ROLE AND REGULATION OF PHOSPHATIDYLINOSITOL 3-KINASE β IN PLATELET INTEGRIN α2β1 SIGNALING

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Running head: PI3Kβ in platelet integrin α2β1 signaling

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SUMMARY

Integrin α2β1-mediated adhesion of human platelets to monomeric type I collagen or to GFOGER peptide caused a time-dependent activation of phosphatidylinositol 3-kinase (PI3K) and Akt phosphorylation. This process was abrogated by pharmacological inhibition of PI3Kβ, but not of PI3Kγ or PI3Kα. Moreover, Akt phosphorylation was undetectable in murine platelets expressing a kinase-dead mutant of PI3Kβ (PI3KβKD), but occurred normally in PI3KγKD platelets. Integrin α2β1 failed to stimulate PI3Kβ in platelets from phospholipase Cγ2 (PLCγ2) knockout mice, and we found that intracellular Ca2+ linked PLCγ2 to PI3Kβ activation. Integrin α2β1 also caused a time-dependent stimulation of the focal kinase Pyk2, downstream of PLCγ2 and intracellular Ca2+. While activation of Pyk2 occurred normally in PI3KβKD platelets, stimulation of PI3Kβ was strongly reduced in Pyk2 knockout mice. Neither Pyk2 nor PI3Kβ were required for α2β1-mediated adhesion and spreading. However, activation of Rap1b and inside-out stimulation of integrin αIIbβ3 were reduced upon inhibition of PI3Kβ, and significantly impaired in Pyk2-deficient platelets. Finally, both PI3Kβ and Pyk2 significantly contributed to thrombus formation under flow. These results demonstrate that Pyk2 regulates PI3Kβ downstream of integrin α2β1, and document a novel role for Pyk2 and PI3Kβ in integrin α2β1-promoted inside-out activation of integrin αIIbβ3 and thrombus formation.
INTRODUCTION

Class I phosphatidylinositol 3-kinases (PI3K) are key signaling enzymes that phosphorylate the inositol ring of membrane phospholipids, and generate different 3-phosphoinositides, important intracellular messengers that regulate several cellular processes through the downstream activation of the protein Ser/Thr kinase Akt.\(^1\) Circulating blood platelets express all the members of the class I PI3K family, which includes the PI3K\(\alpha\), PI3K\(\beta\), PI3K\(\delta\), and PI3K\(\gamma\) isoforms. PI3K activity is essential for platelet aggregation and thrombus formation,\(^1,2\) and therefore these enzymes are potential novel targets for anti-thrombotic agents. For this reason, it is essential to recognize the precise contribution of every single PI3K isoform in platelet activation induced by different extracellular agonists.

Pharmacologic and genetic evidence indicates that PI3K\(\beta\) plays a predominant role in the regulation of platelet function.\(^3-7\) Selective inactivation of PI3K\(\beta\) completely prevents platelet aggregation induced by the collagen receptor GPVI, and reduces occlusive thrombus formation.\(^4,5\) PI3K\(\beta\) is also implicated in the platelet response to agonists that stimulate G-protein coupled receptors (GPCRs), such as ADP or thromboxane A\(_2\) (TxA\(_2\)).\(^3,4,8,9\) While PI3K\(\delta\) has been demonstrated to play a minor role in platelet activation,\(^10\) PI3K\(\alpha\) has been recently proposed to be as important as PI3K\(\beta\) in GPVI signaling.\(^7\) Similarly, several reports have documented that, in addition to PI3K\(\beta\), also PI3K\(\gamma\) is implicated in GPCRs-mediated platelet activation.\(^4,9,11,12\) These observations are indicative of a still poorly appreciated interplay between different PI3K isoforms in selected contexts of platelet activation. PI3K\(\beta\) has also been proposed to be involved in integrin \(\alpha\)IIb\(\beta\)3-mediated outside-in signaling, a process essential for platelet spreading, stable thrombus formation and clot retraction.\(^3-5,13\) By contrast, very little is known about the role and regulation of PI3K downstream of the other major platelet integrin, integrin \(\alpha\)2\(\beta\)1.

