Platelet endothelial cell adhesion molecule-1 inhibits platelet response to thrombin and von Willebrand factor by regulating the internalization of glycoprotein Ib via AKT/glycogen synthase kinase-3/dynamin and integrin αIIbβ3

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Platelet Endothelial Cell Adhesion Molecule-1 inhibits platelet response to thrombin and von Willebrand Factor by regulating the internalisation of Glycoprotein Ib via AKT/Glycogen Synthase Kinase-3/Dynamin and Integrin αIIbβ3

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Running title: PECAM-1 mediated internalisation of GPIb

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Abstract.

Objectives – Platelet endothelial cell adhesion molecule-1 (PECAM-1) regulates platelet response to multiple agonists. How this immunoreceptor tyrosine-based inhibitory motif–containing receptor inhibits G protein-coupled receptor–mediated thrombin-induced activation of platelets is unknown.

Approach and Results – Here, we show that the activation of PECAM-1 inhibits fibrinogen binding to integrin αIIbβ3 and P-selectin surface expression in response to thrombin (0.1–3U/ml) but not thrombin receptor-activating peptides SFLLRN ($3x10^{-7}$–$1x10^{-5}$M) and GYPGQV ($3x10^{-6}$–$1x10^{-4}$M). We hypothesised a role for PECAM-1 in reducing the tethering of thrombin to GPIbα on the platelet surface. We show that PECAM-1 signalling regulates the binding of FITC-labelled thrombin to the platelet surface and reduces the levels of cell surface GPIbα by promoting its internalisation, while concomitantly reducing the binding of platelets to von Willebrand factor under flow *in vitro*. PECAM-1 mediated internalisation of GPIbα was reduced in the presence of both EGTA, and/or Cytochalasin D and Latruculin, but not either individually, and was reduced in mice in which tyrosines 747 and 759 of the cytoplasmic tail of β3 integrin were mutated to phenylalanine. Furthermore PECAM-1 cross-linking led to a significant reduction in the phosphorylation of GSK-3β Ser^{9}, but interestingly an increase in GSK-3α $pSer^{21}$. PECAM-1 mediated internalisation of GPIbα was reduced by inhibitors of Dynamin (Dynasore) and GSK-3 (CHIR99021), an effect that was enhanced in the presence of EGTA.

Conclusions – PECAM-1 mediates internalisation of GPIbα in platelets through dual AKT/AKT/protein kinase B/glycogen synthase kinase-3/dynamin-dependent and αIIbβ3-dependent mechanisms. These findings expand our understanding of how PECAM-1 regulates non-immunoreceptor signalling pathways and helps to explains how PECAM-1 regulates thrombosis.

Nonstandard abbreviations and acronyms	
PMI	PECAM-1 mediated internalisation

Introduction

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a 130-kDa transmembrane glycoprotein expressed on the platelet surface at between 5000 and 8800 copies per cell.[1-3] It is composed of a 574 amino acid residue extracellular portion organised into six immunoglobulin (Ig)-like homology domains, a 19 amino acid transmembrane domain and a cytoplasmic domain that contains an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) ((L/V/I/S/T)XYXX(L/V)) and an Immunoreceptor Tyrosine-based Switch Motif (ITSM) (TxYxx(V/I)).[4, 5] The tyrosine residues (residues 663 and 686) within the ITIM and ITSM become tyrosine phosphorylated by Src or Csk family kinases,[6] following homophilic ligation or stimulation of platelets through protease-activated receptor (PAR)-1 and 4, glycoprotein VI (GPVI), or GPIb in a process that is largely, but not wholly, dependent on integrin αIIbβ3-dependent platelet aggregation.[7-10]

The inhibition by PECAM-1 of immunoreceptor signalling downstream of GPVI is well established. Once phosphorylated these twin ITIM and ITSM tyrosine residues support the recruitment of Src-homology 2 (SH2) domain containing proteins, including the protein-tyrosine phosphatases SHP-2, and to a lesser extent SHP-1,[11] to which the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-K) becomes associated destabilising its association with the linker for activation of T cells (LAT) and the scaffolding molecule Grb-2-associated binding protein-1 (Gab1).[12] By disturbing the LAT-GAB1-PI3K signalling complex PECAM-1, therefore, disrupts the recruitment and activation of phospholipase C (PLCɣ2),[12] thereby inhibiting immune-like signalling downstream of GPVI and reducing the activation of platelets by collagen, the GPVI specific ligand cross-linked collagen-related peptide (CRP-XL) and convulxin (CVX).[10, 13, 14]

More surprising is the ability of PECAM-1 to reduce platelet activation through GPCR mediated signalling pathways following stimulation by thrombin or ADP. Whilst hyper-reactivity to thrombin and ADP has not been detected in PECAM-1 deficient mice,[15] pre-activation of PECAM-1 has been shown to reduce fibrinogen binding to platelets, secretion and calcium mobilisation in response to ADP and thrombin,[14] and in a laser injury model of thrombosis in cremaster muscle arterioles, that is dependent on thrombin generation rather than collagen exposure,[16] thrombi formed more rapidly, were more stable and approximately 35% larger in PECAM-1 deficient mice.[17] The mechanism by which PECAM-1 inhibits GPCR-mediated activation of platelets is as yet unclear.

