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Structure-function relationship of the platelet glycoprotein VI (GPVI) receptor: does it matter if it is a dimer or monomer?

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Abstract

GPVI is a critical signalling receptor responsible for collagen-induced platelet activation and a promising anti-thrombotic target in conditions such as coronary artery thrombosis, ischemic stroke and atherothrombosis. This is due to the ability to block GPVI while having minimal effects on haemostasis, making it a more attractive target over current dual-antiplatelet therapy (DAPT) with acetyl salicylic acid and $P2Y_{12}$ inhibitors where bleeding can be a problem. Our current understanding of how the structure of GPVI relates to function is inadequate and recent studies contradict each other. In this article we summarise the structure-function relationships underlying the activation of GPVI by its major ligands, including collagen, fibrin(ogen), snake venom toxins and charged exogenous ligands such as diesel exhaust particles. We argue that contrary to popular belief dimerisation of GPVI is not required for binding to collagen but serves to facilitate binding through increased avidity, and that GPVI is expressed as a mixture of monomers and dimers on resting platelets, with binding of multivalent ligands inducing higher order clustering.

Introduction

Glycoprotein VI (GPVI) has been an intense area of research since its recognition as the major signalling receptor in platelets for collagen in the late 1990's.[1] Surprisingly however, our understanding about how the structure of GPVI relates to function is not well understood. This is particularly apparent when discussing whether a unique dimeric conformation is required for signal transduction, with studies continuing to contradict each other. In this review, we will summarise the evidence that GPVI is expressed as both a monomer and dimer on platelets, and whether the ability of the dimer to bind selectively to collagen is due to its increased avidity. We discuss this in light of the recent demonstration that the synthetic collagen mimetic, collagen-related-peptide (CRP), which consists of cross-linked glycine-proline-hydroxyproline (GPO) repeats, binds to the D1 immunoglobulin (Ig) domain. Understanding the configuration and conformation of GPVI in platelets is critical for designing therapeutics that effectively target GPVI in thrombosis.

The GPVI-FcRγ-chain complex

GPVI is expressed exclusively on megakaryocyte and platelet membranes, with levels ranging from 3000-4000 copies per human platelet.[1-3] Human and mouse GPVI were first cloned in 1999 and 2000 respectively.[4-6] Human GPVI is composed of 319 amino acids (339 with the signal sequence) and has a molecular weight of 62 kDa following SDS gel electrophoresis of which 34.9 kDa is the amino acid weight. GPVI belongs to the Ig receptor superfamily and has two extracellular Ig domains (D1 and D2), a mucin-rich stalk which contains sites of *O*-glycosylation, a single transmembrane helix and a cytoplasmic tail of 51 amino acids (Figure 1). Human and mouse GPVI share 64% sequence homology at the protein level. Mouse GPVI cytoplasmic tail lacks 24 amino acids that lie C-terminal to the human GPVI proline-rich region.[1,6,7] Close to the extracellular portion of the transmembrane region of GPVI there is a positively charged arginine that is essential for forming a salt bridge with the FcRγ-chain

homodimer.[8,9] Thus each GPVI monomer is associated with two FcRγ-chains.[10] The coupling to the FcRγ-chain is necessary for the expression of GPVI on mouse platelets and interestingly on some but not all cell lines.[8-9,11]

The FcRγ-chain acts as the signalling arm in this complex and contains an immunoreceptor tyrosine-based activation motif (ITAM) defined by the sequence of two YxxL groups (single amino acid code) separated by 12 amino acids (Figure 1). The GPVI-FcRγ-chain-ITAM interaction is critical for early signalling events and downstream signalling. The discovery of the first and most characterised endogenous ligand for GPVI, collagen, came in 1987 through studies on a patient with autoantibodies to GPVI and loss of the glycoprotein receptor on the platelet surface.[12] More recently, many other endogenous and exogenous ligands have been shown to activate GPVI including extracellular matrix proteins such as laminin, vitronectin and fibrin(ogen), a variety of snake venom toxins of which convulxin is the best known, and a miscellaneous group of charged exogenous ligands including diesel exhaust particles (for references, see below).

Mechanism of GPVI signalling: recent updates

There have been many reviews of GPVI signalling.[1,2,6,13] In this section, the key features of the GPVI signalling cascade, and recent updates are summarised. GPVI cross-linking induces tyrosine phosphorylation of the FcRγ-chain ITAM by the Src family kinases Fyn, Lyn and Src (Figure 2).[13,14] Fyn and Lyn are constitutively bound to the proline-rich region of the cytoplasmic tail of GPVI and localised to cholesterol rich regions in the membrane through N-terminal palmitoylation.[13,15] These kinases have been shown to have overlapping roles in regulating GPVI signalling.[16] Lyn plays a dominant role in GPVI-mediated platelet activation with Lyn-deficient platelets showing a delayed onset of aggregation. Fyn or Src-deficient platelets showed only a marginal reduction and unaltered onset of aggregation, respectively.