Together with GPVI, integrin \(\alpha\)2\(\beta\)1 is a platelets receptor for collagen,\(^14\) but it can also interact with other ligands, including decorin and tenascin.\(^15,16\) Although some controversies persist, the role of integrin \(\alpha\)2\(\beta\)1 in adhesion to collagen, platelet activation, and thrombus formation is well documented.\(^14,17-19\) Recruitment of integrin \(\alpha\)2\(\beta\)1 initiates an outside-in signaling pathway leading to platelet spreading on the extracellular matrix, and to activation of integrin \(\alpha\)IIb\(\beta\)3, thus allowing binding of soluble fibrinogen to adherent platelets.\(^20-21,23\) The organization of this intracellular signaling pathway is still poorly understood. It is very well documented that phospholipase C\(\gamma\)2 (PLC\(\gamma\)2) is activated, and leads to intracellular Ca\(_{\text{2+}}\)
increase and protein kinase C (PKC) stimulation.\textsuperscript{20,21} PLC\(\gamma\)2 activation is essential for integrin \(\alpha 2\beta 1\)-mediated spreading, as well as for the cross-talk to integrin \(\alpha I I b\beta 3\).\textsuperscript{20,21} It has also been shown that integrin \(\alpha 2\beta 1\) stimulates tyrosine kinases, including Src and Syk, as well as small GTPases, like Rac and Rap1b.\textsuperscript{20-24} Based on the observation that the PI3K inhibitor wortmannin affects some platelet responses,\textsuperscript{22,23} the stimulation of PI3K by integrin \(\alpha 2\beta 1\) has been hypothesized, but so far this has not been directly demonstrated.

In this study, we adopted pharmacologic and genetic approaches to investigate the regulation and function of PI3K in integrin \(\alpha 2\beta 1\)-mediated adhesion of human and murine platelets. We demonstrate that integrin \(\alpha 2\beta 1\) selectively stimulates PI3K\(\beta\) isoform through a novel mechanism that involves intracellular Ca\(^{2+}\) and the Ca\(^{2+}\)-regulated tyrosine kinase Pyk2. Moreover, we also provide evidence that PI3K\(\beta\) is dispensable for integrin \(\alpha 2\beta 1\)-mediated platelet spreading on collagen, but is required for the inside-out activation of integrin \(\alpha I I b\beta 3\).
MATERIALS AND METHODS

Materials

Monomeric type I collagen was provided by Prof. M.E. Tira (University of Pavia, Italy). GFOGER peptide was provided by Dr. R. Farndale (University of Cambridge, UK). PLCγ2 knockout mice were kindly provided by Dr. J. Ihle (St. Jude Children's Research Hospital, Memphis, TN, USA) through Dr. S.P. Watson (University of Birmingham, Birmingham, UK). Generation and characterization of PI3KβKD, PI3KγKD, and Pyk2-knockout mice is reported elsewhere.25-27 The use of mice for our experimental work was approved by the Ethics Committee of the University of Pavia. The rabbit polyclonal antibodies against Rap1 (121), against Pyk2 (N-19), and against FAK (A17), as well as the monoclonal antibody anti-tubulin (DM1A) were from Santa Cruz Biotechnology (Tebu-Bio, Magenta, Italy). Anti-phosphoAkt(Ser473), and anti-phosphoPyk2(Tyr402) antibodies were from Cell Signaling Technology (Celbio, Pero, Italy). Goat polyclonal anti-pleckstrin antibody was from Abcam (Cambridge, UK). Apyrase, acetylsalicylic acid (aspirin), TRITC-conjugated phalloidin, fibrinogen, AS252424, and carboxyfluorescein succinimidyl ester (CFSE) were from Sigma (Milan, Italy). Biotinylated-fibrinogen was prepared as previously described.28 Bicinchoninc acid assay, and enhanced chemiluminescence substrate were from Pierce (Pero, Italy). RO318220 and 2-APB were from Calbiochem (VWR International, Milan, Italy). Wortmannin, and BAPTA-AM were from Alexis (Vinci-Biochem, Vinci, Italy). TGX-221 was a gift from Dr. Peter R. Shepherd (University of Auckland, New Zealand). PIK-75 was from Axon MedChem (Groeninger, The Netherlands).