While the inhibitory effect of PECAM-1 on individual pathways is modest, corresponding to 5-15% inhibition in platelet function, the physiological importance of PECAM-1 comes from its ability to inhibit multiple activation pathway. The combination of these effects account for a substantial inhibition in thrombus formation.[14, 17]

We have previously reported that although PECAM-1 inhibits the activation of platelets by thrombin, it does not inhibit activation stimulated by TRAP. Here we investigate this disparity and show that the activation of PECAM-1 triggers the internalisation of GPIb by dual AKT/GSK-3/dynamin and αIIbβ3-dependent mechanisms, which result in the reduction in platelet binding of, and hence response to, thrombin, and reduced platelet binding to VWF.

Materials and methods

Materials and Methods are available in the online-only Data Supplement

Results

Activation of PECAM-1 inhibits the binding of thrombin to human platelets and their subsequent activation.

Consistent with our previous work, activating PECAM-1 by cross-linking antibodies that recognise the sixth Ig domain of PECAM-1 (AB468), resulting in PECAM-1 phosphorylation,[12-14] led to a small (5-15%) but significant inhibition in platelet fibrinogen binding and P-selectin exposure in response to thrombin across a range of concentration ($p<0.0001$ and $p=0.001$, respectively, two-way ANOVA) (Figure 1A). Again confirming our previous findings PECAM-1 cross-linking did not inhibit platelet response to either PAR-1 or PAR-4 activating peptides (Figure 1A). The inhibition of thrombin activation of platelets without inhibiting PAR signalling suggests a role for PECAM-1 in modulating the binding of thrombin to platelets. Consistent with this cross-linking PECAM-1 led to a significant reduction in the binding to FITC-labelled thrombin and a concomitant reduction of platelet activation measured by the surface exposure of P-selectin (Figure 1B and Supplementary Figure I).

This reduction of thrombin binding was specific to the activation of PECAM-1. To control for the potential steric inhibition caused by the binding and cross-linking of the PECAM-1 antibody we repeated these experiment using a different PECAM-1 antibody (WM59) that binds PECAM-1 but prevents its activation, thereby providing a control for any steric effects. No reduction in thrombin binding was seen when platelets were incubated with antibodies against the 1st or 2nd Ig domain of PECAM-1 (WM59) (which prevent homophilic ligation and the activation of PECAM-1[18]) and the cross-linking antibody (Figure 1C). Similarly the binding and cross-linking of antibodies against GPIbα, GPVI, PAR-1 and PAR-4 had no effect on the binding of thrombin to the platelet surface (Figure 1C).

PECAM-1 crosslinking reduces the levels of GPIbα at the cell surface.

GPIbα as part of the GPIb/V/IX complex plays an integral role in thrombin activation. It is a high affinity receptor for thrombin[19] and acts as a cofactor for PAR cleavage.[20] Furthermore PECAM-1 plays a role in GPIb signalling, becoming tyrosine phosphorylated upon VWF binding to GPIbα, and PECAM-1 deficient mice show enhanced aggregation in response to VWF.[9] We hypothesised that the reduction in thrombin binding to platelets may be due to reduced surface expression of GPIbα following PECAM-1 activation. PECAM-1 cross-linking resulted in a significant reduction in the binding of antibodies against GPIbα, and a reduction in antibodies against GPIbβ (Figure 2A and Supplementary Figure II). Although the level of GPIbα expressed on the surface of resting platelets varied between subjects the reduction in GPIbα following PECAM-1 cross-linking occurred in all subjects. The action of PECAM-1 appeared to be specific for GPIb and did not cause a reduction in the binding of antibodies against PAR-1 and PAR-4, nor GPVI (Figure 2A).

To confirm that this reduction was due to PECAM-1, PECAM-1 on the surface of WT mouse platelets was cross-linked using antibodies that recognise the sixth Ig domain of mouse PECAM-1 (M-185), and, as with human blood resulted in a significant decrease in GPIbα on the platelet surface. No such decrease was seen in PECAM-1 deficient mouse platelets (Figure 2B). Again to control for the effects of steric inhibitions caused by the binding and cross-linking of the PECAM-1 antibody we repeated these experiments using a different PECAM-1 antibody (WM59) that binds PECAM-1 but prevents its activation. No reduction in GPIbα was seen when platelets were incubated with antibodies against the 1st or 2nd Ig domain of PECAM-1 (WM59) and the cross-linking antibody (Figure 2C).

PECAM-1 activation inhibits platelet binding to VWF

Given these findings we hypothesised that PECAM-1 cross-linking should also reduce platelet adhesion to VWF. The number of platelets in whole blood binding to VWF (100µg/ml) under arterial flow conditions was indeed reduced following PECAM-1 cross-

linking (Figure 2D-G). This supports the role of PECAM-1 in regulating surface GPIbα and is in line with previous reports showing enhanced aggregation in response to VWF in PECAM-1 deficient mice.[9]

PECAM-1 mediated reduction of platelet surface GPIbα is due to internalisation and not cleavage.

