Activation of phosphatidylinositol 3-kinase β by the platelet collagen receptors integrin α2β1 and GPVI: The role of Pyk2 and c-Cbl

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1. Introduction

Blood platelets are activated by a multitude of extracellular factors that trigger shape change, secretion and aggregation by stimulating different classes of membrane receptors, including G-proteins–coupled receptors, integrins and platelet specific glycoproteins such as GPVI and the GP Ib-IX-V complex [1]. The intracellular signaling pathways stimulated downstream of the different platelet receptors display unique features, but they also share a number of key events. In particular, stimulation of two phosphoinositide-metabolizing enzymes, phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI3K), occurs in all the contexts of platelet activation, and thus these enzymes represent crucial mediators of the platelet response.

The mechanism for PLC activation downstream of the different platelet receptors is quite understood: members of the PLC subfamily, namely PLCβ1 and PLCβ3, are stimulated by binding of α subunits (αq and α11) or γ dimers of activated G proteins upon stimulation of PAR1 and PAR4 by thrombin, P2Y1 receptor by ADP, or TPα receptor by thromboxane A2 [23]. Alternatively, integrin ligation by adhesive proteins, interaction of von Willebrand Factor with GP Ib-IX-V, or recruitment of ITAM-bearing receptors, such as GPVI, CLEC-2 and FcyRIa, stimulates the PLCγ2 isoform by direct phosphorylation, and through the contribution of 3-phosphoinositides and the small GTPase Rac [4–6].

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By contrast, the mechanism for PI3K stimulation in activated platelets is still poorly characterized. All four members of class I PI3K, that are responsible for the synthesis of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P$_3$), are expressed in platelets but they contribute differently to platelet activation [7]. While the class IB isoform PI3Kγ appears to be activated downstream of G-proteins–coupled receptors [8], a number of pharmacological and genetic evidence indicates that the class IA member PI3Kα plays a predominant role in platelet activation, being involved not only downstream of G-proteins–coupled receptors, but also upon integrins and ITAM-bearing receptors stimulation [9–11]. The roles of PI3Kδ and PI3Kε are minor or still controversial [12,13].

Class IA PI3K members are heterodimers composed of a 110 kDa catalytic subunit and a 85 regulatory subunit, which keeps the enzyme in an inactive state [14]. As with other members of its class, PI3Kα is generally considered to be activated upon binding of tyrosine phosphorylated proteins to the regulatory subunit p85, which overcomes the constitutive inhibitory effect. In a recent study we have demonstrated that integrin αIIbβ3 ligation in fibrinogen–adherent platelets induces the association of the adaptor protein c-Cbl with the p85 regulatory subunit of PI3K through its SH3 domain, and that p85-associated c-Cbl is subsequently tyrosine phosphorylated by the focal adhesion kinase Pyk2 [15], suggesting a possible mechanism of PI3Kα activation and Akt phosphorylation. It is known that PI3Kα is also activated downstream of the collagen receptors integrin α2β1 and GPVI [9,10,16]. Moreover, integrin α2β1–triggered phosphorylation of Akt was reduced in platelets from Pyk2 knockout mice [16].

Based on this background, we hypothesized that phosphorylation of c-Cbl by Pyk2 and the interaction of c-Cbl with the p85 subunit could represent a general strategy for PI3Kα regulation in platelets. In the present study, we compared the mechanism for PI3Kα stimulation by the two main collagen receptors integrin α2β1 and GPVI. We found that Pyk2 regulation of PI3Kα is restricted to integrin signaling and does not operate upon GPVI stimulation where, in contrast, Pyk2 is downstream of PI3Kδ. Moreover, we found that phosphorylation of c-Cbl is dispensable for both Pyk2-dependent and -independent regulation of PI3Kα by the platelet collagen receptors.

