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Clark, Joanne; Neagoe, Raluca Alexandra Iulia; Zuidscherwoude, Malou; Kavanagh, Deirdre; Slater, Alexandre; Martin, Eleyna; Soave, Mark; Stegner, David; Nieswandt, Bernhard; Poulter, Natalie; Hummert, Johan; Herten, Dirk-Peter; Tomlinson, Michael; Hill, Stephen J; Watson, Steve

DOI:
10.1055/a-1401-5014

Document Version
Peer reviewed version

Citation for published version (Harvard):
Clark, J, Neagoe, RAI, Zuidscherwoude, M, Kavanagh, D, Slater, A, Martin, E, Soave, M, Stegner, D, Nieswandt, B, Poulter, N, Hummert, J, Herten, D-P, Tomlinson, M, Hill, SJ & Watson, S 2021, 'Evidence that GPVI is expressed as a mixture of monomers and dimers, and that the D2 domain is not essential for GPVI activation: GPVI dimerisation is not critical for ligand binding', *Thrombosis and Haemostasis*, vol. 2021, no. 00, pp. 1-26. https://doi.org/10.1055/a-1401-5014

Evidence that GPVI is expressed as a mixture of monomers and dimers, and that the D2 domain is not essential for GPVI activation

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Running title: GPVI dimerisation is not critical for ligand binding

Abstract word count: 216

Word count: 4996

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Abstract

Collagen has been proposed to bind to a unique epitope in dimeric GPVI and the number of GPVI dimers has been reported to increase upon platelet activation. However, in contrast, the crystal structure of GPVI in complex with collagen-related peptide (CRP) showed binding distinct from the site of dimerisation. Further fibrinogen has been reported to bind to monomeric but not dimeric GPVI. In the present study we have used the advanced fluorescence microscopy techniques of single molecule microscopy, fluorescence correlation spectroscopy (FCS) and bioluminescence resonance energy transfer (BRET), and mutagenesis studies in a transfected cell line model to show that GPVI is expressed as a mixture of monomers and dimers, and that dimerisation through the D2 domain is not critical for activation. As many of these techniques cannot be applied to platelets to resolve this issue, due to the high density of GPVI and its anucleate nature, we used Förster resonance energy transfer (FRET) to show that endogenous GPVI is at least partially expressed as a dimer on resting and activated platelet membranes. We propose that GPVI may be expressed as a monomer on the cell surface and forms dimers in the membrane through diffusion giving rise to a mixture of monomers and dimers. We speculate that the formation of dimers facilitates ligand binding through avidity.

Key words: platelets, glycoprotein VI (GPVI) receptor, dimerisation, collagen, single molecule microscopy

Summary Table

What is known on this topic? • GPVI is a receptor for collagen and fibrinogen. • The synthetic collagen CRP binds to the D1 domain of GPVI. • Dimer-specific antibodies show increased binding to activated platelets although it is not known if this is due to a change in conformation or increased dimer formation.
What does this paper add? • GPVI is expressed as a mixture of monomers and dimers in cell lines, and at least partially as a dimer in platelets. • The D2 domain in GPVI mediates dimerisation but is not essential for activation by collagen. • We propose that GPVI dimerisation facilitates activation through increased avidity and not through a dimer-specific epitope.

Introduction

The platelet receptor glycoprotein (GP) VI is a member of the immunoglobulin (Ig) receptor superfamily and a receptor for collagen, the most thrombogenic constituent of the extracellular matrix. GPVI contains two Ig domains, termed D1 and D2, a mucin-rich stalk and a cytoplasmic tail.[1,2] GPVI is linked to the Fc receptor (FcR) γ-chain homodimer by a salt bridge. The FcRγ-chain contains a signalling motif defined by the presence of two YxxL groups (single amino acid code) separated by 12 amino acids, called an immunoreceptor tyrosine-based activation motif (ITAM).[3,4] Collagen binding to GPVI induces phosphorylation of the

conserved tyrosines in the ITAM by Src family kinases. This in turn leads to binding of Syk and initiation of a downstream signalling cascade that activates PLCγ2.[2]

In the original studies, collagen was reported to bind to recombinant dimeric but not monomeric GPVI, and dimer-specific antibodies were shown to block platelet activation, leading to the proposal that collagen binds to a unique epitope in dimeric GPVI. [5-8] Further, the number of GPVI dimers was shown to increase upon platelet activation, serving as a positive feedback step.[6-8] In support of a dimer-specific conformation, the crystal structure of the D1 and D2 domains shows a site of dimerisation through the D2 domains, although the protein does not dimerise in solution, suggesting that this is of low affinity.[9] Dimerisation of GPVI under resting conditions has been shown in transfected cell lines through measurement of bioluminescence resonance energy transfer (BRET) and by co-immunoprecipitation studies of differentially tagged forms of GPVI, and in platelets by use of a cross-linking reagent.[10]

Together, these data have led to a generally accepted model in which GPVI is at least partially expressed as a dimer, and that collagen binds to a unique dimer-specific conformation. Recently, however, several observations have led to a renewed interest in the configuration of GPVI on platelets and challenged the concept that collagen binds to a dimer-specific conformation. Firstly, fibrin and fibrinogen have been identified as ligands for GPVI and have been shown to bind selectively to recombinant monomeric GPVI[11-14], although this is controversial.[15] Secondly, the synthetic collagen, CRP, has since been shown to bind to the D1 domain in GPVI using X-ray crystallography, which is removed from the site of dimerisation (PDB 5OU8 and 5OU9, Feitsma et al.). Thirdly, collagen has now been shown to bind to recombinant monomeric GPVI[11,14,16] suggesting that the original observation of binding to dimeric GPVI was due to increased avidity.