We sought to determine if the PECAM-1 mediated reduction in the surface expression of GPIbα was due to internalisation or cleavage of the receptor. Previous reports have demonstrated the cleavage of GPIbα by ADAM17, generating a ~95kDa fragment and a ~45kDa N-terminal fragment.[21] Using a purified cobra metalloproteinase, Nk (10μg/mL)[22] as a positive control we identified both the ~130kDa intact GPIbα and the ~95kDa and ~45kDa fragments by Western blotting, using antibodies against the C-terminal and N-terminal of GPIbα respectively (Figure 3A). Neither of these cleaved forms of GPIbα were observed in lysates of resting, CRP-XL stimulated or PECAM-1 stimulated platelets, indicating that no detectable cleavage of GPIbα occurred (Figure 3A). Furthermore, there was no evidence of any GPIbα cleavage products in these samples. Although there is a smear in the lane of the PECAM-1 stimulated platelets, this appears at the same height when probing with both the N-terminal and C-terminal antibodies and is present in the antibody control lanes. We therefore conclude that this smear is not due to cleavage of GPIbα.

To further test if GPIbα was cleaved or internalised we measured the binding of GPIbα antibodies to non-permeablised and permeablised platelets. As in all previous experiments the binding of GPIbα antibodies to non-permeablised platelets (i.e. binding solely to GPIbα expressed on the surface of platelets) decreased following PECAM-1 stimulation. There was, however, no reduction in binding of GPIbα antibodies to permeablised platelets (i.e. when the GPIb antibody could access both external and internal pools of GPIb) indicating that there was no loss of GPIbα from the platelets (Figure 3B). Taken together these two pieces of evidence suggest that PECAM-1 mediated reduction of platelet surface GPIbα is due to internalisation and not cleavage.

PECAM-1 internalisation of GPIbα is dependent on cytoskeletal rearrangement and exogenous calcium ions.

The internalisation of GPIbα is well known following platelet activation,[23] and PECAM-1 has, in other cells types, been shown to be involved in non-clathrin mediated endocytosis.[24] To elucidate the mechanism by which PECAM-1 mediated internalisation (PMI) of GPIbα occurs in platelets the reduction in surface expression of GPIbα was measured following PECAM-1 activation in the presence of EGTA, to chelate extracellular calcium ions, and/or Cytochalasin D and Latruculin, to prevent actin polymerisation. Individually none of the compounds significantly inhibited PMI of GPIbα. A combination of EGTA and either Cytochalasin D or Latrunculin did, however, completely abrogate the effect of PECAM-1 activation on the surface expression of GPIbα (Figure 4A). By contrast GPIbα internalisation following stimulation of GPVI by CRP-XL was not inhibited by EGTA alone but was completely inhibited by either Cytochalasin D or Latrunculin (Figure 4B). This indicates that while GPVI mediated internalisation of GPIbα is entirely dependent on cytoskeletal re-arrangement, the activation of PECAM-1 initiates two separate mechanisms, one dependent on cytoskeleton and the other dependent on exogenous divalent cations, both of which are capable of modulating the surface exposure of GPIbα independently.

Chelating extracellular calcium inhibits the activation of integrin αIIbβ3, the activation of which is known to be enhanced by PECAM-1.[15, 25] To establish whether integrin αIIbβ3 is involved in PMI of GPIbα, we used knock-in (KI) mice (DiYF mice) in which tyrosines 747 and 759 of the cytoplasmic tail of the β3 integrin were mutated to phenylalanine, preventing tyrosine phosphorylation, and selectively impairing outside-in αIIbβ3 signalling.[26] Internalisation of GPIbα following PECAM-1 cross-linking was reduced in heterozygous mice

and abolished in KI mice (Figure 4C). In corroboration of our findings in humans, internalisation of GPIbα following stimulation with CRP-XL was unaltered in DiYF mice (Figure4D). There was no significant difference between WT, heterozygous or homozygous KI mice in the surface expression of either αIIbβ3 or GPVI (Figure 4E, F).

PMI of GPIbα occurs in an AKT/GSK-3/Dynamin dependent manner

PECAM-1 clustering has been reported to induce Dynamin-2 dependent endocytosis in endothelial cells,[24, 27] while in platelets dynamin has been shown to be critical to the internalisation of both $P2Y_1$ and $P2Y_{12}$.[28] To investigate the role of dynamin in PMI of GPIbα, platelets were incubated with Dynasore (100μM) prior to antibody mediated PECAM-1 cross-linking. PMI of GPIbα was reduced in the presence of Dynasore and almost abolished in the presence of Dynasore and EGTA (Figure 5A). In part the action of dynamin in endocytosis is regulated by the de-phosphorylation (by calcineurin[29, 30]) and re-phosphorylation (by cdk5 and GSK-3[31, 32]) of two serine residues in the C-terminal proline-rich domain (PRD) of dynamin. While de-phosphorylation of dynamin is required for clathrin-mediated endocytosis in neurons, non-clathrin-mediated bulk endocytosis (of the type previously shown to result from PECAM-1 activation in endothelial cells[24]) requires both de-phosphorylation and re-phosphorylation.[32, 33] We have previously shown that PECAM-1 crosslinking resulted in reduced phosphorylation of AKT at Ser^{473}.[12] This should lead to a reduction in the phosphorylation of GSK-3, which would in turn regulate its kinase activity. Following stimulation of platelets with CRP-XL the phosphorylation of GSK-3β Ser^9 increased compared to the level in unstimulated cells, however, following PECAM-1 crosslinking the phosphorylation of GSK3-β Ser^9 decreased (Figure 5B-C). Interestingly the same pattern was not repeated for GSK-3α. Ser^{21} phosphorylation of GSK-3α did not increase significantly in response to CRP-XL but did increase significantly following PECAM-1 crosslinking (Figure 5B-C). To confirm the role of GSK-3 in PMI we used the highly selective inhibitor of both GSK-3 isoforms, CHIR99021, which inhibited the internalisation of GPIbα following the cross-linking of PECAM-1 (Figure 5D). Together this data suggests that the internalisation of GPIbα following PECAM-1 stimulation occurs in a non-clathrin, AKT/GSK-3/Dynamin dependant manner which may be similar to bulk endocytosis reported in endothelial or neuronal cells.

