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The Role of ITAM- and ITIM-coupled Receptors in Platelet Activation by Collagen

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Key words

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Summary

The major activation-inducing collagen receptor glycoprotein VI (GPVI) has been cloned within the last two years. It is a member of the Ig superfamily of proteins and is constitutively associated with the ITAM-bearing Fc receptor γ-chain (FcR γ-chain). GPVI signals through a pathway that involves several of the proteins used by Fc, B- and T-lymphocyte receptors and which takes place in glycolipid-enriched membrane domains in the plasma membrane known as GEMs. Responses to GPVI are regulated by PECAM-1 (CD31) and possibly other ITIM-bearing receptors. Despite a pivotal role for GPVI, there are important differences between signalling events to collagen and GPVI-specific ligands. This may reflect a role for co-receptors in the response to collagen.

Introduction

The interaction of platelets with collagen supports adhesion to the exposed subendothelium and stimulates activation leading to responses such as aggregation, secretion and expression of procoagulant activity. These events play a fundamental role in cessation of bleeding following tissue injury. The integrin α2β1 (GPIa-IIa) and GPVI are recognised as the major receptors underlying interaction with subendothelial collagen, with other receptors such as CD36 (GPIV) playing undefined roles.

Collagen supports adhesion though direct and indirect interactions. Direct interactions involve α2β1 and GPVI, whereas indirect interactions are mediated via von Willebrand factor (vWF) which bridges subendothelial collagen fibres to platelet GPIb-IX-V. The relative importance of these interactions is dependent on the rate of shear. The interaction with vWF is indispensable for adhesion at high shear, with the fast on rate of association between vWF and GPIb enabling capture of circulating platelets. Rapid dissociation gives rise to limited movement (or rolling) of platelets on a vWF surface, requiring a second subendothelial protein for static adhesion such as collagen or immobilized fibrinogen (which binds to αIIbβ3 in its resting state). α2β1 has been proposed to be the major receptor for collagen underlying adhesion. It is now recognized however that α2β1 undergoes a conformational change to a high affinity state upon activation of GPVI or other receptors, notably those for ADP (1). Binding of collagen to α2β1 is therefore facilitated by the initial activation of GPVI or stimulation by other agonists. These events are summarised in Fig. 1 and have led to revision of the two-site, two-state model of platelet-collagen interaction (2). In this model, platelets interact initially with either GPVI or α2β1, whilst the interaction with the other receptor strengthens adhesion. Other collagen receptors play an undetermined role in this process. The co-operative interaction between vWF and collagen or fibrinogen in generating stable adhesion at high shear may serve to limit thrombotic episodes. A ligand-receptor combination with a fast on rate and slow off rate would give rise to pseudoirreversible binding and place the individual at increased risk of thrombosis. At lower shear rates (<500 s^{-1}), the interaction of platelets with collagen is sufficient to support adhesion.

Several important advances have been made with respect to collagen receptors and their signalling pathways over the course of the last two years. Major developments include cloning of GPVI; progress in signalling via GPVI; design of an α2β1-specific collagen agonist; demonstration that α2β1 exists in different affinity states; and that polymorphisms in α2 that lead to increased expression of α2β1 are risk factors for thrombotic disease. This review focuses on the recent advances in collagen receptor signalling events whereas that by Clemetson and Clemetson in this volume is concerned with other aspects of collagen receptors. A number of other reviews summarise recent developments (2) or the state of the field prior to 1999 (3-9).

Platelet-Collagen Interactions – Patient and Mouse Studies

The clinical importance of α2β1 and GPVI as receptors for collagen was proposed following discovery of patients with mild to occasionally severe bleeding problems associated with reduced levels of either receptor, or presence of autoantibodies to the receptors (5). The first patients were described in the 1980's, and yet only five patients in each group have been identified. This is an extremely low value in comparison with the number of patients with Glanzmann's thrombasthenia and Bernard Soulier Syndrome, caused by deficiencies in the adhesion receptors αIIbβ3 and GPIb-IX-V, respectively. The paucity of patients with deficiences in α2β1 and GPVI raises the possibility that other factors contributed to the excessive bleeding. Thrombocytopenia is an important consideration in patients with autoantibodies to the receptors, whereas the first patient with a deficiency in α2β1 also had defective adhesion to other subendothelial matrix proteins (10). The bleeding phenotype of patients with a deficiency in GPVI is particularly mild

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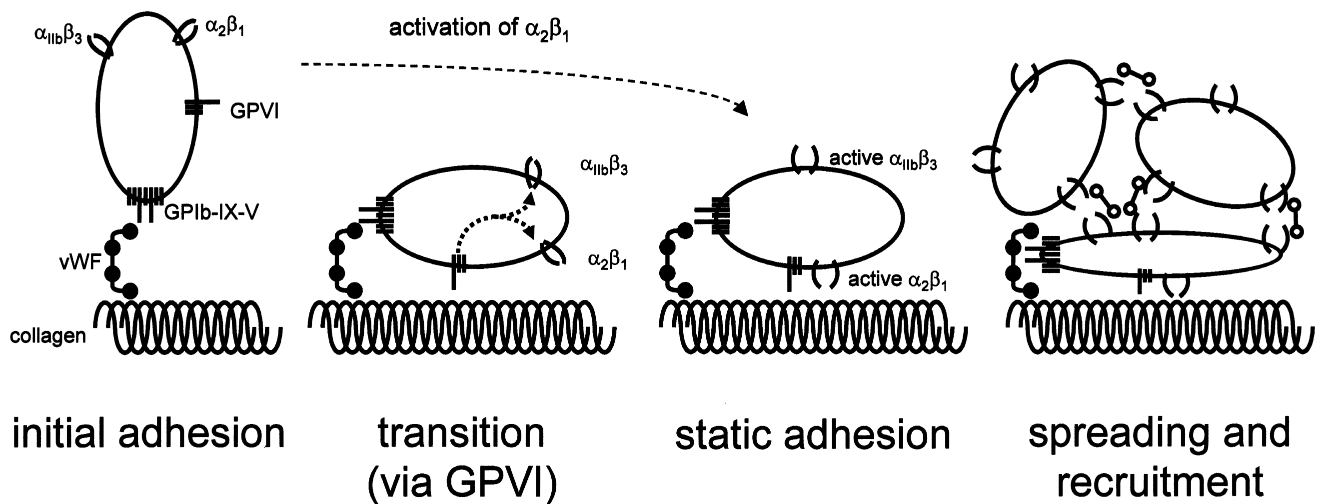

Fig. 1 Role of collagen in platelet adhesion to the vessel wall under high shear. This figure depicts the way in which subendothelial collagen supports platelet adhesion to damaged vessels under high shear conditions. The initial interaction of platelets with the damaged subendothelium is mediated via binding of von Willebrand factor (vWF) to GPIb-IX-V. Collagen fibres play an indirect role in this event by providing a docking site for vWF. The interaction with subendothelial collagen stabilises the adhesion. Whilst it is well established that binding of α2β1 to subendothelial collagen plays a critical role in this pathway, the interaction is facilitated by conversion of the integrin to a high affinity state. Signals from GPVI and released mediators e. g. ADP, and possibly shear, regulate this transition. Stable adhesion of platelets leads to spreading and activation of the integrin αIIbβ3, enabling recruitment of further platelets

and, in general, they have normal life styles without the need for treatment. It therefore seems likely that some individuals with a deficiency in the glycoprotein have gone undetected.

The possibility that the low number of patients with defects in α2β1 and GPVI is a result of a lethal phenotype is unlikely. Mice deficient in α2 appear healthy and have only a modest bleeding phenotype (Zutter and Santoro, personal communication). An earlier report that loss of α2 is an embryonic lethal is incorrect, although this is the case for ablation of β1 (discussed in [2]). Mice engineered to lack GPVI have not been made, but the glycoprotein is absent on platelets from mice deficient in the FcR γ-chain making this equivalent to a "knockout" (11). The loss of GPVI can be explained by the requirement for FcR γ-chain to confer stable expression of the glycoprotein in the surface membrane (12). FcR γ-chain-deficient mice do not exhibit excessive bleeding problems, despite being unable to aggregate in response to collagen.

The observations that deficiencies in α2β1 or GPVI lead to a modest or negligible bleeding phenotype should not be taken as evidence against an important physiological role for either receptor in platelet activation. The cyclooxygenase inhibitor, aspirin, and the ADP receptor antagonist, clopidogrel, do not cause spontaneous bleeding in healthy individuals and yet their use as antithrombotics is established. Additionally, the absence of a major bleeding phenotype in collagen receptor-deficient patients may reflect redundancy within the receptor family. Studies on mice with combined deficiencies in α2β1 and GPVI are of considerable interest in this regard. These arguments also have important implications for the physiological roles of other putative collagen receptors, including CD36 (see below).