In light of these contrasting reports, the aim of the present study was to further investigate the configuration of GPVI in the membrane using several fluorescence-based microscopy methods and mutagenesis. The majority of the work has been performed in cell lines because of the anucleate nature of platelets and the relatively high density of expression of GPVI which hampers interpretation of single molecule microscopy data.[17] In addition, we have used Förster resonance energy transfer (FRET) in resting and activated platelets to investigate the presence of dimeric GPVI. The results show that GPVI is expressed as a mixture of monomers and dimers in transfected cell lines and that it is at least partially expressed as a dimer in platelets, but that dimerisation is not critical for activation by collagen in a transfected cell line model.

Materials and Methods

Detailed methods are described in the supplemental material.

Fluorescence correlation spectroscopy (FCS)

FCS measurements were made using a Zeiss LSM-880 confocal microscope equipped with gallium arsenide phosphide photon detectors (GaAsP) (Carl Zeiss, Jena, Germany). Single-photon excitation with a continuous argon ion laser was performed using a 40x (NA 1.2) C-apochromat water immersion objective. Before each experiment, the microscope was aligned and calibrated using Atto-488 dye (10 nM) in water at 25ºC with 0.1 and 0.2% 488 nm laser power for 30 sec measurement time to determine axial and lateral radii and confocal volumes. Atto-488 has a well-established diffusion coefficient of 400 $\mu m^2\ s^{-1}$ (Picoquant). In HEK293T cells, FCS measurements were taken on the plasma membrane where monitoring the photon counts per molecule in real time (interactive counts/molecule window in the Zeiss software) was performed to achieve optimum positioning in the centre of the observation volume and to find the focal plane corresponding to the maximal photon counts/molecule. Measurements were performed with 0.03% 488 nm laser power at 25ºC for 5 sec measurement time per point for 10 points per cell using Zen Black 2012 (Carl Zeiss, Jena, Germany). Monitoring of time-

dependent fluorescence intensity fluctuations produced autocorrelation decay curves. Details of FCS analysis is in supplemental material.

Single molecule stepwise photobleaching

Single-molecule photobleaching imaging was performed in total internal reflection fluorescence (TIRF) microscopy at using a fully motorised Nikon TIRFM mounted on a NIKON-N-STORM microscope on a Ti-E stand equipped with a Nikon 100x 1.49 NA TIRFM oil objective, Perfect Focus System, Agilent MLC400 laser bed with 405 nm (50 mW), 488 nm (80 mW), 561 nm (80 mW) and 640 nm (125 mW) solid-state lasers and Andor iXon Ultra EM-CCD camera. During the acquisition, at 28^{o}C the sample was continuously illuminated at 488 nm using a 488 TIRF filter for 5000 frames with 5% laser power (40 x 40 μm, 30 ms exposure time, 300 gain). To ensure homogenous illumination, a central 256 x 256 pixel region of interest (ROI) of the chip was used. Microscope control and image acquisition were performed by NIS Elements 5 (Nikon Instruments).

Stepwise photobleaching analysis

Trace extraction and stepwise photobleaching analysis was performed using the python package quickpbsa.[18] In short, fluorescent spots were localised using the ImageJ[19] plugin ThunderSTORM[20] on the average of the first 5 frames in the sequence, extracting the spots centre position and Gaussian width (σ). Spots with a σ below 50 nm and above 250 nm, or within a distance of 3.5 pixels (576 nm) of another spot, were excluded. Image sequences with aberrant imaging conditions were excluded based on the overall fluorescent decay. Photobleaching traces were extracted from a ROI with a 2 pixel radius (329 nm). A ring-shaped ROI with an inner radius of 3.5 pixels (576 nm) and an outer radius of 5 pixels (832 nm) was used for background subtraction. Photobleaching analysis in quickpbsa was run with a step detection threshold of 20 counts and otherwise default parameters. However, to increase the robustness to photophysics the Bayesian refinement step in the quickpbsa analysis was not

used, since this step relies on reproducible brightness of a single fluorophore and could be affected by blinking. This approach is valid for small fluorophore numbers (<10).[18]

FRET in platelets

Human and mouse washed platelets were prepared as previously described.[21,22] Platelets ($5x10^7$/ml) were pre-incubated with human anti-GPVI, 313A10-488 (50 μg/ml), 313A10-546 (50 μg/ml) and 313A10-unlabelled (50 μg/ml) F(ab') fragments or anti-integrin αIIbβ3, MWReg30-488 (10 μg/ml) and anti-GPIX-546 (10 μg/ml) mAb as controls for 10 min at 37ºC followed by 10 min at room temperature with and without CRP (10 μg/ml). 313A10-488 (donor) and 313A10-546 (acceptor) were used as a FRET pair for GPVI. 313A10-488 and 313A10-unlabelled or MWReg30-488 and anti-GPIX-546 were negative controls. We refer to 313A10 F(ab') fragments as A10 throughout the text and figures. For all measurements, a FACSAria flow cytometer (BD-Biosciences) equipped with standard single 488 nm or 546 nm laser was used.

Statistical analysis

Results are shown as mean ± SEM unless otherwise stated and the number of independent experiments is described in Figure legends. Data were analysed using PRISM v8.3.0 (GraphPad, San Diego, CA). For FCS, data sets were first tested for normality using the Shapiro-Wilks test. FCS PCH analysis data were tested by Kruskal-Wallis with Dunn's *post-hoc* test. For stepwise photobleaching experiments statistical analysis was by Epps-Singleton 2 sample test[23] implemented in scipy.[24] Statistical analysis of the adhesion assays were by two-way ANOVA with a Bonferroni *post-hoc* test. For NFAT and nanoBRET assays, Student's two-tailed unpaired *t* tests were used. For FRET assays, one-way or two-way ANOVA with a Bonferroni *post-hoc* test were used. Significance was set at $P \leq 0.05$.

