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Clustering of GPVI dimers upon adhesion to collagen as a mechanism to regulate GPVI signalling in platelets

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Running title: GPVI-dimer clustering on collagen

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Summary (250 words) and Main text (3986 words)

Essentials

- Dimeric high-affinity collagen receptor glycoprotein VI (GPVI) is present on resting platelets.
- Spatio-temporal organization of platelet GPVI-dimers was evaluated using advanced microscopy.
- Upon platelet adhesion to collagenous substrates, GPVI-dimers coalesce to form clusters.
- Clustering of GPVI-dimers may increase avidity and facilitate platelet activation

Summary

Background: Platelet GPVI binding to subendothelial collagen exposed upon blood vessel injury initiates thrombus formation. Dimeric GPVI has high affinity for collagen and occurs constitutively on resting platelets. Objective: To identify higher order oligomerisation (clustering) of pre-existing GPVI-dimers upon interaction with collagen as a mechanism to initiate GPVI-mediated signalling. Methods: GPVI was located using fluorophore-conjugated GPVI-dimer-specific Fab (antigen-binding fragment). Tested substrates include Horm collagen I fibres, soluble collagen III, GPVI-specific collagen peptides and fibrinogen. GPVI-dimer clusters on the platelet surface interacting with these substrates were visualised.

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using complementary imaging techniques: Total Internal Reflection Fluorescence Microscopy (TIRFM) to monitor real time interactions and direct Stochastic Optical Reconstruction Microscopy (dSTORM), providing relative quantification of GPVI cluster size and density. Confocal microscopy was used to locate GPVI-dimer clusters, GPIb, integrin α2β1, and phosphotyrosine. *Results:* Upon platelet adhesion to all collagenous substrates, GPVI-dimers coalesced to form clusters; notably clusters formed along the fibres of Horm collagen. dSTORM revealed that GPVI density within clusters depended on the substrate, collagen III being most effective. Clusters on fibrinogen-adhered platelets were much smaller and more numerous; whether these are pre-existing oligomers of GPVI-dimers or fibrinogen-induced is not conclusive. Some GPVI-dimer clusters colocalized with areas of phosphotyrosine, indicative of signalling activity. Integrin α2β1 localized to collagen fibres close to GPVI-dimer clusters. GPVI-clustering depends on a dynamic actin cytoskeleton. *Conclusions:* Platelet adhesion to collagen induces GPVI-dimer clustering. GPVI-clustering increases both avidity for collagen and proximity of GPVI-associated signalling molecules, which may be crucial for initiation and persistence of signalling.

**Introduction**

Upon blood vessel injury, circulating platelets interact with exposed subendothelial collagen through the collagen receptor glycoprotein VI (GPVI). This 65-kDa immune receptor signals through its associated Fc-receptor γ-chain, which contains an immunoreceptor tyrosine-based activation motif (ITAM) in its intracellular domain. Binding of GPVI to collagen induces phosphorylation of the ITAM residues, which can then bind to Syk, itself becoming phosphorylated and activated. This process initiates signalosome assembly [1], leading to a series of downstream signals, resulting in platelet activation, finally culminating in thrombus formation.
GPVI contains two extracellular Ig-like domains: D1 containing the collagen binding site [2,3] and D2, connected via an O-glycosylated stem to its transmembrane domain and short cytoplasmic tail [4]. GPVI binds to tandem GPO (glycine-proline-hydroxyproline) sequences in collagen [5,6]. Surface plasmon resonance showed that dimerized recombinant GPVI (D1D2-Fc)₂ bound collagen fibres with high affinity but binding of its monomeric form (D1D2) was too low to measure [7]. Monomeric and dimeric recombinant D1D2 showed similar affinity for collagen-related peptide (CRP), a triple-helical peptide containing 10 contiguous GPO triplets, suggesting GPVI-dimer may have a specific conformation that recognizes the higher-order structure of fibrous collagen, beyond simply the GPO sequences. The crystal structure of a D1D2 dimeric assembly [8] allowed docking simulations [3,5,8] which suggested that D1 contained grooves large enough to accommodate the triple-helical CRP.

In 2009, Jung et al. provided direct evidence for the presence of dimers on the resting platelet surface with GPVI-dimer specific, inhibitory m-Fab-F [9]. Later, they reported a non-inhibitory Fab, 204-11, which recognized GPVI-dimers [10], and used it to show that GPVI-dimers were constitutively present on resting platelets. These observations suggested that the first interaction in collagen-induced activation of platelets is collagen binding to GPVI-dimers. Several groups, however, reported that platelet activation induced formation of GPVI-dimers. Arthur et al. (11) provided biochemical evidence for disulphide-linked dimers in activated platelets. Loyau et al., employing GPVI-dimer-specific mab 9E18, reported that GPVI dimerization was induced by soluble agonists or VWF, with almost no dimers detected on resting platelets [12], leading them to propose dimer formation as a means to control collagen-induced platelet activation. Dimerization is an accepted mechanism for cell activation through receptor tyrosine kinases [13], where ligand binding to the receptor extracellular domains induces dimerization, causing a conformational change that brings together the kinase domains in the cytoplasmic tails, facilitating autophosphorylation, thereby initiating intracellular signals. The Src-family kinase Lyn associates with the cytoplasmic tail of GPVI [14], which lacks intrinsic kinase activity, so that FcRy attached to one GPVI...
monomer might be phosphorylated by Lyn associated with a second monomer, brought into proximity by dimerization.