However double deficient Lyn/Fyn and Lyn/Src platelets showed a further delay in CRP-induced aggregation indicating that all three kinases work together to mediate GPVI signalling.[16] ITAM phosphorylation leads to the recruitment and binding of the tyrosine kinase Syk through its tandem SH2 domains where it undergoes autophosphorylation and further Src family kinase-mediated phosphorylation.[13] A study using mouse platelets expressing a mutant Syk (R41A), where the binding of the N-terminal SH2 domain to a phosphorylated YxxL sequence was disrupted, found abolished collagen and CRP-induced platelet aggregation,[17] confirming that the binding of Syk N-SH2 domain is critical for downstream signalling. Following ITAM binding, Syk initiates a downstream signalling cascade leading to the formation of a multi-protein complex termed a signalosome, located at the cell membrane (Figure 2).[13,18] The transmembrane adaptor protein, linker of activated T-cells (LAT) and the cytosolic adaptors SLP-76, Gads and Grb2 are responsible for forming the intracellular framework of the signalosome that regulates the cellular location of effector and kinase proteins.[13,18,19] Grb2 has recently been shown to have a dominant role and the related family member Gads a supportive role.[20] Single-deficient platelets only had partial defects in ITAM signalling due to the redundancy of the two proteins, while Grb2/Gads double-deficient platelets had abolished ITAM signalling. This study also identified a possible positive feedback loop on Syk activation via Grb2/Gads.[20]

Formation of the signalosome allows effector proteins including the tyrosine kinases Btk and Tec to come into contact with their substrate which is key for efficient downstream signal transduction and for the regulation and activation of phospholipase C γ2 (PLCγ2). In humans, inhibitors of Btk such as ibrutinib and acalabrutinib, or the absence of Btk (which gives rise to the immunodeficiency syndrome X-linked agammaglobulaemia), have been shown to inhibit the response to low concentrations of GPVI agonists and to delay the response to higher concentrations despite the marked loss of phosphorylation of PLCγ2.[21] While one explanation

for this could be the presence of the related kinase, Tec, this is also inhibited over similar concentrations by most of the current 'Btk' inhibitors (albeit there are discrepant reports on this[21-23]). Furthermore Btk has been shown to support PLCγ2 activation and GPVI signalling in transfected cell lines by acting as both an adaptor protein and a tyrosine kinase.[21] An inhibitor of Btk that has a greater selectivity over Tec and no other off-target effects is required to resolve these two explanations.

Activation of GPVI by collagens, snake venom toxins, antibodies and additional ligands

GPVI ligands activate GPVI by higher order receptor clustering due to their multivalency (Table 1). The synthetic collagen mimetic, CRP, consists of ten repeats of the GPVI binding motif, GPO, cross-linked by cysteine or lysine residues.[24] Because of this variable cross-linking, the potency of each batch of CRP needs to be tested by bioassay as the final product is a mixture of dimers, trimers and high order polymers. CRP induces powerful activation of human and mouse platelets, with its potency dependent on the number of GPVI receptors. This is illustrated by studies in transgenic mice models with 50% and 20% the normal level of GPVI.[25] The concentration response for aggregation to CRP is shifted tenfold in mice with a 50% reduction in GPVI and aggregation is lost in mice with 20% of the normal level.[26] This illustrates that binding and activation of GPVI by CRP is dependent on the combination of affinity and avidity, with a greater degree of clustering occurring in cells with a greater number of receptors. A similar relationship is not seen with collagen even though it also induces clustering of GPVI as shown using single molecule super-resolution microscopy.[26] This is because in addition to the GPO sequence, which is found throughout most collagens including types I and III which are present in the sub-endothelial matrix, collagen also contains sequences that mediate high affinity binding to a second receptor on platelets, integrin α2β1.[27,28] The binding of collagen to α2β1 strengthens adhesion, as well as modulates signalling, and lessens the dependency on the density of GPVI.

The binding site for CRP on GPVI has recently been shown to reside on the D1 domain using X-ray crystallography (PDB 5OU8 and 5OU9) (Figure 3). The CRP binding site is found within a groove made by the β-3 and β-5 strands and polar contacts are made with the GPVI residues R38, E40, R67, Q71 and W76. This binding site is directly next to the N92 glycosylation site, and although no direct contacts are made with this residue or the sugar, glycosylation could be important for positioning the binding residues in optimal conformations for binding.