Results

NanoBRET shows the D2 domain is critical for GPVI dimerisation in transfected HEK293T cells

Bioluminescence resonance energy transfer (BRET) is a distance and orientation-dependent assay where excited-state energy is transferred from a luciferase donor to a fluorescent acceptor when brought into proximity of <10 nm. BRET has been used to demonstrate dimerisation and higher order oligomerisation of many membrane proteins including GPVI.[10,25,26] NanoBRET is a more sensitive version of BRET that uses the energy transfer from a bioluminescent donor protein (nanoluciferase) to a fluorescent acceptor protein (HaloTag).

Our original studies using BRET were carried out in transfected HEK293T cells using C-terminally-tagged human GPVI and revealed expression as a dimer with no change in signal upon activation by collagen or convulxin.[10] One explanation for the lack of change in signal on ligand engagement is that the fluorescent groups in the C-terminal tails may not be brought sufficiently close (i.e. < 10 nm) for energy transfer. To address this, we generated N-terminally tagged versions of human GPVI (Figure 1Ai) suitable for nanoBRET and co-expressed these in HEK293T cells. For controls, we also co-expressed the constructs with tagged versions of CD28, CD86 and neuropilin-1 (NRP1) [27-30], proteins which are not expressed in platelets and are not reported to associate with GPVI.

NanoBRET saturation assays were performed by transfecting a fixed concentration of Nanoluc-GPVI with increasing concentration of HaloTag-GPVI. The expression of the constructs on the cell surface was confirmed by flow cytometry (Figure 1Aii). The BRET signal showed saturation with increasing concentrations of the HaloTag-GPVI construct, demonstrating a specific interaction (Figure 2Bi), in agreement with our previously published work.[10] In contrast, a linear BRET signal characteristic of a non-specific interaction was seen between

Nanoluc-GPVI and SnapTag-CD86 (Figure 1Bi). Unexpectedly, a specific BRET signal was also detected between Nanoluc-GPVI and SnapTag-CD28, and Nanoluc-GPVI and HaloTag-NRP1, respectively (Figure 1Bi). These results confirm previous reports that GPVI receptors form dimers when expressed in a cell line but demonstrate that the collagen receptor also associates with other membrane proteins.

To further interrogate GPVI dimerisation, we generated GPVI constructs in which the D2 domain was removed. NanoBRET was performed using Nanoluc- and HaloTag-tagged human GPVI-D2-deleted constructs (Figure 1Ai). FACS analysis of transfected HEK293T cells confirmed expression of the two tagged D2-deleted GPVI receptors, although at a reduced level compared to wild-type GPVI (Figure 1Aii). There was no specific BRET signal detected upon deletion of the D2 domain (Figure 1Bii) consistent with this region being critical for GPVI dimerisation in the transfected cell line model. Furthermore, a non-specific BRET signal was detected between Nanoluc-GPVI-D2 deleted and wild-type HaloTag-GPVI or SnapTag-CD86 (Figure 1Biii). The former further demonstrates that the D2 domain is critical for GPVI dimerisation and that two D2 domains are required to facilitate dimerisation.

Studies were carried out to investigate whether CRP stimulates an increase in the BRET signal when measured using Nanoluc-GPVI and HaloTag-GPVI and an intermediate and high concentration of CRP (2 & 10 μg/ml). Both concentrations of CRP induced a similar increase in the BRET signal above untreated dimerisation (Figure 1Biv). Conversely, the addition of CRP (2 μg/ml) to the GPVI D2-deleted receptors failed to increase the BRET signal (Figure 1Bv).

The results show that the D2 domain is crucial for constitutive dimerisation in transfected HEK293T cells and that dimerisation of the wild-type but not the D2-deleted receptor is increased upon ligand binding. The results also show that GPVI associates with two unrelated

proteins in the membrane which are not expressed in platelets. The potential significance of this is discussed in the Discussion.

Fluorescence correlation spectroscopy shows GPVI is a mixture of monomers and dimers in transfected HEK293T cells

The increase in fluorescence signal observed in the nanoBRET studies could be due to dimerisation of GPVI monomers or formation of higher order oligomers. To investigate whether GPVI is expressed as a monomer and as a dimer, or solely as a dimer, on resting cells we have used FCS. This technique is able to distinguish monomeric and dimeric proteins by measurement of the molecular brightness of fluorescently-tagged proteins diffusing through a stationary volume in the membrane.[31] FCS is sufficiently sensitive to detect a two-fold change in brightness but not a change in the rate of diffusion between a monomer and dimer.

We first measured the axial and lateral radii of the confocal volume to ensure that measurements were in the range of previously published work.[32] The axial and lateral radii were 1.82±0.14 μm and 0.23±0.01 μm respectively (Supplementary Figure 1A), and the confocal volume was 0.21±0.04 μm^3 (0.21 fl). FCS measurements were then taken in the membrane of HEK293T cells expressing eGFP-tagged GPVI and compared to those for eGFP-tagged CD86 and eGFP-tagged CD28 (a disulphide-linked homodimer), which serve as controls for monomeric and dimeric proteins, respectively (Figure 2Ai).[27,28] Confocal microscopy was used to demonstrate surface localisation of eGFP-tagged GPVI, CD86 and CD28 (Figure 2Aii).

