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ORIGINAL ARTICLE

GPIbα shedding in platelets is controlled by strict intracellular containment of both enzyme and substrate

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Abstract

Background: A disintegrin and metalloprotease 17 (ADAM17) catalyzes platelet glycoprotein (GP) Ibα ectodomain shedding, thereby releasing glycocalicin in plasma. The spatiotemporal control over the enzyme-substrate interaction and the biological consequences of GPIbα shedding are poorly understood.

Objectives: This study aimed to determine the spatiotemporal control over GPIbα shedding by ADAM17.

Methods: Transmission electron microscopy with immunogold staining, immunoprecipitation, and quantitative western blotting were used.

Results: Immunogold staining showed that all ADAM17 antigen is expressed intracellularly, irrespective of platelet activation. ADAM17 clustered in patches on a tortuous membrane system different from α- and dense granules. Mild activation by platelet adhesion to immobilized fibrinogen did not cause GPIbα shedding, whereas strong and sustained stimulation using thrombin and collagen (analogs) did. Glycocalicin release kinetics was considerably slower than typical hemostasis, starting at 20 minutes and reaching a plateau after 3 hours of strong stimulation. Inhibition of the ADAM17 scissile bond specifically in GPIbα receptors that reside on the platelet's extracellular surface did not prevent shedding, which is in line with the strict intracellular location of ADAM17. Instead, shedding was restricted to a large GPIbα subpopulation that is inaccessible on resting platelets but becomes partially accessible following platelet stimulation. Furthermore, the data show that proteinaceous, water-soluble ADAM17 inhibitors cannot inhibit GPIbα shedding, whereas membrane permeable small molecule ADAM inhibitors can.

Conclusion: The data show that platelets harbor 2 distinct GPIbα subpopulations: one that presents at the platelet's surface known for its role in primary hemostasis and one that provides substrate for proteolysis by ADAM17 with kinetics that suggest a role beyond hemostasis.

KEYWORDS
ADAM17, ectodomain shedding, enzyme, GPIbα, platelet

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1 | INTRODUCTION

Shedding of surface membrane receptors alters interactions with ligands, changes cell adhesive properties, or releases bioactive fragments that subsequently regulate cellular processes like inflammation, tissue remodeling, and cancer [1,2]. On platelets, membrane glycoprotein (GP) Ibα is one of several surface membrane receptors susceptible to receptor shedding. GPIbα is a 140 kDa subunit of the GPIb-IX receptor complex consisting of GPIbα, GPIbβ, and GPIX. The receptor complex is essential for hemostasis as mutations in any of the *GPIBA*, *GPIBB*, and *GPIX* genes can cause Bernard–Soulier syndrome, resulting in loss of function of GPIb-IX antigen and leading to macrothrombocytopenia and clinically relevant bleeding [3]. The GPIbα receptor is a highly abundant transmembrane receptor with an estimated 25 000 to 50 000 copies per platelet [4,5]. GPIbα is the main receptor for von Willebrand factor (VWF) acting in conditions of elevated shear stress [6] and supporting platelet adhesion to the subendothelium. It is well established that a significant subpopulation of GPIbα receptors is expressed intracellularly [7,8]. This subpopulation is present on the internal tortuous invaginations of membranes called the (open) canalicular system [7,9].

GPIbα receptor metalloproteolysis releases the GPIbα ectodomain, called glycocalicin [10], which circulates in plasma at nanomolar concentrations. Studies on platelet concentrates demonstrate a constitutive but slow rate of GPIbα receptor shedding as a function of storage time [11]. The rate of proteolysis can be significantly increased during strong and sustained platelet activation [12] or in situations of physical stress like temperature shock [13,14]. GPIbα shedding is primarily catalyzed by a disintegrin and metalloproteinase (ADAM) 17 [15–17], also known as tumor necrosis factor α-converting enzyme. Mature ADAM17 is membrane bound and consists of an extracellular catalytic, cysteine-rich disintegrin and growth factor-like domain followed by a single transmembrane domain and a cytoplasmic tail. ADAM17 is expressed by many cell types and *Adam17*$^{-/-}$ mice die perinatally [18]. ADAM17 cleaves a range of substrates including tumor necrosis factor α and interleukin receptor 6 on leukocytes, for example [19]. There is no conserved scissile bond amino acid motif in ADAM17 substrates. However, scissile bonds in all known substrates are in close proximity to the plasma membrane, resulting in release of almost the entire ectodomain. This topology requires conformational flexibility to enable substrate docking [20], which, together with spatial localization [21], likely conveys some level of control over ADAM17 activity.

It is unclear why GPIbα is susceptible to shedding at all or what the role of glycocalicin is in health and disease. Although shedding of GPIbα could alter platelet adhesion in theory, there is little evidence that supports this [22,23]. GPIbα shedding influences the rate of platelet clearance, but it is unknown if this relationship is direct or a mere consequence of all collateral alterations in platelets inevitably linked to GPIbα shedding [24]. Mechanistically, it is commonly assumed that both enzyme and substrate reside conjunctively on the platelet's outer surface membrane, although direct evidence for this is lacking.

Essentials

- The enzyme-substrate relationship between GPIbα and ADAM17 is poorly understood.
- ADAM17 spatiotemporal control over GPIbα shedding was investigated *in vitro*.
- ADAM17 is exclusively expressed in the intracellular membranes of platelets.
- An intracellular GPIbα subpopulation provides all substrate for shedding.

In this study, the spatiotemporal regulation of GPIbα shedding by ADAM17 was investigated on platelets using a diverse array of biochemical tools. We focused on characterizing the kinetics of glycocalicin release as well as the spatial location of ADAM17 and differentiated between surface-exposed and subcellular GPIbα subpopulations for assessing susceptibility to shedding. Our data suggest biochemical control over the shedding process by strict intracellular containment of both enzyme ADAM17 and substrate GPIbα.

2 | METHODS

2.1 | Antibodies and reagents

The reagents, buffers, and antibodies used in the study are presented in Supplementary Table S1.

2.2 | Platelet preparation

Platelet concentrates were prepared by pooling 6 buffy coats, derived from whole blood donations, in citrate phosphate dextrose-plasma with 65% (vol/vol) additive solution (SSP+). All donors consented in writing to the use of their products for scientific research. The study was approved by the ethical committee of the University Hospital of Antwerp (18/24/289) and is in accordance with the Royal Decree on biobanks regarding the use of human substances for scientific research purposes (enforced November 1, 2018). Washed platelets were prepared from platelet concentrates as described, with minor adaptations [25].

2.3 | Study groups

Experiments were performed with platelets in suspension or immobilized on fibrinogen-coated 96-well plates. In both setups, untreated platelets were compared to platelets stimulated with an agonist mix supplemented with 2 mM $CaCl_2$. The agonist mix consists of 25 μM protease activated receptor-1 (PAR1) activating peptide, 250 μM protease activated receptor-4 (PAR4) activating peptide and 50 ng/mL convulxin unless otherwise mentioned.

2.4 | Induction of shedding

2.4.1 | In suspension

Washed platelets (50 × 10^3/μL) were preincubated with 150 ng/mL tirofiban to avoid aggregation. Platelets were either untreated (vehicle control) or were stimulated with 2 mM $CaCl_2$ supplemented with agonists (i) 5 μM phorbol 12-myristate 13-acetate (PMA), (ii) 150 μM N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7), (iii) 0.2 U/mL thrombin + 0.25 μg/mL crosslinked collagen-related peptide (CRP-XL) or (iv) PAR1/PAR4/convulxin. Samples were collected at fixed time points with protease and phosphatase inhibitor cocktail (HALT) (Thermo Scientific) and 10 μM marimastat to avoid additional shedding.

