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Journal Title: Journal of Thrombosis and Haemostasis
Volume: Volume 11, Number 12
Publisher: Wiley: 12 months | 2013-12-01, Pages 2155-2162
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1111/jth.12425
Permanent URL: https://pid.emory.edu/ark:/25593/v47v9

Final published version: http://dx.doi.org/10.1111/jth.12425

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Accessed January 22, 2020 10:43 PM EST
Specific inhibition of ectodomain shedding of glycoprotein Ibα by targeting its juxtamembrane shedding cleavage site

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Summary

Background—Ectodomain shedding of GPIbα, a proteolytic event in which metalloprotease ADAM17 cleaves the Gly464-Val465 bond and releases glycocalicin to the plasma, is considered a critical step in mediating clearance of stored platelets. Supporting evidence has largely come from studies using ADAM17 inhibitors. However, the definitive proof is lacking due to the broad substrate specificity of ADAM17.

Objectives—to achieve substrate-specific inhibition of GPIbα shedding.

Methods—Development of monoclonal antibodies that directly bind the sequence around the GPIbα shedding cleavage site and inhibit GPIbα shedding by blocking ADAM17 access to the cleavage site.

Results—Six anti-GPIbα monoclonal antibodies with varying binding affinities were obtained. The prototypic clone, designated 5G6, and its monomeric Fab fragment, bind specifically purified GPIb-IX complex, human platelets, and transgenic murine platelets expressing human GPIbα. 5G6 showed similar inhibitory potency as a widely used shedding inhibitor GM6001 in both constitutive and induced GPIbα shedding in human platelets. It does not recognize mouse GPIbα. Nor does it inhibit shedding of other platelet receptors. Finally, 5G6 binding displays no detectable effect on platelet activation and aggregation.

Conclusion—5G6 specifically inhibits GPIbα shedding with no detectable effect on platelet functions. The method of substrate-specific shedding inhibition by macromolecular binding of the shedding cleavage site can be applicable to many other transmembrane receptors undergoing ectodomain shedding.

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Addendum
XL designed research, performed research, analyzed data and wrote the paper; SRR, SE, LJH, SC, STB, JW performed research and analyzed data; MLK and MCB provided key reagents; RL initiated and designed research, analyzed data and wrote the paper.

Disclosure of Conflicts of Interest
The authors state that they have no conflict of interest.
Keywords
Glycoprotein Ib; Glycocalicin; Platelet transfusion; ADAM proteins; Antibodies, Monoclonal

Introduction
Glycoprotein (GP)Ibα is abundantly expressed on the platelet surface. GPIbα is the platelet receptor for von Willebrand factor (VWF) and other ligands in circulation [1]. In addition to its function in mediating ligand-induced platelet activation during primary hemostasis, GPIbα plays an important role in thrombosis, thrombocytopenia, inflammation and other disease states [2, 3]. GPIbα is continuously proteolyzed in circulating platelets, with its extracellular domain, also known as glycocalicin, released into the plasma [4]. This process, later named ectodomain shedding, has been also reported to occur in platelets activated by chemical or physiological agonists, such as α-thrombin, N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W7, a calmodulin inhibitor that sequesters calmodulin from binding to its ligands), carbonyl cyanide 3-chlorophenylhydrazone (CCCP, a drug that damages the mitochondria and induces apoptosis), and phorbol 12-myristate-13-acetate (PMA) [5–8]. ADAM17 is the physiological sheddase for GPIbα [6]. Broad-spectrum metalloproteinase inhibitors, such as hydroxamic acid-based GM6001 that chelates the zinc ion required for the metalloproteinase activity, strongly inhibit W7- and CCCP-induced GPIbα shedding [7, 8]. Recombinant ADAM17 cleaves GPIbα-based peptides at the Gly464-Val465 peptide bond, suggesting it as the shedding cleavage site in GPIbα [8].