Preparation of human and murine platelets

Human platelets were obtained from healthy volunteers, who had not taken drugs for at least two weeks before the withdrawn, using citric acid/citrate/dextrose (ACD) as anticoagulant (152 mM sodium citrate, 130 mM citric acid, 112 mM glucose). Whole blood was centrifuged at 120g for 10 minutes at room temperature, and apyrase (0.2 U/ml), and PGE1 (1 μM) were then added to the platelet-rich plasma. Platelets were recovered by centrifugation at 720 g for 15 minutes, washed with 5 ml PIPES buffer (20 mM PIPES, 136 mM NaCl, pH 6.5), and finally gently resuspended in HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.9 mM KCl, 12 mM NaHCO3, pH 7.4). The cell count was typically adjusted to 0.4 x 10^9 platelets/ml.
Murine platelets were prepared from blood collected from abdominal *vena cava* using ACD/3.8% Na-citrate (2:1), as anticoagulant. Blood was centrifuged at 180g for 10 minutes, and 0.02 U/mL apyrase, 10 μM indomethacin, and 1 μM PGE₁ were added to the isolated PRP. In order to increase platelet yield, the red blood cells pellet was washed with HEPES buffer and centrifuged at 180g for 10 minutes. The upper phase was collected and pooled with the PRP. Platelets were then recovered by centrifugation at 550g for 7 minutes, washed, and resuspended in HEPES buffer. Platelet count was adjusted to 0.3 X 10⁹ cells/mL, and, upon addition of 5.5 mM glucose, cells were allowed to rest for 30 minutes at room temperature.

**Adhesion assay**

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, or 10 μg/ml GFOGER peptide diluted in PBS. Dishes were washed 3 times with 5 mL PBS, blocked with 2 mL 1% BSA in PBS for 2 hours at room temperature, and then washed again 3 times with PBS. Human or murine platelets (0.5 mL, 4x10⁸ platelets/mL for human platelets, or 3x10⁸ platelets/mL for murine platelets) were added to collagen-coated dishes in the presence of 2 mM MgCl₂ and 1 mg/mL BSA. Apyrase (0.4 U/ml) was also typically added to the platelet suspension, as indicated in the text. After 10, 30, or 60 minutes of incubation at room temperature, non-adherent cells were removed, and dishes were washed 3 times with 5 mL PBS. For whole cell lysate preparation, adherent cells were directly solubilised by addition of 0.5 mL of 2% SDS in HEPES buffer, and then collected. For analysis of Rap1 activation, adherent platelets were recovered by lysis with 1 mL ice-cold RIPA buffer (50 mM Tris/HCl pH 7.4, 200 mM NaCl, 2.5 mM MgCl₂, 1% Nonidet P-40, 10% glycerol, 1 mM PMSF, 1 μM leupeptin, 0.1 μM aprotinin, 0.1 μM Na₃VO₄), and lysates were centrifuged at 18000g for 10 minutes. The protein content in the cleared supernatant was determined by the bicinchoninic acid assay, and aliquots of each sample containing the same amount of proteins (and, thus, deriving from the same number of adherent platelets) were used for immunoblotting analysis or for Rap1b activation assay.

Evaluation of platelet adhesion and spreading was performed using a fluorescence microscopy-based method, upon incubation of platelets to glass coverslips coated with 50 μg/ml monomeric type I collagen in 0.1 M acetic acid, as above described. Adherent platelets were fixed, permeabilized, and stained by TRITC-conjugated phalloidin. Platelets were viewed on a fluorescence microscope (Olympus BX51), and digital images (x40) were
acquired. The number of adherent cells, as well as the average cell area (as an index of platelet spreading), was determined using the ImageJ software. For each specimen, five different fields were analysed by two independent observers.

**Rap1 activation assay**

Analysis of accumulation of active GTP-bound Rap1b in adherent platelets was performed by a pull-down assay, using the glutathione S-transferase-tagged Rap-binding domain of RalGDS, essentially as previously described.\(^{28}\)

**Electrophoresis and immunoblotting**

Aliquots of platelet lysates containing the same amount of proteins were dissociated by addition of 0.5 volumes of SDS-sample buffer 3X (37.5 mM Tris, 288 mM glycine, pH 8.3, 6% SDS, 1.5% DTT, 30% glycerol, 0.03% bromophenol blue), and samples were heated at 95°C for 3 minutes. SDS-sample buffer 2X was also added to precipitated, active Rap1b. Proteins were separated by SDS-PAGE, typically on a 10% acrylamide gel for the analysis of Akt or Pyk2 phosphorylation, or on a 10-20% acrylamide gradient gel for Rap1b detection, and subsequently transferred to PVDF membrane. Membranes were blocked for 2 hours with 5% BSA in Tris buffer saline (20 mM Tris/HCl, pH 7.5, 0.5 mM NaCl), and then incubated overnight at 4°C with the desired primary antibodies diluted in 20 mM Tris/HCl, pH 7.5, 0.5 mM NaCl. In the present study the following antibodies and dilution were used: anti-phosphoAkt(Ser473), 1:500; anti-phosphoPyk2(Tyr402), 1:500; anti-pleckstrin, 1:1000; anti-tubulin, 1:1000; anti-Pyk2, 1:500; anti FAK, 1:200, anti-Rap1, 1:1000. Membranes were then extensively washed with 0.1% Tween 20 in Tris buffer saline, and incubated with peroxidase-conjugated secondary antibody (1:3000 dilution) for 45 minutes. Upon extensive washing, reactive proteins were visualized with a chemiluminescence reaction. The PVDF membranes were then stripped and reprobed with a different antibody (typically, anti-tubulin or anti-pleckstrin) as a control for equal loading. All the immunoblots reported in this manuscript are representative of at least three different experiments giving similar results. Quantification of protein intensity was performed by computer assisted densitometric scanning using image J software.