An alternative activation mechanism would be higher-order receptor clustering [15], which does not preclude the presence of constitutive GPVI-dimers in non-activated platelets. Clustering has been demonstrated for many classes of receptor, including G protein–coupled receptors [16]; adhesion receptors such as platelet integrin αIIbβ3 [17]; platelet CLEC-2 [18] and notably, the discoidin domain receptor, DDR1, a constitutively-dimeric tyrosine kinase receptor for collagen [19].

As GPVI dimers are present on resting platelets we hypothesize that a further level of control is required. We hypothesize that clustering of GPVI-dimers is a plausible mechanism to modulate platelet activation once the high-affinity, but low-copy-number, GPVI-dimers engage the limited number of binding sites on collagen [2,20]. The formation of GPVI clusters could result in increased avidity and may bring associated signalling molecules together to facilitate platelet activation. We used complementary imaging techniques to test this hypothesis: TIRFM (Total Internal Reflection Fluorescence Microscopy), to visualize in real-time GPVI-dimer distribution at the interface between the platelet membrane and immobilized collagenous substrates; dSTORM (Direct Stochastic Optical Reconstruction Microscopy) for relative quantification of GPVI cluster size and density; and confocal microscopy to compare localization of GPVI-dimers and two other receptors involved in the platelet-collagen interaction, GPIb and integrin α2β1, and whether signalling was associated with the clustered GPVI-dimers.

Materials and methods

Non-inhibitory, recombinant dimer-specific 204-11 Fab [10] was derived from clone 204-11 [21] by Kaketsuken (Kumamoto, Japan). Antibodies were fluorescently labelled by a Microscale Protein Labelling kit (Molecular Probes); degree of fluorescent labelling =2–5 dye
molecules/protein molecule. 4G10 (anti-phosphotyrosine; Millipore Merck); Anti-human CD42b/GPIb, clone 486805 (R&D Systems); 16B4 (mouse anti-human CD49b/integrin α2 chain; AbD Serotec); Alexafluor 647–conjugated AffiniPure F(ab’)2 fragment goat anti-mouse IgG, Fcy–specific (Jackson); Gi9 (anti-integrin α2; Abcam), Alexafluor 647–anti-human CD62P (Bio-Rad). Other materials, reagent grade or better, were obtained from commercial sources.

Platelet preparation

Washed platelets were prepared from ACD-anti-coagulated blood from healthy volunteers (22) and resuspended at 3–5 x 10⁷ platelets/ml in Heps-Tyrodes buffer (HT: 134 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM HEPES, 5 mM glucose, pH 7.3).

Preparation of collagenous substrate–coated glass dishes for imaging

35-mm glass (0.7 mm)–bottomed MatTek dishes (MatTek, USA) were coated with 10 µg/ml CRP-XL, III-30, or collagen III, in phosphate-buffered saline (PBS: 0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, pH 7.4 ); Horm collagen, in manufacturer-supplied diluent) overnight at 4°C. The dishes were PBS-washed, 1% bovine serum albumin–blocked (BSA/PBS; heat-denatured,filtered) for 1 h, and then PBS-washed ready for platelet spreading.

TIRFM

Adhesion of Alexafluor 488–204-11 Fab–labelled washed platelets (3 x 10⁷ platelets/ml in HT buffer containing 2 mM MgCl₂) to immobilized collagenous substrate was imaged by TIRFM (Nikon TIRF system mounted on a Nikon Eclipse Ti inverted microscope, with Nikon 60x NA 1.49 TIRF objective). Images were obtained at 5-s intervals for 20–30 min at 37°C, followed by fixation in formalin and confocal imaging. Integrin α2β1–blockade was achieved
by preincubating platelets with 10 μg/ml Gi9 blocking antibody. Platelet morphology was followed using differential interference contrast (DIC) microscopy.

dSTORM

Alexafluor 647-204-11 Fab–labelled washed platelets were allowed to adhere to collagenous-substrate-coated MatTek dishes. Adhered platelets were fixed, permeabilised and stained with Phalloidin-Alexa488. Samples were imaged in switching buffer as described previously (22) on a Nikon Eclipse Ti-E N-STORM system in dSTORM mode using Perfect Focus, with a CFI SR Apochromat TIRF 100x Oil, 1.49NA objective lens and an N-STORM filter cube with excitation using the Agilent Ultra High Power Dual Output Laser bed (170-mW 647-nm laser), and image capture with an Andor IXON Ultra 897 EMCCD camera. 30,000 frames were captured using Nikon NIS Elements v4.2 and reconstructed using STORM analysis module v3.2 with drift correction and Gaussian rendering of data points. Detected points with a photon count of less than 500 were discarded from reconstructed data before further processing. Points in the reconstructed images represent individually identified fluorescent blinking events, which are referred to as molecules.

Confocal imaging

Platelets were preincubated with Alexa 488 or 647–conjugated 204-11 Fab (4 μg/ml) alone or with another fluorescently labelled antibody. In some experiments, platelets were also treated with an inhibitor or inhibitory antibody. The platelets were allowed to adhere to a collagenous substrate, fixed and imaged with an FV300 IX81 Laser-scanning confocal microscope, 60x oil immersion objective (Olympus). Where indicated, platelets were permeabilized (0.1% Triton/PBS) following fixation and stained for actin (Alexafluor 647–Phalloidin) or phosphotyrosine (4G10,5 μg/ml).
Flow cytometry to measure GPVI-dimers

Washed platelets were pre-incubated with DMSO (0.25% final concentration) or actin antagonist (cytochalasin D, latrunculin A, or jasplakinolide; 10 µM; 0.25% DMSO, final concentration), and GPVI-dimer formation was measured by flow cytometry [10].