Two families of snake venom toxins have been shown to activate GPVI, namely C-type lectin-like and metalloproteinase-disintegrins. The C-type lectin-like convulxin, consists of a dimer of cyclic heterotetramers composed of α and β subunits $(\alpha_4\beta_4)_2$.[29] This structure provides binding for up to 8 individual GPVI receptors and is capable of bridging GPVI on adjacent surfaces.[30] Convulxin binds to GPVI with a fast on-rate of association and slow off-rate, with a dissociation constant (K_D) for binding to a single GPVI of 0.8-3 nM giving an overall picomolar affinity because of the added effect of avidity. Convulxin has been proposed to bind to sites on the D1 and D1/D2 interface which are distinct from the binding site of collagen and CRP.[29,30] This is illustrated by the ability of Fabs of the mAbs 1G5 and 12A5 to block CRP- and collagen-induced aggregation but not convulxin-induced aggregation.[31] On the other hand, the Fab fragment of 9O12.2 has been shown to delay platelet aggregation in response to convulxin and to block binding of soluble GPVI to collagen and convulxin suggesting that the binding sites are close to each other (Figure 3).[32,33] Other examples of C-type lectin-like snake toxins include ophioluxin, alboagregin-A and alboluxin, with the latter two also binding to GPIbα. The three toxins are structurally similar to convulxin and, like convulxin induce potent activation of platelets.[34-37] The metalloproteinase snake venom toxin, alborhagin, also causes powerful platelet activation through binding to GPVI.[38]

GPVI can also be activated in mouse and human platelets by mAbs with activation strengthened by cross-linking. In human platelets, activation by whole antibodies can be

dependent on or mediated by the low affinity immune receptor FcγRIIA, and this can be prevented by use of $F(ab)_2$ fragments or inclusion of the FcγRIIA-blocking Fab or $F(ab)_2$ of mAb IV. Al-Tamimi *et al*[31] described the activation of human platelets by seven mAbs, 12H1, 1A12, 12C9, 1G5, 4B8, 12A5 and 12E2, and showed that activation was independent of FcγRIIA demonstrating that dimerisation is sufficient to induce activation of GPVI. Only the Fab fragments of 1G5 and 12A5 were able to inhibit collagen- and CRP-induced aggregation showing that the other antibodies bind to different sites on GPVI and yet all seven can induce activation.[31] Together, these results demonstrate that dimerisation of GPVI is sufficient to mediate activation and that this can occur at epitopes distinct from the binding site of collagen and CRP.

These observations demonstrate that dimerisation is sufficient to mediate activation of GPVI and may help to explain the large number and diversity of endogenous and exogenous ligands that have been shown to activate the glycoprotein receptor (Table 1). These include negatively charged surfaces such as glass and diesel exhaust particles which may bind primarily through an electrostatic interaction, for example through the positive charges at K41, K59, R60 and R61 in GPVI.[39] The list of endogenous ligands include laminin[40], adiponectin[41], vitronectin[42], fibronectin[43,44] and CD147.[45,46] For many of these, the affinity for GPVI is low and of uncertain physiological significance. GPVI can also be activated by amyloid beta peptides[47], the main component of the amyloid plaques in the brains of Alzheimer's patients, and this may have significance in dementia.

Recently, there has been interest in fibrin and fibrinogen as ligands for GPVI due to their central role in haemostasis and thrombosis. Both ligands have been shown to activate GPVI signalling in human platelets and in humanised mouse platelets, with fibrinogen unable to activate wild type mouse platelets suggesting that it does not bind to mouse GPVI.[48-53] The absence of activation of GPVI by fibrinogen in suspension in the blood can be explained by the low affinity

interaction and the inability to induce dimerisation or higher order clustering. While the functional evidence for activation of GPVI by fibrin and fibrinogen is strong, there is controversy, as to whether binding is to monomeric or dimeric GPVI, or indeed to either form of the Ig receptor.[19] In some studies, binding has been reported to occur exclusively to monomeric recombinant GPVI[48,50,51,53] and in others to dimeric GPVI.[49,52] While this controversy may be partly explained by differences in the sequence of recombinant GPVI and other experimental variables, we have been unable to show robust binding of GPVI transfected cells to either ligand (unpublished) despite robust binding to collagen suggesting that binding and/or activation may be dependent on one or more other platelet receptors such as integrin αIIbβ3. The binding to a second receptor could increase binding of low affinity epitopes in fibrin and fibrinogen to GPVI or, more speculatively, induce activation of GPVI by associating in trans or possibly through altering its distribution relative to tyrosine phosphatases and kinases. The latter suggestion, which does not require direct binding to GPVI, may also explain why such a wide spectrum of ligands have been shown to activate GPVI. The mapping of the binding site of fibrin, fibrinogen and the other ligands described above is required to confirm direct activation of GPVI.

While collagen is still considered to be the major endogenous ligand for GPVI, it is clear that a wide spectrum of endogenous and exogenous ligands can activate the Ig receptor as a result of dimerisation and higher order clustering. This can occur through multiple sites of interaction on GPVI and can be facilitated by binding to other surface receptors. In addition, we speculate that GPVI may be activated through an alteration in distribution in the membrane in the absence of direct ligand binding, although there is no direct evidence for this.