**Thrombus formation under flow**

Glass coverslips were coated with monomeric type I collagen (100 μg/ml), and blocked with 1% BSA in PBS pH 7.4. The coverslips were mounted in a 50 μm-deep parallel-
plate flow chamber (RC-31 from Warner Instruments) under a fluorescence microscope, and rinsed with washing buffer (HEPES buffer supplemented with 2 mM CaCl$_2$, 2 mM MgCl$_2$, 5.5 mM glucose, 0.1% BSA, 1 U/ml heparin). PPACK/heparin-treated mouse blood was preincubated with 3 μg/ml CFSE for 5 minutes, and flowed over collagen at 1,000 sec$^{-1}$ for 4 minutes, using a pump system (Harvard Apparatus PHD 2000). Upon perfusion, the flow chamber was rinsed with washing buffer, and at least 10 randomly taken fluorescence microscopic images were collected after 2 minutes and 10 minutes of rinse. Images were analyzed by ImageJ software, and the extent of thrombus formation was calculated as the percentage of platelet covered area.

**Measurement of fibrinogen binding to collagen adherent platelets**

Measurement and quantification of specific binding of biotin-labeled fibrinogen to integrin $\alpha_{IIb}\beta3$ in platelets adherent through integrin $\alpha_{2}\beta1$ was performed according to a procedure that integrates data from multiple determination, as previously described in details.$^{21}$ This procedure allows the calculation of the specific binding of fibrinogen for the same number of adherent cells.
RESULTS

Integrin α2β1 activates PI3Kβ.

We have previously reported that PI3Kβ is essential for GPVI-mediated platelet activation, and is required for platelet spreading on fibrinogen.\textsuperscript{4} Here we investigated the activation of PI3K upon platelet adhesion through integrin α2β1, by measuring the phosphorylation of the downstream effector Akt. Washed human platelets were allowed to adhere to immobilized monomeric type I collagen, or to GFOGER peptide in the presence of 2 mM MgCl₂ for increasing times. We have previously demonstrated that, under the conditions of this assay, monomeric type I collagen promotes platelet adhesion exclusively through integrin α2β1 and does not lead to GPVI stimulation.\textsuperscript{4,24,29} GFOGER peptide is a well characterized specific ligand for integrin α2β1.\textsuperscript{30} Adherent platelets were lysed, and Akt phosphorylation on Ser473 was evaluated by immunoblotting with a phosphospecific antibody. Figure 1A shows that engagement of integrin α2β1 by monomeric collagen or by GFOGER peptide induced a robust time-dependent phosphorylation of Akt. To identify the PI3K isoform involved, platelets were incubated with inhibitors of different PI3K isoforms before adhesion to monomeric type I collagen. Figure 1B shows that integrin α2β1-induced Akt phosphorylation was prevented by wortmannin, and by TGX-221, a selective inhibitor of PI3Kγ, but was unaffected by the PI3Kα inhibitor AS252424, or by the PI3Kβ inhibitor PIK-75. These results indicate that integrin α2β1 activates PI3Kβ. To confirm this observation, we analysed Akt phosphorylation in murine platelets expressing catalytically inactive forms of either PI3Kβ (PI3Kβ\textsuperscript{KD}) or PI3Kγ (PI3Kγ\textsuperscript{KD}). Figure 1C shows that Akt phosphorylation induced by platelet adhesion through integrin α2β1 occurred normally in PI3Kγ\textsuperscript{KD} platelets, but was not detectable in the absence of PI3Kβ activity. These results demonstrated that PI3Kβ is the PI3K isoform activated downstream of integrin α2β1, and responsible for Akt phosphorylation in adherent platelets.

PI3Kβ is regulated by intracellular Ca\textsuperscript{2+} downstream of integrin α2β1.