Direct evidence that α2β1 is a suitable target for antithrombotics has emerged following the realisation that the protein exists in a number of polymorphic forms. The T807/873 allele of α2 leads to a significant upregulation in receptor density and places the individual at increased risk of myocardial infarction, stroke and diabetic retinopathy (summarized in [13]). Although the increase in risk is relatively low, in the order of 2-3 fold, this becomes much greater when combined with other factors such as smoking. Antagonists of α2β1 therefore represent potential antithrombotics which are unlikely to cause excessive bleeding, although side-effects through actions on other cell types may limit their use. The levels of GPVI also differ (Best, Berlanga and Watson, unpublished), possibly contributing to the variation in response between individuals (see ref [14]). The cause of this, and whether it is a factor in thrombotic disease, are important areas for future investigation. The restricted distribution of GPVI is likely to make this a preferred target for antithrombotics relative to α2β1.

GPVI

The evidence that GPVI is the major activation-inducing receptor for collagen is also based on studies with GPVI-specific ligands and platelets from mice deficient in key proteins involved in the GPVI signalling cascade. These include the FcR γ-chain-deficient mouse, which lacks GPVI as discussed above. GPVI-specific agonists, namely collagen-related peptide (CRP), based on a repeat Gly-Pro-HydroxyPro (GPO) sequence, the snake venom toxin, convulxin, and the rat monoclonal antibody JAQ1, stimulate a similar pattern of tyrosine phosphorylation to collagen in platelets and are powerful agonists (14-17). However, there are important differences between the actions of collagen and GPVI-selective agonists as discussed below.

The cloning of human GPVI was reported in October 1999 and murine GPVI in August 2000 (18, 19). Two further isoforms of human GPVI have been described, only one of which has a transmembrane domain (20). GPVI is a member of the Ig superfamily of surface proteins, possessing two extracellular Ig domains, a mucin-like stalk, a transmembrane region with an arginine group, and a short cytosolic sequence (51 amino acids). The original human isoform, GPVI-I, has 339 amino acids, but an apparent molecular size on SDS-PAGE of 62 kDa due to glycosylation. The second isoform with a transmembrane domain, GPVI-II, lacks 18 amino acids in the mucin stalk (20). The mouse protein has 319 amino acids, and an overall homology of only 64% to the human receptor and a shorter tail (27 amino acids). There is one conserved serine in the cytosolic region of murine and human GPVI and no threonine or tyrosine residues; there are no reports of receptor phosphorylation.

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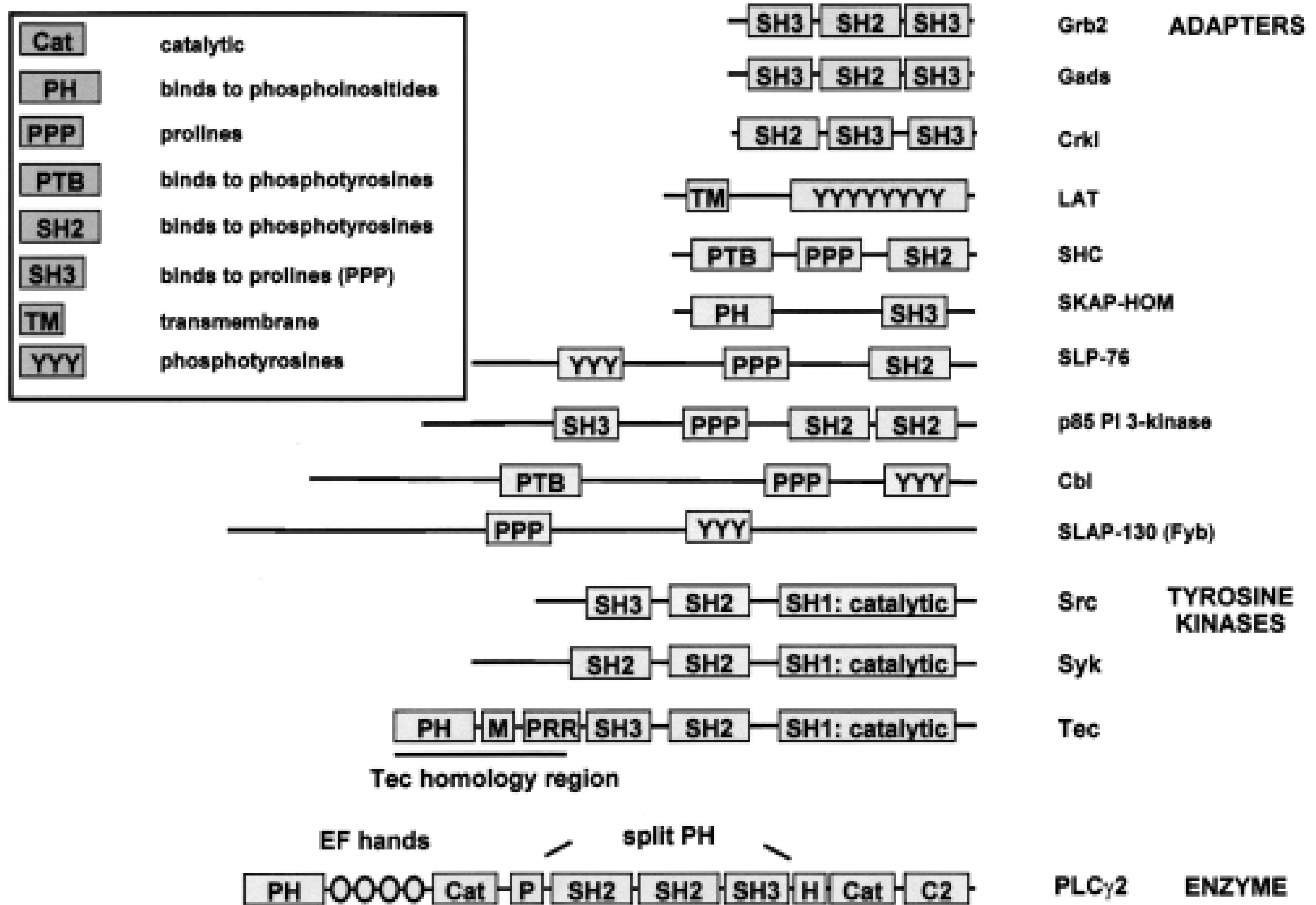

Fig. 2 Proteins and their domains involved in the GPVI signalling pathway

GPVI is restricted to megakaryocytes and platelets (19, 21) and is upregulated towards the end of megakaryocytopoiesis making it an important marker of megakaryocyte maturation (20, 21). The expression of GPVI in megakaryocytes may be the consequence of its role in the platelet as FcR γ-chain-deficient mice, which also lack GPVI, and GPVI-deficient patients have normal platelet counts and responses to other agonists.

It is important to demonstrate that the GPVI-FcR γ-chain complex is sufficient to reconstitute the response to collagen. Clemetson et al. expressed GPVI in the DAMI megakaryocytic cell line and observed potentiation of the response to convulxin and induction of a response to collagen (18). A low level of endogenous GPVI supported the response to convulxin but not to the weaker agonist collagen in control cells. GPVI has since been expressed in a number of haematopoietic and non-haematopoietic cell lines by several groups (19, 20, 22). Expression of GPVI confers binding to convulxin, as demonstrated by flow cytometry and ligand blotting. In addition, biotin-labelled CRP binds to expressed GPVI (20), whilst the interaction of collagen was demonstrated using an adhesion assay (19). Interestingly, none of these studies reported a functional response to GPVI. This is not surprising for non-haematopoietic cells, which lack many of the haematopoietic-specific proteins in the GPVI signalling pathway.

These studies confirm that GPVI is able to bind to convulxin, CRP and collagen, but do not provide definitive evidence that the GPVI-FcR γ-chain complex is sufficient to reconstitute a functional response bearing in mind the presence of endogenous receptor in the DAMI cells used by Clemetson's group. To address this, we have expressed GPVI and FcR γ-chain in the erythroid/megakaryocytic cell line K562 in collaboration with Drs. Moroi and Jung (Kurume, Japan), since neither protein is detectable in control cells. Expression of the GPVI-FcR γ-chain complex reconstitutes tyrosine phosphorylation of Syk in response to convulxin, whereas expression of GPVI alone does not (23). Expression of GPVI and the FcR γ-chain is increased following differentiation along the megakaryocytic lineage by the phorbol ester PMA, leading to an increase in response to convulxin. Expression of a mutant form of GPVI lacking the cytosolic domain uncouples the receptor from FcR γ-chain and causes a loss of functional response (23). These results demonstrate that association with the FcR γ-chain involves the cytosolic tail of GPVI and is essential for signalling through the glycoprotein. Phosphorylation of Syk by convulxin in K562 cells does not lead to phosphorylation of PLCγ2 suggesting the absence of one or more proteins downstream of the kinase. Work is ongoing to identify the nature of this lesion.