Monitoring of time-dependent fluorescence intensity fluctuations (Supplementary Figure 2Ai) produced autocorrelation decay curves (Figure 2Aiii) which were subjected to autocorrelation analysis and photon counting histogram (PCH) analysis to determine the diffusion coefficients and oligomeric state of the receptors respectively. The data were fitted using autocorrelation

analysis (Figure 2Aiii) which showed similar diffusion coefficients for GPVI-eGFP, CD86-eGFP and CD28-eGFP of 0.32±0.02, 0.34±0.02 and 0.40±0.02 $\mu m^2 s^{-1}$, respectively (Figure 2Aiv). Previous studies have shown that FCS cannot detect a significant difference in diffusion coefficients over a narrow range of molecular weights.[33] The fit deviations are displayed in Supplementary Figure 2Aii.

PCH analysis was used to determine the oligomeric state of the receptors (Figure 2Bi). A PCH is generated by analysing the amplitude of the fluorescence fluctuations around the mean intensity to determine the molecular brightness (photon counts per molecule) of a fluorescently-tagged protein.[34,35] PCH analysis showed a significantly greater brightness for dimeric CD28-eGFP compared to the monomeric CD86-eGFP, whereas GPVI-eGFP displayed an intermediate brightness suggestive of the presence of a mixture of monomers and dimers, with the majority (in the absence of CRP) present as monomers (Figure 2Bii). The fit deviations are displayed in Supplementary Figure 2Aiii. Therefore, PCH analysis of GPVI relative to CD86 and CD28 provides evidence that GPVI is expressed as a mixture of monomers and dimers in transfected HEK293T cells. This could therefore indicate that the increase in BRET observed in CRP-stimulated transfected HEK293T cells is due to crosslinking of GPVI by CRP.

Stepwise photobleaching shows GPVI is predominantly monomeric in transfected HEK293T cells when expressed at low level

We used a second advanced microscopy technique, namely single molecule localisation microscopy in transfected HEK293T cells to further investigate the expression of GPVI. Single molecule localisation microscopy is able to resolve proteins to approximately 20 nm which is one order of magnitude below the limit of diffraction-limited microscopy. While this level of resolution and associated brightness is unable to distinguish between monomers and dimers,

this can be addressed through photobleaching and image reconstruction. If a protein is solely monomeric then stepwise photobleaching will result in complete loss of signal in a single spot, whereas it will result initially in partial loss for a dimeric protein. It is crucial to include controls of monomeric and dimeric proteins to enable calibration as results are influenced by overlapping signals and non-fluorescent tags.[18] It is also critical to perform these studies on cells transfected with low levels of receptors to resolve individual monomers and dimers. These considerations prevent similar studies being performed on platelets.

We have performed single molecule localisation microscopy using total internal reflection fluorescence (TIRF) microscopy on the lower plasma membrane of fixed HEK293T cells with low levels of expression of GPVI-eGFP, with CD86-eGFP and CD28-eGFP receptors as controls. TIRF imaging was used to identify single fluorescent protein complexes as individual fluorescent spots (Figure 3Ai). During imaging, eGFP molecules were photobleached over time. Using an automated spot detection algorithm of the entire basal membrane of the cell where detected spots were isolated from background (Figure 3Aii), fluorescence intensity traces for individual spots were extracted and the number of photobleaching steps was determined for each trace (Figure 3Aiii).[18] The number of discrete bleaching steps is equal to the number of eGFP molecules within the protein complex. Data representing all accepted spots for each receptor were pooled into photobleaching step frequency histograms (Figure 3B).

The histograms display a range of photobleaching step frequencies in part because of the presence of non-fluorescent eGFP molecules within the protein complexes due to maturation issues or premature photobleaching, and the presence of overlapping fluorescent spots that cannot be resolved by the automated spot detection algorithms.[18] These occurrences could lead to underestimation or overestimation of bleaching steps and stoichiometry respectively. Therefore the distributions were modelled with functions taking into account a fraction of traces containing overlapping fluorescent spots (double spots) and non-fluorescent eGFP

molecules. Modelling the CD28-eGFP distribution (assuming it is always a dimer) determined the labelling efficiency to be 53% and fraction of double spots to be 33%. (Supplementary Figure 3). This labelling efficiency of eGFP is in the range of previous reports in mammalian cells that have used this approach.[36,37] Modelling the CD86-eGFP distribution (assuming it is always a monomer) determined the fraction of double spots to be 31% consistent with the CD28-eGFP distribution (Supplementary Figure 3). This suggests that just over 30% of spots at this level of expression appear as dimers due to their distribution in the membrane, bearing in mind that CD86 is monomeric.

The majority of CD86-eGFP fluorescent spots bleached in a single step while CD28-eGFP fluorescent spots contained a large proportion of two-step photobleaching. The distributions for CD86 and CD28 were significantly different demonstrating that this technique can distinguish monomers from dimers. The photobleaching step frequency histogram distribution of GPVI-eGFP closely resembled that of CD86-eGFP (Figure 3B) and was significantly different from CD28-eGFP indicating that GPVI is predominately monomeric at this level of expression. These results therefore provide further evidence that GPVI can be expressed on the membrane of HEK293T cells as a monomer.