2.4.2 | Immobilized platelets

Excess anti-GPIbα antibody (clone AK2) was added to washed platelets (500 × 10^3/μL) before addition to fibrinogen-coated 96-well plates. After 30 minutes, unbound platelets were removed and wells were rinsed. Immobilized platelets were incubated with Tyrode's buffer alone (untreated) or Tyrode's buffer containing agonist mix and 2 mM $CaCl_2$ (stimulated). Supernatant was collected at fixed time points.

2.4.3 | Glycocalicin detection by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) western blotting

Cleared releasate samples were diluted in sample buffer supplemented with 40 mM 1,4-dithiotreithol for reduction. Samples were loaded onto anyKD polyacrylamide Tris-Glycine TGX gels (Bio-Rad), and GPIbα was detected with a mouse monoclonal (mAb) anti-GPIbα antibody (clone 8H211) and a secondary peroxidase-conjugated polyclonal goat antimouse antibody in Tris-buffered saline with 5% (wt/vol) skim milk. Membranes were developed with a ChemiDoc MP imaging systems. Glycocalicin levels were determined by densitometry (ImageLab v6.0.1.).

2.5 | Detection and spatial location of ADAM17

Although a range of anti-ADAM17 mAbs are commercially available, only few met quality standards required useful for the biochemical analyses conducted throughout the manuscript (Supplementary Table S2).

2.5.1 | Flow cytometry

Expression of ADAM17 was analyzed using APC-labeled anti-ADAM17 antibody (clone C10) on CD41-positive platelets. The median fluorescence intensity (MFI) in samples containing APC-labeled antibody (SIGNAL) was compared to the MFI obtained by addition of a 50-fold excess of the same unlabeled antibody (NOISE) to verify the specificity of the obtained signal. Platelets were either intact or permeabilized with 100 μg/mL saponin.

2.5.2 | Immunofluorescence microscopy

The cellular location of ADAM17 was visualized using fluorescence and differential interference contrast microscopy (Axio observer A1, Carl Zeiss) with a 100× oil immersion objective (numeric aperture 1.4; final magnification 1000×). Platelets (50 × 10^3/μL) were immobilized on fibrinogen-coated round glass coverslips for 1 hour. The fluorescent signal from platelets incubated with Alexa Fluor 488-labeled anti-ADAM17 mAb (clone C10) was defined as SIGNAL and specificity of the antibody was verified by co-incubation with a 50-fold excess of the same, unlabeled antibody (NOISE). The signal-to-noise ratio was determined for both intact and saponin-permeabilized platelets.

2.5.3 | Transmission electron microscopy

The Tokuyasu method was used for immunogold labeling of ADAM17 on ultrathin cryosections of platelets with brief modifications [26]. Detailed methods can be found in Supporting Information.

2.5.4 | Immunoprecipitation of surface-bound antibodies

Washed platelets (500 × 10^3/μL) were either untreated or stimulated by agonist mix. Samples were incubated with 5 μg/mL anti-ADAM17 or anti-P-selectin mAb for 1 hour using gentle rotation. Supernatant was collected as INPUT. The pellet was washed twice before lysis with radioimmunoprecipitation assay buffer with 2 mM ethylenediamine tetraacetic acid. Samples were prepared for SDS-PAGE western blotting. Detection of anti-ADAM17 or anti-P-selectin mAb was with secondary peroxidase-conjugated goat antimouse antibody. To verify equal loading, blots were reprobed with 0.5 μg/mL anti-glyceraldehyde-3-phosphate dehydrogenase antibody and corresponding peroxidase-conjugated secondary antibody.

2.6 | Glycocalicin release in presence of anti-GPIbα mAb 5G6

Washed platelets (500 × 10^3/μL) were preincubated with 5 μg/mL anti-GPIbα cleavage site blocking mAb (clone 5G6) [27,28] to block all surfaceaccessible GPIbα receptors before stimulation (or vehicle control) or were stimulated in the presence of an excess of this blocking antibody. After 180 minutes of stimulation, the supernatant was collected, and the platelet pellet was washed and lysed. Samples

were prepared for SDS-PAGE western blotting, and detection of 5G6 mAb in both pellet and supernatant at endpoint was with secondary peroxidase-conjugated goat antimouse antibody. As a control, glycocalicin levels in supernatant were determined by quantitative western blotting.

2.7 | Quantification of GPIbα receptors on untreated and stimulated platelets

The number of GPIbα receptors per platelet was determined in 96-well plates by quantification of both the anti-GPIbα mAb (clone AK2) amount per well by ELISA and the number of adhered platelets per well using 5 mM 4-nitrophenyl phosphate disodium salt hexahydrate (p-NPP). Detailed methods can be found in supporting information.

2.8 | Treatment of platelets with *O*-sialoglycoprotein endopeptidase

Washed platelets (10^6/μL) were pretreated with 200 μg/mL *O*-sialoglycoprotein endopeptidase (OSE) (or vehicle control) for 2 hours to cleave off surface-exposed *O*-sialoglycoproteins, predominantly GPIbα. OSE activity was quenched by supplementation with plasma. Both pellet and supernatant fractions were collected following OSE treatment. Platelets were washed twice with Tyrode's buffer by centrifugation (300 *g*, 5 minutes) in the presence of 500 ng/mL prostaglandin E1. Platelets at baseline or after 2 hours of OSE/vehicle treatment were subjected to flow cytometry. Flow cytometric detection with an N-terminal binding anti-GPIbα binding antibody (clone AK2) on CD61-positive platelets was used to confirm the cleavage of the N-terminal part of GPIbα. After OSE/vehicle treatment, platelets were left for 30 minutes before addition of agonist mix with 2 mM $CaCl_2$ for 45 min to induce shedding. Supernatant was collected following activation. SDS-PAGE western blotting was performed with anti-GPIbα mAbs WM23 and 8H211 to evaluate the N- and C-terminal portions.

2.9 | Pulse-chase experiment monitoring anti-GPIbα mAb on immobilized platelets

An anti-GPIbα mAb (clone 8H211) was used to label surface-exposed GPIbα receptors (PULSE). Platelets were preincubated with 5 μg/mL 8H211 for 15 minutes before removal of unbound antibody. Labeled washed platelets (250×10^3/μL) were allowed to adhere to fibrinogen-coated plates for 30 minutes, after which unbound platelets were removed by gentle rinsing. After immobilization, platelets were either untreated (Tyrode's buffer + pacifiers) or were stimulated with agonist mix with 2 mM $CaCl_2$ in the absence/presence of 5 μM metalloproteinase inhibitor GW280264X. After 180 minutes, supernatant was collected and evaluated for levels of glycocalicin and anti-GPIbα antibody (CHASE) by SDS-PAGE western blotting.

This involved making serial dilutions of anti-GPIbα mAb pre-incubated platelets, lysed with radioimmunoprecipitation assay buffer in western blot. The number of platelets per well was determined by 4-nitrophenyl phosphate disodium salt hexahydrate measurements in 96-well plates and expressed as a mean ± 2 SDs. By quantitative western blotting, the optical density in accordance with this maximal platelet number was expressed as 100%. Glycocalicin and GPIbα levels were expressed relative to this value.