Although these data provide strong evidence that GPVI-FcR γ-chain complex is able to reconstitute the response to GPVI stimulation, the explanation for the absence of a response following expression in some haematopoietic cells is not known. We have also been unable to detect a functional response to convulxin following expression in a number of megakaryocytic lines (Berlanga, Snell and Watson, unpublished). An additional protein may be required for optimal signalling in these systems.

Signalling by GPVI. The GPVI/FcR γ-chain complex evokes platelet activation through a pathway that shares many similarities with signalling by immune receptors. This pathway involves proteins that

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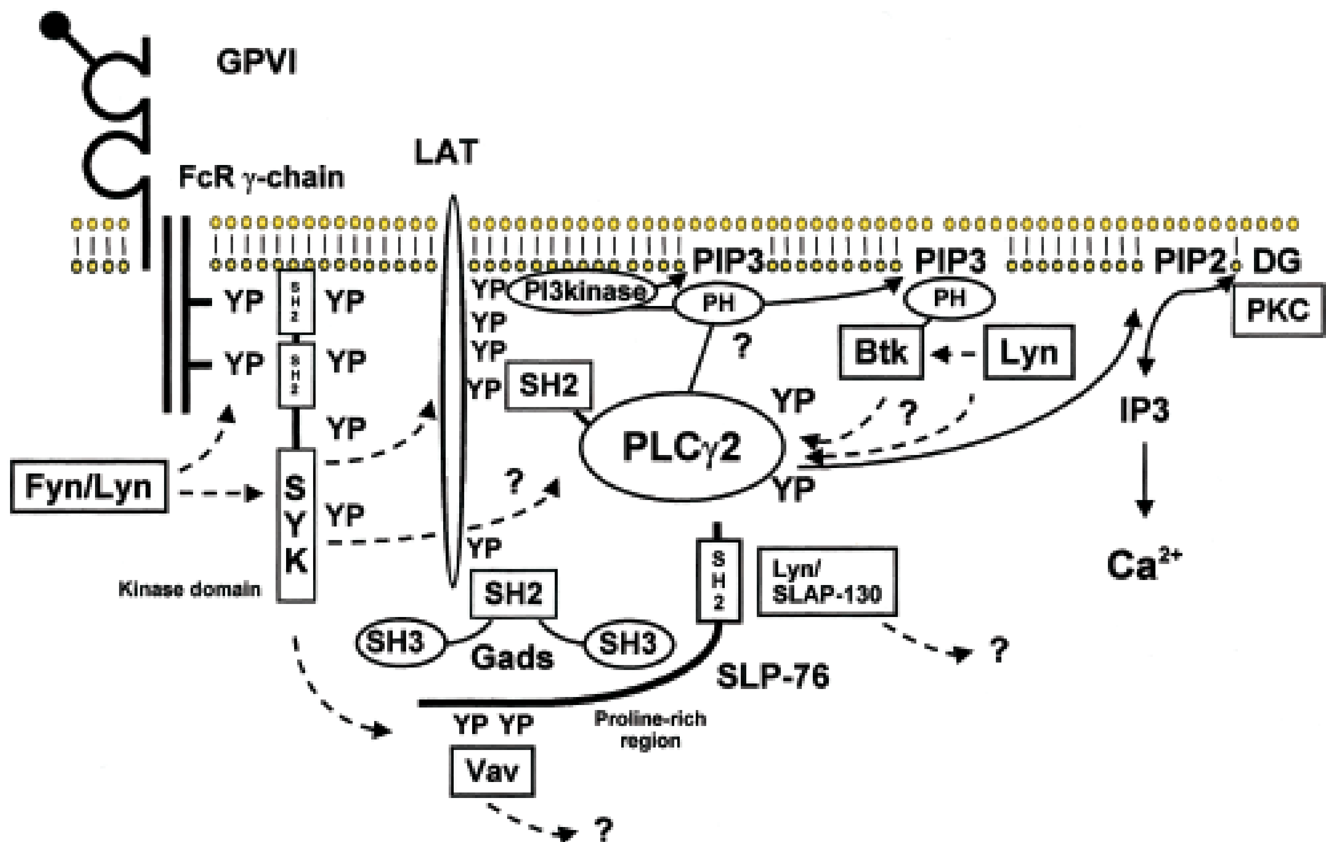

Fig. 3 The role of LAT-SLP-76 signalosome in the GPVI signalling cascade. Schematic of events underlying activation of PLCγ2 by GPVI. Tyrosine phosphorylation of the FcR γ-chain ITAM by the Src kinases Lyn and Fyn leads to recruitment of Syk. Syk undergoes autophosphorylation and is further phosphorylated by Src kinases leading to activation. Phosphorylation of the membrane adapter LAT leads to recruitment of PLCγ2 and a Gads/SLP-76 complex to the membrane. LAT also associates with PI 3-kinase leading to formation of the second messenger PI 3,4,5P3. PI 3,4,5P3 supports recruitment of the Tec family kinase Btk to the membrane, which undergoes autophosphorylation subsequent to phosphorylation by Lyn. In addition, PI 3,4,5P3 supports association of PLCγ2 to the membrane, possibly through an interaction with the N-terminal PH domain. The membrane-associated phospholipase may be phosphorylated in the linker region by Btk and a second tyrosine kinase, possibly Lyn. Although the Lat-SL-76 signalosome and PI 3-kinase support activation of PLCγ2, the phospholipase is also regulated independent of these events. Syk and SLP-76 play critical roles in this second pathway. Several proteins are assembled into the signalling complex have uncertain roles e. g. SLAP-130 and Vav

participate in activation by Fc, B- and T-cell antigen receptors, and may also have unique elements. The key feature linking this class of receptor is tyrosine phosphorylation of an immunoreceptor tyrosine-based activation motif (ITAM) by a Src family kinase leading to recruitment and activation of a Syk family kinase. Much of what we know about the GPVI pathway is based on the understanding of immune receptor signalling, and this has been the subject of a number of reviews (e. g. [24, 25]).

In the case of GPVI, the FcR γ-chain ITAM is phosphorylated by the Src kinases, Lyn and Fyn, leading to recruitment and activation of Syk. Syk initiates a signalling cascade that culminates in activation of the second messenger producing enzymes phosphatidylinositol 3-kinase (PI 3-kinase) and PLCγ2. A remarkable number of proteins are regulated downstream of Syk, including many adapters and the Tec family tyrosine kinase Btk. Adapters are modular proteins that support protein-protein interactions, and are as important as the enzymes themselves in signalling cascades. The domain structure of many of the proteins involved in GPVI signalling are shown in Fig. 2.

Critical roles of the adapters LAT, Gads and SLP-76 in the regulation of PLCγ2. The role of the transmembrane adapter LAT and the cytosolic adapter SLP-76 have been investigated in platelets from genetically-engineered mice. The absence of Syk leads to complete loss of phosphorylation of LAT, SLP-76 and PLCγ2, whereas depletion of SLP-76 leads only to loss of phosphorylation of PLCγ2 (26-29). Intriguingly, in LAT-deficient platelets, tyrosine phosphorylation of SLP-76 is only weakly inhibited and a significant level of tyrosine phosphorylation of PLCγ2 remains (26). These results place Syk upstream of phosphorylation of LAT and SLP-76, both of which have been shown to be substrates for the kinase. In addition, they demonstrate a critical role for SLP-76 in the regulation of PLCγ2 that is mediated partially downstream of LAT. In T cells, the adapter Gads is constitutively associated with SLP-76, and is recruited to LAT upon phosphorylation. In addition, LAT associates with PLCγ1. The three adapters and PLCγ1 are assembled into a LAT/SLP-76 signalosome that is essential for activation of the phospholipase (25). Gads is also constitutively associated with SLP-76 in platelets and recruited to LAT upon activation of GPVI; in addition LAT associates with PLCγ2 (30). The LAT/SLP-76 signalosome may therefore play a similar role in the regulation of PLCγ2 (Fig. 3). The mechanism underlying the LAT-independent pathway of regulation of SLP-76 and PLCγ2 in platelets, described above, is not known and may be unique. The adapter protein that performs a similar function to SLP-76 in B cells, Blnk (also known as SLP-65), is not expressed in platelets (30).