GPVI dimers are present on resting human platelet membranes

The above studies on the HEK293T cells show that GPVI is expressed as a mixture of monomers and dimers, and provide evidence that ligand binding increases the level of dimerisation. While transfection studies cannot be used in platelets due to their anucleate nature, evidence of dimerisation can be generated by labelling of receptors with suitable fluorescent antibodies and analysis by FRET using flow cytometry. FRET occurs when proteins are within 10 nm of each other with the signal decreasing to the inverse sixth-power.[38]

In this assay, we measured the mean fluorescence intensity (MFI) of a donor antibody alone and in the presence of an acceptor antibody. FRET between the two antibodies labelled with fluorophores whose emission and excitation spectrums overlap leads to a decrease in the MFI of the donor. We labelled Fab fragments of the anti-GPVI mAb A10 with AF488 (donor) and AF546 (acceptor) to investigate the possible dimerisation of GPVI in resting platelets and platelets stimulated by CRP. We also labelled platelets with A10-unlabelled Fab as a control as FRET between AF488 and an unlabelled Fab cannot occur. Antibodies were used at saturating concentrations of 50 μg/ml. We measured the MFI of resting and CRP-stimulated platelets labelled with A10-AF488 (donor) in the absence and presence of A10-AF546 (acceptor) or A10-unlabelled (control) (Figure 4A). A significant decrease in the MFI of the donor antibody (A10-AF488) in the presence of the acceptor antibody (A10-AF546) was observed demonstrating a similar level of FRET for both resting and activated platelets. This is in contrast to the absence of FRET with the donor and unlabelled antibodies (A10-AF488 + A10-unlabelled) (Figure 4A). We determined FRET efficiency between A10-AF488 and A10-AF546 for resting and CRP-stimulated platelets to be 32 and 33% respectively (Figure 4B), indicating that dimerisation of GPVI does not significantly increase upon platelet stimulation by CRP. As a control, we labelled two of the most highly expressed proteins on platelets, integrin αIIbβ3 and GPIb-IX-V with a donor and acceptor antibody pair and measured FRET efficiency. Integrin αIIbβ3 mAb MWReg30 was labelled with AF488 (donor) and GPIX with mAb αGPIX-AF546 (acceptor). The FRET efficiency was 7% (Figure 4B) which we interpret as an upper limit for association of two proteins that are not known to associate in platelets.

This demonstrates that GPVI dimers are present on resting human platelet membranes and dimerisation does not significantly increase upon activation. Furthermore, as the FRET level is higher than the FRET that occurs between the very highly expressed integrin αIIbβ3 and

GPIX, it suggests that this is not solely due to the expression level of GPVI. These studies however do not show the proportion of GPVI expressed as a dimer.

The D2 domain is not critical for adhesion to collagen and signalling in transfected HEK293T cells

The dimerisation of GPVI has been shown to occur through the D2 domain using X-ray crystallography.[9] This is in line with the BRET studies in HEK293T cells described above, in which deletion of this domain prevents dimerisation on the cell surface. This therefore raises the question whether dimerisation through the D2 domain is critical for binding to collagen and activation. We measured adhesion of HaloTag-D2-deleted GPVI transfected DT40 B cells to collagen and monitored activation in these transfected cells which, as a haematopoietic cell, express proteins that are crucial for GPVI signalling.[39] The D2-deleted protein supported a similar level of adhesion of transfected DT40 cells to an immobilised collagenous surface as wild-type HaloTag-GPVI, whereas only minimal adhesion was observed in mock-transfected cells and on a BSA-treated surface (Figure 5B) despite a partially reduced level of expression (~34%) compared to wild-type HaloTag-GPVI (Figure 5Aii). We used the nuclear factor of activated T cells (NFAT) reporter assay to determine whether the D2-deleted receptor could signal in response to collagen and CRP. The NFAT reporter assay is an established technique that detects weak sustained signalling, and is a highly sensitive readout of ITAM receptor signalling.[39] We co-transfected an NFAT-luciferase reporter with GPVI and the FcRγ-chain, which is essential for GPVI signalling, in DT40 cells and measured luciferase activity over 6 hours. Collagen and CRP stimulated a 2.1-fold$\pm$0.17 and 2.0-fold$\pm$0.14 increase in NFAT-luciferase activity over basal respectively with HaloTag-GPVI (Figure 5Ci). Collagen and CRP stimulation of the HaloTag-D2-deleted GPVI showed a smaller increase in NFAT-luciferase activity of 1.5-fold$\pm$0.07 and 1.4-fold$\pm$0.08 ($P < 0.05$) over basal, respectively (Figure 5Ci). One possible explanation for this decrease is the partial reduction in expression compared to

wild-type HaloTag-GPVI (Figure 5Aii). This result shows that signalling can still occur in the absence of the D2 domain but does not rule out that it plays a facilitatory role.

Discussion

The present study was undertaken to investigate whether GPVI is expressed as a dimer in transfected cells and in platelets, and whether the D2 domain is critical for adhesion and activation by collagen in transfected cell line models. The results lead us to conclude that (i) GPVI is expressed as a mixture of monomers and dimers on the surface of transfected HEK293T cells, (ii) the degree of dimerisation increases upon stimulation by CRP in transfected cells, (iii) the D2 domain mediates dimerisation in resting cells, (iv) the D2 domain is not essential for adhesion to collagen or signalling and (v) GPVI is at least partially expressed as a dimer on platelets. Together, the results show that dimerisation of GPVI in transfected cell line models is not essential for activation, thereby refuting the argument that activation of GPVI is critically dependent on a dimer-specific conformation. The increased binding of collagen to recombinant dimeric GPVI can therefore be explained by an increase in avidity.