2.10 | Statistical analysis

Results are reported as mean ± SD using Prism version 9.3.1 (GraphPad Software Inc.). Two study groups were compared using 2-tailed paired t-tests, whereas 3 study groups were compared using 1-way analysis of variance (ANOVA) with Tukey's multiple comparisons correction. Glycocalicin release kinetics between 2 study groups as a function of time were compared by 2-way ANOVA with Sidak's multiple comparisons correction. Results from statistical analysis are depicted on top of the panels and are shown as $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$, and $^{****}p < .0001$.

3 | RESULTS

3.1 | GPIbα shedding kinetics

Glycocalicin levels were significantly higher in the supernatants of platelets stimulated with a mix of thrombin and CRP-XL compared with PMA ($p = .033$) or W7 ($p = .015$) (Figure 1A). GPIbα shedding from platelets stimulated with thrombin and CRP-XL progressed over the course of hours rather than minutes both in suspension (Figure 1B, C) as well as when immobilized to coated fibrinogen (Figure 1D). Fibrinogen binding causes substantial platelet activation [29] but without the additional agonists; shedding in this condition was minimal (Figure 1D). Statistically significant increases in glycocalicin levels between stimulated and untreated platelets were found from 60 minutes onwards in suspension ($p < .001$) and from 120 minutes ($p < .0001$) onwards when immobilized to fibrinogen. Single agonist activation using thrombin or CRP-XL alone resulted in significantly slower kinetics compared with combining agonists (Figure 1E). Substituting thrombin for PAR1 and PAR4 activating peptides, combined with either CRP-XL or convulxin, resulted in GPIbα shedding kinetics similar to thrombin/CRP-XL stimulation (Figure 1F, $p > .05$) for platelets adhering to fibrinogen. Sustained and combined activation of platelet PAR and GPVI receptors thus is a potent cue for glycocalicin release.

3.2 | Intracellular location of ADAM17

ADAM17 antigen was not detectable on the surface of untreated platelets by flow cytometry. Following permeabilization with saponin,

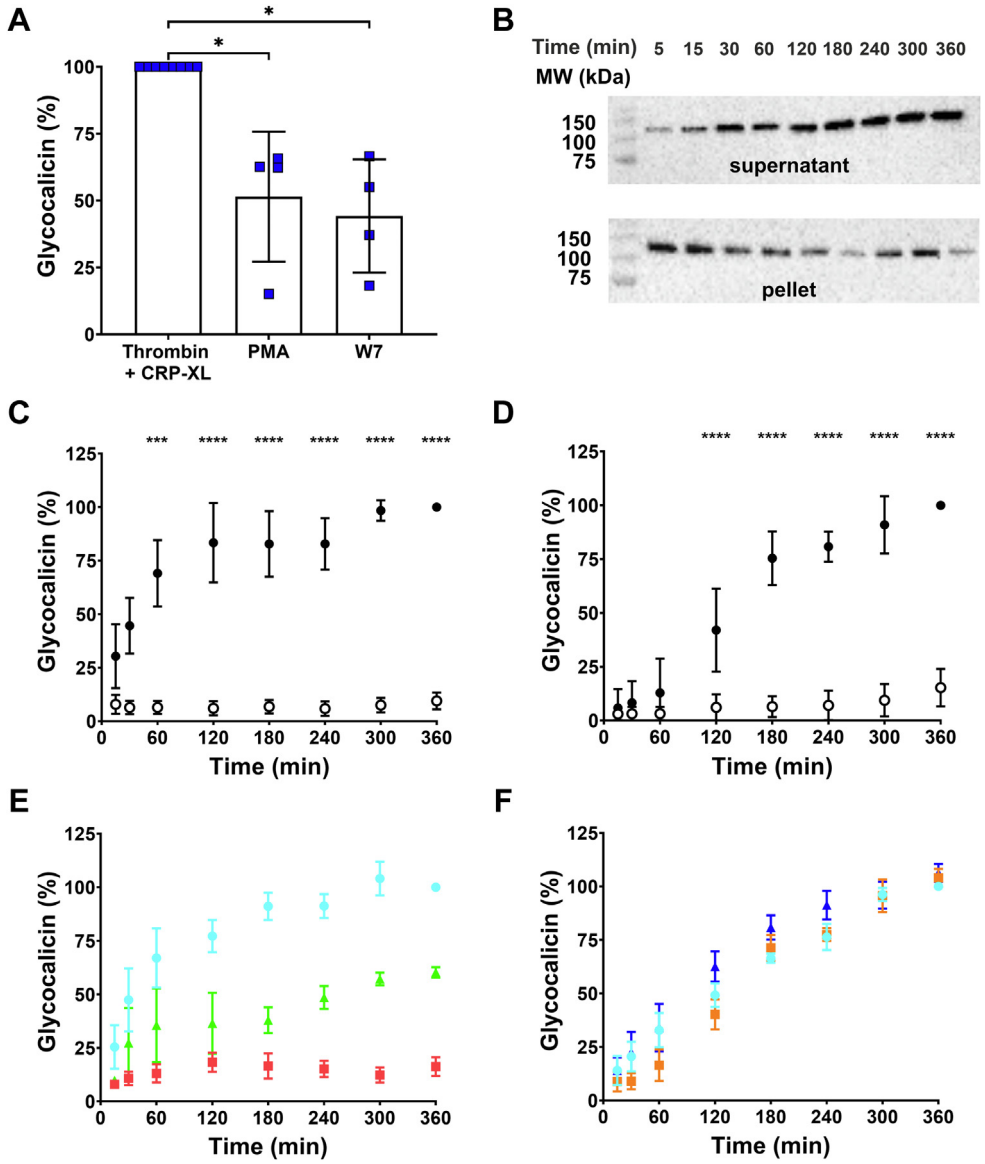

FIGURE 1 GPIbα shedding kinetics measured by glycocalicin levels in the supernatant of stimulated platelets. (A) Glycocalicin levels in platelet supernatant after 360 minutes of stimulation with a combination of thrombin and CRP-XL, PMA alone, or W7 alone. Glycocalicin levels are expressed relative to the level in the thrombin/CRP-XL condition. (B) Representative blots of glycocalicin in the platelet supernatant (*top*) or GPIbα in the platelet pellet (*bottom*). Substrate and product were followed as a function of time in platelet suspensions activated with thrombin + CRP-XL. (C and D) Relative glycocalicin levels in platelet supernatant as a function of time for untreated platelets (◯) or after stimulation with thrombin/CRP-XL (●) for platelets in suspension (C), or when adherent to immobilized fibrinogen (D). (E) Glycocalicin levels in the platelet supernatant of platelets in suspension as a function of time after stimulation with thrombin/CRP-XL (●); CRP-XL alone (▲) or thrombin alone (■) or (F) of platelets immobilized after stimulation with thrombin/CRP-XL (●); PAR1/PAR4/convulxin (▲) or PAR1/PAR4/CRP-XL (■). Glycocalicin levels are expressed relative to the level at endpoint ($t_{360\ min}$) of platelets stimulated with thrombin/CRP-XL. Data were collected from $n \geq 3$ biological repeats and are shown as mean ± SD; * for $p < .05$, *** for $p < .001$, or **** for $p < .0001$. *P* values were calculated using 1-way ANOVA with Tukey's multiple comparisons correction for panel A and 2-way ANOVA with Sidak's multiple comparisons correction for panels C, D, E, and F. CRP-XL, crosslinked collagen-related peptide; GP, glycoprotein; PAR, protease activated receptor; PMA, phorbol12-myristate 13-acetate.