Data analyses

Data analyses, performed with Prism v7 (GraphPad, San Diego, CA), are described in the figure legends. Differences among treatment groups in the flow cytometry experiments were calculated by paired t-test. dSTORM cluster analysis was performed within MATLAB for each 3 x 3 µm ROI (region of interest) as described by Owen et al. [23] with modifications described in Pollitt et al. [18]. Platelets were identified by phalloidin staining of the actin cytoskeleton. ROIs were positioned within the platelet ensuring that the edges of the ROI fell within the platelet boundary. Clusters of <3 points were discounted. The numerical data, processed using Microsoft Excel, were analysed as detailed in the figure legends. The degree of colocalization between the GPVI and phosphotyrosine confocal images was quantified by Image-Pro Premiere 9.2 (Molecular Cybernetics).

Results

TIRFM imaging of GPVI-dimer–cluster formation in live platelets interacting with immobilized collagenous substrates

We investigated the spatial and temporal distribution of GPVI dimers on the surface of platelets interacting with different immobilized collagenous substrates (Table I). Washed platelets labelled for GPVI-dimer were allowed to adhere, and cluster formation and dynamics were visualized by TIRFM (Fig.1; supplemental movie). Discrete clustered GPVI-
dimers can be visualized in spreading platelets, whereas non-clustered GPVI-dimers are poorly-resolved, appearing as diffuse fluorescence over the platelet surface.

GPVI dimers form clusters on platelets adhering to all collagenous substrates tested. Cluster formation on fibrous Horm collagen (Horm) follows a distinct distribution, concentrated along the collagen fibre (Fig. 1A: i, ii), initially forming at the point of contact between the platelet and the fibre, then propagating along the fibre (Fig. 1Aii). Clusters are not restricted to the visible collagen fibre and are also present on the platelet surface presumably due to indirect signal-induced clustering (e.g., outside-in signalling through released substances such as fibrinogen, ADP, VWF, etc.), through contact with smaller (non-visible) fibres in these portions of the surface, or both. Non-fibrous collagenous substrates CRP-XL (Fig. 1B), collagen III (Fig. 1C, Col III), and III-30 (Fig. 1D, III-30) also support formation of GPVI-dimer clusters, each with similar cluster distributions. Small clusters were evident upon first contact of the platelet with the coated surface, eventually forming throughout the platelet surface; some clusters expanded and coalesced.

Different collagenous substrates induce different degrees of clustering

GPVI-clustering in fixed platelets spread on the different collagenous substrates was quantitated using dSTORM, which has a typical x-y resolution of 20–30 nm (24, 25). Widefield TIRF imaging of the platelets labelled for F-actin and GPVI dimer show the same distribution of GPVI as that observed in the live-cell imaging (Fig. 2A). Cluster analysis of dSTORM data is represented by the heat maps in Fig.2A. High and low levels of GPVI-clustering are seen as red and blue, respectively. All collagenous substrates induced more GPVI-clustering than expected to occur randomly; however, the cluster distribution depended upon the specific substrate. Horm induced high degrees of clustering along the fibres, whereas the other substrates induced clusters more evenly distributed throughout the ROI. Quantification shows that there are more GPVI dimers and a greater number of clusters found per unit area on platelets spread on Col III than on the other substrates (Fig. 2B,C). These clusters are small (Fig. 2D) but contain the highest density of GPVI dimers (Fig. 2D).
2E, median values). Horm induced the next highest amount of GPVI dimer per ROI, 42% less than Col III, and Horm-induced clusters were 31% less dense than those on Col III. CRP-XL and III-30 were least effective in dimer formation (each with ~70% fewer molecules detected in the ROI compared to Col III) and cluster densities were also correspondingly reduced when compared to Col III (40% and 37% reduction, respectively). The size of GPVI-dimer clusters formed on Horm and CRP are not significantly different from each other but were significantly larger than those formed on Col III and III-30 (Fig. 2D). In summary, all collagenous substrates cause GPVI dimers to cluster, but different numbers of dimers and densities of GPVI within clusters are formed, depending on the nature of the collagenous substrate.

GPVI dimers are not restricted to the platelet membrane in direct contact with the visible collagen fibres (Figs. 1A, 2A). Potential explanations for this are the existence of “non visible” microfibers in those areas, to which GPVI-dimers can still bind, and/or fibrinogen or other substances released from adhered platelets may induce cluster formation via outside-in signalling. To determine whether GPVI clusters can form by collagen-independent platelet activation, platelets were spread on fibrinogen and clustering of GPVI dimer quantified using dSTORM (Fig 3). GPVI-dimer clusters seen on fibrinogen differ from those seen on Horm collagen: relative density of dimers in platelets spread on fibrinogen is ~50% less when compared to platelets spread on Horm (Fig 3B); there are many more small clusters on the fibrinogen, and the small clusters are ~40% less dense than those formed on Horm (3C–E). Although it is possible that GPVI-dimer clustering can occur by a GPVI-independent mechanism, collagen greatly increases the formation of GPVI dimers, which coalesce into larger and denser clusters.
Relationship between clustered GPVI-dimers and other receptors involved in the platelet–collagen interaction

Localisations of other receptors involved in the platelet-collagen interaction, GPIb (Fig. 4A) and integrin α2β1 (Fig. 4B), were compared to that of GPVI-dimer clusters on platelets adhered to collagenous substrates. GPVI-dimer clusters form along Horm collagen fibres, whereas they appeared throughout the lamellipodia of platelets spread on Col III and III-30. Like GPVI-dimer clusters, α2β1 was also found to localize along Horm collagen fibres, but was confined to the cell body of platelets adhered to Col III and III-30; no co-localisation with GPVI-dimer clusters was observed on the platelet lamellipodia. GPIb (VWF receptor) did not associate with GPVI-dimer clusters in the lamellipodia of any of the collagenous substrates tested, remaining in the platelet cell body.