The original observation of Jung and Moroi[5] that an Fc dimer of GPVI (GPVI-Fc) but not a monomer bound to fibrous collagen raised the possibility of a unique collagen-binding motif in the dimer, although the authors were careful to note that this result could also be explained by an increase in avidity.[5] The development of the dimer-specific antibodies, m-Fab-F[7], 204-11[8] and 9E18[6], provided further evidence for a unique conformation of the dimer, and functional significance of the dimer based on the observation of a reduction in aggregation under flow by mAb 204-11. The conclusion that collagen binds solely to dimeric GPVI has critical implications for the development of blocking antagonists and for understanding GPVI function.

Several observations, including the results of the present study, however, challenge the concept that collagen binds only to a unique conformation in dimeric GPVI. Collagen has now been shown to bind to recombinant monomeric GPVI[11,14,16] and the binding site for CRP on the D1 domain of GPVI is remote from the site of dimerisation. Furthermore, the results of the present study show that collagen is still able to bind to and activate D2-deficient GPVI in transfected cell lines, providing unequivocal evidence against a critical role for dimerisation in the activation of GPVI by collagen. Thus, the greater binding observed by Moroi and Jung to recombinant GPVI-Fc is most likely due to the increase in avidity. However, interestingly, a recent study[40] has found that a small loop in the D2 domain is critical for collagen and CRP signalling. The explanation for this contrasting result is unknown. It is possible that differences in mutagenesis strategies have resulted in differential effects on signalling.

The present study has also investigated the configuration of GPVI on the surface of single HEK293T cells using advanced microscopy and in populations of HEK293T cells and platelets using BRET and FRET respectively. The results demonstrate that GPVI is expressed on HEK293T cells as a mixture of monomers and dimers, with an increase in dimer formation upon stimulation by CRP. The measure of FRET in platelets labelled with donor- and acceptor-labelled antibodies to GPVI shows that the glycoprotein receptor is at least partially expressed as a dimer on resting platelets. The absence of an increase in the FRET signal upon activation by CRP is in agreement with our earlier studies in transfected HEK293T cells in which GPVI was labelled in the cytoplasmic C-terminal tail[10] but not those of the present study in the same cell line when GPVI was N-terminally labelled. This demonstrates that the location of the BRET pairs is critical for detection of a change in energy transfer upon binding of CRP, which can be explained by its exquisite sensitivity to distance (which is inversely proportional to the sixth power of the distance between donor and acceptor). This may therefore explain the lack of change in FRET in activated platelets. An alternative explanation that GPVI is fully

dimerised in platelets seems unlikely given its expression as a mixture of monomers and dimers in transfected cell lines.

The observation that GPVI is expressed as a mixture of monomers and dimers raises the question as to what regulates dimer formation. While this has not been directly addressed in this study, several observations suggest that dimer formation may be regulated by diffusion of GPVI monomers in the membrane and encounters with other GPVI monomers. We presume that there is some affinity between the GPVI monomers, given the importance of clustering in GPVI function[41], and so leads to the formation of a transient dimer. The evidence in this study that leads to this proposal is (i) GPVI is predominantly expressed as monomer in HEK293T cells when expressed at *low level* as shown using single molecule microscopy and photobleaching; (ii) GPVI is mobile in the membrane of HEK293T cells as shown using FCS; and (iii) GPVI associates with two unrelated proteins, CD28 and neuropilin-1, neither of which is expressed in platelets suggesting that there may be a common mode of interaction of membrane proteins. We hypothesise that weak association could occur through low affinity interactions in the membrane due to diffusion and that they are probably short-lived. Measurement of the life time of GPVI dimers on the surface of HEK293T cells and on platelets will help to further investigate this hypothesis.

One of the limitations of this study is the use of transfected cell lines with only the FRET studies being performed on platelets. This was necessary because of the anucleate nature of platelets, which prevents expression of tagged forms of GPVI, and the relatively high level of expression of GPVI which prevents interpretation of single molecule microscopy results, which can only resolve protein location to approximately 20 nm. The relatively high level of expression means that proteins may appear to associate because of their proximity but interpretation of this requires data from proteins that are expressed at a similar level and known to be expressed as monomers or dimers. This was addressed in transfected cell lines using

CD86 and CD28, respectively. Interpretation of results with knock-in mouse models would be challenging due to the lack of tagged monomer and dimer control proteins and the relative high expression of GPVI. Furthermore, techniques that utilise antibody labelling such as stochastic optical reconstruction microscopy (dSTORM) cannot be used to investigate dimerisation due to the multiple blinking events per fluorophore even if the antibody is labelled with a single fluorophore. A further limitation is that most of these studies have been performed in a non-haematopoietic cell line, HEK293T cells, and in the absence of the FcRγ-chain homodimer which associates with GPVI through a salt bridge. It has however previously been shown that GPVI dimerisation in HEK293T cells does not require the FcRγ-chain, and that the FcRγ-chain does not significantly modify GPVI dimeric status.[10] The observation however that collagen and CRP activate the D2-deficient form of GPVI in DT40 cells is in line with the conclusion that dimerisation of GPVI through this region is not required for activation, although this does not rule against a critical role for this domain in supporting optimal activation.

In conclusion, the present observations provide unequivocal evidence that collagen can bind and activate GPVI independent of dimerisation through the D2 domain and that GPVI is predominantly a monomer on the cell surface when expressed at low level. The results also show that GPVI is at least partially expressed in platelets as a dimer. These results have important implications in designing therapeutics that effectively target GPVI in thrombosis. For example, strategies targeting a 'unique-dimeric' conformation of GPVI or the site of dimerisation are unlikely to succeed given that dimerisation is not crucial for activation.