a significant increase in specific fluorescent signal was observed (Figure 2A). Consequently, the signal-to-noise ratio of permeabilized platelets was significantly higher compared with intact platelets (Figure 2B, $p = .0022$). This observation was confirmed in platelets adhering to fibrinogen using immunofluorescence microscopy (Figure 2C). The data suggest that resting as well as fibrinogen-bound intact platelets do not express ADAM17 on the outer cytoplasmic membrane because the fluorescent signal of anti-ADAM17 mAb did not differ between conditions with or without 50-fold excess unlabeled mAb. By contrast, platelets treated with saponin displayed a significantly increased fluorescent signal compared with intact platelets and compared with saponin-treated platelets that were

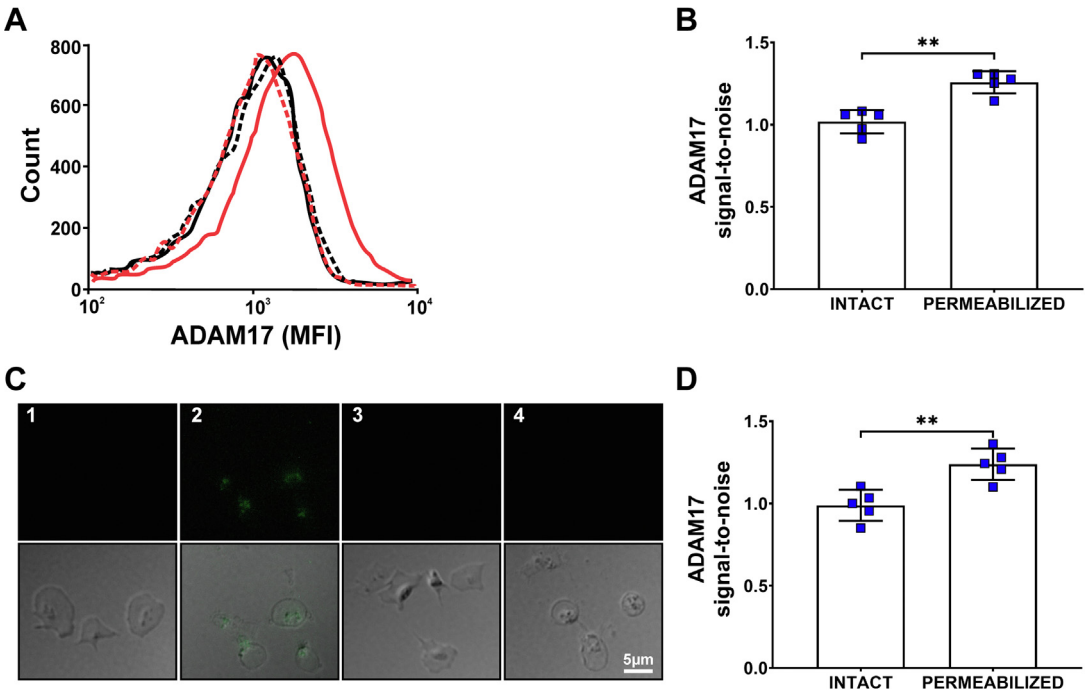

FIGURE 2 ADAM17 antigen detection by flow cytometry and immunofluorescence microscopy on intact and permeabilized platelets. (A) Representative flow cytometry histograms of intact (*black tracings*) or permeabilized (*red tracings*) platelets, in the absence (*full tracings*) or presence of 50-fold excess unlabeled anti-ADAM17 mAb (*dashed tracings*). (B) The specific median fluorescent intensity (MFI) signal of fluorescently labeled anti-ADAM17 mAb binding to platelets is calculated by dividing with the same condition in the presence of 50-fold excess unlabeled mAb. (C) Representative images of ADAM17 antigen detection by immunofluorescence in intact (1 and 3) or permeabilized (2 and 4) platelets in the absence (1 and 2) or presence (3 and 4) of 50-fold excess unlabeled anti-ADAM17 mAb. The top row depicts fluorescence images only, the bottom row depicts field of view overlays of fluorescence images with corresponding differential interference contrast images (original magnification: 1000×). (D) The specific fluorescence signal of fluorescently labeled anti-ADAM17 mAb binding to platelets is calculated by dividing with the same condition in the presence of 50-fold excess unlabeled mAb. Data are shown as mean ± SD (n = 5); ** for $p < .01$. P values were calculated using 2-tailed paired t-test. ADAM17, A disintegrin and metalloprotease 17; mAb, monoclonal antibody.

preincubated with 50-fold excess unlabeled mAb. The signal-to-background ratio of permeabilized platelets was 1.21 ± 0.05 compared with 0.98 ± 0.05 for intact platelets (p = .0022) (Figure 2D). Quality control experiments showed that the used mAb could bind ADAM17 antigen in western blotting, indicating it recognizes a linear epitope and should be insensitive to conformational changes (Supplementary Figure S1A).

Transmission electron microscopy with anti-ADAM17 immunogold labeling shows that ADAM17 antigen is exclusively found intracellularly in both untreated (Figure 3A) and stimulated platelets (Figure 3B). ADAM17 staining was in patches, which is different from typical intracellular GPIbα staining that is homogeneous and dispersed on external and internal membranes [9]. Gold particle counting revealed that 94 ± 3% and 96 ± 2% of all particles were detected inside the platelet's contours, respectively (Figure 3C, $p > .05$). Supplementary Figure S1B shows a representative image of untreated platelets labeled with anti-P-selectin mAb. P-selectin is abundant in α-granules but rare on the surface of resting platelets [30]. Comparison of Figure 3A and Figure S1B suggests that the intracellular sublocation of ADAM17 differs from that of P-selectin, and ADAM17 is not in α-granules.

Next, an immunoprecipitation experiment was designed using platelets as bait and specific mAbs to trace ADAM17 and P-selectin in platelet pellets. As a positive control for inducible expression, anti-P-selectin mAb was successfully captured on stimulated but not on untreated platelets (Figure 3D). In contrast, anti-ADAM17 mAb was not captured by platelets, under either experimental condition. A loading control is given in Supplementary Figure S2. Because anti-ADAM17 mAbs cannot be captured by untreated and stimulated platelets, either an extremely low abundance or an intracellular location is suspected.

Finally, GPIbα shedding was efficiently inhibited by the membrane-permeable small molecules marimastat and KP-457 to 12 ± 9% and 5 ± 2% relative to control (no inhibitor) respectively. These were used at concentrations that favor ADAM17 inhibition and avoid redundancy with other ADAMs [12,31]. However, the membrane-impermeable ADAM17 inhibitory antibody D1(A12) [32] and the recombinant ADAM17 prodomain [33] could not inhibit GPIbα shedding, yielding glycocalicin levels of 89 ± 28% and 107 ± 22% relative to control (no inhibitor), respectively (Supplementary Figure S3A). The inhibitory potency of all 4 molecules was confirmed in a cell-free dose-response experiment using fluorogenic