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Role of Src family kinases in GPVI signalling. Fyn and Lyn were originally proposed to mediate the initial phosphorylation of the FcR γ-chain ITAM (31), a result that has been confirmed through studies on mouse platelets (32). The onset of tyrosine phosphorylation of the ITAM is delayed in Lyn-deficient platelets and reduced in magnitude in Fyn-deficient cells. In Fyn and Lyn-double deficient platelets, the response is further delayed. These results suggest that Lyn initiates phosphorylation, with Fyn having a supporting role. The kinase underlying phosphorylation of the ITAM in the double-deficient platelets is not known, but is likely to be a third member of the Src family since phosphorylation is inhibited completely in the presence of the selective inhibitor of Src kinases, PP1 (32, 33). This kinase is unlikely to play a major role in platelet activation to GPVI, however, in view of the substantial delay in response in the Lyn/Fyn double "knockout". The delay in activation of Lyn-deficient platelets is followed by an increase in rate of response suggesting that Lyn regulates proaggregatory and feedback pathways (32). This is discussed further in the section on ITIM-bearing receptors in platelets. Lyn may in fact play several roles as it also associates with the C-terminal SH2 domain of PLCγ2 and SH2 domain of SLP-76 (28, 34) (Fig. 3).

Role of Tec family kinases and the PI 3-kinase pathway in GPVI signalling. There is strong evidence that Tec family kinases regulate phosphorylation of PLCγ2 and PLCγ1 in B- and T-lymphocytes, respectively, and also Ca^{2+} entry (for review see [35]). The Tec kinases Btk and Tec are also expressed in platelets and undergo phosphorylation in response to CRP and collagen ([36, 37] and unpublished). Aggregation and secretion are substantially reduced in XLA platelets, which express a mutation in the gene encoding Btk, in response to CRP and collagen. This is accompanied by a partial reduction in tyrosine phosphorylation of PLCγ2, and to a lesser degree, SLP-76 (36), whereas increases in phosphorylation of the majority of proteins is unaltered. These observations suggest a role for Btk in mediating phosphorylation of the phospholipase. The partial nature of the reduction of phosphorylation of PLCγ2 in XLA platelets may be due to expression of Tec, whose phosphorylation is not altered in the absence of Btk (37). Alternatively, PLCγ2 may be phosphorylated through a distinct pathway involving Src or Syk-related kinases. These explanations may also account for the negligible reduction in phosphorylation of PLCγ2 in platelets from Xid mice, which express a naturally occurring mutant of Btk with a non-functional PH domain, in response to GPVI (38).

Studies with inhibitors of PI 3-kinase also demonstrate a role for this second messenger pathway in the regulation of PLCγ2 and provide evidence for a pathway of phosphorylation of the enzyme which is independent of Tec family kinases. The two structurally distinct inhibitors of PI 3-kinase, wortmannin and LY294002, cause substantial but incomplete inhibition of PLCγ2 activation by GPVI which results in inhibition or a delay in onset of functional responses (29, 39). The two inhibitors also block platelet activation by the platelet low affinity immune receptor, FcγRIIA, which signals through an ITAM-based pathway (40). Inhibition is mediated at the level of Btk and PLCγ2. Phosphatidylinositol 3,4,5-trisphosphate (PI3,4,5-P3) binds with high affinity to the PH domain of Btk, promoting its migration to the membrane where it is phosphorylated by a member of the Src family of kinases, probably Lyn, causing activation (37). In addition, the PI 3-kinase pathway is required for translocation of PLCγ2 to the plasma membran independent of Btk (41).

The observation that tyrosine phosphorylation of PLCγ2 is maintained in the presence of wortmannin and LY294002 supports a pathway of phosphorylation that is independent of Tec kinases (29, 39). It also raises the possibility that phosphorylation of PLCγ2 may take place in the cytosol, although it is perhaps more likely that there is an increased rate of dissociation from the membrane subsequent to phosphorylation. The role of the phosphoinositide pathway may therefore be to keep the phosphorylated (and thereby activated) PLCγ2 at the membrane.

Paradoxically, absence of the 5'phosphatase SHIP leads to an increase in the levels of PI3,4,5P3 and sustained tyrosine phosphorylation of Btk in murine platelets without a corresponding change in phosphorylation or activity of PLCγ2 in response to CRP (38). Whilst this may be partly due to differences in the role of Btk in murine and human platelets, it is surprising that there is no direct effect on PLCγ2. It is possible that 3-phosphorylated lipids are not rate-limiting in the regulation of the phospholipase, although this is not consistent with observations supporting a role of this pathway in potentiation of collagen signalling by thrombopoietin (42). Determination of the sites of elevation of 3-phosphorylated lipids relative to Btk and PLCγ2 in the SHIP-deficient platelets may shed further light on this.

Regulation of PLCγ2. Studies on PLCγ2-deficient mice demonstrate a critical role for the phospholipase in aggregation and secretion responses to collagen (43). This is consistent with previous studies showing that PLCγ2 and PLCγ1 are regulated in distinct ways in platelets and megakaryocytes, in that only the former undergoes phosphorylation and translocation to the membrane (39, 44).

PLCγ2 is cytosolic and migrates to the plasma membrane of the platelet and megakaryocyte upon activation of GPVI (41). The domains within PLCγ2 that support translocation to the plasma membrane and the sites and role of phosphorylation are not established. Several domains have the potential to mediate this translocation, including an N-terminal PH domain, tandem SH2 domains preceding an SH3 domain, a split PH domain flanking the SH2 and SH3 domains and a C2 domain in the C terminus (Fig. 2). Tyrosine phosphorylation of the phospholipase itself may also play a role. The ability of wortmannin and LY294002 to block translocation could be mediated through inhibition of binding of 3-phosphorylated lipids to the N-terminal PH domains or tandem SH2 domains as proposed for PLCγ1 (see [45]). We have shown, however, that these domains do not translocate to the plasma membrane when GFP fusion proteins were expressed in PC12 cells and following activation of PI 3-kinase by EGF (41). However, this does not rule out the possibility that they support association to the membrane when combined with other domains within the phospholipase. A weak interaction of several domains with the membrane might facilitate correct orientation and provide multiple pathways of regulation. Studies on full length PLCγ2 with mutations in individual domains are required to investigate this. Tyrosine phosphorylation of residue 783 in the linker region between the tandem SH2/SH3 domains and C-terminal catalytic domain has been shown to be critical for activation of PLCγ1 (46). The corresponding sites of phosphorylation in PLCγ2 have not been mapped and, although they cannot be identified on the basis of sequence homology, it is notable that there are three tyrosines within the linker region. Although the major role of PLC isoforms is to generate the second messengers, IP3 and 1,2-diacylglycerol, there is evidence that PLCγ1 may have an additional role, perhaps by serving as an adapter (47). A similar situation may also apply to PLCγ2. There is increasing evidence that domain-containing enzymes can have functions that are independent of their enzymatic group. The role of PLCγ isoforms in haematopoietic signalling has been the subject of a recent review (45).

Role of other proteins in the GPVI cascade. A number of the proteins in the GPVI signalling cascade do not appear to have major functional roles in platelet activation. For example, murine platelets

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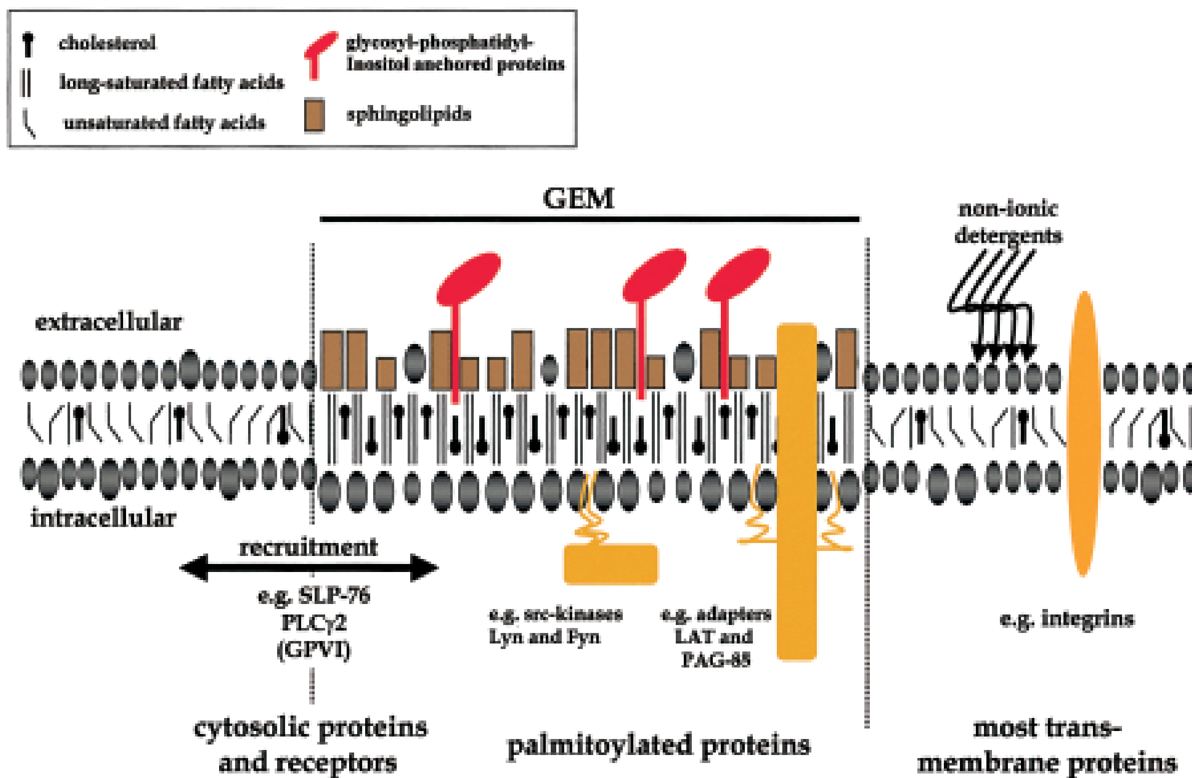