Authors' contributions: J. C. Clark performed experiments, generated constructs, analysed data, wrote and edited the manuscript. R. A. I. Neagoe performed FRET experiments, analysed data, wrote and edited the manuscript. M. Zuidscherwoude performed experiments, generated constructs, provided supervision and molecular biology training and analysed data. D. M. Kavanagh designed experiments and performed FCS and photobleaching experiments, and analysed data. A. Slater, E. M. Martin and M. G. Tomlinson designed constructs and experiments and edited the manuscript. M. Soave provided reagents, constructs and expertise for nanoBRET study design and analysis. D. Stegner, B. Nieswandt and N. S. Poulter have provided reagents, supervision and edited the manuscript. J. Hummert and D. Herten have analysed data and developed the algorithms for photobleaching image analysis and edited the manuscript. S. J. Hill provided supervision, concept, funding and contributed to manuscript editing. S. P. Watson provided supervision, funding, study design and concept, reviewed data, wrote and edited manuscript. All authors have read the manuscript.

Acknowledgement: We thank Laura Kilpatrick and Chloe Peach for providing the HaloTag-NRP1 construct for nanoBRET experiments. The authors would like to acknowledge the Imaging Suite at the University of Birmingham for support of imaging experiments. Imaging facilities used in this project were funded by University of Birmingham, COMPARE and the British Heart Foundation (IG/18/2/33544).

Source of Funding: This study was supported by a grant from the Centre of Membrane Proteins and Receptors, University of Birmingham and University of Nottingham, Midlands, UK and British Heart Foundation (RG/13/18/30563). RN receives funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement (No 766118). SPW is a British Heart Foundation Professor (CH 03/003).

Conflict of Interests: The authors declare no conflict of interest.

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Figure captions

Figure 1. NanoBRET shows GPVI dimers on the cell membrane. (Ai) Schematic representation of N-terminal human nanoluciferase (Nanoluc/NL)-GPVI, HaloTag (HT)-GPVI, Nanoluc-GPVI-D2-deleted and HaloTag-GPVI-D2-deleted constructs. (Aii) The expression of NL-GPVI, HT-GPVI, NL-GPVI-D2-deleted (D2del) and HT-GPVI-D2-deleted (D2del) in HEK293T cells measured by flow cytometry using anti-GPVI HY101 antibody (1:400) with anti-mouse alexa fluor-647 secondary staining (1:400). Black histograms show non-specific secondary staining alone. (Bi) GPVI dimer formation was investigated using transient transfection with a fixed concentration of Nanoluc-GPVI (10 ng/well) and increasing concentrations (2.5-200 ng/well) of HaloTag-GPVI or SnapTag-CD28 or SnapTag-CD86 or HaloTag-neuropilin-1 (NRP1) cDNA (n=3-9). (Bii) GPVI dimer formation was investigated using transient transfection with a fixed concentration of Nanoluc-GPVI or Nanoluc-GPVI-D2-deleted cDNA (10 ng/well) and increasing concentrations (2.5-200 ng/well) of HaloTag-GPVI or HaloTag-GPVI-D2-deleted cDNA (n=3-9). (Biii) GPVI D2-dependent dimerisation was investigated using transient transfection with a fixed concentration of Nanoluc-GPVI or Nanoluc-GPVI-D2-deleted cDNA (10 ng/well) and increasing concentrations (2.5-200 ng/well) of HaloTag-GPVI or SnapTag-CD86 (n=3-9). (Biv) The effect of CRP (2 & 10 μg/ml) on GPVI dimerisation was investigated using transient transfection with a fixed concentration of Nanoluc-GPVI (10 ng/well) and increasing concentrations (2.5-200 ng/well) of HaloTag-GPVI cDNA (n=3-9). (Bv) The effect of CRP (2 μg/ml) on D2-deleted Nanoluc and HaloTag-GPVI interactions was investigated using transient transfection with a fixed concentration of Nanoluc-GPVI or Nanoluc-GPVI-D2-deleted (10 ng/well) and increasing concentrations (2.5-200 ng/well) of Halotag-GPVI or HaloTag-GPVI-D2-deleted cDNA (n=3-9). Significance of the highest concentration was measured with Student unpaired *t* test where $P \leq 0.05$. * = statistical significance compared to wild-type Nanoluc-GPVI + HaloTag-GPVI and # = statistical significance compared to Nanoluc-GPVI D2del + HaloTag-GPVI D2del. Representative data presented as mean±SEM. Each experiment performed in triplicate**.**

Figure 2. Characterisation of the membrane dynamics and molecular brightness of GPVI, CD86 and CD28 using fluorescence correlation spectroscopy (FCS). (Ai) Schematic representation of C-terminal CD86-eGFP monomer control, CD28-eGFP dimer control and human GPVI-eGFP. eGFP tags have an A206K mutation to prevent eGFP dimerisation. (Aii) Representative confocal microscopy images of transfected HEK293T cells showing membrane localisation of CD86-eGFP, CD28-eGFP and GPVI-eGFP (field of view = 52 x 52 μm) (scale bar = 5 μm). (Aiii) Representative raw autocorrelation data for CD86 (red), CD28 (blue) and GPVI (green) with the autocorrelation 1-component fits (black curves). Mean diffusion coefficients (D, $\mu m^2\ s^{-1}$) and count rates (CR, kHz, the average fluorescence intensity) for CD86, CD28 and GPVI are displayed. (Aiv) Box plot of CD86, CD28 and GPVI diffusion coefficient data in HEK293T cells. The diffusion coefficients were calculated from the derived autocorrelation fits. (B) Oligomerisation of CD86, CD28 and GPVI was determined by photon counting histogram (PCH) analysis. (Bi) Representative raw PCH distributions for CD86 (dotted red line), CD28 (dotted blue line) and GPVI (dotted green line) with 1-component PCH model fitting (black lines). Mean molecular brightness (ε, counts per molecule per second (cpm s^{-1})) and number of fluorescent molecules within the volume (N) for CD86, CD28 and GPVI are displayed. (Bii) Box plot of CD86, CD28 and GPVI molecular brightness data in HEK293T cells. The molecular brightness data were calculated from the derived PCH fits. For all box plots, centre lines represent the median; box limits indicate the 25^{th} and 75^{th} percentiles and whiskers extend to minimum and maximum points. Significance was measured with Kruskal-

Wallis with Dunn's *post-hoc* where $P \leq 0.05$. FCS measurements were taken in 59-66 cells (n=6-7).