Despite abundant information obtained over the past few years, the biological significance of GPIbα shedding remains to be defined. Recently it was suggested that GPIbα shedding plays a critical role in clearance of damaged platelets. Like human platelets, mouse platelets that had been stored in vitro or treated with CCCP to simulate cell damage were observed to shed a significant amount of GPIbα, and they were cleared rapidly upon infusion [7]. Incubation of these platelets with GM6001, or a small-molecule inhibitor of p38 MAPK that is required for ADAM17 activity, blocked shedding of GPIbα and improved the post-transfusion recovery and survival of these platelets [7, 9]. These results suggest that blocking GPIbα shedding can hamper the clearance of stored platelets. However, ADAM17 has broad substrate specificity [10, 11]. With a relatively shallow substrate-binding groove exposed on the surface of its catalytic domain and the ability to adapt the binding pocket to the shape of a substrate or an inhibitor, ADAM17 can recognize and cleave a substrate with an extended backbone conformation that is not strictly dependent on any particular side chain [12, 13]. ADAM17 has been shown to cleave physiologically GPIbα, TNF-α and many other substrates including GPV [14]. Thus, the evidence reported so far cannot rule out the possibility that a receptor on the platelet surface other than GPIbα that is also a shedding substrate is the cause for platelet clearance. To definitively determine whether GPIbα shedding is actually the trigger for platelet clearance or merely an inconsequential indicator for damaged and to-be-cleared platelets, a reagent that specifically inhibits shedding of GPIbα but not other receptors will be required.

In the present study we report novel anti-GPIbα monoclonal antibodies (mAbs) that specifically inhibit shedding of human GPIbα in platelets.

Materials and methods
Materials and animals
Immunization of C57BL mice and production of monoclonal antibodies against GPIbα were carried out by Green Mountain Antibodies (Burlington, VT). CCCP, L-cysteine and BSA

J Thromb Haemost. Author manuscript; available in PMC 2014 December 01.
were from Sigma-Aldrich (St. Louis, MO). GM6001, W7 and PMA were from Calbiochem (La Jolla, CA). The anti-GPV mAb SW16 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated antibody was prepared using sulfo-NHS-biotin (Thermo Scientific, Rockford, IL) and following manufacturer’s instruction. Transgenic IL4Tg and hTg mice have been described [15]. All animal procedures have been performed in accordance with institutional guidelines and approval.

**Preparation of washed human platelets**

Human whole blood was obtained from healthy human volunteers. The informed consent and related protocols were approved by Emory University Institutional Review Boards. Platelet-rich plasma (PRP) was isolated by centrifugation at 140 g. 10 ml of PIPES-buffered saline with prostaglandin E1 (1 µM) was then mixed with PRP followed by centrifugation at 1,900 g for 8 min. The platelet pellet was resuspended in a modified Tyrode’s buffer without calcium (134 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 1 mM MgCl₂, 5 mM glucose, 12 mM NaHCO₃, 20 mM HEPES, pH 7.35). Platelet counts were measured using a HemaTrue hematology analyzer (HESKA, Loveland, CO).

**Preparation of Fab fragment**

Purified mAb (10 mg/ml in PBS) was incubated with immobilized papain (Thermo Scientific) in the presence of 20 mM L-Cysteine at 37 °C overnight. After papain was removed by centrifugation, the generated Fab fragment was purified using protein A beads (Invitrogen, Carlsbad, CA).