2. Materials and methods

2.1. Materials

PI3KαKO, PI3KγKO, Pyk2 and c-Cbl knockout mice were generated and characterized as reported elsewhere [17–20]. The use of mice for our experimental work was approved by the Ethics Committee of the University of Pavia. Monomeric type I collagen was provided by Prof. M.E. Tira (University of Pavia, Italy). Convolxin was from Centerchem, Inc. (Norwalk, CT, USA), and collagen–related peptide (CRP) was provided by Dr. R. Fardanhe (University of Cambridge, UK). The rabbit polyclonal antibodies against Pyk2 (N-19), as well as the mouse monoclonal antibody anti-tubulin (DM1A) were from Santa Cruz Biotechnology (Tebu-Bio, Magenta, Italy). Anti-phosphoPyk2(Tyr402), anti-phosphoAkt(Ser473), anti-phosphoThr308, and anti-phosphoSer PKC substrates, anti-phosphoSyk(Tyr525/526), anti-Akt, and anti-β-actin antibodies were from Cell Signaling Technology (Celbio, Pero, Italy). Anti-phosphotyrosine (Clone 4G10) and anti-PI3 kinase(p85) antibodies were from Millipore (Prodotti Gianni, Milano, Italy). Anti-n-Cbl monoclonal antibodies was from BD Biosciences (Milano, Italy). Anti-pleckstrin was from Abcam (Prodotti Gianni, Milano, Italy). Appropriated peroxidase-conjugated anti-IgG antibodies were from Bio-Rad (Milano, Italy). BAPTA-AM, U73122, PP2, wortmannin, AS252424 and TGX-221 were from Alexis Biochemicals (3VChemica, Roma, Italy). Fura-2-AM was from Calbiochem (VWR, Milano, Italy).

2.2. Preparation of washed mouse platelets

Blood platelets preparation from wild type and transgenic mice was performed essentially as previously described [21] according to a protocol approved by the Ethics Committee of the University of Pavia. Animals were anesthetized, and blood was withdrawn from the abdominal vena cava using ACD/3.8% sodium citrate (2:1) as anticoagulant. Anticoagulated blood was diluted with HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.9 mM KCl, 12 mM NaHCO$_3$, pH 7.4) up to 2 ml and centrifuged for 10 min at 180 g to obtain platelet-rich plasma (PRP). PRP was then transferred to new tubes and the remaining red blood cells were diluted with HEPES buffer to a final volume of 2 ml and centrifuged again at 180 g for 7 min. The upper phase was added to the previously collected PRP, and 0.02 U/ml aspirase plus 1 μM PGE$_1$ were added before centrifugation at 550 g for 10 min. Platelets were washed with 1 ml PIPES buffer (20 mM PIPES, 136 mM NaCl, pH 6.5), and finally resuspended in HEPES buffer.

2.3. Platelet adhesion and stimulation

Washed murine platelets were finally resuspended at 0.3 × 10$^9$ cells/ml and, upon addition of 5.5 mM glucose, cells were allowed to rest for 30 min at room temperature. Polystyrene dishes (60-mm) were coated overnight at room temperature with 50 μg/ml monomeric type I collagen diluted in 0.1 M acetic acid. Dishes were washed 3 times with 5 ml PBS, blocked with 2 ml 1% BSA in PBS for 2 h at room temperature, and then washed again 3 times with PBS. Murine platelets (0.5 ml) were added to collagen-coated dishes in the presence of 2 mM Mg$_2$O, and 1 mg/ml BSA. Aspirase (0.4 U/ml) was also typically added to the platelet suspension, as indicated in the text. After 30 or 60 min of incubation at room temperature, non-adherent cells were removed, and dishes were washed 3 times with 5 ml PBS. For whole cell lystate preparation, adherent cells were directly solubilized by addition of 0.5 ml of 2% SDS in HEPES buffer, and then collected. For immunoprecipitation experiments, cells were lysed with 0.3 ml ice-cold immunoprecipitation buffer (20 mM Tris/HCl pH 7.4, 100 mM NaCl, 0.2% Nonidet P-40, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na$_3$VO$_4$). Lysates were centrifuged at 18,000 g for 10 min, and the protein content in the cleared supernatant was determined by the bicinchoninic acid assay. Aliquots of each sample containing the same amount of proteins (and, thus, deriving from the same number of adherent platelets) were used for further analysis.

Stimulation of platelets in suspension (0.3 × 10$^9$ cells/ml, 400 μl) was typically performed with 0.5 μg/ml CRP at 37 °C under constant stirring. Platelet lysis for subsequent analysis was performed by addition of an equal volume of 2% SDS in HEPES buffer or 2× immunoprecipitation buffer.