Fig. 4 Structure of GEMs. Immune receptor signalling is believed to take place in specialised regions of the membrane known as glycoplipid-enriched membrane domains (GEMs). They are rich in GPI-linked extracellular proteins and palmitoylated cytosolic proteins, but many proteins are excluded from this region. Additional proteins are recruited into GEMs upon receptor activation. For further information, see text

deficient in the GTP exchange factor Vav (Wilde, Turner and Watson, unpublished) or human platelets deficient in the cytoskeletal protein WASP (48), respond relatively normally to GPVI despite the fact that both proteins undergo phosphorylation upon activation of control cells. Explanations for this apparent paradox including the possibility that their role is restricted to the megakaryocyte or that the appropriate response was not measured. Alternatively, it may reflect redundancy between members of the family. For example, it is noteworthy that Vav2 but not Vav1 plays a critical role in B cell receptor signalling (49) and vice versa in T cells. It is also possible that phosphorylation is an epiphenomenon of platelet activation, with the irreversible nature of platelet aggregation preventing "unnecessary phosphorylation events" being selected against during evolution.

Studies on platelets from patients with chronic myeloid leukemia (CML) provide an alternative approach to addressing the role of protein phosphorylation in platelet activation by GPVI and again provide an example where phosphorylation does not appear to have a physiological consequence. CML platelets have constitutive tyrosine phosphorylation of the adapter Crkl which coprecipitates with a second tyrosine phosphorylated adapter, Cbl (50). Responses to GPVI are not significantly altered in CML platelets despite the fact that both proteins undergo increases in tyrosine phosphorylation upon activation of GPVI (51).

Many other proteins undergo tyrosine phosphorylation and/or assembly into signalling complexes upon activation of GPVI including the adapters SLAP-130 (also called Fyb), Shc, SKAP-HOM and Grb2 (e. g. [28, 30]). As discussed above, this does not necessarily reflect a role in the GPVI signalling cascade, and experiments on genetically-modified mice and other approaches are required to address this.

Signalling through GEMs. It is now recognized that signalling via immune receptors takes place in specialised regions of the membrane known as GEMs (also known as rafts, digs, drms etc) (24, 52). GEMs are rich in cholesterol and sphingolipids, and also in many of the signalling proteins involved in the ITAM-dependent signalling pathway (Fig. 4). Extracellular proteins are localised to the GEMs via glycerophosphoinositol (GPI)-anchors, whereas many intracellular and transmembrane proteins are attached via palmitoylation on cysteine. LAT and the Src kinases Fyn and Lyn are examples of palmitoylated proteins that are present in GEMs. GEMs are attached to the cytoskeleton and so localise cytoskeletal proteins to sites of receptor signalling. Signalling proteins and receptors are recruited to GEMs following receptor activation as a consequence of protein-protein interactions.

The evidence for a role of GEMs in GPVI signalling is from biochemical analyses of membranes and use of the cholesterol-depleting reagent, β-methyl cyclodextrin. Isolation of GEMs by sucrose density centrifugation in the presence of non-ionic detergents such as Brij58 or Triton-X-100 reveals that more than 90% of LAT and the Src kinases Fyn and Lyn are present in this region, whereas the integrin β1 subunit is excluded (Wonerow and Watson, unpublished). Stimulation by convulxin leads to recruitment of further proteins to the GEM fraction including tyrosine-phosphorylated PLCγ2 and SLP-76, both of which associate with LAT. Tyrosine phosphorylated forms of these two proteins are also found in the soluble fraction, but not in association with

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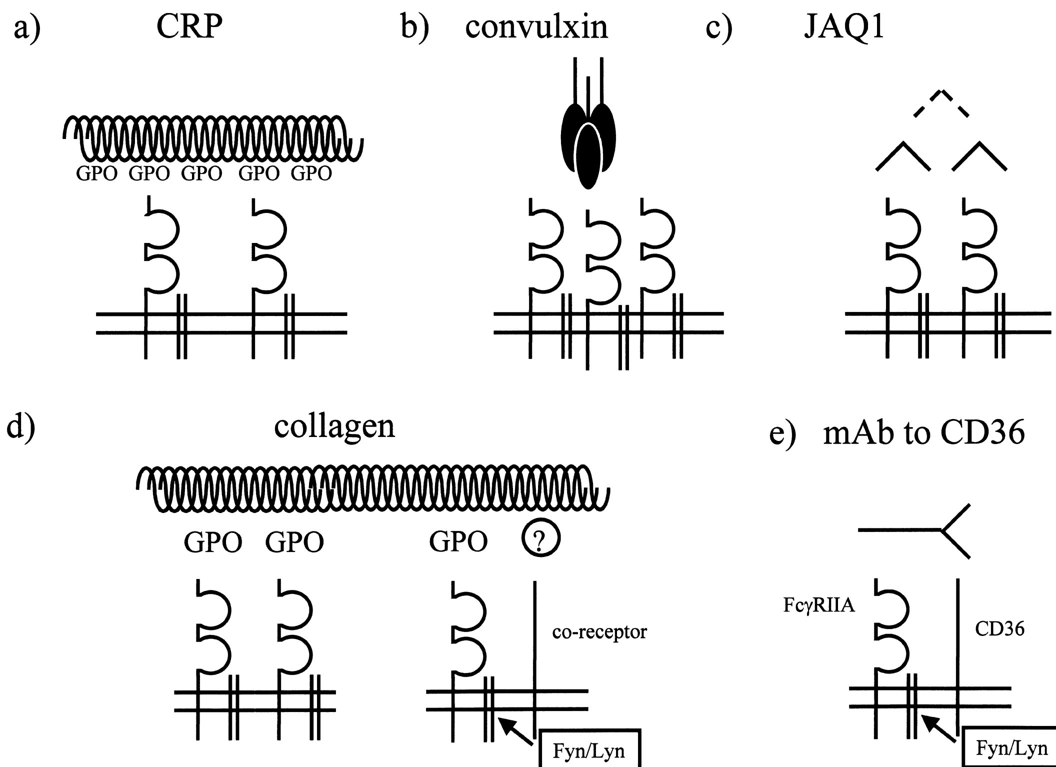

Fig. 5 Models of activation of GPVI by collagen and other receptors. This figure depicts the way in which collagen and related agonists at GPVI induce crosslinking. (a) CRP is made up of a backbone of GPO repeats giving rise to the potential crosslinking of many GPVI receptors. (b) The α3β3, hexameric snake venom toxin convulxin has the potential to bind three molecules of GPVI. (c) Antibodies to GPVI have two binding sites. (d) The sequence GPO is approximately 10% of the collagen sequence and appears primarily as single repeats, although it is present as a continuous sequence of five or more in some collagen α chains. The model depicts two ways in which collagen can induce crosslinking of GPVI, namely by crosslinking two GPVI receptor or by crosslinking to a co-receptor, such as CD36 or α2β1. (e) Proposed way in which antibodies to CD36 stimulate platelet activation

LAT. It is possible that this reflects a role in the LAT-independent signalling pathway discussed above or dissociation from the LAT/SLP-76 signalosome. β-methyl cyclodextrin disrupts GEM function through depletion of cholesterol leading to loss of LAT and partial inhibition of aggregation to GPVI-specific agonists (Wonerow and Watson, unpublished).