Evidence of dimerisation of GPVI

There has been considerable debate on the configuration of GPVI in the membrane of resting and activated platelets, including whether it is a monomer or dimer, whether the dimer has a

unique conformation and whether the level of the dimers increases upon activation. Understanding the configuration on the platelet surface is important for the design of high affinity inhibitors that potentially selectively block receptor binding of endogenous and pathological ligands. Below, we present the evidence in support of dimerisation and formation of a distinct epitope and then critically review this in light of the recent finding the CRP binds to the D1 domain.

In 2002, the group of Jung and Moroi[54] reported that an Fc dimer of GPVI (GPVI-Fc) bound to fibrous collagen with a K_D of 576 nM but that fibrous collagen had no affinity towards monomeric GPVI. They further showed that GPVI-Fc but not monomeric GPVI was able to inhibit collagen-induced platelet aggregation.[54] From these studies, they concluded that collagen only showed high affinity for a dimeric form of GPVI, possibly due to increase in avidity or the presence of a unique epitope. The structure of the human GPVI D1 and D2 extracellular domains was solved by X-ray crystallography in 2006, and revealed a potential site of dimerisation between the β-strands within the D2 domain.[55] Recombinant monomeric GPVI was however found to be monomeric in solution even at high concentration suggesting that the dimerisation may have been driven by the massively increased local concentration of GPVI during crystallisation. The fact that GPVI *can* dimerise is not surprising given that it is activated by clustering.

Our own studies using C-terminal tagged versions of GPVI provided further support for the presence of a dimer and/or higher order clusters of GPVI in transfected cell lines.[56] Using bioluminescence resonance energy transfer (BRET) of GPVI, we reported that GPVI produced a specific BRET signal in transfected HEK293T cells that was intermediate between that of a known monomer (CD2) and dimer (CTLA-4), with the degree of dimerisation only marginally increased by expression of the FcRγ-chain.[56] This suggests that GPVI is expressed as a mixture of monomer and dimers and that this is not dependent on the FcRγ-chain. One surprising

observation from this study however was that neither collagen nor convulxin were able to increase the BRET signal suggesting that binding may only have occurred to the dimeric form of the receptor or that clustering of the extracellular domain does not bring the intracellular tails close enough together to increase the signal. In addition, in the same study we provided additional evidence of dimerisation through the co-immunoprecipitation with myc- and flag-tagged versions of GPVI and CD2-GPVI chimeras, with dimerisation being mediated by the extracellular domain of GPVI, and by the use of a chemical cross-linker which demonstrated the presence of dimers and higher order oligomers in platelets.[56] These observations strongly suggest that GPVI is expressed as a combination of monomers and dimers in a transfected cell line and raises the surprising possibility that ligands only bind to the dimeric form.

In addition to above, the activation of GPVI in platelets has also been shown to lead to formation of an inter-molecular disulphide bond via C338 within its intracellular tail[57], providing a separate mechanism of dimerisation which may be a consequence of ligand-induced dimerisation. However, this cannot explain the dimerisation in resting cells and the overall significance of this result remains unclear as the C-terminal cysteine is absent in mouse GPVI.

In 2009, the group of Moroi and Jung provided direct evidence for expression of a dimeric form of GPVI on platelets through the generation of a dimer-specific Fab by phage display.[58] The monomeric Fab, termed m-Fab-F, bound to GPVI-Fc but not to monomeric GPVI, and bound to platelets as shown by flow cytometry. Furthermore, high concentrations of m-Fab-F (60 μg/ml) partially inhibited platelet aggregation by collagen. These data provided the first direct evidence of a unique conformation in GPVI-Fc that is also present in platelets and which is recognised by collagen. Furthermore, in a follow up study, Jung *et al* reported that m-Fab-F blocked collagen adhesion under flow showing that dimerisation is critical for platelet

activation.[59] However it was not possible to quantitate the degree of dimerisation of GPVI on platelets using m-Fab-F due to its low affinity (K_D = 408 nM).

Jung and Moroi have since identified a second Fab to GPVI, 204-11, which although raised against the monomer, bound selectively to GPVI-Fc with high affinity (K_D = 1 nM). mAb 204-11 binding increases the binding of m-Fab-F demonstrating that the two antibodies bind to distinct sites on GPVI and that mAb 204-11 must therefore induce a conformational change.[58] Significantly, Jung and Moroi reported increased binding of m-Fab-F and Fab 204-11 upon activation of platelets by CRP and thrombin suggesting that the number of dimers increases upon activation. Using Fab 204-11, they estimated that approximately 29% of GPVI is dimeric in resting platelets and that this increases to 40% and 44% upon stimulation by CRP and thrombin, respectively.[59]

A study from the group of Jandrot-Perrus *et al*[60] in the same year also described a potent dimer-specific antibody, 9E18, that exhibited over 200-fold selectivity to dimeric over monomeric GPVI. The K_D for binding of 9E18 to dimeric GPVI-Fc was estimated to be 0.36 nM. Strikingly, 9E18 bound to less than 2% of total GPVI in whole blood but this value increased to 14% in washed platelets and by up to 36% upon stimulation by TRAP, ADP or PMA, whereas binding of a second antibody to GPVI, 3J34 was not altered.[60] The degree of binding of 9E18 was also increased in shear activated platelets. The increase in binding was reversed by cAMP and cGMP-elevating agents, including forskolin, PGE1 and the NO donor SNAP. The molecular basis of this is not known but this does not appear to be of functional relevance as elevation of cAMP has no effect on GPVI signalling[61], suggesting that the conformation recognised by 9E18 is distinct from that recognised by the two antibodies from the group of Moroi and Jung.

