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Impact of the PI3-kinase/Akt pathway on ITAM and hemITAM receptors: haemostasis, platelet activation and antithrombotic therapy

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ABSTRACT

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases that are activated in response to various stimulants, and they regulate many processes including inflammation; the stress response; gene transcription; and cell proliferation, differentiation, and death. Increasing reports have shown that the PI3Ks and their downstream effector Akt are activated by several platelet receptors that regulate platelet activation and haemostasis. Platelets express two immunoreceptor tyrosine based activation motif (ITAM) receptors, collagen receptor glycoprotein VI (GPVI) and Fcγ receptor IIA (FcγRIIA), which are characterized by two YxxL sequences separated by 6–12 amino acids. Activation of an

ITAM receptor initiates a reaction cascade via its YxxL sequence in which signaling molecules such as spleen tyrosine kinase (Syk), linker for activation of T cells (LAT) and phospholipase C γ2 (PLCγ2) become activated, leading to platelet activation. Platelets also express another receptor, C-type lectin 2 (CLEC-2), which has a single YxxL sequence, so it is appropriately called a hemITAM receptor. ITAM receptors and the hemITAM receptor share many signaling features. Here we will summarize our current knowledge about how the PI3K/Akt pathway regulates (hem)ITAM receptor-mediated platelet activation and haemostasis and discuss the possible benefits of targeting PI3K/Akt as an antithrombotic therapy.

Abbreviation

PI3K, phosphoinositide 3-kinases; ITAM, immunoreceptor tyrosine based activation motif; GPVI, glycoprotein VI; FcγRIIA, Fcγ receptor IIA; Syk, spleen tyrosine kinase; LAT, linker for activation of T cells; PLCγ2, phospholipase C gamma 2; CLEC-2, C-type lectin; vWF, von Willebrand factor; TxA_2, thromboxane A_2; GPCR, G protein-coupled receptor; TCR, T cell receptor; Lck, leukocyte-specific kinase; Zap-70, zeta-chain-associated protein kinase 70; CRP, collagen-related peptides; HEV, high endothelial venule; Grb2, growth factor receptor-bound protein-2; Gads, growth factor receptor-bound protein-2 adaptor downstream of Shc; PTEN, phosphatase and tensin homologue deleted on chromosome 10; DAG, diacyl glycerol; SHIP, SH2 domain-containing inositol polyphosphatase; PKC, protein kinase C; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; SNP, single-

nucleotide polymorphism; GSK3, glycogen synthase kinase 3; LiCl, lithium chloride; MEK, MAPK/ERK kinase; ERK, extracellular signal-regulated kinase

Keywords

Protein kinase B; Phosphatidylinositol 3-kinases; Platelets; C-type lectin; Glycoprotein VI

1. Introduction

Platelets are released from megakaryocytes in the bone marrow and have a short life, circulating in blood for about 9–10 days. Because they are fragments of megakaryocytes, they have no nucleus. Platelet activation is a vital step in both haemostasis: a physiological response that prevents significant blood loss following vascular injury and thrombosis: a pathological extension of normal haemostasis [1]. Platelets circulate in healthy vessels, but when a vessel wall is damaged, platelets become activated when they interact with the newly exposed subendothelial collagen. Platelets are tethered onto the site of injury through interaction of the glycoprotein (GP) Ib-V-IX complex with von Willebrand factor (vWF), which is bound to exposed collagen. This interaction enables collagen to interact with glycoprotein (GP) VI, thus initiating signaling events that lead to the conformational change (activation) of integrins αIIbβ3 and α2β1, enabling the former to bind to vWF and fibrinogen and the latter to bind to collagen. Following activation of these receptors, secondary mediators such as ADP and thromboxane A_2 (TxA_2) are secreted from platelets and activate ADP receptors and TxA_2 receptors, further supporting the haemostatic and thrombotic function of platelets. Dysregulation or dysfunction of platelet signaling events

can lead to bleeding disorders or formation of arterial thrombosis; thus it is important to understand platelet signaling pathways so we can treat these disorders appropriately [1]. PI3K and its downstream effectors are activated by several platelet receptors, however inhibition of PI3K isoforms or their downstream effectors leads to only partial blockade of platelet activation and only has a significant effect in response to low agonist concentrations [1-3]. This characteristic therefore makes them very attractive targets for novel antithrombotic therapy reducing the risk of potential bleeding side effects, which current strategies have to negotiate.

2. Platelet receptors

Platelets are known to express a wide variety of receptors: G protein-coupled receptors (GPCRs) (thrombin receptors PAR-1 and PAR-4), integrins (αIIbβ3, α2β1, α5β1, α6β1, αvβ3), leucine-rich repeat (LRR) family receptors (GPIb/IX/V), ADP receptors ($P2Y_1$ and $P2Y_{12}$), TxA_2 receptors (TPα and TPβ), immunoglobulin superfamily receptors (GPVI, FcγRIIA), and C-type lectin-like receptors (P-selectin and CLEC-2) [4] (Table1). Many of these receptors have had their signaling mechanisms investigated with data suggesting they share a high degree of similarity with the receptors activated under growth factors or hormones in nucleated cells. The common signaling pathways are activation of phospholipase C (PLC) and elevation of intracellular Ca^{2+}, leading to secretion of secondary mediators to facilitate platelet activation and conversion of inactive integrin via a conformational change to its ligand-binding active state (inside-out signaling). Among

these signaling events, phosphoinositide signaling is active in platelets and has been shown to play a critical role in platelet activation [1].

3. Platelet ITAM receptors

The immunoreceptor tyrosine based activation motif (ITAM) is a highly conserved sequence defined by Yxx(L/I)x_{6-12}Yxx(L/I) which can be found in the cytosolic domain in a number of receptors and adaptor proteins. When phosphorylated, ITAMs serve as docking sites for SH2 domain containing tyrosine kinases which are critical for initiation of signaling cascades. GPVI is the major collagen receptor on platelets. Through association with the Fc receptor γ (FcRγ) chain, which contains two YxxL motifs, signal transduction is initiated demonstrating that the GPVI-FcRγ complex is essential for GPVI signaling [5]. Other ITAM receptors of C-type lectin-like receptor (CLR) family such as dendritic cell-associated C-type lectin-2 (Dectin-2) which is predominantly expressed in myeloid cells is also known to interact with FcRγ and signal through the YxxL sequences in FcRγ. GPVI/FcRγ is the only ITAM receptor expressed in mouse platelets, but human platelets express both GPVI/FcRγ and FcγRIIA both of which signal through a tandem YxxL sequences. Platelet ITAM receptors utilize the same family of signaling proteins as ITAM receptors in other hematopoietic cells such as T cell receptor (TCR) and B cell receptor (BCR). GPVI uses predominantly Lyn and Syk and the PLCγ2 isoform, whereas the TCR uses leukocyte-specific kinase (Lck) and zeta-chain-associated protein kinase 70 (Zap-70) and the PLCγ1 isoform. Because of these similarities, it is possible to study the signaling pathways of the platelet ITAM receptors by transfecting platelet signaling proteins into T and B cell lines.