Contribution of collagen receptor integrin α2β1 to platelet adhesion and GPVI-dimer cluster formation

Platelets labelled with Alexafluor 488–204-11 Fab with or without Gi9 (α2β1-blocking antibody), were allowed to settle onto immobilized non-fibrous collagen III or Horm fibres, and monitored by TIRFM. Gi9 treatment prevented platelet adhesion to collagen III, but merely decreased the extent of GPVI-dimer cluster formation on Horm fibres (Fig. 4C). Gi9 had no effect on platelet adhesion/cluster formation on the GPVI-specific ligands CRP-XL and III-30 (data not shown).

Effect of Src-family and Syk kinase inhibition on GPVI cluster formation

Confocal images of platelets adhered to Horm, Col III, III-30, or CRP-XL show that areas rich in phosphotyrosine co-localize with some of the GPVI-dimer clusters (Fig. 5A), suggesting signalling is occurring in these regions. Degree of colocalization was analysed using Pearson’s correlation (Fig. 5B, right). To investigate whether protein tyrosine phosphorylation is important in GPVI cluster formation, platelets were treated with the Syk
inhibitor PRT-060318 or Src-family kinase inhibitor PP2. Both inhibitors, at concentrations demonstrated to inhibit spreading and phosphorylation in human platelets [26], inhibited adhesion on Horm and Col III. Both inhibitors reduced platelet spreading on all collagenous substrates (Supplementary Fig. 1 shows corresponding DIC images that enable visualisation of platelet morphology in the presence and absence of the inhibitors). Despite having a reduction in spreading, discrete GPVI-dimer clusters were visible in platelets adhered to CRP XL and III-30 in the presence of PRT-060318 or PP2 (Fig. 6).

Effect of disrupting actin dynamics on GPVI-dimerisation and clustering
Flow cytometry was used to quantify effects of drugs that inhibit actin dynamics on GPVI-clustering: Cytochalasin D (cyt) and Latrunculin A (lat), actin polymerization–blockers, and Jasplakinolide (jas), F-actin stabilizer. All three inhibitors (10 μM) significantly decreased GPVI-dimer levels in resting platelets (Fig. 7A,B). To avoid total abolition of adhesion, we used low-dose (2 μM) actin antagonist to assess the role of the actin cytoskeleton in clustering of GPVI-dimers on Horm, Col III, CRP-XL, and III-30 (Figs. 7C–D, respectively). Low-dose Latrunculin A disrupted the actin cytoskeleton and severely depressed cluster formation, with platelets that did adhere not spreading. Low-dose Jasplakinolide had similar but more severe effects on cluster formation, with no apparent F-actin staining, and fewer adhered, but non-spread platelets.

Discussion
GPVI-dimers constitutively present on resting platelets are the collagen-binding form of this receptor, having over 100-fold higher affinity for collagen than the monomer [9,10]. The constitutive presence of dimers suggests that a mechanism beyond the formation of dimers may be necessary to initiate signalling through GPVI. One mechanism may be the formation of higher-order oligomers (clusters) which has been demonstrated to play an important role in the amplification, maintenance and termination of receptor signalling in many cell types.
We propose that GPVI clusters which may recruit signalling molecules to facilitate platelet activation. To explore this hypothesis, we used complementary imaging methods: TIRFM, dSTORM, and confocal microscopy.

TIRFM enabled the temporal visualisation of GPVI-cluster formation at the surface of platelets spreading on collagenous substrates. After the initial contact of GPVI-dimer with the substrate, the number of GPVI-dimer clusters increases rapidly. Notably, in platelets adhered to fibrous Horm collagen, discrete clusters form along the fibres first, then throughout the surface of the spread platelet (Fig. 1A). This is consistent with GPVI-dimers interacting with the surface of the collagen fibre, forming clusters at those points, followed by signalling that induces more cluster formation in regions of the platelet not in direct contact with the visible fibre. A different clustering pattern is observed on the immobilized non-fibrous collagenous substrates, CRP-XL (Fig. 1B) and III-30 (Fig. 1D), which exclusively bind GPVI, and soluble Col III (Fig. 1C), which binds GPVI and α2β1. Immobilized non-fibrous substrates are randomly-orientated triple-helical molecules and would be expected to support a different clustering pattern than Horm collagen fibers, which contain a highly organised parallel assembly of triple-helical tropocollagen molecules within the microfibrils. This structure dictates that GPVI-binding sites will be distributed on the surface of a fibre at fixed lateral and axial intervals, and suggests that a GPVI-dimer would be able interact with neighbouring tropocollagen molecules presenting either separate or a composite binding sites [20].