Together, these results strongly suggest the presence of a GPVI dimer on platelets that can be recognised by three dimer-specific antibodies, with at least two of these, 204-11 and Fab-F, binding to distinct sites. All three antibodies demonstrate an increase in GPVI dimers on activated platelets by up to 44% and, in the case of 9E18, that dimerisation is inhibited by cyclic nucleotides, although this appears to have no functional significance.

Taken together, it is clear that GPVI exists on platelets in a mixture of monomers and dimers, and potentially higher oligomers. The dimerisation of GPVI in basal conditions may explain the weak phosphorylation of the FcRγ-chain that is seen in washed platelets and the ability of GPVI to signal constitutively in transfected cell lines measured using a NFAT reporter.[62] The observation that dimerisation is inhibited by cAMP and cGMP provides a potential mechanism to counter this constitutive signalling and helps to prevent platelet activation in the vasculature. Even so, it is interesting to note that elevation of cAMP has no effect on the ability of GPVI to signal.[61]

The significance of dimerisation in the activation of GPVI

The observation that GPVI exists as a mixture of monomers and dimers on the platelet surface, and that the dimer presents one or more additional epitopes, does not mean that dimerisation is a prerequisite to binding of collagen. Indeed, the observations that GPVI may exist predominantly as a monomer in whole blood (as shown using mAb 9E18), that CRP binds at the D1 domain, which is remote from the site of dimerisation, and that a variety of agents can activate GPVI through binding to distinct sites, suggests that the creation of a unique conformation in the dimer for activation (and indeed for binding to collagen) is not critical. Rather the existence of the dimer might be due to the density of GPVI receptors in the membrane and the complementarity of its D2 domains which enables dimerisation to occur. Such complementarity is essential for a receptor that has to be cross-linked for activation. This is consistent with the distribution of GPVI on platelets as shown using single molecular super-

resolution microscopy. This shows a mixture of monomers, dimers and higher order clusters as might be expected for a highly expressed protein rather than a uniform distribution.[26] Furthermore, GPVI surface expression may also depend on other platelet receptors such as GPIb-IX-V where direct association of the two receptors has been reported and collagen and CRP-induced aggregation is blocked by the anti-GPIbα-specific mAb, SZ2.[63] Additionally in a recent study using the nanoBRET assay, a specific BRET signal was observed between GPVI and two unrelated proteins, neuropilin-1 and CD28 (Clark et al., submitted). This raises the possibility that the relative surface density of other receptors could indirectly affect GPVI dimerisation. The regulation of GPVI surface density could also be affected by ectodomain shedding of GPVI in platelets which recently has been shown to be associated with increased bleeding risk.[64]

We feel it is therefore justified to propose that monomeric GPVI is functional and can bind to ligands including collagen which induces cross-linking and higher order receptor clustering due to their multivalent nature. Dimerisation in the resting membrane would provide a mechanism to actively bring binding sites on GPVI closer together rather than form a unique epitope thereby facilitating activation through the combination of affinity and avidity.

Concluding remarks and targeting GPVI in disease

The ability to bind to and multimerise GPVI either as a monomer or as a dimer has important implications for the design of receptor-specific inhibitors. We now know where CRP binds to GPVI and it seems likely that this is where collagen also binds as the two ligands compete in binding to GPVI. Therefore there is no need to introduce the idea of a unique conformation that binds to collagen with the higher affinity of GPVI-Fc over monomeric GPVI being attributed to avidity. It is also clear that GPVI can be cross-linked by a wide spectrum of reagents which bind at distinct sites. While this opens up the possibility of selectively blocking each interaction, the sites of binding need to be mapped and, for the most part, the significance

of activation other than by collagen needs to be proven. There is also the possibility of targeting GPVI by prevention of clustering by blocking the interaction between the two D2 domains. However, attempts to prevent activation by mutagenesis or deletion of this region have proved unsuccessful (Mike Tomlinson, personal communication) suggesting that the site of interaction is relatively large and therefore not drugable by small molecule inhibitors. In addition the observation that GPVI is activated by clustering, and that the binding region of GPVI with CRP covers a large area, makes it very unlikely that we will be able to identify small molecules with affinities of nanomolar and below and slow off-rates of association to out compete the multimeric nature of collagen. Thus, Fabs of blocking antibodies or nanobodies are likely to remain the major route of specific inhibition of GPVI.