3.1. GPVI

GPVI is a platelet-specific collagen receptor that is expressed on the platelet surface. Genetic deletion of GPVI in mice abolished the platelet response to collagen but the mice only exhibited a mild defect in haemostasis [6, 7]. Similarly, patients with a GPVI defect only show mild bleeding disorders [8, 9]. GPVI has two extracellular Ig domains, a mucin-rich stalk and short cytoplasmic domain. The cytosolic tail of GPVI has a sequence for binding to calmodulin and one that binds to the SH3 domain of Src family kinases such as Fyn and Lyn. It is non-covalently associated with the FcRγ-chain homodimer, which contains an ITAM. Both GPVI and FcRγ are essential for collagen-induced platelet activation [5]. Once phosphorylated on two conserved tyrosines after GPVI engagement by collagen, the FcRγ chain is responsible for signal transduction. Phosphorylation of FcRγ chain leads to binding of the tyrosine kinase Syk, inducing downstream signaling cascades, which are similar to those of other immune receptors as described above[10]. FcRγ is also used by other ITAM receptors in the C-type lectin-like receptors such as Dectin-2 and MINCLE. In these cases, activation of Syk, PLCδ, CARD9-Bcl10-Malt-1 and MAPK pathways induce cytokine production in myeloid cells which is important for host defence [11]. However, these cell surface receptors including Dectin-2 are not expressed in platelets and thus the role of CARD9-Bcl10-Malt-1 pathway downstream of GPVI and C-type lectin-like receptor stimulation in platelets is unknown.

Collagen is a powerful activator of platelets that activate both GPVI and integrin α2β1. A number of GPVI-specific ligands have been used to delineate the signaling pathways, including collagen-related peptide (CRP), which contains GPVI recognition motif GPO

(glycine-proline-hydroxyproline); GPVI-specific antibodies; and the snake venom toxin convulxin [12].

3.2. Fcγ Receptor IIA (FcγRIIA)

FcγRIIA is the second ITAM receptor in human platelets. Unlike GPVI, FcγRIIA has its ITAM within its cytoplasmic tail. Since mouse platelets lack a gene equivalent to FcγRIIA, transgenic mice expressing human FcγRIIA have been generated. Mouse platelets expressing human FcγRIIA have enhanced spreading on fibrinogen and thrombus formation in vivo, and it has been proposed that this is due to enhanced integrin outside-in signaling [13]. FcγRIIA signals through the same pathways as GPVI; therefore, in this commentary we will focus on the signaling through GPVI as an ITAM receptor.

4. Platelet hemITAM receptors

The C-type lectin-like receptor CLEC-2 (encoded by the Clec1b gene), and the closely related receptor, Dectin-1 which is found on dendritic cells, are founder members of a novel class of signaling receptors defined by a single YxxL sequence and known as a hemITAM (ITAM-like) receptor. CLEC-2 is the only hemITAM receptor in platelets.

4.1. CLEC-2

CLEC-2 is a C-type lectin-like receptor that is highly expressed in platelets and to a lesser extent, in other cell types such as activated dendritic cells and B cells [14]. The first ligand to be identified for CLEC-2 was rhodocytin, which was purified from the Malayan pit viper,

Calloselama rhodostoma, by the groups of Huang [15] and Morita [16] in the 1990s. To date, the only endogenous ligand found for CLEC-2 is podoplanin. Podoplanin is a sialomucin-like glycoprotein expressed in a variety of cells but is absent from vascular endothelial cells and is ordinarily not expressed in the vasculature.

In vitro studies have shown that treating platelets with CLEC-2 agonists activate platelets, inducing aggregation and secretion, identifying CLEC-2 as a platelet activating receptor [17]. We and others have previously shown using murine models that homozygous loss of CLEC-2 results in developmental defects in brain vasculature, lung inflation and lymphatics, and is associated with perinatal lethality [18, 19]. However, the role of CLEC-2 in haemostasis and thrombus formation seems to be limited; CLEC-2-deficient mice show no or minimal elongation of tail bleeding times which is also seen in GPVI-deficient mice [19, 20]. These data indicate that mechanisms independent of CLEC-2 and GPVI mediate platelet activation or that platelet activation by other agonists such as thrombin and ADP are sufficient to form haemostatic clots in the tail.

More recently, Herzog et al. showed that platelet CLEC-2 is important for maintaining high endothelial venule (HEV) barrier function during chronic inflammation [21]. Through interaction with podoplanin expressing-fibroblastic reticular cells, CLEC-2 dependent pathways were required to prevent vascular leak in HEV in reactive lymph nodes. Moreover, CLEC-2 has been reported to be involved in capturing HIV-1 virus particles and transferring of HIV-1 infection by platelets together with other receptors [22]. This is the only report implicating CLEC-2 in pathogen recognition. It would be interesting to

speculate whether CLEC-2 plays a more general role in pathogen recognition since the structurally related receptor Dectin-1 is a known pattern recognition receptor (PRR). This family of proteins serve as protection against fungi recognising conserved microbial structures known as pathogen associated molecular patterns (PAMPs) leading to the activation of transcription factor nuclear factor-kappa B (NF-κB). Taken together these reports suggest CLEC-2 has a number of roles which are not related to haemostasis.

5. Comparison of ITAM and hemITAM receptors in platelets

From the studies detecting signaling events and proteomic analysis it has been revealed that the CLEC-2 shares many of its signaling cascades with GPVI and signals through activation of Syk, Src and PLCγ2 using adaptor proteins such as LAT and SH2-containing leukocyte protein of 76kDa (SLP-76) [23]. Moreover, in vivo experiments on GPVI/CLEC-2-double knockout mice indicated that loss of both receptors resulted in severe defects in haemostasis and arterial thrombus formation, whereas deletion of GPVI or CLEC-2 alone only resulted in a minor or negligible defect in haemostasis and thrombus stability. This suggests that there is considerable redundancy in the function of these two receptors in these events [24].

Common signaling pathways in ITAM and hemITAM receptors in platelets are illustrated in Fig. 1. Upon ITAM and hemITAM receptor stimulation, the tyrosine residue in the YxxL motifs in both receptors becomes phosphorylated enabling Syk to bind through its two SH2 domains and undergo both autophosphorylation and further phosphorylation by Src family

kinases [25]. These events occur after a delay of 10 seconds upwards after receptor stimulation.

The next step in the signaling cascade is recruitment and phosphorylation of LAT by activated Syk. Phosphorylated LAT then serves as a platform that binds growth factor receptor-bound protein-2 (Grb2), growth factor receptor-bound protein-2 adaptor downstream of Shc (Gads) and PLCγ2. Adaptor protein SLP-76 is recruited to LAT by binding to Gads or Grb2 which are critical for full activation of PLCγ2. PI3K is also recruited to LAT and generates phosphatidylinositol (3,4,5)-triphosphate (PIP3) from phosphatidylinositol (4, 5)-bisphosphate (PIP2). Adaptor proteins Vav1 and 3 have also been shown to be recruited to the LAT signalosome by binding to SLP-76 and Syk to mediate PLCγ2 activation. The binding of PLCγ2 to phosphorylated LAT and PIP3 on the plasma membrane further stabilizes its localization to LAT. In addition, PIP3 recruits the tyrosine kinases Btk and Tec which phosphorylate PLCγ2 leading to its activation. Activated PLCγ2 generates secondary messengers, inositol-1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG), which release Ca^{2+} from the intracellular store and activate protein kinase C (PKC), respectively. These pathways lead to a common platelet activation cascade, resulting in platelet aggregation and dense granule secretion, which release the positive feedback mediator ADP and TxA_2 formation. In addition, activation of PKC also leads to activation of Raf-1/MEK/ERK pathways; however, unlike Raf-1 pathway activation in Dectin-1, it is not clear if this leads to NF-κB activation.