2.4. Immunoprecipitation

For immunoprecipitation experiments, lysates of resting or stimulated, non adherent or adherent platelets containing equal amount of proteins, were incubated with 0.8 μl whole antisum anti-PI3 kinase(p85) or with 1 μg anti-c-Cbl for 2 h at 4 °C, and then 80 μl protein A-Sepharose (50 mg/ml stock solution) or 80 μl protein G-Sepharose, respectively, were added. Immunoprecipitates were recovered, washed and finally resuspended with 25 μl SDS-sample buffer (25 mM Tris, 192 mM glycine, 2% SDS, 0.5% DTT, 10% glycerol, 0.01% bromophenol blue, pH 8.3), as previously described [22].

2.5. Electrophoresis and immunoblotting

Aliquots of platelet lysates containing the same amount of proteins, or immunoprecipitated proteins were separated by SDS-PAGE, typically on 7.5% acrylamide gels, and proteins were transferred to PVDF membrane.
After blocking for 2 h with 5% BSA in TBS (20 mM Tris/HCl, pH 7.5, 0.5 mM NaCl), membranes were incubated overnight at 4 °C with the desired primary antibodies diluted in TBS. In the present study the following antibodies and dilution were used: anti-phosphoAkt(Ser473), anti-phosphoAkt(Thr308), 1:500; anti-phosphoPyk2(Tyr402), 1:500; anti-tubulin, 1:1000; anti-Akt, 1:1000; anti-Pyk2, 1:500; anti-phosphoTyro, 1:1000; anti-PI3 kinase(p85), 1:1000; anti-c-Cbl, 1:1000; anti-phospho(Ser) PKC substrates antibodies, 1:1000; and anti-pleckstrin 1:1000. Membranes were washed, incubated with appropriate peroxidase-conjugated secondary antibody (1:3000 dilution), and proteins were visualized with a chemiluminescence reaction, as described [23]. Typically, the PVDF membranes were treated with 1% NaN₃ in TBS for 4 h at room temperature and reprobed with a different antibody as a control for equal loading. All the experiments reported in this study were repeated at least three times, and comparable results were obtained. The blots reported in the figures are representative images. When the observed differences deserved to be quantified, analysis of band intensity was performed by computer assisted densitometric

![Fig. 1. Pyk2, but not c-Cbl, mediates PI3K activation by integrin α2β1](image-url)

Platelets from wild type (WT) and Pyk2 KO mice were allowed to adhere to immobilized monomeric collagen for 30 or 60 min. Adherent cells, as well as samples of non-adherent cells (NA) at 60 min, were collected and lysed. (A) Akt phosphorylation on Ser473 and Thr308 was analyzed by immunoblotting with specific phospho-antibodies as indicated. A representative immunoblot is shown in (i), where reprobing with anti-Akt (lower panel) was performed as control for equal loading. Histograms in (ii) and (iii) report the quantitative analysis of Akt phosphorylation on Ser473 and Thr308, respectively, in wild type (black bars) and Pyk2 KO platelets (grey bars). Data are the mean ± SD of three different experiments. (B) The p85 regulatory subunit of PI3K was immunoprecipitated from adherent and non-adherent platelets (NA) from wild type (WT) and Pyk2 KO mice. Lysates of adherent platelets were also immunoprecipitated with a non-related antibody (NR Ab), as control. Immunoblotting analysis was performed with anti-c-Cbl, followed by reprobing with anti-p85 antibodies, as indicated on the right. The reported immunoblot is representative of three different experiments that produced comparable results. (C) c-Cbl was immunoprecipitated from murine (i) or human (ii) platelets adherent to monomeric collagen for 30 or 60 min. As control, c-Cbl was also immunoprecipitated from untreated (BAS) or CRP-stimulated platelets (CRP 0.5 μg/ml, 1 min). Immunoblotting analysis was performed with anti-phosphorytosine antibody, followed by reprobing with anti-c-Cbl antibody. An immunoblot representative of three similar experiments is reported. (D) Platelets from wild type (WT) and c-Cbl KO (cCbl−/−) mice were allowed to adhere to immobilized monomeric collagen or to bovine serum albumin (BSA) for 45 min. Analysis of Syk and Akt phosphorylation was performed by immunoblotting with phosphospecific antibodies. Phosphorylated Syk(Tyr525/526), Akt(Ser473), and Akt(Thr308) are indicated by the arrows on the left in the representative immunoblot reported in (i). Immunoblotting with anti β-actin was performed as control for equal loading. Quantitative analysis of protein phosphorylation is reported in (ii) as mean ± SD of three different experiments. * = p < 0.05; ** p < 0.005.
scanning using Image J software. Statistical analysis was performed with the Student t-test.