Figure 3. Stepwise photobleaching analysis shows GPVI is predominately a monomer. (Ai) Representative total internal reflection fluorescence (TIRF) microscopy images with average intensity projections of the first 100 frames of the basal plasma membrane of transfected HEK293T cells expressing CD86-eGFP, CD28-eGFP and human GPVI-eGFP showing individual fluorescent spots (field of view = 40 x 40 μm) (scale bar = 10 μm). (Aii) Representative image of the fluorescent spot detection following application of an automated spot detection algorithm of the entire basal membrane of the cell expressing GPVI-eGFP. Detected spots included in the analysis are shown in orange and detected background is shown in yellow in panels 1 and 2 (scale bar = 5 μm). (Aiii) Example fluorescence intensity decay traces (grey) for individual spots showing discrete photobleaching steps determined following application of the algorithm (orange line). (B) Photobleaching step frequency histograms determined from all accepted spots for CD86-eGFP (red), CD28-eGFP (blue) and GPVI-eGFP (green) in HEK293T cells. Significance of the distributions was measured with Epps-Singleton 2 sample test where $P \leq 0.05$. * = statistical significance compared to CD86-eGFP and NS = not significantly different compared to CD86-eGFP. GPVI-eGFP was also significantly different from CD28-eGFP. Data pooled from 348-464 traces.

Figure 4. GPVI is partially expressed as a dimer on platelets. The FRET efficiency was calculated by dividing the mean fluorescence intensity (MFI) of the donor fluorophore in the presence of the acceptor fluorophore by the MFI of the donor alone. (A) Resting human platelets (red) were labelled with anti-GPVI F(ab') fragments, A10-AF488 (donor) (50 μg/ml) alone and together with A10-AF546 (acceptor) (50 μg/ml) or A10-unlabelled (control) (50 μg/ml) and activated samples (blue) were stimulated with CRP (10 μg/ml). The MFI's (FL1 channel) of the samples were measured by flow cytometry. (B) The FRET efficiency of GPVI under resting (red) and activated (CRP, 10 μg/ml) (blue) conditions and integrin αIIbβ3 + GPIX control pair was calculated as described above. GPVI was labelled as described in (A). Resting mouse washed platelets were labelled with anti-integrin αIIbβ3 mAbs, MWReg30-AF488 (donor) (10 μg/ml) and anti-GPIX mAb-AF546 (acceptor) (10 μg/ml). The MFI's (FL1 channel) of the samples were measured by flow cytometry and the FRET efficiencies were calculated (n=2-3). AF= Alexa Fluor. Significance was measured with either a two-way for (A) or one-way for (B) ANOVA with a Bonferroni *post-hoc* test where $P \leq 0.05$. NS= not significant. Data are presented as mean±SEM.

Figure 5. The D2 domain is not critical for adhesion to collagen and signalling. (Ai) Schematic representation of N-terminal human HaloTag-GPVI and HaloTag-GPVI-D2-deleted receptors. (Aii) The expression of HaloTag (HT)-GPVI and HT-GPVI-D2-deleted (D2del) in DT40 chicken B cells was measured by flow cytometry using anti-GPVI HY101 antibody (1:400) with anti-mouse alexa fluor-647 secondary staining (1:400). NFAT-luciferase reporter and FcRγ-chain constructs were also co-transfected. Black histograms show non-specific secondary staining alone. (B) Mock transfected or wild-type (HT-GPVI) or D2-deleted (HT-GPVI D2del) GPVI with FcRγ-chain transfected DT40 cells ($2x10^6$/ml) were allowed to adhere to collagen-coated coverslips or BSA-coated coverslips for 1 h at 37ºC. Three fields of view per condition per experiment were captured with an EVOS FL cell imaging inverted microscope and adherent cells were counted manually (field of view = 1152 x 864 μm). (Bi) Representative zoomed-in images of mock or the indicated GPVI construct transfected DT40 cells adhered to collagen (field of view = 567 x 432 μm) (scale bar: 200 μm). (Bii)

Quantification of DT40 cell adhesion. # = statistical significance compared to control collagen condition and * = statistical significance compared to counterpart collagen condition (n=3). (Ci) DT40 cells were transfected with an NFAT-luciferase reporter construct, FcRγ-chain and either 2 μg of wild-type GPVI (HT-GPVI) or D2-deleted (HT-GPVI D2del) GPVI constructs. Cells were either unstimulated or stimulated for 6 h and then lysed and assayed for luciferase activity. Luciferase activity normalised for basal values for wild-type HaloTag-GPVI and HaloTag-GPVI D2del unstimulated and stimulated with collagen (10 μg/ml) or CRP (10 μg/ml). Each experiment performed in triplicate. * = statistical significance compared to basal. Significance was measured with either a two-way ANOVA with a Bonferroni *post-hoc* test for (Bii) or Student two-tailed *t* test for (Ci) where $P \leq 0.05$. Data are presented as mean±SEM (n=3-9).