Syk signaling can also activate NF-κB via CARD9/Bcl10/MALT1 downstream of other hemITAMs, such as Dectin-1, in myeloid cells. Although MALT1 and Bcl10 are expressed in platelets, it is currently unclear if following (hem)ITAM receptor activation this signaling pathway plays a direct role in platelet activation [26].

Although there are similarities between GPVI and CLEC-2 signaling, there are also quantitative and qualitative differences between these pathways (Fig. 2). First, GPVI uses the YxxL sequences in the associated FcRγ chain, whereas CLEC-2 uses YxxL sequence in its own cytoplasmic domain. Secondly, as described above, ITAM receptors use two YxxLs, whereas the hemITAM receptor CLEC-2 only has a single YxxL sequence motif. As a consequence, in ITAM receptors Syk binds to two YxxLs in a single receptor, whereas in hemITAM receptors, Syk binds to two phosphorylated receptors bridging them via its tandem SH2 domains [27]. Thirdly, there are differences in proximal signaling events; Suzuki-Inoue et al. have reported that platelets from mice deficient in SLP-76, which is an adaptor protein that binds to LAT, do not respond to GPVI agonists. This is consistent with the role of SLP-76 in other hematopoietic cells, including T cells where SLP-76 deficiency leads to a failure in T cell development [17, 28]. However, in CLEC-2 signaling, although platelets from SLP-76-deficient mice do not respond to a low concentration of CLEC-2 agonist, this can be overcome by adding a high concentration [17]. Similar results were observed for platelets from Vav1/Vav3 double-deficient mice; they fail to respond to high concentration of GPVI-specific agonist, but although unresponsive to CLEC-2 agonist at low concentration, they respond normally to high concentration [17]. Taken together these results demonstrate that SLP-76 and Vav1/Vav3 are essential for GPVI signaling whereas

the requirement of these adaptor proteins are overcome at high agonist concentrations in CLEC-2 signaling. Moreover, these data may suggest that CLEC-2 may use other, as yet unknown, adaptor proteins for signaling. A fourth difference is in the role of secondary mediators used to reinforce platelet activation following GPVI or CLEC-2 activation. Pollitt et al. reported that blocking secondary mediators such as ADP and TxA_2 can block platelet activation at a low concentration of GPVI agonists but this is overcome at higher concentrations. In contrast, under the same conditions, platelet activation through CLEC-2 signaling is severely inhibited, including activation of Syk and downstream signaling events. This was insurmountable using high concentrations of agonists in human platelets [29] but not in mouse platelets [30]. However, the exact mechanism of how these secondary mediators reinforce CLEC-2 signaling is unknown and ADP or TxA_2 stimulation itself does not activate CLEC-2. Also, in this paper, they reported that blocking actin polymerization by cytochalasin D had no effect on maximum aggregation in GPVI-mediated platelet activation but CLEC-2-mediated platelet activation is inhibited. These data suggest that actin polymerization plays a key role in CLEC-2 signaling possibly by retaining CLEC-2 signaling molecules near CLEC-2 [29]. Although it is reported in T cells that actin polymerization is important for immunological synapse formation, the role of actin polymerization on CLEC-2-mediated adhesion to other cells has not yet been identified. These observations demonstrate that although ITAM and hemITAM signaling in platelets share many signaling features, the regulation and importance of a number of signaling molecules downstream of Syk differ between these receptors.

To date there have not been any reports describing Syk-independent signaling events that occur during GPVI and CLEC-2-mediated platelet activation. This is in contrast to the hemITAM receptor Dectin-1 where Raf-1 can activate NF-κB independent of Syk in myeloid cells. Indeed, it has been reported that disruption of Syk activity completely blocks platelet aggregation and secretion in response to GPVI or CLEC-2 agonists [31]. However, it is noteworthy that Syk deficiency does not affect haemostasis whereas the GPVI/CLEC-2 double knockout mouse has a severe defect in haemostasis suggesting that a Syk independent pathway may exist or that this is the consequence of loss of adhesion [24, 32].

6. PI3K and Akt

The PI3K/Akt pathway is activated in response to various types of stimulation including growth factors, cytokines, integrin, hormones and extracellular matrix (ECM) proteins; and this pathway regulates a wide spectrum of cellular processes including cell cycle progression and cell survival. This is illustrated by the correlation of gain of function mutations in PI3K or Akt resulting in cancer development. Furthermore, inhibitory mutations on the phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10), which is a negative regulator for PI3K/Akt, are also found in cancer.

PI3Ks are family of lipid kinases that in platelets are reported to be activated and regulate platelet activation [33]. PI3Ks catalyze the conversion of plasma membrane lipid PIP2 to PIP3. There are three families of PI3K: class I, II and III. Class I PI3Ks phosphorylate PIP2 to generate PIP3. They are heterodimeric proteins consisting of a catalytic subunit (p110α, p110β, p110δ and p110γ) and a regulatory subunit. According to their catalytic subunit,

class I PI3Ks are further divided into class IA that is composed of p110α, p110β and p110δ, each associating with a SH2-containing regulatory subunit (p50, p55 or p85) that binds to phosphorylated tyrosine residues in a Y(P)xxM motif. Class IB consists of one catalytic subunit p110γ associated with a regulatory subunit (p101 and p84), which is regulated by binding to G protein βγ subunits following GPCR activation. Human and mouse platelets express all class I PI3Ks but express p110δ at low level [34, 35]. Class II PI3Ks produce phosphatidylinositol (3) phosphate (PI3P) from phosphatidylinositol (PI) and also contribute to phosphatidylinositol (3,4) bisphosphate (PI(3,4)P2) production. Three monomers have been identified: ubiquitous PI3K-C2α and C2β and the liver specific PI3K-C2γ. Finally, in class III, the only member is vascuolar protein sortin 34 (Vps34) that is a PI specific kinase that only generates PI3P and is ubiquitously expressed. The role of class I PI3Ks have been studied in platelets, however the role of class II and III PI3Ks are not known. Platelets also express PIP3 phosphatases that are known to regulate the level of PIP3 by converting PIP3 back to PIP2; PTEN, SH2 domain-containing inositol polyphosphatase 1 (SHIP1), and SHIP2 [33].

One of the major downstream effectors from PI3K is the serine/threonine kinase Akt (also known as protein kinase B). Mouse and human platelets express all three isoforms of Akt, namely Akt1, Akt2 and Akt3 [2, 36-39]. The activation of Akt is mainly mediated by PI3K, generating PIP3 that recruits Akt and the other PH domain-containing proteins to the plasma membrane, keeping Akt in proximity to its upstream kinases. The activity of Akt is regulated through phosphorylation of two residues, Thr308 and Ser473. Phosphorylation of Thr308 is mediated by phosphoinositide-dependent kinase 1 (PDK1), and phosphorylation

of Ser473 is mediated by mTORC2 [40-43]. Consistent with this, platelets from PDK1-deficient mice have impaired platelet activation and Akt phosphorylation only at Thr308 and have impaired thrombin-mediated platelet activation [42]. However, the importance of phosphorylation at Ser473 is unknown in platelets since an mTORC2 inhibitor had no effect on thrombin-mediated platelet activation [43]. In contrast, in other ITAM receptors such as TCR, T cells from mTORC2 deficient mouse has significant reduction in phosphorylation at Ser473 after stimulation and this leads to reduction in the Th2 cell response such as IL-4 production suggesting that Ser473 phosphorylation is indispensable for Akt activity [44].