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Declaration of interest

The authors report no conflict of interest.

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Figure legends

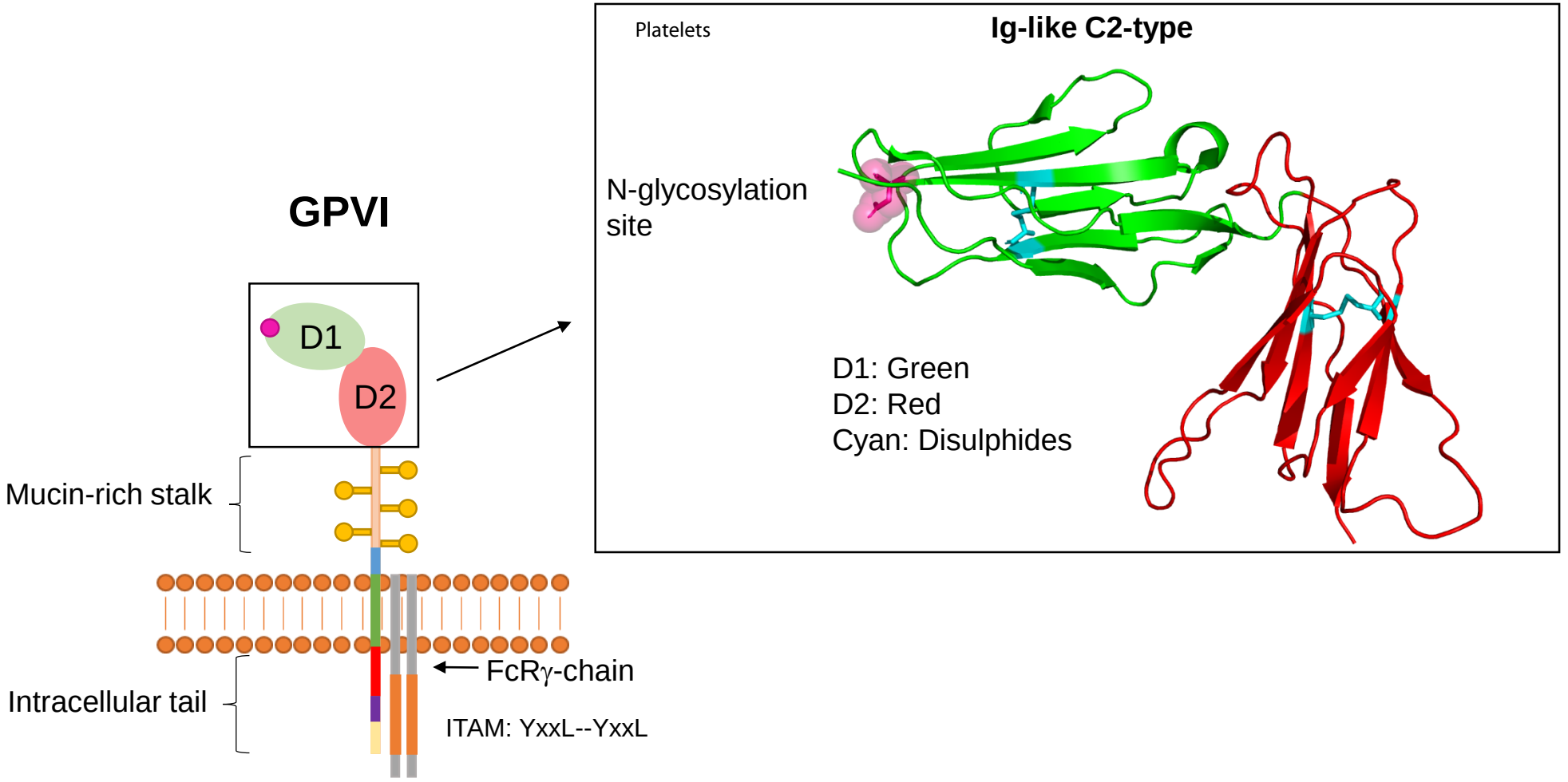
Figure 1. The structure of GPVI. A cartoon representation of GPVI consisting of two extracellular C2-type immunoglobulin (Ig) D1 and D2 domains, a mucin-rich O-glycosylated stalk, a transmembrane region and a short intracellular tail. The transmembrane region and tail are divided into five regions, each with distinct sequence motifs. GPVI is coupled to the FcRγ-chain via a salt bridge which contains an ITAM signalling motif by disulphide bridges. The crystal structure of D1,D2 (PDB:2GI7) is also shown highlighting disulphide bridges and the N-glycosylation site.

Figure 2. GPVI signalling cascade. GPVI cross-linking results in tyrosine phosphorylation of the ITAM found in the FcRγ-chain by Src family kinases (SFK) which are bound to the proline-rich region of GPVI. This leads to Syk engagement and phosphorylation at the FcRγ-chain and initiation of a Syk-dependent signalling cascade where a LAT signalosome consisting of adaptor, effector and kinase proteins is formed. PLCγ2 is then activated which catalyses the formation of DAG and IP_3 from PIP_2, triggering elevation of intracellular Ca^{2+}, PKC activation and ultimately platelet activation.