dSTORM allows single fluorophore molecules to be detected and located with very high spatial precision. Combined with cluster analysis, dSTORM allows quantitation of the number of GPVI dimer molecules and cluster number, size and density in ROIs within a spread platelet. This information permits the relative differences in GPVI clusters to be compared in platelets spread on different substrates. Horm collagen fibres induced a high degree of clustering by virtue of their structure, outlined above. The spacing and orientation of the two proposed collagen-binding grooves of GPVI-dimer might allow it to bind with increased avidity to sites on adjacent tropocollagen molecules, allowing clustering to occur

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The ability of immobilized III-30, CRP-XL, and Col III to bind GPVI will depend on the density and relative orientation of the immobilized substrate helices. CRP and III-30 contain only GPVI-binding motifs, the former having more GPVI-reactive GPO triplets per molecule, whereas immobilized soluble Col III binds both GPVI and α2β1, which may support cooperative platelet binding, since motifs for each receptor are located close together. Such differences may explain the variation in cluster density observed between the different collagenous substrates. Indeed, both Horm and Col III had significantly higher GPVI cluster density than III-30, and Col III clusters were also significantly denser than those on CRP-XL, suggesting a measurable role for α2β1 in these events.

On platelets adhered to non-fibrous Col III and III-30, GPIb (Fig. 4A) and α2β1 (Fig. 4B) do not co-localize with the majority of the GPVI-dimer clusters, which are located in the lamellipodia of the spread platelet. However, α2β1 is indispensable for platelet binding to soluble Col III, since adhesion is severely reduced by the blocking anti-integrin α2β1 antibody Gi9. Synergism between GPVI and α2β1 has also been observed using immobilized model peptides in flowing blood thrombus deposition studies [31]. In contrast, Gi9 did not prevent platelet adhesion/cluster formation on fibrous Horm collagen, indicating that GPVI binding to the collagen fibre is much stronger than to non-fibrous substrates. These observations are consistent with GPVI being sufficient to support platelet binding to collagen fibres but not to soluble collagen [32]. Integrin α2β1 was also located along Horm collagen fibres in bound platelets, and the magnified confocal images (Fig. 4B) suggest that α2β1 binds close to some of the GPVI-dimer–clusters on the fibre. The model proposed by Herr and Farndale [20] suggests that α2β1 and GPVI might bind ~10nm apart in collagen III, and inspection of the collagen I sequence suggests that similar considerations would apply to Horm collagen. Although non-activated α2β1 may bind its high affinity motif, GFOGER, in Horm fibres [33], this is not essential for GPVI-dimer binding and clustering. However, α2β1 may serve to accelerate platelet adhesion to the collagen fibre [34], thus facilitating further GPVI-clustering. Under the present static adhesion conditions, however, the distribution of

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GPIb differed from that of the GPVI dimer–clusters on collagen fibres, although GPIb was observed in the platelet cell body, adjacent to the fibre (Fig. 4A). A Mab against GPIb (SZ2) was reported to block CRP-XL–induced platelet aggregation, and GPIb to co-precipitate with GPVI in both resting and thrombin-stimulated platelets, suggesting an interaction between the two receptors [35]. Platelet adhesion to collagen under high shear requires VWF multimers, via GPIb, to tether the platelet to collagen, but our experiments are performed under static conditions in the absence of plasma VWF, so GPIb is less important.

What induces GPVI clustering? One possibility is that platelet activation induced by the initial interaction of GPVI with collagen leads to GPVI oligomerisation. However, in spite of severe inhibition of platelet spreading by the Syk inhibitor PRT-060318, limited cluster formation on all four immobilized collagenous substrates was still observed (Fig. 6, right). The marked inhibition of cluster formation on Col III (which lacks the high-affinity motif, GFOGER) would be due to blocking α2β1 activation, hence removing the contribution of its high affinity form to stabilize platelet binding to collagen. Src-family kinase inhibitor PP2 also inhibited platelet adhesion, but to a lesser extent than PRT-060318 (Fig. 6, middle). Alternatively, blockade of both Src and Syk might potently inhibit secretion [36,37], preventing release of active substances (including fibrinogen), thus reducing clustering via secondary signaling pathways. These results suggest that, whilst platelet adhesion and spreading is fully dependent on GPVI-mediated signalling, GPVI-cluster formation is only partly so.

Movement of membrane proteins is controlled, in part, by the cytoskeleton in other cells [38]. Inhibitors of actin dynamics at 10 μM inhibited GPVI-dimer formation in resting platelets and prevented the GPVI-dimer increase in CRP-XL– or thrombin-activated platelets (Fig. 7A,B). At 2 μM, a threshold inhibitory concentration, there is severely limited adhesion of non-spread platelets on all 4 tested substrates, but there is evidence of residual cluster formation and disturbed actin filament distribution. These results suggest the contribution of
the peripheral membrane cytoskeleton to GPVI cluster formation, a topic for future investigation.