**Binding of mAbs to synthetic peptide and purified GPIb-IX**

Human GPIb-IX complex was purified as described [16] from outdated and de-identified leukoreduced apheresis-derived platelets obtained from Blood and Tissue Services at Children’s Healthcare of Atlanta. Synthetic peptides (New England Peptide, Gardner, MA) and purified GPIb-IX in PBS (both 6 µg/ml) were immobilized in separate microtiter wells (Costar cat# 3690, Corning Inc, Corning, NY) at 4 °C overnight. After incubation in the blocking buffer (20 mM HEPES, 0.2 M NaCl, 0.05% Tween-20, 2% BSA, pH7.4) at room temperature (RT) for 2 h, different doses of normal mouse IgG or purified mAb dissolved in the blocking buffer were added to the wells and incubated for 1 h. The wells were then washed 3 times with the blocking buffer without BSA and incubated for another 1 h with biotinylated goat anti mouse IgG (1:50 in the blocking buffer). After washing three times, the wells were incubated for 1h with streptavidin alkaline phosphatase (1:2000 in the blocking buffer). After 3 washes, bound antibodies were quantified by adding the alkaline phosphatase substrate (Bio-Rad, Hercules, CA) and recording the optical density at 405 nm in a SpectraMax Plus³⁸⁴ microplate reader (Spectramax Molecular Devices, Sunnyvale, CA). The absorbance at 405 nm was measured in three independent experiments and analyzed as described before [16].

**Expression and purification of GFP-lactadherin C2 domain (GFP-Lact-C2)**

The DNA fragment encoding the GFP-Lact-C2 fusion protein was amplified from the pEGFP-Lact-C2 plasmid (Addgene, Cambridge, MA). The fragment was further appended with a fragment encoding the hexahistidine tag by another round of PCR. The product was ligated in the pET-22b(+) vector (Novagen, Madison, WI) as an Ndel/EcoRI fragment and transformed into E. coli BL21(DE3) cells. Protein expression and purification by Ni-affinity chromatography followed essentially the published protocol [17]. Purified protein was dialyzed extensively against 50 mM Tris, 150 mM NaCl, pH 7.4 before being stored at −80 °C. The protein concentration was determined using the molar extinction coefficient of 55,000 M⁻¹·cm⁻¹ at 480 nm.

*J Thromb Haemost*. Author manuscript; available in PMC 2014 December 01.
Western blot
Washed human platelets (1.2×10⁷ cells/ml) in modified Tyrode's buffer containing 2.1 mM CaCl₂ were incubated with 150 µM W7 or 100 µM CCCP for 2 h. Immunoprecipitation of glyocalcicin from the supernatant and lysis of platelets were performed as described [18]. The proteins were resolved in an 8% Bis-Tris SDS gel under reducing conditions, transferred to PVDF membranes, and blotted with WM23 (for glyocalcicin and GPIbα), 6B12 (GPVI) and anti-actin antibody.

Flow Cytometry
Washed platelets (1.2×10⁷ cells/ml) were treated with antibodies or reagents targeting specific proteins or lipid at RT for 20 min, washed when necessary, and analyzed on a Becton-Dickinson FACS Canto II instrument. The signal was quantitated by the mean fluorescence intensity (MFI) for the entire cell population (10,000 cells).

Platelet Aggregometry
Agonist-induced platelet aggregation was monitored in a dual-channel Chrono-Log aggregometer (Havertown, PA). PRP was prepared by centrifugation of whole blood at 170 g for 15 min. Autologous PPP was prepared by centrifugation (after removal of PRP) at 2400 g for 20 min, and used to adjust the final platelet count in PRP to 2.5×10⁸ cells/ml. Aggregation was initiated in a 250 µl stirred PRP by addition of noted agonists. When required, the antibody was added to PRP and incubated for at least 5 min before stimulation with agonists.

Results
Generation of monoclonal antibodies targeting the shedding cleavage site in human GPIbα
If a reagent is to inhibit shedding of GPIbα but not that of any other receptor, it needs to act directly on GPIbα the shedding substrate rather than ADAM17 the sheddase. We reasoned that one way to achieve substrate-specific shedding inhibition was to bind the shedding substrate with sufficient binding affinity such as to block its access to the sheddase. Therefore, an antibody targeting the sequence flanking the shedding cleavage site in human GPIbα could inhibit its shedding.