Figure 3. GPVI crystal structure (PDB:2G17) with all known or proposed ligand binding sites highlighted as spheres. D1 and D2 domains are coloured in green and red respectively, and all known ligand binding sites currently reside at D1. The D2 dimer interface is coloured in yellow. The CRP binding site (blue) has been mapped by x-ray crystallography whereas key 9O12.2 binding residues (purple) have been identified through mutagenesis studies.

Table 1. A summary of GPVI ligands, their multivalency and the conditions and nature of their activation of GPVI. ND = Not determined

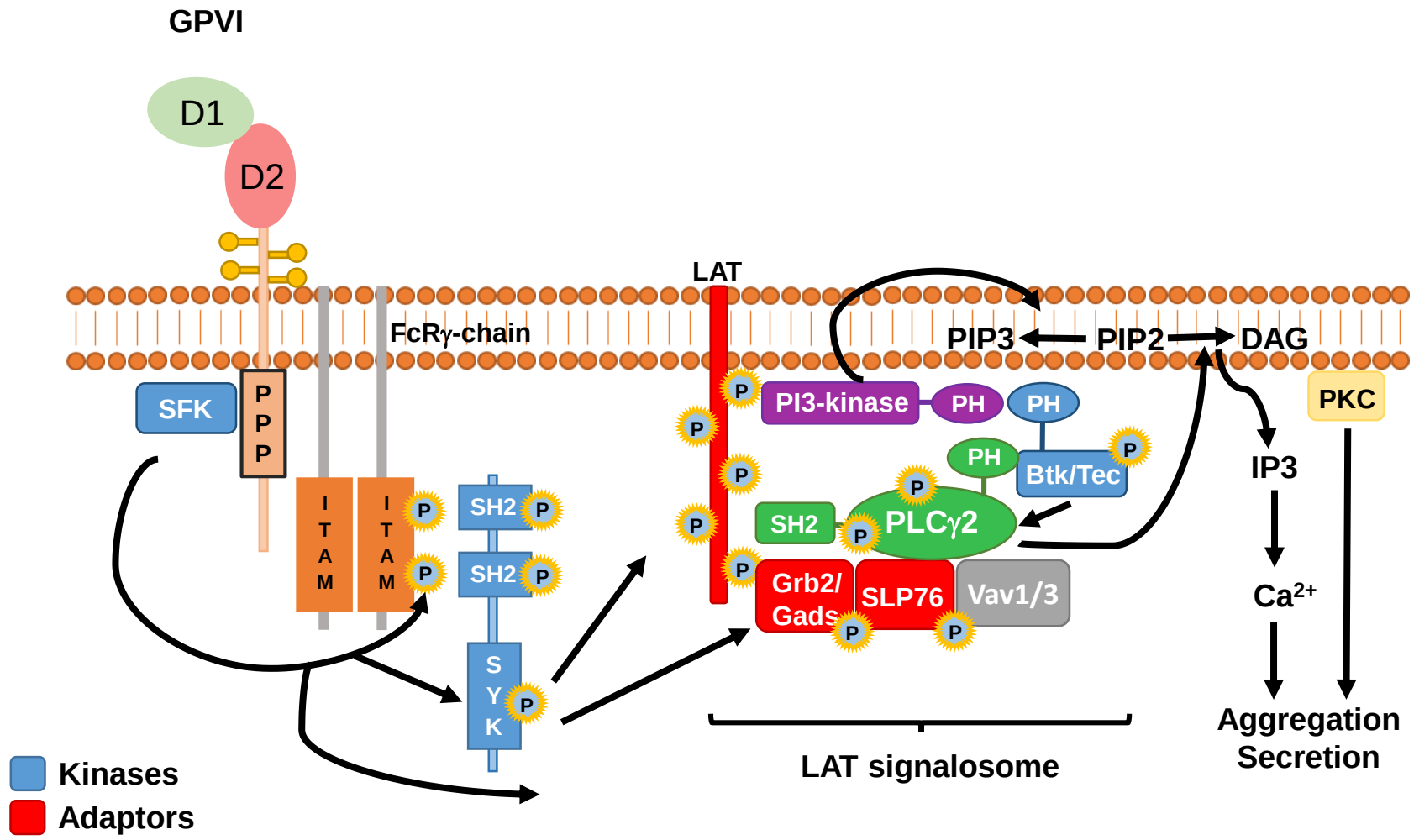
GPVI ligand	Multivalent structure	Activate in solution	Activate on a surface	Nature of activation of GPVI	Binding site on GPVI
Collagen & CRP-XL[24,26,27]	Yes	Yes	Yes	Cross-linking and clustering by binding	D1
Snake venom toxin C-type lectins-like: Convulxin[29,30]	Yes	Yes	Yes	Large cross-linking and clustering by binding	Proposed: D1 & D1/D2 interface
Snake venom toxin Metalloproteinases: alborhagin[38]	Yes	Yes	Yes	Large cross-linking and clustering by binding	Unknown
Cross-linking antibodies: JAQ1[65], 1G5[31]	Yes	Yes – when cross-linked	ND	Cross-linking and clustering by binding	Unknown
Fibrin[48-53]	Polymerised – Yes Monomeric – No	Polymerised – Yes Monomeric – No	Yes	Cross-linking and clustering – unknown if it directly binds	Unknown
Fibrinogen[48-53]	No	No	Yes	Cross-linking and clustering – unknown if it directly binds	Unknown
Laminin[40]	Yes	No	Yes	Unknown	Unknown
Fibronectin[43,44] & Vitronectin[42]	Yes	ND	Yes	Unknown	Unknown
Adiponectin[41]	Yes	Yes	ND	Unknown	Unknown
Aβ42 peptides[47]	Yes	Yes	ND	Unknown	Unknown
EMMPRIN[45,46] (CD147, basigin)	No	ND	Yes	Unknown	Unknown
Exogenous peptides and charged particles[39]: DEPs, polysulfated sugars	Yes	Yes	ND	Possible electrostatic interactions and indirect cross-linking by other receptors	Unknown