2.6. Measurement of cytosolic Ca\(^{2+}\) concentration

Intracellular Ca\(^{2+}\) concentration was measured in Fura-2-AM loaded platelets essentially as previously described [24]. Briefly, human or mouse platelets in PRP were incubated with 3 μM Fura-2-AM for 40 min at 37 °C in the dark. Apprarse and PGE\(_1\) were then added and platelets were recovered by centrifugation, washed with PIPES buffer, and finally resuspended in HEPES buffer containing 0.5% BSA. For these experiments, the final platelet concentration was adjusted to 2 × 10\(^8\) cells/ml, and stimulation was performed at 37 °C on 0.4 ml samples under gentle stirring in a PerkinElmer Life Sciences LS3 spectrofluorometer in the presence of 1 mM EGTA.

3. Results

3.1. The role of Pyk2 and c-Cbl in PI3K\(\beta\) activation downstream of integrin \(\alpha_{IIb}\beta_3\)

We have recently demonstrated that the tyrosine kinase Pyk2 is required for PI3K\(\beta\) activation downstream of integrin \(\alpha_{IIb}\beta_3\), and that the adaptor protein c-Cbl is phosphorylated by Pyk2, which then associates with the p85 regulatory subunit of PI3K [15]. The present study was aimed to investigate whether this pathway represents a recurrent

![Diagram A](image)

![Diagram B](image)

![Diagram C](image)
strategy for PI3Kβ activation in platelets. It is known that PI3Kβ is also 
stimulated upon platelet adhesion to specific ligands of integrin α2β1, such as the GFOGER peptide or monomeric collagen, and that integrin 
α2β1-induced Akt phosphorylation on Ser473 is impaired in platelets 
lacking Pyk2 [9,10,16]. To further investigate the mechanism linking 
Pyk2 to PI3Kβ activation, we analyzed Akt phosphorylation in platelets 
from wild type and Pyk2 knockout mice adherent to monomeric type I 
collagen, under conditions that have been previously demonstrated to 
recruit exclusively integrin α2β1 [6,25]. In agreement with previous 
findings, we confirmed the importance of Pyk2 for PI3Kβ activation, as 
Akt phosphorylation on both Ser473 and Thr308 was reduced, although 
not completely abrogated, in the absence of Pyk2 (Fig. 1A). To investi-
gate the possible involvement of c-Cbl in Pyk2-mediated activation of 
PI3Kβ downstream of integrin α2β1, the p85 regulatory subunit was 
immunoprecipitated from platelets adherent to monomeric collagen, 
as well as from non-adherent cells. The presence of c-Cbl in the immu-
noprecipitates was investigated by immunoblotting. Fig. 1B shows that 
platelet adhesion through integrin α2β1 caused the association of c-Cbl 
with the p85 subunit both in wild-type and in Pyk2-deficient platelets. To 
verify whether c-Cbl is actually tyrosine phosphorylated upon integrin 
α2β1 recruitment, the protein was directly immunoprecipitated from 
murine platelets adherent to monomeric collagen and analyzed by 
immunoblotting with the anti-phosphotyrosine antibody. Surprisingly 
we found that integrin α2β1 engagement failed to induce c-Cbl tyrosine 
phosphorylation, which was clearly detected upon stimulation with 
CRP (Fig. 1C). The inability of integrin α2β1 to induce c-Cbl tyrosine 
phosphorylation was not restricted to murine platelets, as it was also 
confirmed using human platelets adherent to monomeric collagen 
(Fig. 1C). Since this observation argues against a role of c-Cbl phosphor-
ylation in integrin α2β1-induced PI3Kβ activation, we analyzed Akt 
phosphorylation in platelets from c-Cbl knockout mice. Fig. 1D shows 
that platelet adhesion to monomeric collagen caused phosphorylation 
of Akt on Ser473 and Thr308 both in wild type and c-Cbl-deficient 
platelets. Accurate quantitative analysis revealed that, as for the tyrosine 
kine Syk, phosphorylation of Akt was slightly increased in the absence 
of c-Cbl. These results clearly demonstrate that Pyk2-mediated activation 
of PI3Kβ in integrin α2β1 signaling does not require c-Cbl.