Discussion

Two of the enduring questions surrounding the role of PECAM-1 in regulating platelet response are how this ITIM containing molecule inhibits non-immunoreceptor signalling, and how a molecule with what appears to be a moderate impact on individual signalling pathways can have such a profound effect on thrombus formation *in vitro* and *in vivo.*[14, 17] By investigating the disparity between the action of PECAM-1 on platelet response to thrombin and TRAP we have identified a PECAM-1 mediated receptor internalisation pathway mediated via dual AKT/GSK-3/Dynamin and αIIbβ3 dependent mechanisms that result in the internalisation of GPIbα, a reduction in thrombin binding to, and activation of, platelets and reduced platelet binding to VWF. The physiological impact of PMI of GPIbα on the binding of platelets to vessel-bound VWF is uncertain as it is unlikely that PECAM-1 is activated before platelets bind to VWF and adhere to the site of vessel damage. This novel role for PECAM-1 in platelets does, however, explain how PECAM-1 inhibits GPCR-mediated activation of platelets by thrombin, and why it has such a potent impact on thrombosis. By simultaneously reducing thrombin stimulation through PMI of GPIb, and GPVI signalling through its action as an ITIM containing receptor, PECAM-1 directly regulates two of the major pathways by which platelets become activated and propagate thrombus formation.

PMI of GPIbα appears to occur via a distinct mechanism that is separate from the internalisation of GPIbα that follows GPVI stimulation. This was seen in the internalisation of

GPIbα in response to CRP-XL in PECAM-1 KO mice, and the ability of Cytochalasin D and Latrunculin alone to inhibit CRP-XL mediated internalisation of GPIbα, but not PMI. It is likely, however, that *in vivo* these two mechanisms work in concert, particularly as PECAM-1 becomes phosphorylated following GPVI stimulation, and as PECAM-1 and GPVI have opposing effects on AKT and hence GSK-3 phosphorylation. Why such opposing signalling events result in such similar outcomes is not yet known. The importance of PMI during thrombus formation may in part come from the activation of PECAM-1 by homophilic ligation, which only occurs during close platelet-platelet contact. Under these circumstances stimulation of receptor internalisation or endocytosis by PECAM-1 at the site of contact may be a mechanism for recycling receptors that are not occupied or bound into focal adhesion complexes. Trafficking these receptors away from the site of contact to areas of the platelet that are not in contact with other cells provides an attractive mechanism by which to maximise the efficiency of platelet response to multiple simultaneous stimuli and orchestrate thrombus growth.

The mechanism by which integrin β3 regulates PMI is as yet unknown. Its effect, however, appears to be critical. One explanation for this, perhaps lies with filamin. Filamin binds to GPIbα in resting platelets anchoring it to the cytoskeleton,[34] helping to maintain the structure and integrity of the plasma membrane following platelet tethering to VWF under high shear.[35] Upon stimulation filamin becomes detached from the cytoplasmic tail GPIbα allowing its translocation.[36] By contrast filamin binds to αIIbβ3 following platelet activation. One tempting hypothesis is that PECAM-1 plays a role, either directly or indirectly, in the decoupling of filamin from GPIbα and its subsequent binding to integrin β3. We have as yet been unable to confirm or refute this hypothesis but it will be the focus of future work.

Of the two isoforms of GSK-3 it is the β form that is thought to be predominant in platelets.[37] Proteomic analysis has, however, identified that the copy number per platelet for each isoform is similar, 1300 for GSK-3β and 1000 for GSK-3α.[38] Both isoforms are constitutively active in resting platelets but are inhibited by phosphorylation of Ser^{21} (GSK-3α) or Ser^{9} (GSK-3β) by PKC or AKT upon platelet stimulation.[39] It is thought that GSK-3 acts as a negative regulator of platelets; GSK-3 inhibitors increase platelet responses, GSK-3β +/- mice display increased aggregation and thrombus formation, and platelets from mice in which GSK-3α Ser^{21} and GSK-3β Ser^{9} have been mutated to Ala show reduced response.[37, 39] The reduction on GSK-3β $pSer^{9}$ following PECAM-1 cross-linking fits with this understanding of platelet GSK-3. PECAM-1, which has a well-established inhibitory effect on platelet signalling, reduces AKT phosphorylation and therefore reduces the phosphorylation and inhibition GSK-3β, thereby enhancing its inhibitory effect, some of which may be mediated through Dynamin and translocation of surface receptors, as we have shown to be the case with GPIb. The increase in GSK-3α phosphorylation following PECAM-1 cross-linking does not, however, fit with our current understanding. Part of the reason for this is that all previous work on GSK-3 in platelets has focused either on GSK-3β alone, as in the case of the GSK-3β +/- mice, or has assumed that both isoforms work in a similar way and have therefore inhibited both isoforms. In the case of dual inhibition the effect of GSK-3α may be masked by GSK-3β making any interpretation of the physiological relevance of GSK-3α impossible. Although there is 85% similarity in the amino acid sequence between the isoforms and 98% homology in the kinase domain[40] there is growing evidence that the two isoforms of GSK-3 have distinct roles.[41-43] Quite why the activation of PECAM-1 has such contrasting effects on the two GSK-3 isoforms and what significance GSK-3α phosphorylation has in PECAM-1 signalling are as yet unknown.