It is known that PI3Kβ can be activated by GPCRs.\textsuperscript{1,3,4} Therefore we considered that released ADP and/or generated TxA\textsubscript{2} could contribute to Akt phosphorylation upon platelet adhesion through integrin α2β1. Figure 2A shows that Akt phosphorylation was unaltered in aspirin (ASA)-treated platelets, indicating that TxA\textsubscript{2} does not contribute to PI3K activation. By contrast, neutralization of secreted ADP by apyrase, as well as blockade of both P2Y1 and
P2Y12 receptor with the selective antagonists MRS2179 and AR-C69931MX, strongly reduced, but did not abolish, integrin α2β1-induced phosphorylation of Akt. When added together, apyrase and aspirin had no additive effects. Therefore, secreted ADP, but not TxA2 contributed to integrin-mediated PI3Kβ activation. Phosphorylation of Akt induced by integrin α2β1-mediated platelet adhesion was not reduced in the presence of RGDS, rather a small increase was detected, and was still partially sensitive to apyrase and ADP receptors antagonists (figure 2A). These results exclude a significant contribution of platelet autocrine stimulation by integrin αIIbβ3 due to binding of potentially secreted fibrinogen. In order to focus on the direct link between integrin α2β1 and PI3Kβ, all the subsequent experiments were performed in the presence of apyrase.

It is known that outside-in signaling through integrin α2β1 involves PLCγ2 activation, leading to stimulation of PKC and intracellular Ca2+ increase.20,21 We have recently shown that integrin α2β1-mediated PLCγ2 activation occurs through Src-dependent, and independent mechanisms.29 Therefore, we investigated the contribution of Src kinase and intracellular messengers generated by PLCγ2 on PI3Kβ activation. Figure 2B shows that inhibition of Src kinases by PP2 only partially prevented PI3Kβ activation (22 ± 3 of inhibition, n=3). By contrast, Akt phosphorylation was completely suppressed by intracellular Ca2+ chelation with BAPTA-AM, but not upon inhibition of PKC with RO318220. Interestingly, Akt phosphorylation was also suppressed by 2-APB, an inhibitor of the IP3 receptor, and thus an antagonist of IP3-mediated Ca2+ release. These findings place PI3Kβ downstream of PLC and intracellular Ca2+ in integrin α2β1 signaling. Therefore, we analyzed PI3Kβ activation in platelets from PLCγ2 knockout mice. Figure 3C shows that engagement of integrin α2β1 in PLCγ2-deficient platelets failed to induce Akt phosphorylation, indicating that PLCγ2 is required for PI3Kβ stimulation.

Role of the tyrosine kinase Pyk2 on the Ca2+-dependent stimulation of PI3Kβ.

Multiple mechanisms have been so far proposed to activate PI3Kβ, including binding to phosphorylated tyrosine kinases through the SH2 domains-containing regulatory subunit, G-protein βγ dimers, and Ras.1 In order to characterize the mechanism for the novel Ca2+-mediated regulation of PI3Kβ in platelet integrin α2β1 signaling, we hypothesized the involvement of the focal adhesion kinase Pyk2, which is known to be activated both by Src-dependent phosphorylation, and by intracellular Ca2+.31 Pyk2 is expressed in platelets and is activated by several soluble agonists.32-34 However, its implication in integrin α2β1 signaling...
is still unknown. Using a phospho-specific antibody able to detect Pyk2 autophosphorylation on Tyr402, we evaluated the activation of this kinase in platelets adherent to monomeric collagen or to GFOGER peptide. Figure 3A shows that integrin α2β1 promoted the time-dependent activation and autophosphorylation of Pyk2. Activation of Pyk2 did not require PI3K activity, as it occurred normally in the PI3KβKD platelets (figure 3B), but was regulated by intracellular Ca^{2+}, because it was inhibited by BAPTA-AM, and upon blockade of IP3-mediated Ca^{2+} release by 2-APB (figure 3C). Importantly, integrin α2β1-induced activation of Pyk2 was completely dependent on PLCγ2, as it failed to occur in PLCγ2-deficient platelets (figure 3D). Therefore, as for PI3Kβ, also Pyk2 activation is downstream of PLCγ2 and cytosolic Ca^{2+}.

To further investigate the role of Pyk2 in PI3Kβ activation we analysed platelets from Pyk2 knockout mice. These cells did not express Pyk2, but contained normal amounts of the related focal adhesion kinase FAK (Canobbio et al., manuscript in preparation). Pyk2 knockout platelets were let to adhere to monomeric collagen, and phosphorylation of Akt was evaluated after 30 and 60 minutes. We observed a strong and statistically significant inhibition of integrin α2β1-mediated phosphorylation of Akt in Pyk2-deficient platelets (figure 4). Therefore we conclude that the Ca^{2+}-dependent tyrosine kinase Pyk2 links PLCγ2 activation to PI3Kβ downstream of integrin α2β1.