A synthetic peptide that corresponds to human GPIbα sequence Glu455-Phe478 was used as the antigen for mouse immunization. Hybridoma clones obtained from immunized mice were screened in ELISA assays for binding activities to the ovalbumin-conjugated antigen peptide and shorter shedding-site peptide that corresponded to GPIbα residues Lys461-Leu470. Positive clones were further screened for their abilities to bind purified human GPIb-IX complex by ELISA [16], and to inhibit W7-induced GPIbα shedding in human platelets by flow cytometry [18]. In the end, 6 positive single clones were selected to produce monoclonal antibodies. All 6 purified mAbs exhibited strong binding to washed human platelets but they bound to immobilized GPIb-IX to various degrees (Fig. 1A,B). Two mAbs, 5G6 and 15C6, with the fitted K₄ of 3.29 ± 0.56 nM and 28.18 ± 3.17 nM, respectively, showed much higher affinities for GPIb-IX than the other 4. It is noteworthy, however, that the other 4 mAbs displayed higher binding than the negative control, suggesting that their binding affinity for GPIb-IX may be weaker and beyond the detection range in our ELISA assay. Further characterization revealed that these mAbs exhibited strong binding to multivalent ovalbumin-conjugated shedding-site peptide but disparate binding to monovalent shedding-site peptide that was directly immobilized in the microtiter plate (Fig. 1C,D). 5G6 binding of multivalent shedding-site peptide was similar to its binding to washed platelets, suggesting that mAbs bind GPIbα in human platelets via
multivalent binding, supported by the high expression level of GPIbα in platelets [19] and the bivalent structure of a mAb.

**5G6 binds specifically to the shedding cleavage site of human GPIbα in platelets**

With the highest $K_d$ for GPIb-IX, mAb 5G6 was henceforth characterized in detail. Whether 5G6 specifically recognizes the shedding cleavage site of human GPIbα in platelets was examined first. Like WM23, a well-documented anti-GPIbα mAb [20], 5G6 exhibited strong binding to fresh human platelets as detected by flow cytometry using FITC-conjugated goat anti-mouse IgG (Fig. 2A,1B). The binding reached saturation at 12 nM and 36 nM for 5G6 and its Fab fragments, respectively (Fig. 2B,C). The binding affinity of 5G6 for human platelet was similar to that obtained using the purified GPIb-IX complex (Fig. 2C). Moreover, immunoblotting of platelet lysate with 5G6 produced essentially the same protein bands as those blotted with WM23 (Fig. 2D), indicating that 5G6 specifically recognizes GPIbα. Finally, premixing the shedding-site peptide blocked binding of 5G6 to human platelets, confirming that the 5G6 epitope is located at the shedding cleavage site of GPIbα (Fig. 2E).

The sequence flanking the GPIbα shedding cleavage site is not conserved across species. Only 2 residues are conserved between human GPIbα Lys461-Leu470 and its murine counterpart sequence. Since 5G6 was raised to recognize the shedding cleavage site in human GPIbα, it did not, as expected, bind either wild-type (WT) murine platelets, or those expressing a mutant GPIbα in which its extracellular domain was replaced by that of IL-4Rα (IL4Tg). Instead, it bound transgenic murine platelets expressing only human GPIbα (hTg) (Fig. 2F), confirming that 5G6 was directed against the shedding cleavage site of human GPIbα, not mouse GPIbα, nor any other receptors in the platelet.