The recognition of the significance of GEMs in signalling by GPVI has provided a potential explanation for the mechanism of activation of the Src kinases Lyn and Fyn. In T-lymphocytes, PAG-85 (also known as CBP) is constitutively associated with the regulator of Src kinases, Csk (53, 54). Phosphorylation of Src kinases by Csk leads to intramolecular binding of the SH2 domain to the newly phosphorylated tyrosine and a reduction in kinase activity. Activation of the T-cell receptor causes dephosphorylation of PAG-85 and dissociation of Csk. This is believed to lead to dephosphorylation of the Src kinase Lck on its regulatory subunit and an increase in kinase activity. PAG-85 is also localised to GEMs in platelets and undergoes dephosphorylation in response to convulxin (Wonerow, Leo, Schraven and Watson, unpublished). CHK, a protein that shares close homology with Csk, is present in platelets and is localised in the membrane with CD36, which is found in GEMs (55). It seems reasonable to suggest that dephosphorylation of PAG-85 in response to GPVI leads to loss of binding to CHK and activation of the Src kinases.

Despite the evidence for a role of GEMs in platelet activation, there are more questions than answers for this specialised region of the membrane. The protein composition of GEMs varies with the detergent used to preserve their structure, possibly reflecting a competing need to maintain structure and yet cause solubilisation from other membrane regions and the cytoskeleton. GEMs may also not be uniform in composition and size. Additionally, although β-methyl cyclodextrin is widely used to disrupt GEMs, its selectivity is uncertain. The presence of GPVI and other collagen receptors such as α2β1 in the non-GEM fraction also suggests that signalling events may take place outside of this region.

Differences between Collagen and GPVI-Specific Agonists: Evidence for Other Receptors for Collagen

There are a number of important differences in responses to collagen and GPVI-mimetics in platelets. GPVI selective agonists like CRP and convulxin induce a much greater degree of tyrosine phosphorylation and stimulation of the PI 3-kinase pathway relative to collagens. This is exemplified by the ability of convulxin to stimulate tyrosine phosphorylation and Ca^{2+} mobilisation in megakaryocytic cell lines that express endogenous GPVI, whereas collagen is inactive (2, 18). Additionally, CRP induces a tenfold greater increase in the levels of the PI 3-kinase derived lipids, PI 3,4,5P3 and PI 3,4P2, than collagen in platelets (39, 42). Responses to CRP also show a greater reduction in magnitude in XLA platelets, which are deficient in Btk, or in the presence of inhibitors of PI 3-kinases in comparison to collagen. This demonstrates that the differences between GPVI-specific agonists and collagen are qualitative as well as quantitative.

The presence of other receptors for collagen and the relatively low frequency of the GPVI-specific sequence GPO in collagen are likely to

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account for these differences. The sequence GPO makes up approximately 10% of most collagens, and is seldom present as repeats of two or more, although this may be compensated to some extent by assembly of the three α chains into a superhelical structure. In contrast, GPO forms the backbone of CRP, while convulxin has three binding sites for GPVI and antibodies have two (Fig. 5). Collagen will therefore crosslink GPVI to other receptors such as the integrin α2β1 or CD36, and in addition, will also crosslink these receptors to each other and to themselves (Fig. 5). This is illustrated, for example, by the ability of antibodies to α2β1 to block platelet activation by collagen but not to GPVI-specific agonists (56). It is unclear whether this is due to (i) reduced binding to GPVI; (ii) loss of crosslinking of GPVI to α2β1 and (iii) the inability of α2β1 to signal on its own. The location of α2β1 in non-GEM regions is also likely to be a factor. The potential role of collagen receptors other than GPVI in signalling events is discussed below.

Is there a second receptor for collagen coupled to FcR γ-chain? One possible explanation for the difference between collagen and GPVI-mimetics is the presence of a second receptor coupled to FcR γ-chain. Evidence in support of this has come from studies using the rat mAb to murine GPVI, JAQ1, although the results are also consistent with the presence of a second epitope within collagen (other than GPO) which can bind to GPVI. JAQ1 completely inhibits aggregation to the GPO-rich peptide CRP, but only blocks aggregation to low concentrations of collagen (17). The knowledge that aggregation to collagen is abolished in FcR γ-chain-deficient mice demonstrates that these results are due either to binding to a distinct region within GPVI or to a separate receptor that is also coupled to the FcR γ-chain. The response to high concentrations of collagen in the presence of JAQ1 is associated with tyrosine phosphorylation of Syk, SLP-76 and PLCγ2, but only minimal phosphorylation of LAT (17). The second epitope may selectively regulate the LAT-independent pathway of regulation of SLP-76 and PLCγ2 described above. This pathway is not exclusive to this epitope, however, as it is also regulated by CRP. It is noteworthy that convulxin binds to two sites on platelets and that CRPs displace binding by only 30% (57). It is not known whether this is due to the presence of two epitopes for convulxin within the glycoprotein.

Is there a second collagen receptor that induces tyrosine phosphorylation? Evidence for a second receptor for collagen that regulates tyrosine phosphorylation of Syk and PLCγ2 is based on studies in platelets from FcRγ-chain-deficient mice, which also lack GPVI (58), and in GPVI-deficient platelets (59). The observation that tyrosine phosphorylation of PLCγ2 by collagen is abolished in Syk-deficient murine platelets, in contrast to the results in FcR γ-chain-deficient platelets, supports a role for this kinase in the second pathway. The limited tyrosine phosphorylation in platelets from FcR γ-chain-deficient mice is not sufficient to support aggregation and secretion, whereas the response in the GPVI-deficient patients is accompanied by extremely weak activation of αIIbβ3 (60), and may even be due to residual expression of GPVI.

α2β1 integrin. We have shown that an α2β1-selective collagen-based peptide, or antibodies to α2 and β1 subunits, some of which have been shown to stimulate tyrosine phosphorylation in other cells, are unable to stimulate tyrosine phosphorylation in platelets (61, 62). Additionally, we have reported that antibodies to α2β1 do not stimulate tyrosine phosphorylation in platelets activated by G protein-coupled receptor agonists (62), which convert the integrin into its high affinity conformation (63). Thus, it would appear that the integrin is unable to stimulate tyrosine phosphorylation in either its low or high affinity conformations.

Evidence for a role of α2β1 in stimulating tyrosine phosphorylation has come from studies on snake venom toxins. Trimucytin and rhodocytin (also known as aggretin) activate platelets and induce tyrosine phosphorylation through a pathway that is inhibited by antibodies to α2β1. In the case of trimucytin, however, the inhibitory action of the antibodies is incomplete and, moreover, aggregation is maintained following cleavage of the β1 subunit using the protease jararhagin (62). This provides strong evidence that the response to trimucytin is mediated through an additional receptor that works in combination with α2β1. Trimucytin stimulates a similar pattern of tyrosine phosphorylation to that induced by convulxin raising the possibility that the receptor is GPVI.

The response to rhodocytin is distinct from that to collagen in that it is maintained in platelets deficient in the FcR γ-chain and is associated with a distinct pattern of tyrosine phosphorylation (64, 65). However, it has recently been reported that rhodocytin does not bind recombinant α2β1 (66), although it remains unclear whether this is the case for endogenously expressed protein bearing in mind that it exists in two conformational states. It is therefore unclear whether the venom induces activation through a pathway that is of relevance with respect to collagen.

CD36. CD36 was proposed as a receptor for collagen in the late 1980's, but has received relatively little attention following recognition that it is absent in 5% of the Japanese population and that this group do not have major haemostatic problems and exhibit only marginal reductions in platelet responses to collagen (discussed in [9]). The importance of CD36 may however have been overlooked in consideration of the arguments presented above in relation to the modest phenotype of patients deficient in α2β1 or GPVI. Redundancy between receptors for collagen is an important consideration and it is possible that CD36 would have a much greater role in patients deficient in α2β1 or GPVI.

It is important to consider whether CD36 contributes to intracellular signalling events and whether these are of physiological relevance. CD36 is localised to GEMs in platelets where it is associated with the Src family kinases Lyn, Fyn and Yes (67, 68). Crosslinking of CD36 has been shown to cause activation of Src family kinases in other cells (69). It is well established that antibodies to CD36 induce platelet activation through a pathway that is dependent on crosslinking to the low affinity immune receptor, FcγRIIA (Fig. 5). There are no reports however that crosslinking of CD36 using $F(ab')_2$ fragments of anti-CD36 antibodies stimulates tyrosine phosphorylation. The similar mechanisms of platelet activation between GPVI and FcγRIIA raises the possibility that crosslinking of CD36 to GPVI will also generate an intracellular signal. In this way, CD36 would contribute to platelet activation by collagen but only in the presence of GPVI (Fig. 5). Results in our laboratory show that crosslinking of CD36 has little effect on its own but increases the response to the GPVI-specific agonist convulxin (Snell, Nichols and Watson, unpublished) suggesting that it may be a co-receptor for collagen, rather than a receptor in its own right. It is also of interest to consider whether other receptors for collagen including α2β1 may function as a co-receptor to GPVI.