PI3Kβ is required for the cross-talk between integrin α2β1 and integrin αIIbβ3.

We next addressed the question as to the functional relevance of the Pyk2/PI3Kβ pathway in platelet adhesion through integrin α2β1. We have previously shown that PI3Kβ is required for platelet spreading on fibrinogen.4 By contrast, we failed to detect any significant difference in platelets adhesion or spreading mediated by integrin α2β1 between wild type and PI3KβKD platelets (figure 5A). This observation was also supported by pharmacologic studies with specific inhibitors. As shown in figure 5B, integrin α2β1-mediated platelet adhesion and spreading was not affected by wortmannin, TGX-221 or AS252424. Similarly, no significant differences in integrin α2β1-mediated adhesion and spreading were detected between wild type and Pyk2 deficient platelets (figure 5C), indicating that, as for PI3Kβ, neither Pyk2 is required for these processes.

Platelet adhesion through integrin α2β1 leads to the inside-out activation of integrin αIIbβ3 through a signaling pathway that involves PLCγ2 and the small GTPase Rap1b.21,29
Therefore, we investigated the contribution of Pyk2 and PI3Kβ on the cross-talk to integrin αIIbβ3. Figure 6A shows that adhesion-dependent activation of Rap1b was strongly reduced upon inhibition of PI3Kβ by wortmannin or TGX-221. Moreover, a similarly impaired Rap1b activation induced by integrin α2β1 was also observed in the Pyk2-deficient platelets. Therefore, Pyk2 and PI3Kβ are important regulators of Rap1b activity downstream of integrin α2β1.

We next directly evaluated the inside-out activation of integrin αIIbβ3 by measuring the specific binding of fibrinogen to collagen-adherent platelets. Figure 6C shows that fibrinogen binding was strongly inhibited by wortmannin and by the specific PI3Kβ inhibitor TGX-221, and was also significantly reduced in the absence of Pyk2. Therefore, we conclude that Pyk2 and PI3Kβ play a role in the cross-talk between integrins α2β1 and αIIbβ3.

Under flow conditions, activation of integrin αIIbβ3 in collagen adherent platelets is important for the growth and stabilization of the thrombus. Therefore, we analysed the role of Pyk2 and PI3Kβ in thrombus formation under flow. Fluorescently labeled platelets in whole blood were perfused for 4 minutes at a shear rate of 1,000 sec\(^{-1}\) over immobilized monomeric collagen, to favour integrin α2β1-initiated platelet adhesion and thrombus formation. The stability of the formed thrombus was then evaluated upon secondary perfusion with HEPES buffer for 10 minutes. Figure 7 shows that thrombus formation was strongly reduced when blood from either Pyk2-deficient or PI3Kβ\(^{KD}\) mice were perfused. The defective thrombus formation was more evident in the absence of catalytically active PI3Kβ (89.28 ± 0.67 % of reduction of the covered area compared to control, n=4), than in the absence of Pyk2 (69.06 ± 7.27 % of reduction, n=4). After extensive perfusion of buffer we did not detect, under our experimental conditions, any significant reduction of the covered area by platelets from wild type and Pyk2 KO mice. A small, but significant reduction of the covered area was detected, however, in the absence of catalytically active PI3Kβ, which more likely reflects the detachment of adherent platelets, since basically no thrombi of relevant size were detected in these samples. These results indicate that perfusion of blood over monomeric collagen triggers the formation of stable platelet thrombi, supported by the Pyk2/PI3Kβ signaling pathway.
DISCUSSION

In this work we have investigated the role and the regulation of PI3K in platelet integrin α2β1 signaling. We have demonstrated that integrin α2β1 selectively stimulates PI3Kβ downstream of PLCγ2, through a mechanism that involves the Ca2+-dependent tyrosine kinase Pyk2. Moreover, we have described that PI3Kβ is not required for integrin α2β1-mediated spreading, but is important for the activation of the small GTPase Rap1b and for the cross-talk to integrin αIIbβ3, leading to fibrinogen binding to collagen-adherent platelets.

Although the role of integrin α2β1 in platelet adhesion to collagen, and its ability to trigger platelet activation are well documented, little is known about the outside-in signaling pathways activated by this integrin. For instance, the involvement of PI3K has been hypothesized based on indirect evidence with inhibitors,22,23 but has never been directly documented. By measuring Akt phosphorylation we have filled this gap of information, as we have directly demonstrated the effective stimulation of PI3K by integrin α2β1, and we have also identified the isoform implicated.