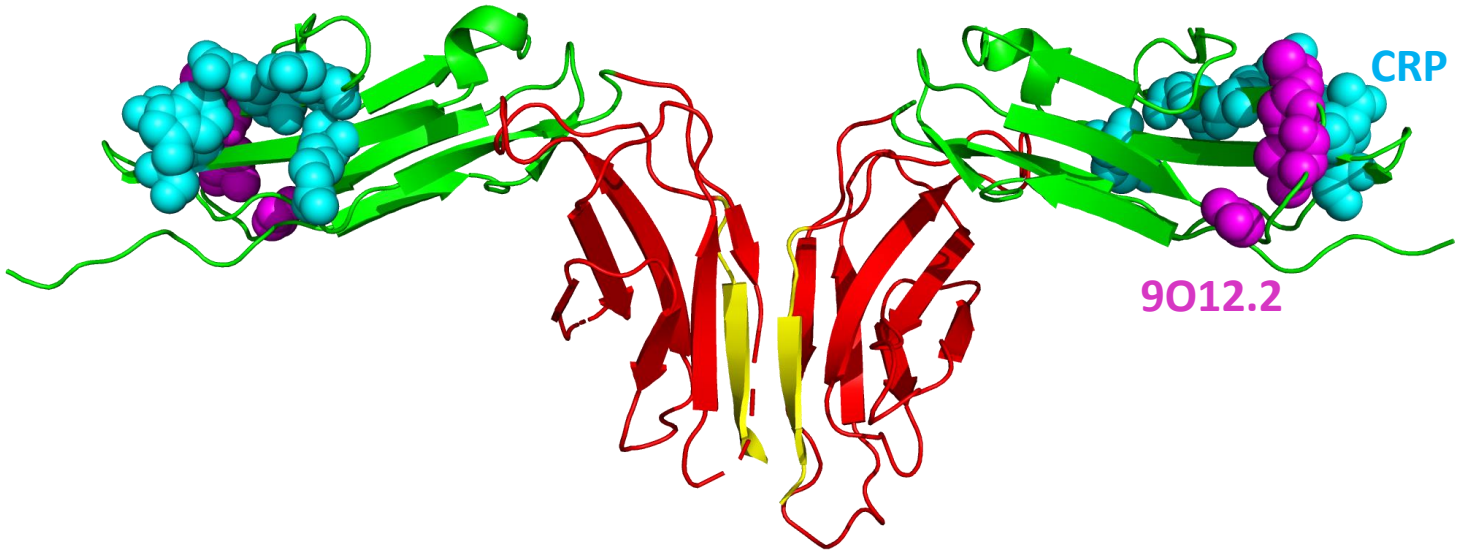


- Extracellular cleavage site
- Transmembrane Helix: interacts with FcRγ
- Basic region: binds calmodulin and interacts with FcRγ
- Proline-rich region: interacts with Fyn and Lyn
- C terminal: absent in mouse GPVI

ITAM: **i**mmunoreceptor **t**yrosine-based **a**ctivation **m**otif

- *O*-glycosylation site
- *N*-glycosylation site


GPVI
D1
D2
FcRγ-chain
LAT
SFK
P
P
P
ITAM
ITAM
SH2
SH2
SYK
PI3-kinase
PH
PH
PH
Btk/Tec
SH2
PLCγ2
Grb2/
Gads
SLP76
Vav1/3
PIP3
PIP2
DAG
PKC
IP3
Ca2+
Aggregation
Secretion
LAT signalosome
Kinases
Adaptors


CRP
9O12.2