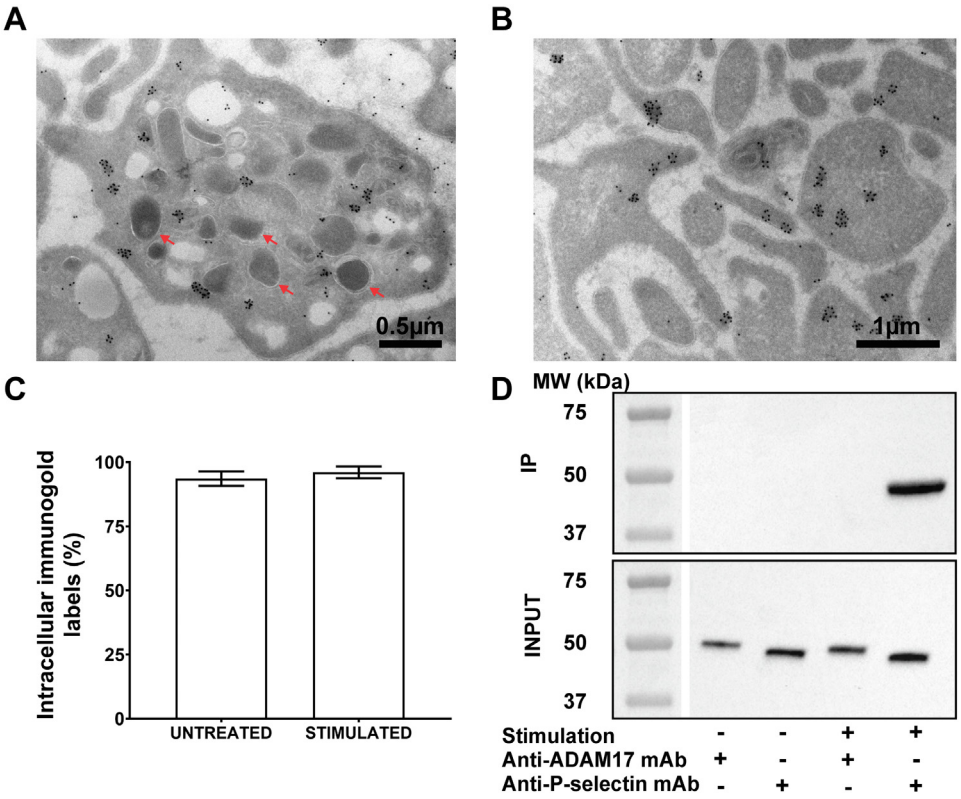

FIGURE 3 ADAM17 antigen detection in platelets using transmission electron microscopy (TEM) and immunoprecipitation. (A) Representative TEM images of untreated and (B) stimulated platelets using immunogold labeling of anti-ADAM17 mAb (original magnification: 15 000×). Arrows (→) indicate suspected α-granules based on Supplementary Figure S1B. (C) Gold particles inside platelet contours were counted and expressed relative to total in the view field ($n \geq 25$ view fields, $n \geq 500$ gold particles per condition). Data are shown as mean ± SD. *P* values were calculated using 2-tailed paired t-test. (D) (**IP**) Immunoprecipitation followed by SDS-PAGE and western blotting under reducing conditions to detect anti-ADAM17 or anti-P-selectin mAbs coprecipitated from washed platelets under indicated experimental conditions. (**INPUT**) The signals of anti-ADAM17 or anti-P-selectin mAb in the input sample supernatant following primary incubation. Part of the molecular weight (MW) marker is shown to the left and labeled in kDa. ADAM17, A disintegrin and metalloprotease 17; mAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

substrate and soluble recombinant human ADAM17 (Supplementary Figure S3B–E).

3.3 | A distinct GPIbα subpopulation provides substrate for shedding

To investigate how intracellular ADAM17 would "find" surface expressed GPIbα, untreated platelets were preincubated with the anti-GPIbα mAb 5G6, followed by removal of excess 5G6 by washing. This mAb directly binds to the scissile bond on GPIbα thereby preventing ADAM17 from chipping off the ectodomain. Although 5G6 is a potent inhibitor of GPIbα shedding [27,28], glycocalicin release was unaffected in these experimental conditions, releasing 92 ± 6% glycocalicin compared with the control condition without 5G6 (Figure 4A). This indicates that GPIbα shedding proceeded to near completion for platelets preincubated with scissile bond blocking anti-GPIbα mAb 5G6. A control experiment confirmed that 5G6 remained bound to these platelets until endpoint (Figure 4B). By contrast, glycocalicin release was partially inhibited (48 ± 6% of control) when 5G6 was not removed by washing but instead remained present throughout the entire experiment, including stimulation (p = .0064, Figure 4C). Because the presence of an intracellular subpopulation of GPIbα receptors had been described previously [9,34], we designed a stoichiometry experiment to quantify total GPIbα receptor number per platelet (Supplementary Figure S4). We detected 24 174 ± 7 474 GPIbα molecules per untreated platelet compared with 43 840 ± 12 890 (mean ± SD, p = .0035) molecules in stimulated platelets (Figure 4D). These experiments collectively suggest that surface-exposed GPIbα on untreated platelets is not a substrate for ADAM17 and that anti-GPIbα mAbs cannot access the entire GPIbα population in resting platelets. Instead, a GPIbα subpopulation that is cryptic in untreated platelets becomes (partially) accessible to anti-GPIbα mAbs during strong, sustained activation. We hypothesized that this subpopulation is the substrate for ADAM17 proteolysis.

To test this hypothesis, the N-terminal portion of GPIbα was cleaved by incubating platelets with the membrane impermeable OSE, thus targeting the surface-expressed GPIbα subpopulation only. This yields a truncated membrane-anchored GPIbα fragment of 70 kDa, schematically presented in Supplementary Figure S5A. The western