P65. The three transmembrane protein P65 was cloned in 1997 and shown to be a receptor for type I but not type III collagen (70, 71). Nothing has been published on P65 since the initial two reports and its sequence is not available in the human genome database available within the public domain. We have not been able to identify the presence of P65 in megakaryocytic cell lines or platelets using PCR, although a restricted distribution is not unique for platelet-specific proteins. Nothing is known about the ability of P65 to stimulate intracellular signalling events. Advances in this field require the development of specific ligands or its reconstitution into relevant cell lines.

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P68-72. Monnet and Lafève reported the partial purification of a receptor for type III collagen in platelets, P68-72, using, a peptide derived from type III collagen, KOGEOGPK (72). The signalling events regulated by P68-72 have not been characterised. Further developments await the cloning of the protein.

The Presence of ITIM-Bearing Proteins in Platelets

In recent years, it has been recognised that receptors signalling through ITAM-dependent pathways can be selectively regulated by transmembrane proteins containing ITIMs (immunoreceptor tyrosine-based inhibition motifs) (for review see [73]). The ITIM sequence is defined as [(Ile/Val/Leu/Ser)-X-Tyr-X-X-(Leu/Val), where X denotes any amino acid. It is closely related to the ITAM sequence but has only one conserved tyrosine and shows a much greater diversity in surrounding amino acids. The power of genomics has identified more than 30 proteins containing this sequence in human, many of which are localised to haematopoietic cells. Phosphorylation of the conserved tyrosine in the ITIM leads to recruitment of the SH2 domain containing tyrosine phosphatases, SHP-1 and SHP-2, or the SH2 domain-containing inositol 5'-lipid phosphatases, SHIP (also known as SHIP1) and SHIP2, to the plasma membrane. The SH2 domains of these proteins have different affinities for ITIMs enabling specificity in recruitment. Recruitment of SHP-1 and SHP-2 leads to dephosphorylation of key proteins in ITAM-dependent signalling cascades. The targets of the phosphatases may include the ITAM and Syk, although this has been difficult to establish because dephosphorylation of one protein can lead to dephosphorylation of others. SHIP and SHIP2 removes the 5-phosphate from the second messenger PI 3,4,5P3 to generate another second messenger, phosphatidylinositol 3,4-bisphosphate. The two lipid messengers recruit distinct subsets of protein to the membrane via their PH domains, including Btk and PLCγ2 (see above). There is also evidence for additional pathways of signalling through this class of receptors, although the proteins involved have not been identified.

Evidence for a role of ITIM-bearing receptors and their signalling proteins in platelets and megakaryocytes is beginning to emerge. We have reported that the lectin wheat germ agglutinin is a powerful platelet agonist, but in contrast, selectively inhibits responses to FcγRIIA in megakaryocyte-like cell lines (74). This led us to propose the presence of an inhibitory receptor in early stages of megakaryocyte development whose function was to prevent premature activation. Additional evidence for the presence of an inhibitory receptor is the (delayed) potentiation of response to GPVI observed in Lyn-deficient platelets, described above, bearing in mind that Lyn mediates phosphorylation of ITIM-bearing receptors in other haematopoietic cells.

Platelet/endothelial cell adhesion molecule-1 (PECAM-1; also known as CD31) is the only ITIM-bearing protein that has been described in human platelets. It is also expressed on the surface of monocytes, neutrophils, T lymphocytes and endothelial cells. PECAM-1 is highly expressed on the platelet surface, with estimates ranging between 5,000-10,000 copies per platelet with approximately 30% on intracellular membranes. PECAM-1 has six extracellular Ig domains and a 118 amino acid cytoplasmic tail containing two ITIMs. Phosphorylated peptides based on the two ITIMs bind to SHP-2 with a ten fold selectively over SHP-1 (75). PECAM-1 was originally reported to undergo tyrosine phosphorylation and association with the SH2 domains of SHP-2 upon platelet aggregation (76). Subsequently, PECAM-1 was shown to undergo phosphorylation in response to thrombin and convulxin independent of αIIb-β3 engagement, and also in response to crosslinking with specific antibodies (77, 78). The association of PECAM-1 with SHP-2 leads to an increase in activity of the phosphatase (77).

The functional role of PECAM-1 in platelets has been the subject of considerable research (for review see [79]). Nevertheless, its role in the platelet remains unclear. PECAM-1 undergoes an homophilic interaction leading to the proposal that it may support aggregation, although studies on αIIbβ3-deficient platelets suggest that this has little physiological significance. It has also been proposed to be a co-stimulatory molecule for integrin-mediated adhesion and aggregation in platelets (80). Bivalent antibodies directed to Ig domain 6 but not to other regions of the glycoprotein potentiate ADP and PAF-induced aggregation responses. On the other hand, crosslinking of PECAM-1 with a different antibody inhibits aggregation to low concentrations of thrombin and convulxin (81). It is conceivable that both of these effects are brought about through association with SHP-2, which has both stimulatory and feedback actions in other cells. This cannot be tested in SHP-2-deficient mice as ablation of the phosphatase causes lethality at day 9.5 because of suppression of haematopoeitic development.

Further insight into the role of PECAM-1 has come from the engineering of mice deficient in the glycoprotein. The mice are healthy with normal platelet counts and no obvious vascular defects (80). Platelet aggregation induced by ADP *in vitro* is unaltered confirming that PECAM-1 is not essential for platelet-platelet interactions. The mice, however, exhibit prolonged tail bleeds that are not corrected by reconstitution with control bone marrow, thereby demonstrating that it is not platelet-derived and may be due to a defect in the endothelium. A recent study, however, did not observe a significant difference in the tail bleeds in PECAM-1-deficient mice (82). This may reflect differences in methodology bearing in mind the potential role of the coagulation cascade in this assay. The same group also used a murine model of endothelial cell injury-based vascular thrombosis and reported no alteration in PECAM-1-deficient mice (82). Caution is also required in regard to this result in light of recent observations that responses to threshold concentrations of collagen and CRP are potentiated in PECAM-1-deficient mice, whereas those to the G protein-coupled receptor agonist thrombin are not (83). This provides the first direct evidence for the selective regulation of an ITAM-coupled receptor by an ITIM-bearing receptor in platelets. It is therefore possible that a deficiency in PECAM-1 is only likely to be prothrombotic in situations where collagen plays a significant role.

The presence of a second ITIM-bearing receptor, paired immunoglobulin receptor B (PIR-B), has been reported in murine platelets and megakaryocytes (84). PIRs are type I glycroproteins with six Ig domains and two alternative cytosolic sequences, giving A and B isoforms (85). They are expressed only on haematopoeitic cells, including B-lymphocytes and mast cells. PIR-A has a short cytosolic tail and an arginine in its transmembrane domain enabling it to bind to FcR γ-chain. In contrast, PIR-B has a long cytosplasmic tail with four ITIMs. PIR-B has been shown to selectively inhibit signalling by immune receptors through recruitment of SHP-1 and SHP-2 (for references see [84]). The ligands for PIRs are not known, although they are thought to be related to MHC-class I proteins.

PIR-B is constitutively phosphorylated on tyrosine in murine platelets and associates with unidentified tyrosine phosphorylated proteins of 38, 73 and 80 kDa following activation by thrombin or through GPVI. However, there is no evidence for an association with SHP-1, SHP-2 or SHIP. Crosslinking of PIR-B with a specific antibody does not alter platelet activation by GPVI-mimetics. It is noteworthy, however, that a similar observation has been made with some antibodies to

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PECAM-1 (see above). Studies on mice-deficient in PIR-B would be of considerable interest in this regard.

PIR-B is not present in the human genome, but is present on a region of mouse chromosome 7 that is syntactic with a region, q3, in human chromosome 19 (86). This region encodes for members of the ITIM-bearing LIR (also known as ILT) family of surface receptors raising the possibility that one or more of these may be expressed in human platelets.

Platelets may also express other ITIM-bearing proteins. Several tyrosine phosphorylated proteins are precipitated by the tandem SH2 domains of SHP-1 and SHP-2 from GPVI-stimulated platelets, including an unidentified 130 kDa band and several bands which migrate in the range of 28-32 kDa (77, 87). The heavily phosphorylated bands of 28-32 kDa proteins are of particular interest because they do not appear to be substrates for the phosphatases. This, and their size, suggests that they may be novel transmembrane ITIM-bearing proteins (77).