7.1. PI3K in platelet activation

Studies on the role of PI3K in platelets have used genetically modified mice lacking PI3K subsets and pharmacological approaches employing specific inhibitors for PI3Ks. Most of the studies investigating the role of PI3K in platelets have used two structurally distinct inhibitors, wortmannin and LY294002 that both inhibit class I and class II PI3Ks. From these studies, it has been reported that PI3Ks are expressed in platelets and play an important role in platelet activation in many processes by affecting Ca^{2+} mobilization, aggregation and platelet spreading in response to several agonists such as collagen, CRP or convulxin [45, 46], ristocetin/vWF [47], thrombin [36] and ADP [48]. However, the role of each PI3K isoform differs depending on the agonist.

During GPVI signaling, it has been appreciated that PI3Ks are activated in two ways: directly by GPVI signaling and indirectly via the production of secondary mediators and

subsequent activation of the ADP and TxA_2 receptors. In the presence of secondary mediator inhibitors to block indirect activation of PI3Ks, and the PI3K inhibitors wortmannin or LY294002, Akt phosphorylation, IP3 accumulation, Ca^{2+} elevation, P-selectin expression and thrombus formation of human platelets were all suppressed demonstrating that direct activation of PI3K/Akt by GPVI signaling is important for platelet activation [46]. As discussed in Section 5, CLEC-2 signaling is highly dependent on secondary mediators. Therefore inhibition of platelet activation secondary mediators leads to an almost complete loss of CLEC-2 activation making it difficult to ascertain whether direct or indirect signaling is important for PI3K activation during CLEC-2 signaling.

The catalytic subunit for PI3Kα, p110α, is frequently mutated in various types of cancer, however its role in platelets is not well known. Using a PI3Kα specific inhibitor, PIK-75, it has been demonstrated that blocking PI3Kα partially inhibited Akt phosphorylation after GPVI stimulation [46, 49]. Moreover, it has been reported that both PI3Kα and PI3Kβ have a non-redundant function under GPVI stimulation,with both isoforms being required for full activation of PLCγ2, IP3 formation, Ca^{2+} elevation, thrombus formation and Rap1 activation, supporting distinct roles for both isoforms during platelet activation [46].

PI3Kβ is reported to be the major PI3K isoform regulating platelet activity downstream of several platelet receptors including GPCRs, such as $P2Y_{12}$, and integrins through regulation of PLC activation and Ca^{2+} elevation. Consistent with these reports, collagen-induced aggregation, Ca^{2+} elevation, Rap1 activation and thrombus formation are impaired in mice

lacking the p85α regulatory subunit (inhibiting class IA PI3Ks) [35] or p110β catalytic subunit of PI3Kβ [50]. This impairment of platelet activation was not regulated by the function of PI3K as a scaffold protein since platelets from mice having the kinase dead mutation of PI3Kβ also had impaired platelet activation [51]. Studies using PI3Kβ specific inhibitors, TGX-221 and AZD6482, have also shown that it inhibits GPVI agonist-mediated platelet activation including Ca^{2+} elevation [46].

Studies to investigate the role of PI3Kγ in platelet function suggest activation of the kinase is specific to ADP-induced platelet activation, with platelets from mice lacking p110γ catalytic subunit of PI3Kγ demonstrating impaired aggregation to ADP, whereas the response to other agonists including collagen were normal [52].

PI3Kδ is highly enriched in leukocytes however expression in platelets is low compared to other PI3K isoforms. It has been reported that mouse platelets lacking the catalytic subunit of p110δ had slightly reduced aggregation to submaximal concentrations of CRP but had a normal response to GPCR agonists and spread normally on collagen [3]. This suggests that PI3Kδ plays a minor role in GPVI signaling, partially reducing aggregation only at low agonist concentrations. This minor role in platelet activation is in contrast to its role in BCR and TCR signaling where the p110δ kinase dead mutant markedly impaired B cell and T cell maturation [53]. These studies on each PI3K isoform show that there is a distinct regulation of PI3K activation between different ITAM receptors in different hematopoietic cell types. The importance of each PI3K isoform in CLEC-2 signaling has not yet been

analyzed but it is important to investigate whether there is different dependency to that on GPVI.

7.2. Akt in platelets

As mentioned above, Akt is the main downstream effector for PI3K [7] and in most cases, including GPVI signaling, the activation of Akt is amplified by secretion of ADP in platelets [49, 54]. Additionally, PI3K-independent pathways of Akt regulation have been reported. Akt is phosphorylated by PKC and by Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) in platelets; and in thrombin stimulation, early Akt phosphorylation was poorly sensitive to PI3K inhibitors [36, 55]. In contrast to these reports, under GPVI stimulation, treating platelets with wortmannin or LY294002 completely blocked the phosphorylation of Akt with or without ADP, demonstrating that Akt activation is initiated in a PI3K-dependent manner [49]. CLEC-2-mediated Akt activation is partially inhibited by a PKC inhibitor, whereas wortmannin and LY294002 completely blocked phosphorylation. This demonstrates that PI3K is the main upstream inducer for Akt activation in both GPVI and CLEC-2 signaling, but that in CLEC-2 signaling, PKC also partially supports activation of Akt by sustaining Akt phosphorylation at later timer point. It has also been reported that PKC inhibition reduces Akt phosphorylation in GPVI-mediated activation [49]. However, PKC inhibition also blocks ADP secretion therefore it is not clear if this is due to inhibition of direct phosphorylation of Akt by PKC or indirect inhibition by loss of ADP-dependent Akt phosphorylation.

Studying the function of Akt in platelets is predominantly achieved by using mice models with deletion of individual Akt isoforms. Among the genetically modified mice generated, only Akt1 deficient mice demonstrated a significant defect in response to collagen. Specifically, Akt1 deficiency reduced platelet aggregation and secretion after collagen stimulation, with prolonged tail bleeding times suggesting deletion of Akt1 is sufficient to impair haemostasis [2, 56]. In contrast, deletion of Akt2 resulted in a reduced platelet response to thrombin or TxA2 but a normal respond to collagen [2, 39, 57]. An enhanced defect was seen in mouse platelets lacking two Akt isoforms, accomplished by Akt2 knockdown in Akt1-deficient mice since the double knock-out is lethal [9, 10]. These data indicate that Akt isoforms have both non-redundant and redundant roles in supporting platelet activation. Of importance to this review, during collagen-mediated platelet activation, Akt1 is the isoform preferentially activated and also contributes more directly to haemostasis.

It has been reported that Akt3 deficient mice also respond normally to collagen stimulation, while having a reduced response to thrombin and TxA2 [39]. However, the role of Akt3 in platelet activation is controversial with Moore et al. reporting that although Akt3 is expressed in platelets, they were unable to detect activation of Akt3 after agonist stimulation [37].

Pharmacological inhibitors for each isoform have not yet been developed however the role of Akt has also been studied using the pan Akt inhibitor MK2206. It has been shown to significantly suppress human platelet activation under both GPVI and CLEC-2 stimulation

in human platelets [37]. However, for GPVI, the Akt inhibitor did not completely block platelet aggregation even at a low agonist concentration. In contrast, during CLEC-2 signaling, the Akt inhibitor almost completely blocked the aggregation and secretion at low concentrations of agonist. These differences of Akt dependency between GPVI and CLEC-2 may be attributable to CLEC-2 being highly dependent on secondary mediator signaling compared to GPVI, as discussed earlier.