**5G6 inhibits specifically GPIbα shedding in human platelets**

To address whether 5G6 inhibits GPIbα shedding, WM23 was used to monitor the level of GPIbα on the platelet surface and the amount of glycocalcin released from the platelet after incubation of fresh human platelets with 5G6. Since both 5G6 and WM23 are of mouse origin, biotin-labeled WM23 and FITC-conjugated streptavidin were used for GPIbα detection by flow cytometry. W7 or CCCP was added to induce GPIbα shedding in platelets. Incubation of washed platelets with 5G6 or its Fab fragment at the saturating concentration inhibited not only constitutive but also W7- or CCCP-induced down-regulation of GPIbα expression on the platelet surface (Fig. 3A). The extent of inhibition was comparable to that by GM6001. Consistently, immunoblotting of the platelet lysate and supernatant showed that both 5G6 and its Fab fragment inhibited W7- and CCCP-induced decrease of GPIbα in human platelets and concurrent increase of glycocalcin released into the supernatant (Fig. 3B). Overall, these results demonstrated that 5G6 as well as its Fab fragment could inhibit GPIbα shedding in human platelets.

To address the specificity of 5G6 inhibition of GPIbα shedding, the effect of 5G6 treatment on the shedding of GPVI and GPV in human platelets was tested [8, 14, 21]. Since anti-GPVI mAb 6B12 does not work well in flow cytometry and anti-GPV mAb SW16 is not amenable to Western blot, shedding of GPVI and GPV were monitored by Western blot and flow cytometry, respectively. Figure 4A shows that W7-induced GPVI shedding was significantly inhibited by GM6001, but not 5G6 nor its Fab fragment. Since SW16 is a mouse antibody, human platelets were pre-incubated with only 36 nM 5G6 Fab prior to shedding stimulation. Figure 4B shows that GPV was down-regulated by W7 or PMA stimulation, which was inhibited by GM6001. But 5G6 Fab fragment did not inhibit W7- or PMA-induced GPV shedding. Overall, these results demonstrated the substrate specificity of 5G6 inhibition.
5G6 does not affect platelet activation and aggregation

Many, but not all, antibodies targeting the extracellular domain of GPIbα cause platelet activation or have pathogenic effects on platelets, although the underlying mechanisms remain to be defined [22–24]. Moreover, there have been reports suggesting an association of GPIb-IX complex with FcγRIIA [25]. Thus, it is important to establish whether 5G6 affects platelet activation or causes platelet apoptosis. Addition of 5G6 to PRP did not induce aggregation (Fig. 5A). Nor did it significantly affect the aggregation induced by ristocetin (Fig. 5A), suggesting that 5G6 does not impact GPIbα association with VWF. At the molecular level, treatment of washed platelets with 5G6 did not induce activation of integrin αIIbβ3 nor increase expression of P-selectin (Fig. 5B). Unlike ionomycin, 5G6 did not cause the exposure of phosphatidylserine (Fig. 5B). Overall, these results indicated that 5G6 has no detectable effect on platelet activation and aggregation.

Discussion

GPIbα is a major receptor complex in platelets. It is continuously shed from the platelet surface. The biological significance of GPIbα shedding remains to be defined, although it has been linked to platelet clearance [7, 9]. Here we have obtained, for the first time, a reagent that specifically inhibits shedding of GPIbα. The monoclonal antibody 5G6 targets the shedding cleavage site in human GPIbα, and is able to block shedding of human GPIbα in platelets (Fig. 2,3). The antigen specificity of the monoclonal antibody limits the inhibitory effect of 5G6 on only GPIbα shedding (Fig. 4). Moreover, 5G6 binding neither activates platelets nor affect platelet activation induced by ristocetin (Fig. 5) and other agonists (data not shown). These results provide a solid foundation for the utilization of 5G6 or its derivatives in future investigations of the functional significance of GPIbα shedding and for potential therapeutic development.

Ectodomain shedding affects a diverse array of transmembrane proteins [26, 27]. With the well-documented broad substrate specificity of ADAM sheddases, it is not surprising that modulating the activity of ADAM sheddase, even specifically that of ADAM17, often results in systemic changes or unwanted toxicity [11]. Modulating the ADAM activity also does not provide the definitive answer about the significance of shedding of individual receptors. Through this study, we have demonstrated that targeting directly the shedding cleavage site of a shedding substrate can achieve substrate-specific inhibition of ectodomain shedding. It is similar to a recent report of therapeutic antibodies that specifically stabilize the NRR domain of individual Notch proteins and thus protect the shedding cleavage site that lies within the NRR domain from being exposed and cleaved [28].

