_q$, Flag-PLCβ-3 fusion protein, or $G\alpha_{i2}$ respectively. (C) HEK293 cells transfected (Tx) with a full-length, Flag-tagged PLCβ-3 (Flag-PLCβ-3) along with WT $G\alpha_q$ (pcDNA3.1-$G\alpha_q$) or G_q^{G188S} (pcDNA3.1-G_q^{G188S}). Proteins were precipitated with an anti-Flag antibody and then probed for $G\alpha_q$ and Flag-PLCβ-3. *Right* Lysates were prepared from cells transfected with Flag-PLCβ-3 in the presence of WT $G\alpha_q$ or G_q^{G188S} and probed with PLCβ-3 and $G\alpha_q$. The vertical line indicates that the input was run on separate gels and then probed with anti-$G\alpha_q$ or anti-$G\alpha_{i2}$ antibody, respectively. (D) The predicted interface of $G\alpha_q$ (green) in complex with PLCβ-3 (blue) overlaps with the binding interface of RGS18 (red). The G188S mutation (orange) lies at the interface between RGS18 and PLCβ-3, and mutations surrounding this residue are predicted to be highly destabilizing via computational alanine scanning. All structures are shown twice, with the second rotated 45° about a vertical axis running through the center of $G\alpha_q$.