3.2. PI3Kβ activation by GPVI does not require Pyk2

Previous works by our group and others have demonstrated that 
PI3Kβ plays a master role in platelet activation by GPVI, but the implica-
tion of Pyk2 and c-Cbl has not been characterized [9,10]. In the present 
study, we used CRP or convulxin as a selective GPVI agonist. We demon-
strated that, in addition to Ser473, also phosphorylation on Thr308 of 
Akt was completely undetectable in platelets from mice expressing a 
catalytically inactive form of PI3Kβ (Fig. 2A). However, in contrast to 
what was observed upon integrin recruitment, CRP-induced phosphor-
ylation of Akt on both Ser473 and Thr308 occurred normally in platelets 
lacking the tyrosine kinase Pyk2 (Fig. 2B), indicating that PI3Kβ activa-
tion downstream of GPVI is Pyk2-independent. Since CRP stimulation 
was performed in a platelet suspension, while integrin engagement 
required platelet adhesion to an immobilized ligand, we verified whether 
the different implication of Pyk2 in PI3Kβ activation could reflect the 
different experimental setting. We thus analyzed Akt phosphorylation 
in wild type and Pyk2-deficient platelets upon adhesion to immobilized 
CRP for 30 and 60 min. We found that, even in this condition, ligation of 
GPVI triggered Akt phosphorylation on Ser473 and Thr308 was compa-
rable in wild-type and Pyk2-deficient platelets (Fig. 2C).

c-Cbl has been reported to be tyrosine phosphorylated upon GPVI 
recruitment by a SFK- and Syk-dependent mechanism [26]. We there-
fore investigated the role of Pyk2 in c-Cbl phosphorylation downstream 
of GPVI. Fig. 3A shows that c-Cbl phosphorylation in CRP-stimulated 
platelets occurred normally in Pyk2-deficient platelets, indicating that 
this event is independent of Pyk2. Moreover, coimmunoprecipitation 
experiments failed to detect any association of c-Cbl with the p85

![Fig. 3](image-url)

**Fig. 3.** c-Cbl is not involved in PI3Kβ activation downstream of GPVI. (A) Wild type (WT) and Pyk2-deficient (Pyk2KO) platelets were stimulated with 0.5 μg/ml CRP for the indicated times. c-Cbl was immunoprecipitated with a specific antibody, and its phosphorylation was evaluated by immunoblotting with the anti-phosphotyrosine antibody (upper panel). The membrane was then reprobed with anti-c-Cbl antibody (lower panel) to verify the efficiency of the immunoprecipitation. Quantitative analysis of c-Cbl phosphorylation from three different experiments is reported in panel (ii); black bars: wild type platelets; grey bar: Pyk2KO platelets. Data are the mean ± SD of three different experiments. (B) Platelets from wild type (WT) and Pyk2KO mice were stimulated with 0.5 μg/ml CRP for the indicated times, and the p85 regulatory subunit of PI3K was immunoprecipitated. As positive control, p85 was immunoprecipitated from wild type platelets adherent to immobilized fibrinogen. Immunoblotting analysis was performed with anti-c-Cbl and anti-p85 antibodies, as indicated on the right. The reported image is representative of three different experiments producing identical results. (C) Analysis of Syk(Tyr525/526) and Akt(Ser473) phosphorylation in wild type (WT) and c-Cbl-deficient (c-Cbl−/−) platelets stimulated with the GPVI agonist convulxin (100 ng/ml) for the indicated times. Immunoblotting was performed with specific phospho-antibodies, as indicated on the left, and anti-α-actin was used for equal loading control. Quantification of Syk and Akt phosphorylation is reported in (ii) and (iii), respectively. Results are the mean ± SD of three different experiments. **p < 0.005.
regulatory subunit of PI3K in platelets stimulated with CRP (Fig. 3b). Importantly, GPVI-induced phosphorylation of Akt occurred normally in platelets from c-Cbl knockout mice (Fig. 3C). In agreement with previous findings indicating that c-Cbl is a negative regulator of GPVI signaling [26], Fig. 3C shows a strong potentiation of Syk phosphorylation in the absence of c-Cbl, which, however, did not impact on PI3Kβ activation and Akt phosphorylation (Fig. 3C). Altogether these results demonstrate that, differently than for integrin signaling, neither c-Cbl nor Pyk2 contributes to PI3Kβ regulation by GPVI.