Although the GPVI-dimers on resting platelets are competent to bind collagen, they are not exposed to it in an uninjured vessel and the low density of GPVI-dimers in resting platelets (≈1500/platelet) [10] suggests that they may be too far apart to induce efficient signalling; thus platelets remain inactive, the GPVI-dimers requiring both receptor ligation and proximity to activate platelets. However, upon vessel injury, subendothelial collagen fibres are exposed to the bloodstream, and efficient platelet activation is necessary to prevent bleeding. Once a vessel is injured, the binding sites on fibres of collagen types I and III become accessible to the receptors involved in the platelet–collagen interaction GPVI, integrin α2β1, and GPIb. The proximity of GPVI-dimer binding sites on the fibre surface enable clustering of GPVI-dimers, increasing avidity and bring together necessary signalling components to initiate signalling and lead to efficient platelet activation and thrombus formation. We examined GPVI-dependent signalling in the vicinity of GPVI clusters, and whether inhibitors of GPVI-mediated signalling affected clustering. The proximity of phosphotyrosine to some of the GPVI clusters (Fig. 5), particularly on the Horm fibres, suggests local tyrosine kinase activity. Jamasbi et al. (39) recently reported that GPVI-Fc (Revacept) bound to collagen fibres can be clustered by the addition of anti-Fc, consistent with dimers binding in close enough proximity in the collagen fiber to allow cluster formation. Activation induces formation of more dimers, as evidenced by the increased dimer numbers in CRP-XL- or thrombin-activated platelets [10], enabling formation of even more clusters. Moreover, activation of platelets leads to activation of α2β1, increasing its affinity for collagen, causing firm adhesion. Our present study suggests that platelets have two layers of activity regulation through GPVI—conversion of monomers to high affinity dimers, and clustering of the GPVI-dimers. Clustering would serve to increase both avidity for collagen and signalling molecule recruitment, leading to efficient platelet activation during thrombus formation.
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Authors have no conflicts of interests to disclose.

Addendum

N. S. Poulter designed experiments, performed dSTORM imaging, analysed and interpreted data, made figures, and wrote the paper. A. Y. Polllit designed experiments, performed TIRFM, analysed and interpreted data, made figures, and wrote the paper. D Owen provided the MATLAB cluster analysis algorithm and provided expert advice. E. E. Gardiner provided antibodies and critically read the manuscript. R. K. Andrews, H. Shimizu, and D. Ishikawa provided antibodies. D. Bihan synthesized Toolkit peptide III-30. M. Moroi designed and performed the flow cytometry analyses, made figures, and interpreted data. R. W. Farndale and S. P. Watson discussed and interpreted data and critically read the manuscript. S. M. Jung, the corresponding and senior author, designed and performed experiments (TIRFM, confocal imaging), analysed and interpreted data, coordinated and wrote the paper, and made figures.

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**Figure Legends**

**Fig. 1.** GPVI forms clusters when platelets spread on immobilized collagenous substrates. TIRF microscopy time course of washed human platelets, labelled with Alexafluor-488-conjugated, dimer-specific 204-11 Fab, interacting with 10 µg/ml immobilized collagenous substrates at 37°C. (A (i)) Horm collagen, (B) CRP-XL, (C) non-fibrous collagen type III and (D) peptide III-30. The position of Horm collagen fibres are indicated on a DIC image by the dashed lines (A (ii)). Images are a single platelet representative of three independent experiments. See Supplemental Movie 1. Time stamp: seconds. Scale bar: 2µm.

**Figure 2: dSTORM analysis of GPVI clustering on collagenous substrates**

A) Platelets spread on the collagenous substrates indicated were labelled for dimeric GPVI using the Alexa-647 conjugated Fab 204-11 (magenta) and F-actin using Alexa-488-Phalloidin (green) and imaged by TIRF (top row). The GPVI was also imaged by dSTORM with the localised points (molecules) shown in the second row. The cluster heat map of the GPVI dSTORM data in the 3µm x 3µm region of interest (ROI; dashed box in images) is shown in the third row where red indicates high degrees of clustering. The threshold value of
a cluster was set to L(r)=100. Quantitative analysis of GPVI dSTORM clustering shows the number of molecules detected in the 3µm x 3µm ROI (B), the number of clusters in the ROI (C), the size of the clusters (in nm²; (D)) and the density of the molecules within the clusters (E). All graphs have the median and interquartile range indicated in red. Statistical analysis: Non-parametric Kruskal-Wallis ANOVA with Dunn’s multiple comparison. Green lines indicate significance of P<0.001, Blue is P<0.01, Orange P<0.05, no line indicates no significance. Scale bar: 5 µm. A total number of n≥70 ROIs taken from 3 or 4 independent experiments were analysed for each collagenous substrate.

**Fig. 3: Comparison of GPVI clustering on Horm collagen and fibrinogen**

A) Platelets spread on the Horm or fibrinogen as indicated on top were labelled for dimeric GPVI using the Alexa-647 conjugated Fab 204-11 (magenta) and F-actin using Alexa-488-Phalloidin (green) and imaged by TIRF. The GPVI was also imaged by dSTORM with the localised points (molecules) shown in the second row. The cluster heat map of the GPVI dSTORM data in the 3µm x 3µm region of interest (ROI; dashed box in images) is shown in the third row where red indicates high degrees of clustering. The threshold value of a cluster was set to L(r)=100. Quantitative analysis of GPVI dSTORM clustering shows the number of molecules detected in the 3µm x 3µm ROI (B), the number of clusters in the ROI (C), the size of the clusters (in nm²; (D)) and the density of the molecules within the clusters (E). All graphs have the median and interquartile range indicated in red. Statistical analysis: If data passed a normality test then a t-test was performed (For ‘number of clusters’). If data was not normally distributed a non-parametric Mann-Whitney test was used. ** is where P<0.001. Scale bar: 5 µm. A total number of n≥90 ROIs taken from 3 independent experiments were analysed for each substrate.
Fig. 4. Comparison of distributions of GPVI-dimer clusters, GPIb, and integrin α2β1 on adhered platelets and the effect of inhibiting integrin α2β1 on cluster formation and adhesion.