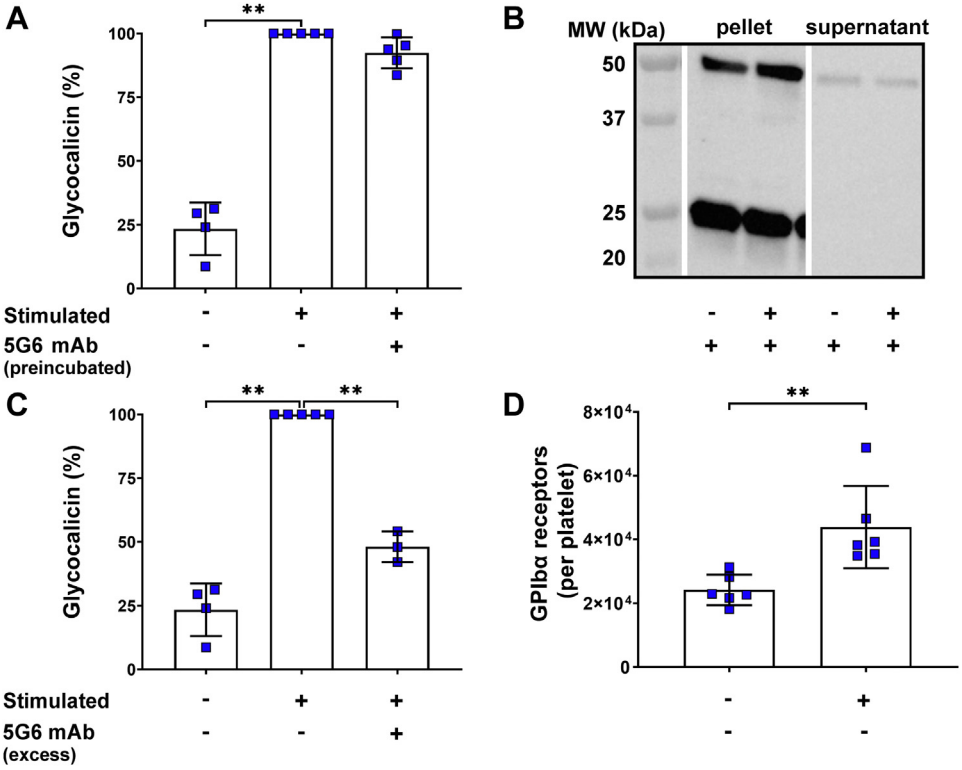

FIGURE 4 Inhibition of GPIbα shedding by mAb 5G6 differs when preincubated or in excess. (A) Relative glycocalicin level in platelet supernatant after 180 minutes of stimulation with a combination of thrombin and CRP-XL. Platelets were first preincubated with mAb 5G6 for 15 minutes and then washed to discard excess mAb prior to stimulation. (B) SDS-PAGE western blotting for murine immunoglobulin was used to chase mAb 5G6 in both the pellet and supernatant samples used in panel A. The 25 kDa and 50 kDa band represent the light and heavy immunoglobulin chain, respectively, in reducing conditions. The molecular weight (MW) marker in kDa is shown to the left. (C) A similar experiment as in panel A, here including a condition with excess 5G6 that was not washed off but instead kept in the sample during stimulation. (D) The number of GPIbα receptors per platelet was estimated on stimulated and untreated platelets using stoichiometry. Data are shown as mean ± SD ($n \geq 6$); ** for $p < .01$. *P* values were calculated using 1-way ANOVA with Tukey's multiple comparisons correction (panel A and C) or 2-tailed paired t-test (panels D). CRP-XL, crosslinked collagen-related peptide; GP, glycoprotein; mAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

blot in Figure 5A confirmed a significant reduction of circa 50% in full length GPIbα molecules (140 kDa) and the presence of a truncated fragment (70 kDa) in the platelet pellet using the anti-GPIbα mAb WM23 that targets the extracellular macroglycopeptide membrane anchored fragment. The supernatant consequently contained the soluble 70 kDa GPIbα fragment as detected by the N-terminal anti-GPIbα mAb 8H211. Truncated GPIbα receptor fragments were not detected in vehicle control samples. Detection of GPIbα in flow cytometry using fluorescently labeled anti-GPIbα N-terminal specific mAb AK2 suggested near complete removal of all receptors, confirming that the outer subpopulation is preferentially targeted by OSE (Figure 5B and Supplementary Figures S5B–D, $p = .0067$). Next, the OSE-treated platelets were subjected to strong and sustained stimulation, and we found that glycocalicin release was still substantial (Figure 5C). Glycocalicin levels were equally increased relative to background in OSE-treated versus vehicle-treated platelets (Figure 5D). Of note, glycocalicin detection in Figure 5C was with WM23 to allow detection of both full length glycocalicin (130 kDa) as well as the putative 60 kDa product that would be released if ADAM17 would cleave the truncated GPIbα fragment on OSE-treated platelets (Figure 5A). The absence of this truncated glycocalicin fragment in platelet supernatant confirms that glycocalicin is released from a GPIbα receptor subpopulation that is not accessible to OSE on platelets.

3.4 | Glycocalicin is generated exclusively from an intracellular GPIbα subpopulation

A pulse-chase experiment was designed to determine the relative size of the GPIbα subpopulation that is susceptible to shedding (Figure 6A). Hereto, resting platelets were preincubated with 8H211 mAb (PULSE) to label surface-exposed GPIbα only, followed by removal of excess unbound mAb. Following strong and sustained stimulation, platelet supernatant was interrogated for both mAb (CHASE) and for glycocalicin. The obtained results indicated substantial release of glycocalicin but minimal release of the preincubated, surface-bound mAb (Figure 6B, C). Quantitative analysis using densitometry of blotted bands and internal standards (Supplementary Figure S6) showed that 81 ± 14% of total GPIbα was released from stimulated platelets, and

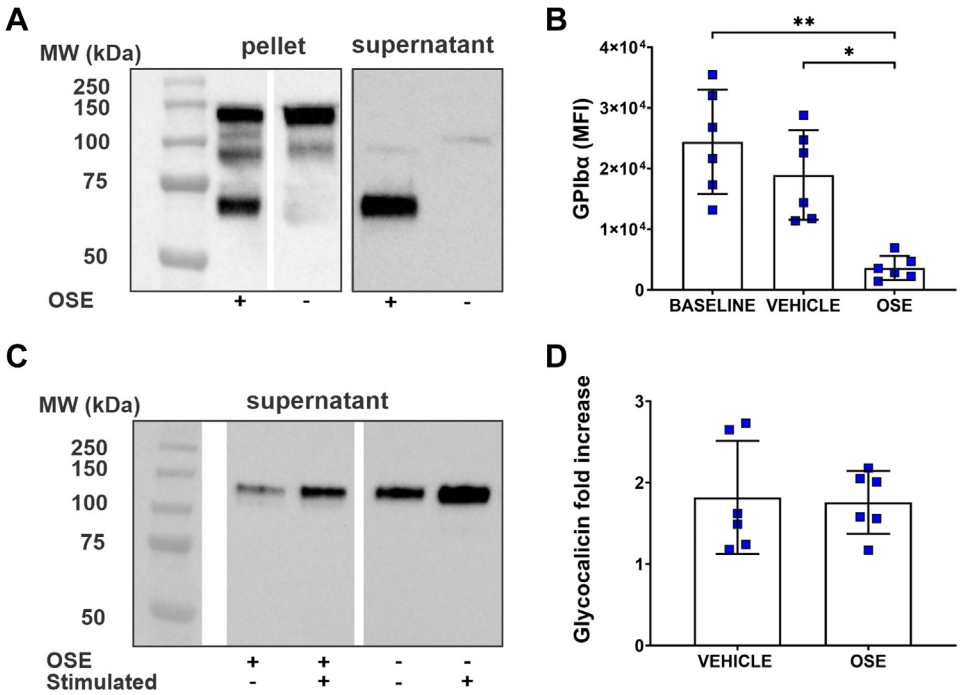

FIGURE 5 GPIbα shedding in platelets pretreated with OSE to remove part of the GPIbα receptor. (A) SDS-PAGE western blotting of GPIbα in platelet pellet and supernatant in reducing conditions using WM23 and 8H211, respectively, as detection mAb. Platelets were treated with buffer or OSE for 120 min to remove an N-terminal portion of surface-exposed GPIbα receptors. The molecular weight (MW) marker in kilodalton (kDa) is shown to the left. (B) Relative surface expression of GPIbα is determined by flow cytometry using an N-terminal fragment binding GPIbα mAb. The MFI is given at the start of the experiment (baseline) and after 120 minutes of buffer (vehicle) or OSE treatment. (C) SDS-PAGE western blotting of glycocalicin in supernatant of platelets that were treated with OSE or vehicle. Detection was with WM23. Platelets were either untreated or stimulated. (D) Glycocalicin levels in panel C were quantified by densitometry. The data from stimulated platelets are expressed as fold increase relative to resting platelets for both vehicle and OSE-treated samples. Data are shown as mean ± SD ($n = 6$); * for $p < .05$ and ** for $p < .01$. *P* values were calculated using 1-way ANOVA with Tukey's multiple comparisons correction (panel B) or 2-tailed paired t-test (panel D). GP, glycoprotein; MFI, mean fluorescence intensity; OSE, *O*-sialoglycoprotein endopeptidase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

this was significantly higher than for untreated platelets (18 ± 6%, $p < .0001$) or stimulated platelets with ADAM inhibitor GW280264X (13 ± 8%, $p < .0001$) (Figure 6D). Only 11 ± 10% of anti-GPIbα mAbs bound to the platelet surface were co-released and that was not statistically different from untreated platelets (3 ± 3%) or from stimulated platelets with GW280264X (3 ± 2%) (Figure 6E). Similar results were obtained with platelets in suspension that released negligible quantities of preincubated anti-GPIbα mAb compared with significant glycocalicin quantities (Supplementary Figure S7). Supplementary Figure S7A (pellet) furthermore confirms that anti-GPIbα mAb remains bound to the platelet surface even following GPIbα shedding.