Functional roles of SHP-1 and SHIP in platelets. At present, we do not know the nature of the ITIM-bearing proteins that recruit SHP-1 and SHIP to the membrane. However, their existence seems likely bearing in mind that SHP-1 and SHIP undergo tyrosine phosphorylation upon platelet activation by GPVI or thrombin (38, 87).

SHP-1 associates with Syk and Lyn in CRP-stimulated platelets along with the unidentified tyrosine phosphorylated proteins of 28 and 32 kDa, described above, and an uncharacterised tyrosine phosphorylated band of 130 kDa (87). The association of Syk and Lyn with SHP-1 raises the possibility that the two kinases are substrates for the phosphatase *in vivo*. Evidence for a role of SHP-1 in the GPVI signalling pathway has emerged through studies on platelets from mice deficient in the phosphatase. SHP-1-deficient mice are described as *motheaten* due to their appearance and general state of ill-health. They do not survive more than two weeks because of defects in immunity and haematopoiesis. *Motheaten* viable mice have a mutation in the SHP-1 gene that results in almost complete loss of catalytic activity. These *motheaten* viable mice *(mev)* survive for several weeks, although older animals are thrombocytopenic. Surprisingly, *mev* platelets exhibit a reduction in tyrosine phosphorylation of Lyn and Syk which is associated with a reduction in expression of P-selectin in response to CRP (87). This reduction is associated with a marked increase in tyrosine phosphorylation of a novel protein of 26 kDa which migrates below those of 28-32 kDa described above. Further studies are required to identify this protein and the basis of the reduction in response to GPVI, including the possibility that this is due to a defect in megakaryocyte development.

Several, uncharacterised tyrosine phosphorylated proteins associate with SHIP in platelets activated by GPVI or thrombin. Tyrosine phosphorylation of SHIP, and association with other proteins, are inhibited by the intracellular Ca^{2+} chelator, BAPTA-AM. The functional role of SHIP in platelets has been investigated in mice deficient in the 5'-phosphatase. As described above, platelets from SHIP-deficient mice show increased responsiveness to CRP in the presence of extracellular Ca^{2+}. This is mediated through a novel pathway of Ca^{2+} entry which is regulated through the binding of PI3,4,5P3 to Btk, and which is independent of PLCγ2 (38). Physiologically, this pathway appears to work in combination with capacitative entry to regulate the intracellular levels of the cation. The role of SHIP would therefore appear to be to prevent excessive activation of this novel pathway of Ca^{2+} entry, consistent with its regulation by the cation.

SHIP2 has a wider distribution than SHIP, and has been found in all haematopoeitic and non-haematopoietic tissues that have been investigated (88). It is also selectively recruited to ITIMs (89). The possibility that SHIP2 participates in platelet activation requires investigation.

Is the Pathway of Platelet Activation by Collagen Unique?

We originally proposed that collagen is a unique platelet agonist in that it is the only physiologically relevant stimulus to induce activation at sites of damage to the vasculature through an ITAM-dependent pathway. This hypothesis has been challenged by the observations that the GPIb-selective snake venom toxin, alboaggregin A, and von Willebrand factor in the presence of ristocetin stimulate tyrosine phosphorylation of FcR γ-chain, Syk and PLCγ2 (90). Ristocetin is required to convert vWF into a form that binds GPIb in the absence of shear. There are however important differences between the actions of alboaggregin A and vWF/ristocetin on platelets. Alboaggregin A stimulates a much greater level of whole cell tyrosine phosphorylation than vWF/ristocetin and is similar in magnitude to that seen with convulxin (91). Further, aggregation by alboaggregin A is preceded by shape change and is associated with activation of PLC, whereas the response to vWF/ristocetin under the same conditions is not. Alboaggregin A-induced aggregation is also partially inhibited by the Src kinase inhibitor PP1 and by a mAb to GPIb, 6D1, and both reagents are required to produce full inhibition. In contrast, aggregation to vWF/ristocetin is blocked completely by mAb 6D1 but is not altered by PP1. Evidence for a role of a second receptor in the response to alboaggregin A is provided by studies on Bernard-Soulier Syndrome platelets, that lack detectable GPIb (91). The pattern of aggregation to alboaggregin A is similar to that induced by convulxin, whereas the response to vWF/ristocetin is abolished. Alboaggregin A also activates the GPVI/FcR γ-chain complex when expressed in K562 cells which lack detectable GPIb, whereas untransfected cells are unresponsive (91). These results demonstrate that alboaggregin A activates GPVI and raises the possibility that the same venom toxin is able to bind to two receptors.

The physiological significance of the increase in tyrosine phosphorylation of proteins in the GPVI pathway, including FcR γ-chain, Syk and PLCγ2, by vWF/ristocetin is not clear. The response to vWF/ristocetin is weaker than that to convulxin despite a far greater number of receptors for GPIb than GPVI (25,000 compared with 2,000). In addition, in our hands the increase in tyrosine phosphorylation does not appear to lead to significant activation of PLCγ2 and αIIbβ3-dependent aggregation (92), although other studies have reported functional responses to the glycoprotein. Whilst the explanation for these differences is not known, it is clear that vWF/ristocetin is a weak stimulus of this pathway. This suggests either that the receptor is poorly coupled to the FcR γ-chain pathway, thereby giving rise only to a subliminal level of signalling, or that only a subpopulation of the receptor underlies the increase in tyrosine phosphorylation. It is not known whether the adhesion molecule elicits a larger increase in response under the physiological condition of high shear.

A number of receptors activate the tyrosine kinase Syk in platelets, including the integrin αIIbβ3, several G protein-coupled receptor agonists and the thrombospondin-derived peptide, KRFYVVMWK. However, αIIbβ3 does not induce a corresponding increase in phosphorylation of the FcR γ-chain, whilst phosphorylation by G protein-coupled receptor agonists is small. Additionally, the Src family kinase inhibitor PP1 has a negligible effect on the initial phase of platelet aggregation to G protein coupled receptor agonists (33), whereas a delayed effect is thought to be due to inhibition of outside-in signalling through $\alpha IIb\beta_3$ (93). Thus the FcR γ-chain-Syk pathway is unlikely to have a major role in the response to thrombin and other G protein coupled receptor agonists.

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The peptide, KRFYVVMWKK, derived from the C-terminus of thrombospondin, stimulates platelet activation and marked phosphorylation of Syk through the integrin-associated receptor (IAP, also known as CD47) (94). Additionally, the related peptide, KRFYVVMWK, stimulates tyrosine phosphorylation of FcR γ-chain (Tulasne and Watson, unpublished). Phosphorylation of Syk and functional responses are inhibited in FcR γ-chain-deficient platelets in response to KRFYVVMWK demonstrating a causal relationship. Studies on GPVI expressed in K562 cells, however, demonstrate that this is not mediated by direct binding to the glycoprotein receptor. The physiological significance of this pathway is unclear as a similar response is not seen with thrombospondin itself (unpublished).

Outstanding Questions on Collagen Receptors and Future Developments

We are only just beginning to understand the signalling events that underlie platelet responses to collagen. Whilst it is clear that the GPVI-FcR γ-chain pathway plays a fundamental role in the response to collagen, with critical roles for FcR γ-chain, Syk, LAT, SLP-76 and PLCγ2, there are many other proteins in this cascade whose roles are undefined. It will be important to determine the role of these proteins in the sequence of events that lead to activation of PLCγ2 and PI 3-kinase, including all protein-protein interactions and the domains through which these are mediated. This will involve mapping sites of phosphorylation and identification of relevant kinases. The genetic engineering of mice deficient in one or more proteins and the emerging technologies of proteomics and genetic engineering of megakaryocytes *in vivo* will play an increasing role in the development of this field over this time.

It is also important to identify the role of other receptors for collagen in the response to the adhesion molecule, and whether they function as co-receptors. There is particular interest in identifying the receptor which is able to induce tyrosine phosphorylation in the absence of GPVI. It is also not known why platelets express a large number of receptors for collagen, although a need to fulfill distinct roles such as adhesion and activation is likely to form part of the explanation. In addition, they may be receptors for distinct collagen types. Nineteen different types of collagen are found in man, of which seven are present in the vasculature. Important quantative and qualitative differences between the effects of different collagens have been reported (95), and it is presently unclear whether all collagens induce activation through GPVI.

Important questions are outstanding regarding the physiological significance of platelet collagen interactions and whether antagonists of GPVI and α2β1 would show efficacy in prevention of thrombosis. Considerable advances can also be expected in the emerging field of ITIM-coupled receptors on the platelet surface, including the possibility that they represent novel targets for therapeutic blockade of GPVI signalling.

Note added in proof

Zhen et al. have recently reported that expression of GPVI in rat rbl mast cells is unable to reconstitute responses to collagen or CRP whereas convulxin elicits a full response with a similar EC50 to that observed in the platelet (96).

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