A. (GPIb) and B (integrin α2β1): Confocal images of adhered platelets prelabelled with Alexa-fluor 488–204-11 Fab (anti-GPVI-dimer, 4 μg/ml, green) and Alexafluor 647–anti-human CD42 clone 486805 (anti-GPIb, 5 μg/ml, red) or Alexafluor 647–16B4 (anti-integrin α2β1; 5 μg/ml, red) and then allowed to adhere on Col III, III-30, or Horm coated dishes. Transmitted light images are included to show degree of spreading and magnified images of the platelet(s) indicated by white arrows are included. Distributions of GPIb are different from those of the GPVI-dimers for all three collagenous substrates and are not associated with the Horm fibres. Integrin α2β1 bound to the Horm collagen fibres, following a similar pattern as the GPVI-dimer clusters, coinciding with GPVI-dimer clusters at some points, but not associated with the lamellipodia of the platelets spread on Col III or III-30. C: TIRF images (15-min time point, upper row in each 4-image group) and DIC images (lower row in each 4-image group). Platelets were prelabelled with Alexa-fluor 488–204-11 Fab, treated with Gi9 (anti-integrin α2β1, 5 μg/ml; + Gi9) or an equal volume of PBS (No Gi9), and allowed to adhere on Col III or Horm under TIRF monitoring for 30 min. Gi9 treatment decreased but did not prevent adhesion and GPVI-dimer clustering on fibrous Horm collagen, but little or no adhesion was seen on collagen type III even at the 30-min time point.

Fig. 5. Phospho-tyrosine staining and GPVI-dimer clustering.

To determine if signalling reactions may be occurring in the vicinity of GPI-dimer clusters, washed platelets were prelabelled with Alexa-fluor 488–204-11 Fab and allowed to adhere on collagenous substrate, followed by formalin fixation, permeabilization with 0.5% Triton/PBS, and staining with 4G10 (anti-phosphotyrosine)/Alexa-fluor-647 conjugated anti-mouse Fc. The experiments shown are performed with platelets of one donor, on the same
day, and same imaging conditions. A: Confocal images show that visually, phospho-tyrosine (P-tyr, red) was found among but not necessarily coincident, with the GPVI-dimer clusters (green) for platelets adhered to Col III, CRP-XL, and III-30. Notably, the P-tyr staining in platelets adhered to Horm collagen very closely followed the pattern of GPVI-dimer staining, which was mainly confined to the fibres. The morphology of spread platelets was identified by transmitted light.

B: Calculation of colocalization. In the left-side set of figures, colocalised pixels are presented as a binary threshold mask, calculated by Image Pro Premier ver. 9.2 (Media Cybernetics); they correspond to the respective merged images in 5A. The right-side graph shows the calculated Pearson’s correlation coefficients for co-localization (mean ±SD), calculated from 9–11 images, and are as follows: Horm collagen (0.176 ± 0.016); collagen type III (0.106 ± 0.046), CRP-XL (0.216 ± 0.024), and toolkit peptide III-30 (0.079 ± 0.057); the Pearson coefficients of the representative images shown in Fig. 5A are 0.178, 0.166, 0.179, 0.074, for Horm collagen, Col III, CRP-XL, and III-30, respectively. The Pearson’s coefficient for Horm collagen is significantly different from that of Col III (**P<0.001) and III-30 (**P<0.001), but not different from that of CRP (paired t-test, Prism v7). These values suggest that only some GPVI-dimer clusters are localized with regions of high signalling activity; it is notable that under the limitations of the resolution afforded by the confocal microscope, both GPVI-dimer clustering and signalling can be seen along the Horm collagen fibers.

Fig. 6. Effect of signalling inhibitors on GPVI-dimer cluster formation in live platelets adhering to immobilized collagenous substrate.

Alexafluor-488–204-11 labelled platelets were reacted with DMSO (vehicle), 10 μM PP2, or PRT (5 μM) and then their adhesion to immobilized collagenous substrate was followed by TIRF. PRT markedly inhibited platelet adhesion to all substrates, but the platelets that did...
adhere still showed GPVI-dimer cluster formation. The effect of PP2 was weaker but similar to that of PRT.

**Fig. 7. Effect of actin antagonists on GPVI dimerization and GPVI-dimer clustering.**

A: Raw flow-cytometry data: Effect of the actin antagonists latrunculin A (Lat A) and jasplakinolide (Jas) on the level of dimers in resting and activated washed platelets were determined by flow cytometry (Accuri C6) using FITC-labeled mFab-F (dimer-specific). Panel A provides a representative histogram and dot-plots of the control (Control, FITC-anti-human Fab), non-treated resting platelets (No inhibitor, vehicle DMSO at 0.2% final concentration), and Lat A (10 μM)–treated resting platelets (+ Lat A). There are no obvious differences in the platelet region (SSC-A vs. FL1-A plot) in the Lat-A–treated and non-treated resting platelets. However, the histogram (upper graph in panel A, FLA-1 vs. count) shows that Lat A treatment markedly decreases the level of GPVI-dimers, as evidenced by the clear leftward shift of the Lat A–treated platelets relative to the untreated resting platelets. B: Comparison of effects of actin-antagonists on GPVI-dimer level in resting and activated platelets. Washed platelets were treated with the vehicle DMSO or an actin antagonist at 10 μM: cytochalasin D (cyt), laetrunculin A (lat), or jasplakinolide (jas) and then added with Hepes Tyrodes buffer (resting), CRP, or thrombin. The samples were processed for flow cytometry using FITC–mFab-F or FITC-anti-human Fab (control). Differences among treatment groups were calculated by paired t-test, by PRISM ver. 7. All tested actin inhibitors decreased GPVI-dimers (MFI, mean ±SEM) in resting platelets [cyt, lat, jas, each P<0.05, n = 5, compared to the vehicle alone (0.2% DMSO, final concentration, n = 8)]; CRP-XL–induced platelets [cyt, lat, jas, each P< 0.05 (n = 5), compared to 0.2% DMSO]; and thrombin-induced platelets [cyt and jas, each P<0.05 (n = 5), and lat, P<0.005 (n = 5), compared to 0.2% DMSO). C – F: Confocal images of GPVI-dimers and F-actin in non-treated and actin-antagonist–treated platelets adhered to Horm (C), Col III (D), CRP-XL (E), and III-30 (F). Washed platelets labeled with Alexa-fluor 488–204-11 Fab (green), with or without treatment with Lat A or Jas, were allowed to adhere on immobilized collagenous substrate, formalin-fixed, permeabilized,