Preincubation of resting platelets with anti-GPIbα mAbs only labels surface-exposed GPIbα. Hence, the relative amount of mAb recovered in Figure 6E is ~11% but expressed relative to total mAb bound on resting platelets and thus linked to surface-exposed GPIbα only. When expressed relative to the total GPIbα receptor population—instead of surface-exposed GPIbα alone—the amount of mAb released must consequently be lower than 11%. Therefore, we estimated the maximal proportion of surface-exposed GPIbα receptors released during shedding using our raw dataset and the derived equations given in the data supplement (Supplementary Figure S8). This shows that maximally ~2% of GPIbα is both surface exposed and a substrate for shedding.

Our set of experiments provides ample evidence that practically all glycocalicin released from stimulated platelets is formed during ectodomain shedding of a GPIbα subpopulation that is confined to the interior of untreated platelets, which is in line with a strict intracellular location of ADAM17.

4 | DISCUSSION

In 2003, Bergmeier et al. identified ADAM17 as the primary enzyme responsible for GPIbα shedding in mice [4,35]. A few years later, this was confirmed in human platelets [16]. Although GPIbα shedding and subsequent glycocalicin release have been known for decades, the mechanism, kinetics and biological relevance still remain elusive [17]. Most studies were on mouse platelets, and translation to human platelets may not be straightforward, for instance, because glycocalicin levels in plasma differ significantly between species [36]. Susceptibility of GPIbα to shedding and kinetics are increased in mice

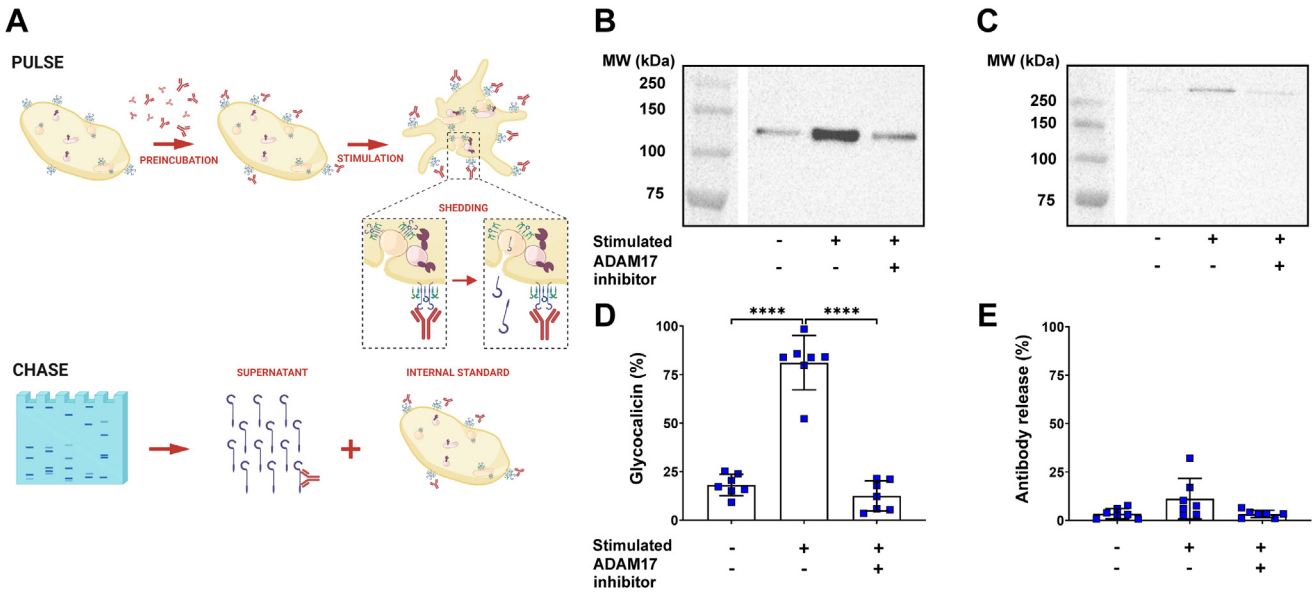

FIGURE 6 Pulse-chase experiment to determine the relative proportion of GPIbα subpopulation susceptible to shedding. (A) Platelets were adhered to immobilized fibrinogen and preincubated with 8H211 mAb to label surface-exposed GPIbα (PULSE). After removal of excess mAb by washing, platelets were stimulated for 180 minutes to induce shedding. Both glycocalicin and pulse mAb were subsequently chased in platelet supernatant (CHASE). Following detection, glycocalicin and pulse mAb were quantified using an internal standard consisting of a serial dilution of pulsed platelets. (B) Western blotting of glycocalicin in supernatant of untreated or stimulated platelets (180 minutes) with or without the ADAM17 inhibitor GW280264X. Samples were analyzed in reducing conditions. (C) Western blotting of pulse mAb in the same conditions analyzed in nonreducing conditions. The molecular weight (MW) marker in kilodalton (kDa) is shown to the left. Integration times of the CCD camera used for developing the blots were similar in panels B and C. (D) Glycocalicin levels in supernatant expressed relative to total GPIbα present in paired source platelets. (E) mAb levels expressed relative to total mAb originally bound to paired source platelets. Data are shown as mean ± SD (n = 7); **** for $p < .0001$. P values were calculated using 1-way ANOVA with Tukey's multiple comparisons. ADAM17, A disintegrin and metalloprotease 17; GP, glycoprotein; mAb, monoclonal antibody.

compared with humans [17]. Furthermore, GPIbα shedding was mainly induced using nonphysiological activators like the calmodulin inhibitor W7 [37], the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone [17] or the phorbol ester PMA [38] that act directly on intracellular signaling pathways in platelets. Others used indirect receptor-ligand engagement, for instance, by VWF modulators such botrocetin [39], which induces binding of VWF to GPIbα. We deliberately chose to work with physiological agonists to induce outside-in signaling and subsequent shedding on human platelets to make the results as relevant as possible using *in vitro* experiments.

Platelet GPIbα shedding has been postulated to be a relatively rapid process on the order of seconds to minutes, depending on the agonists or methods of detection used [38]. These kinetics are however often inferred from studies of GPVI shedding [40–42]. The actual sheddases were therefore assumed to constitutively reside on the outer surface [38] because targeted membrane bound molecular transportation from subcellular compartments generally do not fit with rapid phenomena. This was, however, speculative because there is no evidence (so far) that GPVI and GPIbα shedding mechanisms are similar. When physiological agonists are used, slow kinetics of glycocalicin release is found, and a plateau is reached only after hours of stimulation, as demonstrated by others [12]. Consequently, an intracellular location of ADAM17 in platelets cannot be excluded, and we propose this could be the rate-limiting reason for the observed GPIbα shedding kinetics.