For future investigations, it would be worthwhile to study the roles of the individual Akt isoforms in human platelets because it has been reported that a single-nucleotide polymorphism (SNP) in Akt2 is associated with reduced P-selectin expression after CRP stimulation in platelets [58]. However, the fact that Akt isoforms have a common domain organization and high sequence homology makes it a challenge to develop specific inhibitors for each isoform. Taken together these results using Akt isoform deficient mice or pharmacological inhibitors for Akt demonstrates a role of Akt in (hem)ITAM receptor mediated platelet activation. However, the precise mechanism by which Akt supports platelet activation remains poorly characterized and several candidates have been suggested as Akt substrates which is discussed in Section 8.

8. PI3K and Akt effectors in platelet activation

In ITAM and hemITAM signaling, several candidates are proposed as an effector under the PI3K/Akt pathway (Fig. 3). Akt is known to phosphorylate more than 100 substrates. Glycogen synthase kinase 3β (GSK3β) is known to be one of the main substrates for Akt in a wide range of cell types. GSK3β is reported to be a negative regulator for platelet

activation, inhibitors potentiating platelet aggregation and secretion, and being developed as a potential drug to treat diabetes, stroke, Alzheimer's and other diseases. Phosphorylation of GSK3 at its N-terminal negative regulatory site (Ser21 and Ser9 in GSK3α and GSK3β, respectively) inhibits GSK3α/β activation [59]. Deficiency of Akt isoforms has been reported to partially inhibit GSK3β phosphorylation, suggesting that each isoform has a redundant role in regulating GSK3β. Several groups have reported that GSK3α/β inhibition potentiates platelet activation. The GSK3α/β inhibitors LiCl and SB216763 have been shown to potentiate thrombin-induced platelet activation but to inhibit collagen-stimulated platelet activation, suggesting an opposite role for GSK3α/β under GPCR and ITAM receptors [39, 60, 61]. Moreover, using a new specific inhibitor for GSK3α/β, CHIR-99021, Moore et al. have shown that thrombin-mediated platelet activation was potentiated in inhibitor-treated platelets. This was in contrast to CRP-mediated platelet activation which was unaltered, further suggesting the differential regulation of platelet activation by GSK3α/β, resulting in a negative role in thrombin receptor signaling but positive or negligible role in GPVI signaling in platelets [37]. These discrepant results from the studies on GPVI signaling using GSK3α/β inhibitors which show a negative or no effect in GPVI-mediated platelet activation may be due to the different specificity of these inhibitors since LiCl and SB216763 inhibit GSK3α/β and several other kinases at the concentrations used in these studies [62]. Alternatively, collagen may regulate GSK3α/β downstream of integrin α2β1, which is not activated by CRP. In CLEC-2 signaling, the role of GSK3α/β appears to be inhibitory and GSK3α/β inhibitors potentiate platelet activation to rhodocytin.

Another potential effector under PI3K activation is ERK (extracellular signal-regulated kinase), one of the MAPKs (mitogen-activated protein kinases). A series of studies have reported that, dependent on the conditions, activation of MAPK plays a minor role in platelet activation by GPCRs and GPVI [63, 64]. Studies on the involvement of MAPK in platelet function have been performed using pharmacological inhibitors. The MEK (MAPK/ERK kinase) inhibitors U0126 and PD98059 that inhibit ERK activation have been shown to inhibit platelet aggregation and secretion [64, 65]. On the other hand, Börsch-Haubold et al. demonstrated that the MEK inhibitor PD98059 had no effect on platelet aggregation induced by collagen [65]. This discrepancy could be explained by the experimental conditions as the latter study was performed in the presence of a cyclooxygenase inhibitor that blocks TxA_2 to counter the inhibitory effect of PD98059 against this class of enzyme. These results however do not rule out the possibility that ERK may contribute to platelet activation by GPVI through the regulation of TxA2 formation (which is why ERK inhibitors had no effect under TxA2 blockage). Wortmannin treatment was shown to inhibit the ERK phosphorylation in collagen-stimulated platelets at a later time point [66]. These observations suggest a role of PI3K in phosphorylating ERK at a later time point which could sustain ERK activation and support platelet activation following both GPVI and CLEC-2 stimulation.

In addition to activating Akt and ERK, PIP3 generation by PI3K can lead to the recruitment of PLCγ2, Btk and Tec, inducing PLC activation which is a key player in platelet activation as described in Section 5 [67-69]. This inhibition of PLC activation by PI3K inhibition can lead to impaired Ca^{2+} elevation, integrin inside-out signaling and PKC activation,

influencing more diverse pathways than just Akt or ERK activation. Consistent with this, it has been reported that PI3K inhibitors inhibit PLCγ2 activation as shown by impaired Ca^{2+} elevation in LY294002 or wortmannin treated platelets after GPVI stimulation demonstrating PLCγ2 is regulated by PI3K [45, 46].

9. PI3K/Akt pathways in human diseases and drug development

As mentioned above, Akt is the main downstream effector for PI3K and has been demonstrated to play a role in platelet activation in (hem)ITAM receptors and haemostasis. Consistent with these reports using mouse models, expression of a SNP in the Akt2 associated with human platelet response in a screening of 500 healthy European individuals suggests a role of Akt in human thrombosis or bleeding disorders [58]. Moreover, in the PI3K family, a SNP associated with PI3Kγ expression was also found to associate with changes in mean platelet volume and annexin V binding [70]. These observations further suggest a role for the PI3K/Akt pathway in human bleeding disorders and cardiovascular diseases. However, the functional redundancy of each isoform of PI3K or Akt could make it rather unlikely that a defect in a single isoform would result in the development of a disease.

In the regulation of ITAM and hemITAM receptors in platelets, patients lacking GPVI display only a mild bleeding tendency and mice with either a GPVI or CLEC-2 deficiency have only a mildly prolonged tail bleeding time and reduced thrombus stability. These minor effects of GPVI and CLEC-2 on general haemostasis make them a good therapeutic target for anti-thrombotics without bleeding side effects. To date, Akt seems to be a good

therapeutic target that is differentially involved in GPVI and CLEC-2 signaling, having a partial effect on GPVI signaling and blocking CLEC-2 signaling. Moreover, molecules involved in the PI3K/Akt pathway are known to be frequently mutated in cancer patients [71], and cancer patients are at a high risk of developing venous thromboembolism, making it essential to treat these patients with anti-thrombotic drugs. An Akt inhibitor may be ideal for treating cancer patients since it has a relatively mild effect on GPVI- and CLEC-2-mediated platelet activation in haemostasis and also can block development of the cancer. In PI3K-regulating therapy, an inhibitor targeting PI3Kβ would seem to be ideal for this purpose based on the data obtained from PI3K-deficient mice and inhibitors. However, the PI3K β inhibitor TGX-221, which has been reported to reduce adhesion to fibrinogen and to reduce thrombosis in a rat injury model, had no effect on haemostasis as assessed by tail bleeding time in rats [72]. Moreover, as a treatment against cancers and tumors, the pan Akt inhibitor MK2206 is now in a phase 2 trial; further, combination of this inhibitor with other anticancer agents such as receptor tyrosine kinase inhibitors, has been found to augment the efficiency of this antitumor therapy [73].

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Figure Legends

Figure 1. (hem)ITAM receptor signaling

Activation of ITAM receptor (GPVI) and hemITAM receptor (CLEC-2) induces the recruitment and subsequent activation of Syk. Activated Syk induces activation of signaling cascades that lead to the activation of PLCγ2 and elevation of intracellular Ca^{2+} level for which the adaptor protein LAT serves as a platform for this signaling cascade. This leads to granule secretion, integrin activation and aggregation of platelets which reinforces platelet activation.