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and then stained for F-actin with Alexa-fluor 647–phalloidin. Lat A (2 μM) and Jas (2 μM) were used at threshold inhibitory concentrations, so that platelet adhesion is not completely prevented. Images were obtained on a confocal microscope; the following images are shown: grey scale images of the GPVI-dimer and F-actin; merged images (green = GPVI-dimer clusters and red = F-actin), and transmitted light images. Lat A severely inhibited the formation of large GPVI-clusters, but a limited number of small clusters could still be observed for platelet adhesion to all tested collagenous substrates. The spreading is clearly inhibited on all the collagenous substrates. Jas produced more severe inhibition of F-actin and no F-actin staining could be observed in the Jas-treated platelets. In the Jas-treated platelets, some green fluorescence could still be observed in the adhered cells, but it is not clear whether this corresponds to small GPVI-clusters or is due to the higher density of residual GPVI-dimers due to the much more compact size of the non-spread platelets. Note that in spite of the cytoskeleton being severely compromised in the inhibitor-treated platelets and the total inhibition of spreading, there is residual ability for the platelets to adhere to the collagenous substrates, possibly due to GPVI-dimers, and this is most evident in the platelets adhered along the Horm collagen fibers in the Lat A– or Jas- treated preparations.

Table I. Collagenous substrates used in this study

<table>
<thead>
<tr>
<th>Collagenous substrate</th>
<th>Abbr</th>
<th>Description</th>
<th>Structure</th>
<th>Collagen receptor specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>a Collagen-related peptide-XL</td>
<td>CRP-XL</td>
<td>Triple helical, crosslinked</td>
<td>*GCO-(GPO)_{10}GCOG-NH\textsubscript{2}</td>
<td>GPVI</td>
</tr>
<tr>
<td>b Collagen Toolkit III peptide 30</td>
<td>III-30</td>
<td>Triple helical</td>
<td>*GPC-(GPP)<em>{5}-GAOGARQGA-GPOGPEGGKGAAGPOGPO-(GPP)</em>{5}-GPC-NH\textsubscript{2}</td>
<td>GPVI</td>
</tr>
<tr>
<td>c Horm collagen (equine, type I)</td>
<td>Horm</td>
<td>Fibrous Type I</td>
<td>[\alpha1(\text{I})]_{2}\alpha2(\text{I})</td>
<td>GPVI Integrin α2β1</td>
</tr>
<tr>
<td>d Bovine collagen type I</td>
<td>Col I</td>
<td>Non-fibrous</td>
<td>[\alpha1(\text{I})]_{2}\alpha2(\text{I})</td>
<td>GPVI Integrin α2β1</td>
</tr>
<tr>
<td>e Bovine collagen type III</td>
<td>Col III</td>
<td>Non-fibrous</td>
<td>[\alpha1(\text{III})]_{3}</td>
<td>GPVI Integrin α2β1</td>
</tr>
</tbody>
</table>

*GPVI-binding GPO triplets are shown in bold print for CRP and III-30  
* Cross-linked Collagen Related Peptide (CRP-XL) was synthesized as described by Morton et al. [29].

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Triple-helical peptide III-30 is from the Collagen Toolkit III, a set of overlapping triple helical peptides encompassing the entire Col domain of human collagen III; it was synthesized as described by Raynal et al. [30].

Nycomed Pharma GmbH (1 mg/ml; Munich, Germany).

Koken Co., Ltd. (3 mg/ml; Tokyo, Japan)

Supplementary Movie

GPVI forms clusters when platelets spread on immobilized collagenous substrates. TIRF microscopy time course of washed human platelets, labelled with Alexa 488 labelled dimer-specific 204-11 Fab, interacting with 10μg/ml immobilized collagenous substrates at 37°C. (A (i)) Horm collagen, (B) CRP-XL, (C) peptide III-30 and (D) non-fibrous collagen III. Movies are a single platelet representative of three independent experiments. Frames were taken every 5 seconds. Time stamp: seconds. Scale bar: 2 μm.

Supplemental Fig. 1. DIC images corresponding to the fluorescence images in Figure 6. Platelets were reacted with DMSO (vehicle), 10 μM PP2, or PRT (5 μM) and then their adhesion to immobilized collagenous substrates was followed by DIC. PRT markedly inhibited platelet adhesion to all substrates, but the platelets that did adhere still showed GPVI-dimer cluster formation. The effect of PP2 was weaker but similar to that of PRT.
A. GPIb

B. Integrin α2β1

C. Effect of Gi9

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A. 

B. 

C. Horm collagen

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