3.3. Pyk2 is activated downstream of PI3Kβ in GPVI signaling

Since Pyk2 deficient platelets aggregate normally upon GPVI stimulation [21], we questioned whether Pyk2 was actually activated by CRP. Fig. 4A shows that phosphorylation of Pyk2 on Tyr402, an autophosphorylation site and a marker of its activation [27], is stimulated by CRP in a time-dependent manner. Interestingly, activation of Pyk2 was impaired in platelets expressing a catalytically inactive form of PI3Kβ, but was not altered upon genetic ablation of PI3Kγ catalytic activity. These results...
indicate that, in GPVI signaling, Pyk2 is not upstream, but downstream of PI3Kβ.

It is known that Pyk2 can be activated by both SFK and Ca2+ [27]. Fig. 4B shows that CRP-induced Pyk2 activation was completely abrogated in platelets treated with the Src family kinase (SFK) inhibitor PP2, or incubated with the intracellular Ca2+ chelator BAPTA-AM. Upon activation of GPVI, intracellular Ca2+ increase is consequent to activation of PLCγ2, which is stimulated through SFK-mediated phosphorylation [5]. By using the PLC inhibitor U73122, we found that CRP-induced Pyk2 phosphorylation was downstream of PLCγ2 activation (Fig. 4B). Since it is known that the lipid products of PI3K are required for efficient activation of PLCγ2, we evaluated PLCγ2 activity by measuring the phosphorylation of pleckstrin, the main PKC substrate and a very well standardized marker for PLC activation [28]. Fig. 4C shows that CRP-induced pleckstrin phosphorylation was completely suppressed in platelets from PI3KβKD mice, but was normal in PI3KγKD platelets. Moreover, we observed that tyrosine phosphorylation of PLCγ2 in murine platelets stimulated with the GPVI agonist convulxin was strongly inhibited in the absence of PI3Kβ, but not PI3Kγ, catalytic activity (Fig. 4D). Finally, to confirm the regulation of PLCγ2 activation by PI3Kβ downstream of GPVI, we compared the release of Ca2+ from intracellular stores in control and PI3KβKD platelets. Fig. 4E shows that catalytic inactivation of PI3Kβ virtually abrogated the convulxin-stimulated increase of cytosolic Ca2+. These results indicate that PI3Kβ is absolutely required for PLCγ2 activation downstream of GPVI and that PLCγ2-mediated intracellular Ca2+ increase links PI3Kβ to Pyk2 activation.

Finally, we sought to verify whether Pyk2 and PLCγ2 regulation by PI3Kβ observed in murine platelets could also be translated to humans. Fig. 5A shows that Pyk2 phosphorylation in human platelets stimulated with convulxin was significantly inhibited by wortmannin and by the PI3Kβ selective inhibitor TGX-221, but not by the PI3Kγ inhibitor AS252424. Moreover, TGX-221 and wortmannin, but not AS252424, caused a strong, albeit not complete, inhibition of pleckstrin phosphorylation induced by convulxin in human platelets (Fig. 5B), as well as mouse platelets (data not shown). Similarly, PI3Kβ inhibition by TGX-221 significantly reduced the intracellular Ca2+ release in convulxin-stimulated human platelets (Fig. 5C). Altogether, these results confirm that also in human platelets PI3Kβ regulates PLCγ2 activity and Pyk2 phosphorylation downstream of GPVI stimulation.
4. Discussion

PI3Kβ is recognized to play a crucial role in platelet activation, and is predominantly involved in platelet responses triggered by integrin outside-in signaling and GPVI engagement [9,10]. We have recently described a signaling pathway activated downstream of integrin αIIbβ3, that links the tyrosine kinase Pyk2 and the adaptor protein c-Cbl to PI3Kβ stimulation. In the present work, we demonstrate that this pathway appears restricted to integrin αIIbβ3, as it is not shared by other platelet receptors that stimulate PI3Kβ. In particular, we compared the mechanisms for PI3Kβ activation downstream of the two major collagen receptors in platelets: integrin α2β1 and GPVI. We confirmed that PI3Kβ activation by integrin α2β1 is regulated by Pyk2, but does not involve c-Cbl, as previously hypothesized. Moreover, we demonstrated that in platelets stimulated with GPII ligands, PI3Kβ does not lie downstream but, rather, upstream of Pyk2 phosphorylation. Therefore, our results demonstrate the existence of different mechanisms regulating PI3Kβ by integrins or by GPVI in platelets.