Figure 2. Comparison of GPVI and CLEC-2 signaling

(i) ITAM receptor GPVI interacts with the covalent dimer of FcR-γ chain, in which each monomer contains an ITAM sequence. Activation of GPVI receptor leads to a one-to-one interaction of FcR-γ chain with Syk to activate Syk. The hemITAM receptor CLEC-2 requires two phosphorylated receptors to activate Syk. (ii) SLP-76 is essential for GPVI signaling, whereas in CLEC-2 signaling, a high dose of agonist can overcome SLP-76 deficiency. (iii) Platelets from Vav1/Vav3 double knockout mice fail to respond to high dose of GPVI agonist, whereas they respond to high dose of CLEC-2 agonist. (iv) Blocking actin polymerization almost completely blocked CLEC-2-mediated platelet aggregation, but it does not change the maximum aggregation in GPVI-mediated platelet aggregation. (v) At high dose of agonist, secondary mediators are dispensable in GPVI signaling whereas they are indispensable in CLEC-2 signaling.

Figure 3. Effectors under the PI3K/Akt pathway

Activation of ITAM receptor (GPVI) and hemITAM receptor (CLEC-2) induces activation of PI3K which generates PIP3 in the plasma membrane. This induces the activation of Akt, which then phosphorylates GSK3. Inhibition of GSK3 by Akt suppresses platelet activation in GPVI signaling but potentiates activation in CLEC-2 signaling. PI3K also directly or indirectly activates MEK and ERK, supporting platelet activation. PIP3 generation by PI3K can also lead to activation of PLCγ2 by recruiting PLCγ2, Btk and Tec to the plasma membrane to induce calcium elevation, PKC and platelet activation.

Table 1 Overview of the receptors expressed in platelets

Receptor	Ligand	Receptor type
PAR1 (only in human)	Thrombin, PAR1 peptide (synthetic)	G protein-coupled receptor
PAR4	Thrombin, PAR4 peptide (synthetic)	G protein-coupled receptor
$P2Y_1$	ADP	G protein-coupled receptor
$P2Y_{12}$	ADP	G protein-coupled receptor
TP	TxA_2, U46619 (synthetic)	G protein-coupled receptor
GPVI	Collagen, Laminin, CRP (synthetic), Convulxin (snake toxin), JAQ1 (antibody)	Ig
α2β1	collagen	Integrin
α6β1	Laminin	Integrin
GPIb-XI-V	vWF, Ristocetin (snake toxin)	Leucine-rich
αIIbβ3	Fibrinogen, vWF	Integrin
$P2X_1$	ATP	Ca^{2+} channel
CLEC-2	Podoplanin, Rhodocytin (snake toxin)	C type lectin-like