The shedding cleavage site in GPIbα is located in a juxtamembrane stalk region that is thought to be structurally flexible [29]. In the platelet, GPIbα associates closely with GPIbβ, GPIX and GPV, mostly through their transmembrane domains, to form the GPIb-IX-V complex [30, 31]. While the detailed structural information is lacking, the GPIbα stalk region is expected to locate next to, and likely also interact with, GPIbβ and GPIX extracellular domains that are tightly integrated with one another [29, 32]. Nonetheless, 5G6 and other selected mAbs are able to bind the shedding cleavage site in the full-length functional complex, indicating that the cleavage site in GPIbα is accessible to antibody binding. Since many transmembrane receptors undergoing shedding contain a juxtamembrane stalk region, our method of substrate-specific shedding inhibition through specific macromolecular binding of the shedding cleavage site may be generally applicable.
Acknowledgments

We thank Wolfgang Bergmeier for helpful discussion, and the Emory Children’s Pediatric Research Center Flow Cytometry Core for technical support. This work was supported in part by NIH grants HL082808 and HL097226 from National Heart, Lung and Blood Institute and UL1TR000454 from the National Center for Advancing Translational Sciences.

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Figure 1. Binding of selected mAbs to (A) purified GPIb-IX, (B) washed human platelets, (C) unconjugated and (D) ovalbumin-conjugated shedding-site peptide (A,C,D) GPIb-IX or the peptide was immobilized in microtiter plates. Purified mAbs, each identified by the clone name and colored as indicated, and negative controls, in the form of mouse IgG or BSA, were added to the coated wells. The bound Ab was detected with biotinylated goat anti-mouse IgG. Data are presented as the mean ± SD (n=3). (B) Binding plots of various mAbs to washed platelets. Human platelets were incubated with each mAb at indicated concentrations for 20 min, and washed once. Binding of mAb was detected by flow cytometry using FITC-conjugated goat anti-mouse IgG, and quantitated by MFI. The plots are representative of three independent experiments.
Figure 2. 5G6 binds specifically to human GPIbα in platelets