Determining the cellular location of ADAM17 in platelets is challenging. Quantitative proteomics suggests a total of only 670 copies of ADAM17 antigen per platelet. This is 10- to 20-fold lower than the abundant platelet specific receptors GPIb-IX or integrin $\alpha_{IIb}\beta_3$ [5]. The low ADAM17 copy number requires detection tools with high affinity and high specificity. Using flow cytometry or immunofluorescence microcopy, ADAM17 could not be detected in or on platelets. Following permeabilization, however, faint but specific staining was found. Strict intracellular location of ADAM17 was confirmed directly using immunogold labeling in transmission electron microscopy (TEM) and indirectly by demonstrating that shedding could only be inhibited using membrane permeable ADAM inhibitors [21,31]. In addition, immunogold staining suggested a patchy distribution of ADAM17 antigen. These clusters of ADAM17 antigen appeared at sites distinct from platelet dense (δ-) and α-granules. In addition, ADAM17 could not be inhibited by biological ADAM17 inhibitors D1A12 or prodomain, suggesting the enzyme is hidden in membrane structures that are less accessible than its substrate, which is accessible for mAb binding during platelet activation. From the TEM images, we believe ADAM17's location resembles that of protein disulfide isomerase that resides in the dense tubular system [43]. This topography suggests limited direct interaction of ADAM17 clusters in secondary membrane systems with GPIbα in the continuous membrane system formed by the cytoplasmic membrane and the canalicular system. It supports the notion of a well-controlled enzyme-substrate spatiotemporal

separation. Determining the exact intracellular ADAM17 location at higher resolution could be a first step toward unraveling how ADAM17 and GPIbα meet inside the platelet.

An additional and crucial experiment that supports the idea that ADAM17 is not present on the platelet surface was by immunoprecipitation of anti-ADAM17 and anti-P-selectin (as a control) mAbs. Anti-P-selectin mAb was readily coprecipitated with stimulated and not resting platelets, whereas anti-ADAM17 mAbs were not coprecipitated at all. This suggests very low copy numbers of ADAM17 antigen on the surface of both untreated and stimulated platelets. The detection limit of this experiment was ~200 ADAM17 molecules per platelet. If ADAM17 copy number is at 670 molecules per platelet, this suggest that maximally 30% would be present on the surface before a signal would be detected in our experiment. Our TEM data, however, point to an even lower surface expression of <5%. However, even low levels of ADAM17 may fundamentally accomplish GPIbα shedding of surface membrane receptors, but then the enzyme should be very efficient and highly mobile. Our data suggest that surface membrane GPIbα is not shed, and the literature on receptor mobility indicates that hop diffusion—movement between compartments—restricts mobility of membrane dwelling molecules [44].

The discovery of the intracellular location of ADAM17 in platelets actively shedding glycocalicin raises the question as to how enzyme and substrate find each other. GPIbα movement to the platelet interior is one potential mechanism that has been described [45] but is debated, and multiple studies do not support GPIbα internalization and suggest that the GPIb-IX complex is not mobile under physiological conditions [46–50]. This is confirmed in the 5G6 preincubation experiment here, where GPIbα internalization would cotransport the inhibitory antibody toward ADAM17's location and interfere with proteolysis, which is not in line with our observations. In addition, truncation of surface-exposed GPIbα by OSE treatment did not impair glycocalicin release. Following OSE treatment, the truncated form of GPIbα could readily be detected. However, the fragment that would consequently be produced by ADAM17 cleavage was absent from the supernatant. Only intact 130 kDa glycocalicin could be detected, suggesting that a GPIbα subpopulation that is inaccessible for OSE on resting platelets—and thus residing intracellularly—is cleaved by ADAM17. It is possible that OSE treatment renders truncated GPIbα receptors on the surface membrane resistant to shedding. In addition, following OSE treatment, a small fraction of intact GPIbα remains on the outer surface membrane.

The existence of an intracellular GPIbα subpopulation is widely accepted, although its proportion relative to the total GPIbα copy number is unknown. Depending on the technique used, the number of that subpopulation fluctuates between 25 000 (equal to the surface-exposed subpopulation) [4,9] and 150 000 copy numbers per platelet [34]. Data from our pulse-chase experiment suggest an intracellular GPIbα subpopulation substantially larger than the surface-exposed subpopulation because ~80% of all GPIbα receptors are shed at 180 minutes of stimulation. This is apparently at odds with the stoichiometry experiment in Figure 4D using AK2 on stimulated and untreated platelets, which suggested roughly equal GPIbα copy numbers between both compartments. However, the experiment in Figure 4C using 5G6 as an inhibitor suggests that not all intracellular GPIbα is fully accessible for anti-GPIbα mAbs because inhibition of shedding was incomplete, resulting in release of ~40% of glycocalicin, even with excess 5G6. This indicates the inhibitor was unable to bind to all intracellular GPIbα. This seems reasonable as mAbs must diffuse the entire platelet's canalicular system in order to bind all intracellularly located substrate. We conclude that the intracellular substrate subpopulation of GPIbα outnumbers that on the surface. In addition, this smaller outer subpopulation is entirely untouched by ADAM17 based on our quantitative analysis, showing that maximally 2% of glycocalicin can be derived from it. Our findings are in line with the observations of Aktas et al. [51] who induced shedding of GPIbα by infusion of high dose aspirin in murine circulation. This caused significant GPIbα shedding because increased glycocalicin levels were detected in the plasma of these mice compared to control. Unexpectedly, the GPIbα receptor surface levels on platelets remained unaltered. Although further evidence is necessary, the concept of shedding of an internal GPIbα subpopulation by ADAM17 fits with these *in vivo* data.

ADAM proteins show substrate preferences (eg, ADAM10 for GPVI and ADAM17 for GPIbα) but (partial) redundancy cannot be excluded [12,52]. The inhibitors GW280264X and KP-457, selective for ADAM10/17 and ADAM17, respectively, were used at concentrations [31,53] that should preferentially inhibit ADAM17, although some redundancy with ADAM10 cannot be ruled out. GPIbα shedding is not solely the consequence of sustained platelet activation. It can be shear-induced [39] or be the consequence of a genetic mutation causing type 2 von Willebrand disease [54] in which cases the process appears faster. It may be that "tugging" on GPIbα works as a catalyst for shedding, either by the process we have described or by an entirely separate mechanism involving proteinases other than ADAM17.

Our observations are novel and have implications for the field. The slow kinetics of glycocalicin release in response to physiological agonists suggests a role for GPIbα shedding beyond prompt hemostasis. In addition, because the ADAM17 susceptible GPIbα subpopulation is not accessible on untreated platelets, shedding is unlikely to have a major influence on primary platelet adhesion to GPIbα ligands like VWF. There is growing evidence that platelets have roles in processes beyond hemostasis including inflammation and wound healing [55,56]. The kinetics of GPIbα shedding fit better with tissue inflammation, proliferation, or remodeling than with quick hemostasis.

In conclusion, our study finds a strict intracellular location for ADAM17 within platelets. We show that surface-accessible GPIbα receptors are not susceptible to shedding, essentially providing evidence for the existence of 2 separate GPIbα subpopulations in platelets. These basic findings have implications for studying platelet function in general and GPIbα mediated activation and shedding in particular, in hemostasis and transfusion biology, and fields beyond.

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AUTHOR CONTRIBUTIONS

V.C., H.B.F., and K.R.S. coordinated the study. K.R.S. and H.B.F. designed the study. K.R.S., C.D., and M.V. performed laboratory experiments and data collection. R.D. performed TEM experiments. E.E.G. provided essential reagents. K.R.S., M.V., and H.B.F. performed data interpretation. K.R.S. performed statistical analysis. K.R.S. and H.B.F. drafted the manuscript. All authors read and approved the final version of the paper.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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