Monomeric type I collagen has been mainly used in this work as a reliable, cheap, and easy to obtain ligand for integrin α2β1, because previous studies have clearly demonstrated that under these conditions no activation of GPVI occurs.4,24,29 However, stimulation of PI3Kβ activity by integrin α2β1 was also confirmed using a different specific ligand, the collagen related peptide GFOGER. Moreover, although the majority of the experiments reported in this study were performed using type I monomeric collagen, as integrin α2β1 ligand, many of the results have been confirmed in experiments with the GFOGER peptide (data not shown).

By using a combination of pharmacologic and genetic approaches we have identified PI3Kβ as the PI3K isoform stimulated by integrin α2β1. Among all the members of the class I PI3K, PI3Kβ is emerging as a major regulator of platelet activation. We and others have previously shown that PI3Kβ is activated by GPVI, as well as by GPCRs, and is important for platelet spreading on fibrinogen.3-7 The finding that PI3Kβ is also activated by integrin α2β1 further extends the role and importance of this isoform in platelet function. PI3Kβ is also stimulated downstream of the P2Y12 receptor for ADP,3,4,8 and we have here demonstrated that secreted ADP actually contributes to integrin α2β1-mediated Akt phosphorylation. However, we have also demonstrated that integrin α2β1 can directly stimulate PI3Kβ even in
the absence of secondary released agonists, confirming that PI3Kβ can also be directly regulated by integrin α2β1 engagement.

A central event in platelet adhesion through integrin α2β1 is the stimulation of PLCγ2. Analysis of platelets from PLCγ2 knockout mice, revealed that PLCγ2 is absolutely required for integrin α2β1-induced stimulation of PI3Kβ. Moreover we found that PLCγ2 regulation of PI3Kβ occurs through intracellular Ca2+ increase rather than PKC activation. Our findings are supported by a recent study reporting that Ca2+ is implicated in the P2Y12-independent PI3K activation in thrombin-stimulated platelets.35 Our results move forward these observations, as we have here demonstrated that Ca2+-dependent activation of PI3K occurs in integrin signaling, and we have identified PI3Kβ as the Ca2+-regulated PI3K isoform. Interestingly, when platelets are stimulated through the other main collagen receptor, GPVI, PI3Kβ stimulation lies upstream of PLCγ2, and is actually required for efficient PLCγ2 activation.36 By contrast, our results demonstrate that in the integrin α2β1 outside-in signaling, engagement of PI3Kβ is completely downstream of PLCγ2. Moreover, we also observed that, in this context, the PI3K inhibitor wortmannin did not affect PLCγ2 activation induced by integrin α2β1 (data not shown). These observations outline another important difference in the signaling pathways activated by the two main platelet collagen receptors. Although PLCγ2 is typically considered to be activated through Src-mediated phosphorylation, we have previously demonstrated that integrin α2β1 adopts multiple mechanisms, and is able to stimulate PLCγ2 even in the absence of Src-mediated phosphorylation.29 Consistently with these findings, inhibition of Src kinase by PP2 reduced, but did not abolish PI3Kβ activation.

In the experimental model adopted in this study, PLCγ2-dependent activation of PI3Kβ can be detected only after 30 minutes of adhesion, while these processes are supposed to occur within seconds in vivo. This consideration, that holds true also for a large number of other previous studies, clearly represents an intrinsic limitation in the investigation of the signaling processes associated to platelet adhesion, and the relevance of the experimental observations relies on the assumption that those events that in vitro need time to reach detectable levels, actually occurs much more rapidly under physiological conditions in vivo.