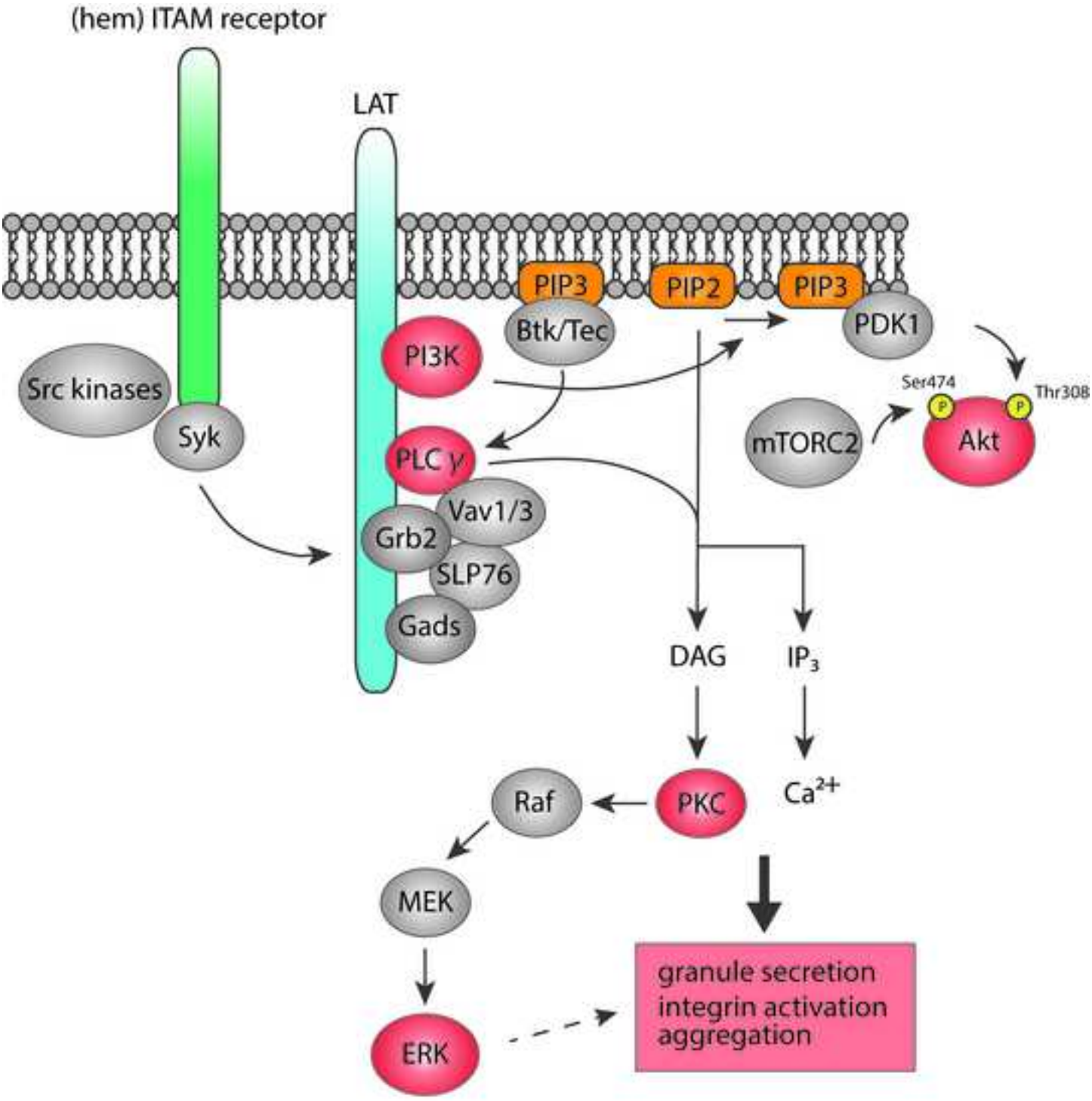


Figure 2

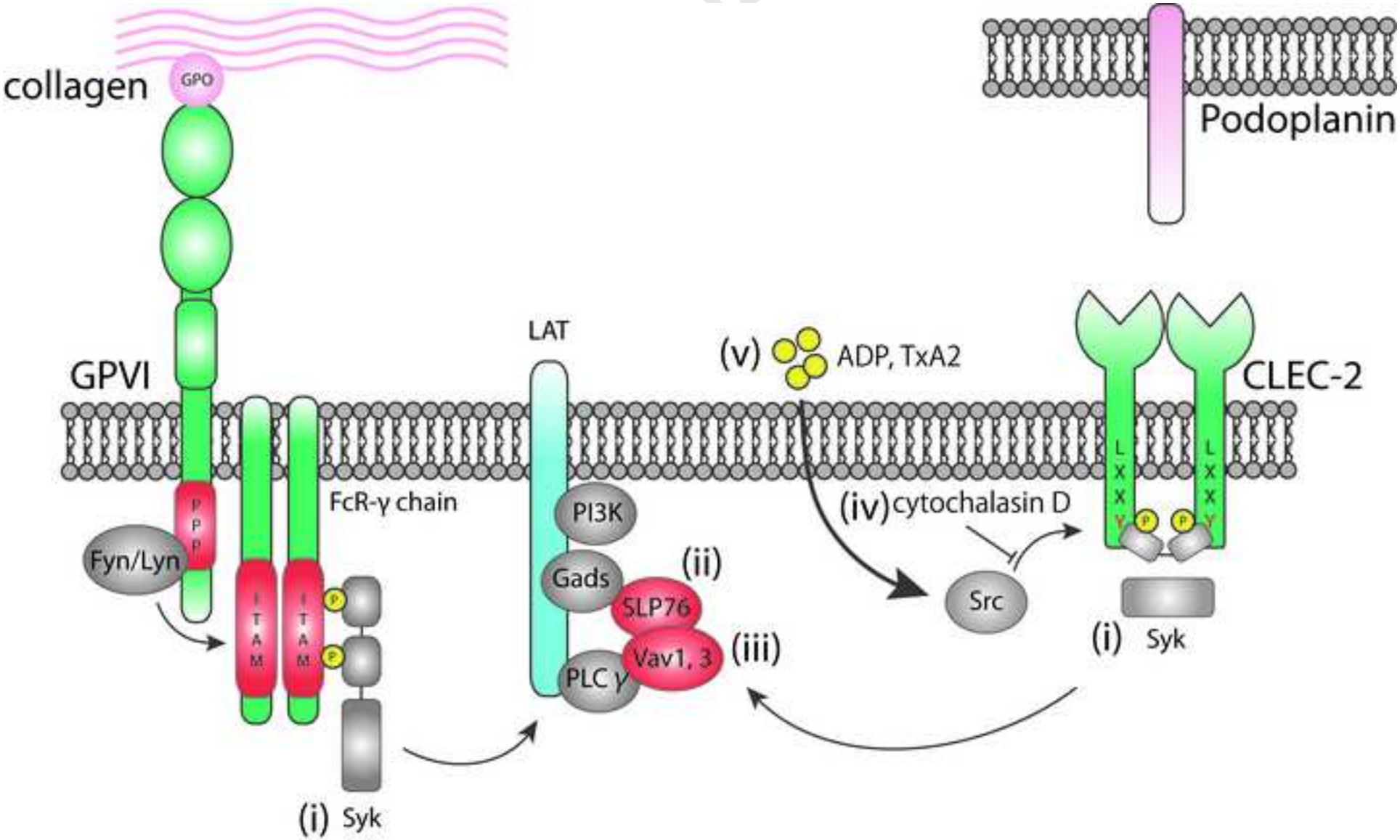
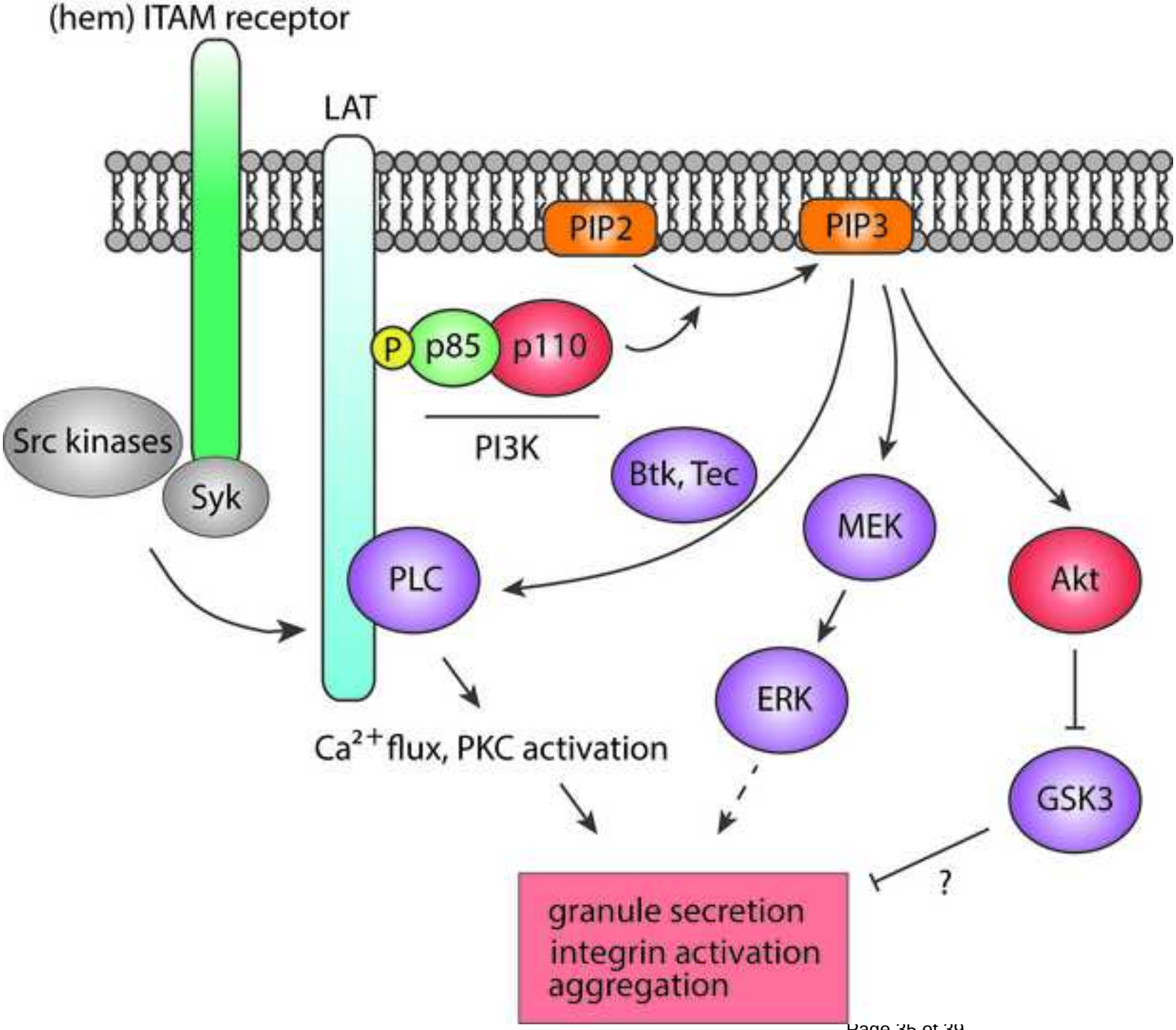

collagen
GPO
Podoplanin
GPVI
LAT
(v) ADP, TxA2
CLEC-2
FcR-γ chain
PI3K
(iv) cytochalasin D
Fyn/Lyn
Gads
(ii)
SLP76
Src
(i) Syk
Vav1, 3
(iii)
PLC γ
(i) Syk

Figure 3


(hem) ITAM receptor
LAT
PIP2
PIP3
P
p85
p110
PI3K
Src kinases
Syk
Btk, Tec
MEK
Akt
PLC
ERK
GSK3
Ca2+ flux, PKC activation
?
granule secretion
integrin activation
aggregation