It is generally accepted that the p110 catalytic subunits of class IA PI3K members, including PI3Kβ, are maintained in an inactive state

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Fig. 5. Regulation of Pyk2 and PLCγ2 by PI3Kβ in human platelets. Human platelets were treated with 0.5 μM TGX-211, 0.5 μM AS232424, 100 nM wortmannin of with an equivalent volume of DMSO (none) for 10 min and then stimulated with 100 ng/ml convulxin for 5 min. A. Immunoblotting analysis of Pyk2 phosphorylation was performed on whole cell lysates with anti-Pyk2(Tyr402) antibody and equal loading control was performed by reprobing with anti-Pyk2 antibody, as indicated on the right. A representative immunoblot is reported in (i), and quantification of phosphorylation, expressed as mean ± SD of three different experiments is reported in (ii). * = p < 0.05. B. Analysis of pleckstrin phosphorylation was performed with immunoblotting with an anti-(phospho)PKC substrates (P-PKCsub), and the amount of pleckstrin in each lane was verified by immunoblotting with a specific antibody, as indicated on the right. A representative immunoblot is reported in (i), and quantification of the results from three different experiments is reported in (ii), as the mean ± SD. * = p < 0.05. C. Intracellular Ca2+ concentration was measured in Fura-2-AM-loaded human platelets incubated with DMSO (none) or 0.5 μM TGX-221 for 10 min, and then stimulated with 100 ng/ml convulxin. Results are the mean ± SD of three different determinations. ** = p < 0.01.
through the constitutive and strong interaction with the regulatory subunit, mainly p85, and that activation occurs when this constitutive inhibition is relieved by binding of tyrosine phosphorylated proteins to the SH2 domain of p85. Activated growth factor receptors, cytosolic tyrosine kinases, as well as phosphorylated adaptor proteins can interact with p85 and cause the activation of p110 catalytic subunit [14]. Among these, the adaptor protein c-Cbl is a known binding partner and activator of PI3K [29–34], and has been shown to associate with PI3K in integral cubitil3 outside-in signaling, and to be tyrosine phosphorylated by Pyk2 (15). Here we demonstrated that upon integrin α2β1 engagement c-Cbl interacts with PI3K but is not tyrosine-phosphorylated. Moreover, integrin α2β1-mediated phosphorylation of Akt on both Ser473 and Thr308 occurred normally in platelets lacking c-Cbl. We conclude that, differently from what proposed for integral cubitil3 [15], Pyk2 is important for PI3K-α2β1 activation by integrin α2β1, but this effect is not mediated by c-Cbl.

In addition to integrin α2β1, GPVI is an important platelet collagen receptor essential for thrombus formation, which relies on PI3K-mediated signaling to stimulate platelet responses. In the present work we have demonstrated that the regulation of PI3Kγ by Pyk2 is restricted to integrin α2β1, and that Pyk2 is not required for PI3K-α2β1 activation upon GPVI stimulation. Moreover, platelet stimulation by CRP, a specific GPVI ligand, did not cause the association of c-Cbl with the p85 regulatory subunit, and GPVI-promoted phosphorylation of c-Cbl was not mediated by Pyk2. In addition, phosphorylation of Akt downstream of GPVI was increased exclusively downstream of integrin ligation, but PI3Kγ activation is suppressed, as demonstrated by the lack of PKC-directed protein phosphorylation and by the reduced release of Ca2+ from internal stores. Importantly, using selective and specific pharmacological inhibitors, we found that the regulation of Pyk2 and PLCγ2 by PI3Kγ also occurs in human platelets. These observations provide important hints to understand the mechanism for the previously reported crucial role of PI3Kγ in GPVI-mediated platelet activation and aggregation.

In conclusion, our study recognizes that multiple mechanisms are indeed involved in PI3Kγ regulation in platelets, and identifies Pyk2 as a regulator of PI3Kγ exclusively downstream of integrin ligation, but not upon GPVI stimulation. Since PI3Kγ is currently regarded as a promising target for anti-platelet drugs, our results suggest novel strategies to differentially target PI3Kγ activity in selected contexts of platelet activation.

**Transparency document**

The Transparency document associated with this article can be found, in the online version.

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**References**