The inhibition of platelet activation by PECAM-1 is well known but the mechanisms by which this ITIM-containing receptor inhibits platelet response to thrombin were not. Here we have shown that the activation of PECAM-1 results in the internalisation of GPIb by dual GSK-3/Dynamin and αIIbβ3 dependent mechanisms, which result in the reduction in platelet binding of, and hence response to, thrombin, and reduced platelet binding to VWF.

Furthermore, we have for the first time shown the differential regulation of GSK-3α/β by PECAM-1 in platelets. This novel role for PECAM-1 expands our understanding of how this promiscuous molecule regulates multiple diverse signalling pathways and why it has such a potent impact on thrombosis. It may also suggest a role for PECAM-1 in regulating conditions where the generation of thrombin, and the response of platelets to thrombin is central to pathology.

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Disclosures

None.

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Significance.

We show two new facets of PECAM-1 biology in platelets. 1) That PECAM-1 mediates the internalisation of GPIbα leading to a reduction in the activation of platelets by thrombin and a reduction in their binding to VWF. 2) That PECAM-1 differentially regulates the phosphorylation of the two isoforms of GSK-3. In so doing this article answers a number of the outstanding questions concerning the regulation of platelets by PECAM-1; how this ITIM containing molecule inhibits non-immunoreceptor signalling, and importantly, how PECAM-1 has such a profound effect on thrombus formation *in vitro* and *in vivo*.

Figure 1 – Activation of PECAM-1 inhibits the binding of thrombin to human platelets and their subsequent activation. (A) Fibrinogen binding (top row) and P-selectin expression (bottom row) to human platelets in response to thrombin, PAR-1 or PAR-4 activating peptides in the presence (black) or absence (grey) of PECAM-1 (AB468) crosslinking and activation was measured by flow cytometry (n=5). P-values compare the difference between the two curves over the entire concentration range using Two-way ANOVA with Bonferroni post-test analysis of the difference at individual concentrations. (B) To ascertain if reduced platelet response to thrombin was due to reduced thrombin binding, the reduction in the binding of FITC-labelled thrombin to human platelets (left) and the resultant surface exposure of P-selectin (right) was measured following PECAM-1 crosslinking. The binding of FITC-thrombin to platelets was measured by flow cytometry and is presented as the median fluorescent intensity (MFI) (left hand axis - presence (black line) or absence (grey line) of PECAM-1 (AB468) crosslinking) and the percentage reduction in median fluorescent intensity (MFI) in PECAM-1 cross-linked samples compared to the isotype control (right hand axis) (n=5). (C) To check that this reduction in thrombin binding was specific to the activation of PECAM-1 and not the binding of antibodies to the platelet surface, the binding of FITC labelled thrombin to human platelets in the presence of cross-linked isotype control, PECAM-1 (WM59 - inhibitory), GPIbα, GPVI, PAR-1 or PAR-4 antibodies was measured (n=3). In all cases *p<0.05, **p=<0.01, ***p<0.001.