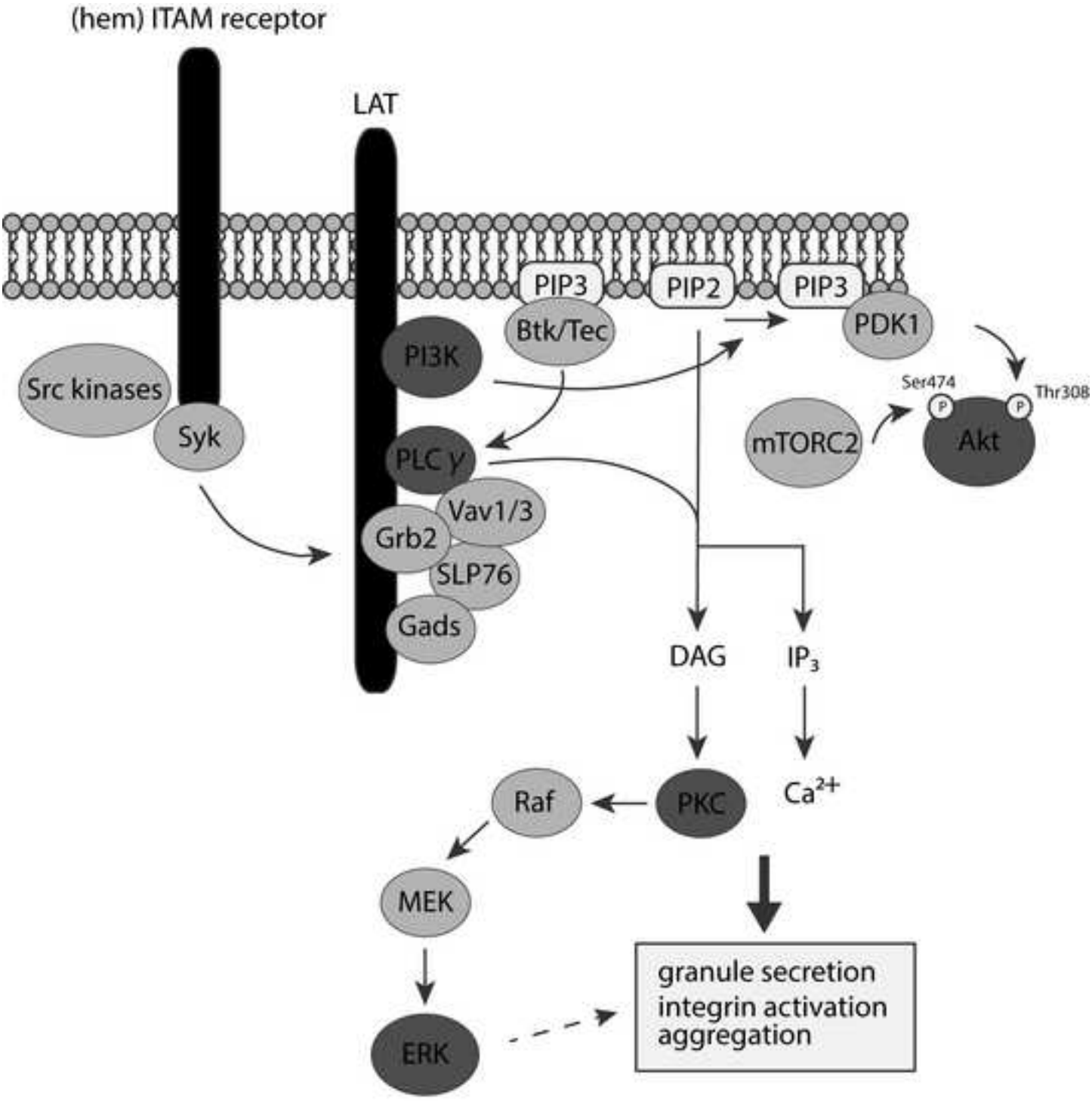


Figure 2 grayscale

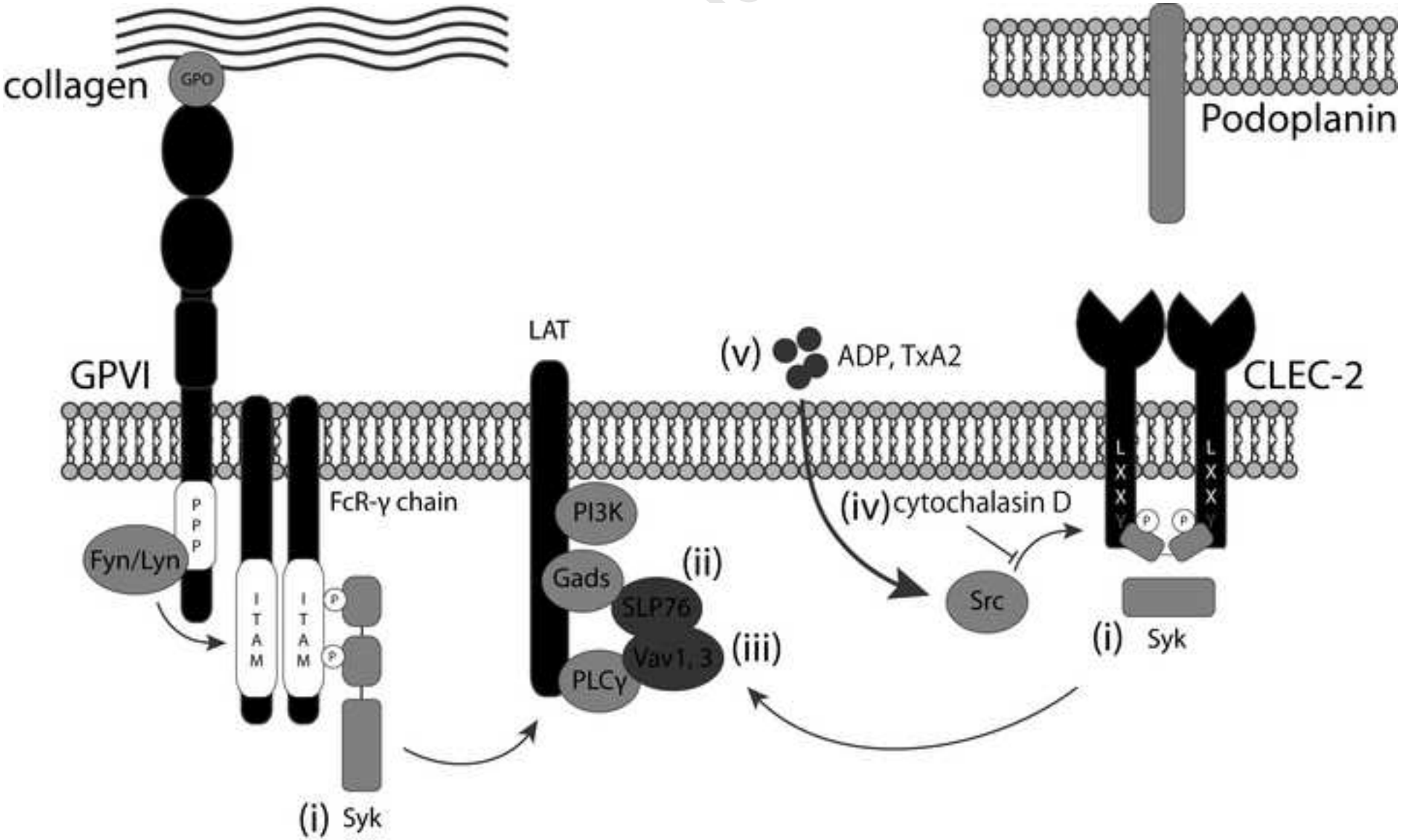


Figure 3 grayscale