(A) Overlaid flow cytometry histograms showing binding of 5G6 to washed human platelets. Human platelets were incubated with 12 nM of mouse IgG (IgG, green trace), 5G6 (red), or WM23 (black) for 20 min. Binding was detected as described in Figure 1C. Platelets treated with only FITC-conjugated goat anti-mouse IgG (control, gray) were included as a negative control. (B) Purity of 5G6 and its Fab fragment shown by 10% Bis-Tris SDS gel electrophoresis under non-reducing (n.r.) and reducing (r.) conditions. Molecular weight markers (M) are shown and labeled in kDa on the left. (C) Binding plots of 5G6 and its Fab fragment to washed human platelets. Human platelets were incubated with 5G6 (•) and biotin-labeled 5G6 Fab (<) at various concentrations for 20 min. Binding
of antibodies was detected by flow cytometry using FITC-conjugated goat anti-mouse IgG (for 5G6) or FITC-conjugated streptavidin (for 5G6 Fab), and quantitated by MFI. (D) 5G6 recognizes specifically GPIbα in western blot. Total lysates of human platelets were immunoblotted with either WM23 or 5G6. The blots were overexposed to visualize all possible bands blotted by 5G6. Molecular weight markers in kDa are indicated on the left. (E) Overlaid flow cytometry histograms showing the competitive inhibition of 5G6 binding to washed human platelets by the shedding-site peptide. Platelets were incubated individually with 7 µM shedding-site peptide (pept, green trace), 12 nM 5G6 (red) for 20 min, or 7 µM shedding-site peptide for 20 min followed by 12 nM 5G6 for another 20 min (pept+5G6, black). Binding was detected as described in Figure 1C. Negative control was the same as in Figure 2A. (F) Overlaid flow cytometry histograms showing binding of biotin-labeled 5G6 to transgenic hTg mouse platelets (red trace), but not WT (black) or IL4Tg (blue) mouse platelets. Mouse whole blood was incubated with 4 µg/ml biotin-labeled 5G6 for 20 min followed by FITC-conjugated streptavidin. The binding to platelets was analyzed by flow cytometry with gating specific on platelets. Each figure or histogram is a representative of three independent experiments.
Figure 3. 5G6 inhibits GPIbα shedding in human platelets
(A) Overlaid flow cytometry histograms showing the inhibition of constitutive and induced shedding of GPIbα by 5G6. Washed human platelets were treated with 5% DMSO (left panel), with 150 µM W7 (middle) or 100 µM CCCP dissolved in 5% DMSO (right) in the absence (platelet, green trace) or presence of 12 nM 5G6 (red), 36 nM 5G6 Fab fragment (blue) or 100 µM GM6001 (GM, black) at RT for 2 h. The platelets were then washed and probed for the surface GPIbα expression level using biotin-labeled WM23 and FITC-conjugated streptavidin. Platelets treated only with FITC-conjugated streptavidin (control, gray) as a negative control. (B) Inhibition of GPIbα shedding by 5G6 assessed by western blot. Platelets were treated as described in Figure 3A. Platelet lysate containing full-length GPIbα, and the culture supernatant containing the released glycocalicin (GC), were immunoblotted with WM23. Actin blot of the platelet lysate was included for equal loading control. Each figure or histogram is a representative of three independent experiments.
Figure 4. 5G6 does not inhibit shedding of GPV or GPVI in human platelets

(A) 5G6 does not inhibit W7-induced shedding of GPVI. Platelets were treated with W7 as described in Figure 3A. Platelet lysates were immunoblotted anti-GPVI mAb 6B12 [33]. Actin blotting indicates equal sample loading. (B) Overlaid flow cytometry histograms showing that 5G6 does not inhibit GPV shedding. Washed platelets were treated with 150 µM W7 (left panel) or 10 µM PMA (right) in the absence (platelet, green trace) or presence of 36 nM 5G6 Fab fragment (Fab, blue) or 100 µM GM6001 (GM, black) at RT for 2 h. The platelets were then washed and probed for GPV surface expression using anti-GPV mAb SW16 and FITC-conjugated goat anti-mouse IgG. Negative control was same as described in Figure 2A. Each figure or histogram is a representative of three independent experiments.
Figure 5. 5G6 exhibits no detectable effect on platelet aggregation and activation
(A) 5G6 does not affect platelet aggregation in PRP. Left panel, aggregation traces of fresh human PRP after the addition of saline (PRP, blue trace) or 20 nM 5G6 in saline (PRP+5G6, black). Right, ristocetin-induced aggregation of PRP that had been pretreated with either saline (blue) or 20 nM 5G6 (black). Ristocetin to a final concentration of 1 mg/ml was added at the time point indicated by arrows to induce aggregation. (B) Overlaid flow cytometry histograms showing that treatment of washed human platelets with 5G6 does not induce platelet activation. Platelet activation is indicated by binding of mAb PAC-1, which is specific for activated integrin αIIbb3 (left panel), increased P-selectin expression on the platelet surface (middle), and binding of GFP-LactC2, which is specific for exposure of phosphoserine lipids (right). Washed platelets were treated with 12 nM of mouse IgG (control, gray traces) or 5G6 (red) at RT for 10 min and then probed using FITC-conjugated PAC-1, APC-conjugated anti-P-selectin Ab or GFP-LactC2. Platelets treated with 0.5 U/ml thrombin or 1 µM ionomycin were included as positive controls (black traces). The histograms are representative of three independent experiments.