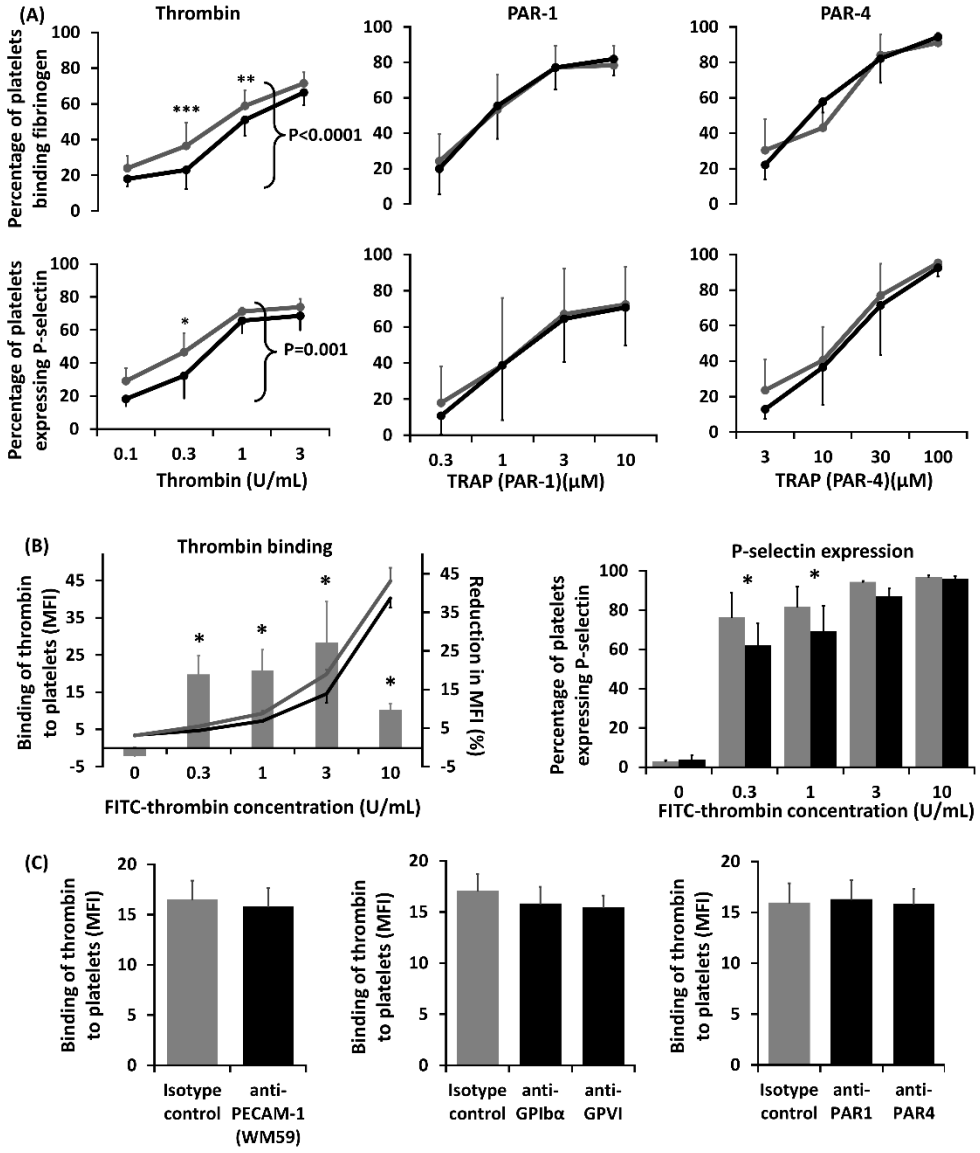

Figure 2 – Activation of PECAM-1 reduces the surface expression of GPIbα and reduces platelet binding to vWF. (A) Human platelet surface expression of GPIbα, GPIbβ, GPVI, PAR-1, PAR-4 in the presence (black) or absence (grey) of PECAM-1 (AB468) crosslinking was measured by flow cytometry (n=12 for anti-GPIbα, and n=3 for all other experiments). (B) To confirm that this was PECAM-1 specific the expression of GPIbα on the surface of mouse platelets from WT (n=4) or PECAM-1 KO (n=6) mice following the crosslinking of isotype control (grey) or PECAM-1 stimulatory antibodies (black) was measured. (C) To check that this reduction in the surface of GPIbα was specific to the activation of PECAM-1 and not the binding of antibodies to the platelet surface, human platelet surface expression of GPIbα was measured in the presence of cross-linked isotype control (grey), PECAM-1 (WM59 - inhibitory)(black)(n=4). The difference in MFI between (A) and (C) are because they were performed on different flow cytometers, a FACSCalibur and an Accuri C6, respectively. (D-G) Given the importance of GPIbα in platelet tethering to VWF we assessed the impact of PECAM-1 crosslinking on the binding of platelets to VWF under flow. (D) Representative images showing human platelets binding to VWF (100µg/ml) under flow conditions following the crosslinking of isotype control or PECAM-1 stimulatory antibodies, or, as a positive control, antibodies that block the binding of GPIb to VWF (Haematologic Technologies Inc). (E) The number of human platelets binding to VWF (100µg/ml), (F) the area covered by, and (G) the sum fluorescence intensity of these platelets following the crosslinking of isotype control (grey) or PECAM-1 (black) stimulatory antibodies (n=3). *p<0.05, **p=<0.01, ***p<0.001

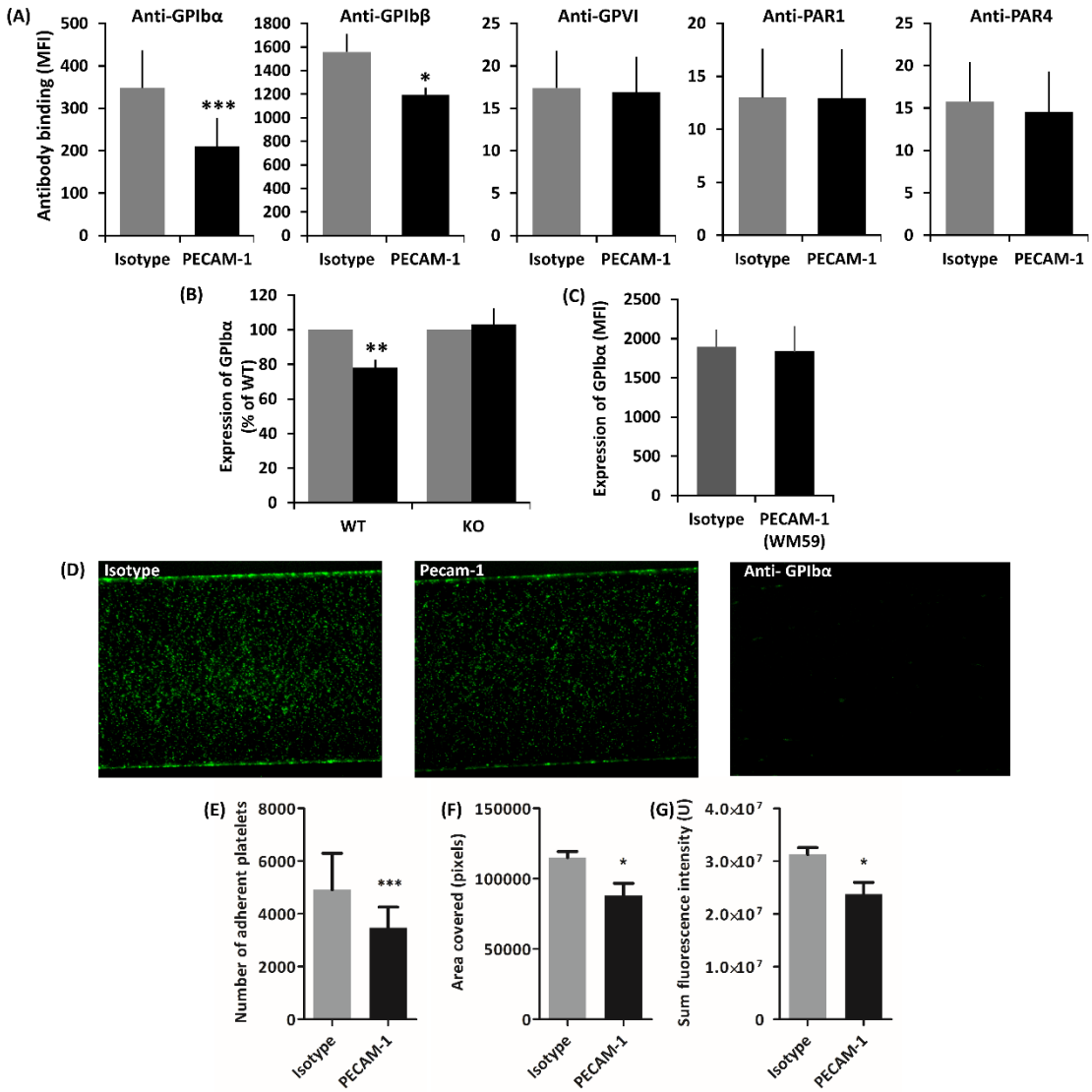

Figure 3 – GPIbα is internalised and not cleaved following PECAM-1 activation. (A) To determine whether GPIbα is cleaved following PECAM-1 activation, human platelets that had been stimulated for 90 or 600 seconds with CRP-XL (µg/mL), or undergone PECAM-1 activation via antibody crosslinking, or been exposed to purified cobra metalloproteinase, Nk (10µg/ml), were probed by western blotting using antibodies that recognise either the C or the N-terminal domain of GPIbα. Cleavage products were clearly visible in samples treated with Nk but not CRP-XL or PECAM-1 stimulated platelets. The smear present in the PECAM-1 stimulated samples appears at the same height when probing with both the N-terminal and C-terminal antibodies and appears when the antibodies are run alone in the absence of platelets. We therefore conclude that it is the result of protein in the stimulating antibodies rather than a result of cleavage. (B) To identify the internalisation of GPIbα following PECAM-1 activation the binding of GPIbα antibodies to non-permeablised human platelets (i.e. binding to GPIbα expressed on the surface of platelets) or to permeablised platelets (i.e. binding to all GPIbα, internal and surface expressed) following PECAM-1 cross-linking was measured by flow cytometery (n=3). *p<0.05.

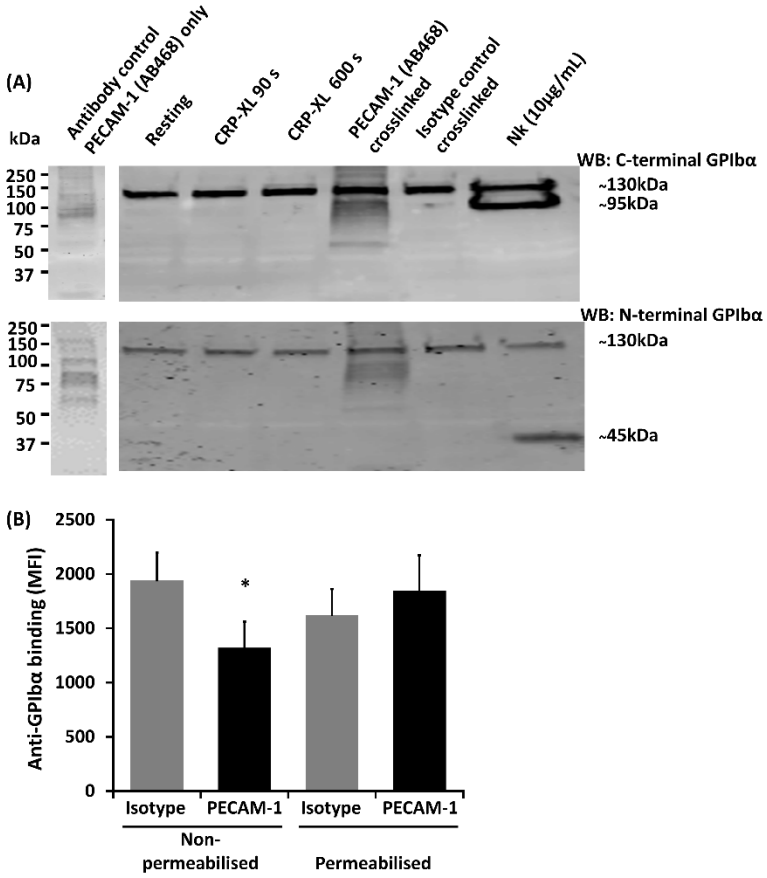

Figure 4 – Actin polymerisation and αIIbβ3 are required for PECAM-1 mediated internalisation of GPIbα. Surface expression of GPIbα on human platelets following (A) PECAM-1 crosslinking or (B) stimulation with CRP-XL in the presence of EGTA (2mM), Cytochalasin D (50μM) or Latrunculin (8.5μM) was measured by flow cytometry (n=5). To test the involvement of αIIbβ3 in PMI of GPIbα, platelets from DiYF mice, in which tyrosines 747 and 759 of the cytoplasmic tail of the β3 integrin were mutated to phenylalanine, underwent (C) PECAM-1 crosslinking or (D) CRP-XL stimulation followed by measurement of surface GPIbα. The surface expression of (E) αIIbβ3 and (F) GPVI were also measured in these mice and showed no significant difference between the genotypes in resting or CRP-XL stimulated conditions (WT: n=5, Het: n=9, KI: n=5). Asterisks indicate significant difference from isotype control or resting platelets in all cases. *p<0.05, **p=<0.01, ***p<0.001

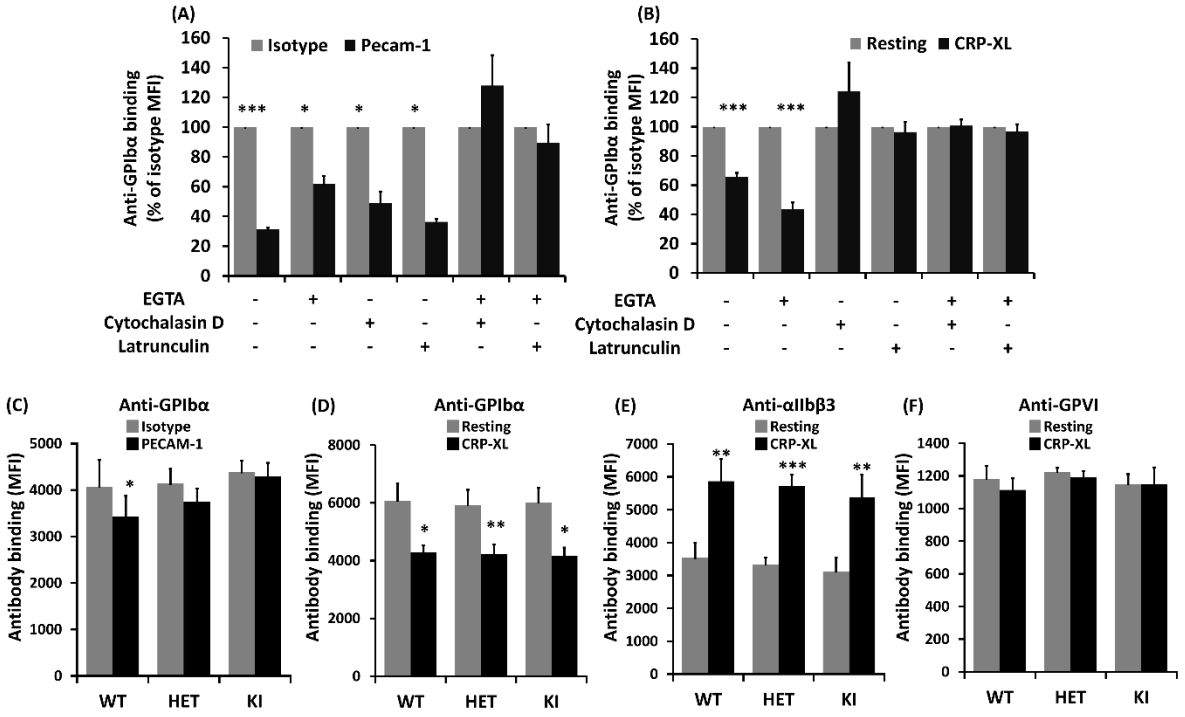

Figure 5 – PECAM-1 mediated internalisation of GPIbα is dependent on AKT/GSK-3/Dynamin signalling. (A) The reduction in the expression of GPIbα on the platelet surface resulting from PECAM-1 crosslinking was measured in the absence or presence of Dynasore (100μM) or Dynasore and EGTA together (n=8). (B) A representative image showing phosphorylation of GSK-3α Ser21 and GSK-3β Ser9, and total GSK-3α and β following platelet stimulation by CRP-XL (1μg/mL) or PECAM-1 cross-linking. (C) Quantification of Western blots showing phosphorylation of GSK-3α Ser21 and GSK-3β Ser9, and total GSK-3α and β following platelet stimulation by CRP-XL (1μg/mL) or PECAM-1 cross-linking (n=3). (D) The reduction in the expression of GPIbα on the platelet surface resulting from PECAM-1 cross-linking was measured in the absence or presence of the GSK-3 inhibitor CHIR99021 (3μM), or CHIR99021 (3μM) and EGTA (2mM) together (n=3). *p<0.05, **p=<0.01, ***p<0.001