PI3Kβ is typically considered to be activated by receptor or non-receptor tyrosine kinases, which provide phosphotyrosine residues able to bind the SH2 domains of the regulatory subunit p85, thus relieving a constitutive inhibitory action on the p110β catalytic subunit.1 In addition, PI3Kβ has been found to be also activated by G-proteins βγ dimers, and
by the small GTPase Ras, through mechanisms that are not completely understood yet.\(^1\) Here, we propose a novel mechanism for PI3K\(\beta\) stimulation that involves elevation of intracellular Ca\(^{2+}\) downstream of PLC\(\gamma\)\(2\). We have also demonstrated that this effect is, at last partially, mediated by the tyrosine kinase Pyk2. Pyk2 belongs to the focal adhesion kinases family, and can be activated both by Src-mediated phosphorylation and by binding of Ca\(^{2+}\) to the N-terminal FERM domain.\(^3\),\(^37\) Pyk2 is highly expressed in platelets, and it has been shown to be activated by many soluble agonists through both Ca\(^{2+}\)-dependent and -independent pathways.\(^3\),\(^2\),\(^3\) The role of Pyk2 in platelet function is still poorly characterized, and its involvement in integrin outside-in signaling has never been directly investigated. Here we have demonstrated that Pyk2 is activated upon integrin \(\alpha2\beta1\) engagement, and that this process requires PLC\(\gamma\)\(2\) activity, intracellular Ca\(^{2+}\) increase, but not PI3K\(\beta\). By contrast, we have clearly demonstrated that Pyk2 regulates PI3K\(\beta\), because integrin \(\alpha2\beta1\)-mediated phosphorylation of Akt was strongly impaired in Pyk2-deficient platelets. Generation of Pyk2-knockout mice allowed a better understanding of the role of this kinase in many physiological context, including macrophage migration and osteoclasts activation.\(^2\),\(^3\),\(^8\),\(^9\) A comprehensive and detailed analysis of platelet functions in Pyk2-knockout mice is described in a separated manuscript, which documents the importance of this kinase for platelet aggregation and thrombus formation (Canobbio et al, manuscript in preparation). In the present study, we report that Pyk2-deficient platelets show a defective activation of PI3K\(\beta\) upon integrin \(\alpha2\beta1\)-mediated adhesion. A possible role for Pyk2 in the regulation of platelet PI3K activity has been previously hypothesized on the basis of some circumstantial evidence.\(^4\),\(^1\) Our results definitively demonstrate that Pyk2 is an essential regulator of PI3K\(\beta\) in platelet integrin \(\alpha2\beta1\) signaling. We have been unable to document a direct association between Pyk2 and PI3K\(\beta\) in collagen-adherent platelets by co-immunoprecipitation experiments (data not shown). This evidence suggests either that the interaction occurs transiently and with a low affinity, or that Pyk2-mediated stimulation of PI3K\(\beta\) involves an addition, still unidentified, molecule. This possibility is currently under investigation. It should be noted, however, that while integrin-mediated PI3K\(\beta\) activation is totally suppressed in the absence of PLC\(\gamma\)\(2\) or upon chelation of intracellular Ca\(^{2+}\), it is reduced, but not abolished, in the absence of Pyk2. This implies that intracellular Ca\(^{2+}\) regulates PI3K\(\beta\) not only through activation of Pyk2, but also through other mechanisms that remain to be identified. In this study the analysis of Pyk2 KO mice has been essential to demonstrate the role of this kinase in integrin signaling in mice, and we can simply assume, as in the case of...
many studies performed with other transgenic mice lacking specific signaling intermediates, that Pyk2 plays a similar role also in humans. In the absence of any pathology associated to a deficiency of Pyk2, this assumption could only be confirmed pharmacologically. We have tested some commercially available Pyk2 inhibitors, but we have detected a number of non-specific effects that precluded any further reliable investigation (data not shown).

In this work, we have also addressed the question as to the role of the Pyk2/PI3Kβ pathway in platelet adhesion through integrin α2β1. We have found that adhesion and spreading on monomeric collagen occurred normally in the absence of catalytically active PI3Kβ. Interestingly, we have previously shown that adhesion and spreading on immobilized fibrinogen was severely compromised in PI3KβKD platelets. Therefore, it is clear that PI3Kβ plays different roles downstream of integrins α2β1 and αIIbβ3. This conclusion is not related to a difference in ligand density, which, for instance, has been shown to affect outside-in signaling through integrin αIIbβ3, because we observed normal adhesion and spreading in the presence of PI3K inhibitors even after coating with much lower concentration of monomeric collagen like 0.1μg/ml (data not shown). Moreover, in agreement with the role of Pyk2 in PI3Kβ activation by integrin α2β1, we also found that platelets lacking Pyk2 were able to normally adhere and spread on monomeric collagen. It can be concluded that the previously reported role of PLCγ2 and intracellular Ca²⁺ on integrin α2β1-mediated spreading involves alternative signaling pathways. However, we were able to recognize a crucial role for Pyk2 and PI3Kβ in the inside-out activation of integrin αIIbβ3, allowing fibrinogen binding to collagen-adherent platelets. The defective integrin αIIbβ3 activation in the absence of the Pyk2/PI3Kβ pathway results in an impaired thrombus formation. Perfusion of whole blood on immobilized collagen under a moderate shear rate revealed that both Pyk2 and PI3Kβ are essential for correct thrombus formation. The stronger reduction of thrombus formation by PI3KβKD compared to Pyk2 KO platelets is consistent with our observation that some residual activation of PI3Kβ still takes place in the absence of Pyk2. We have previously shown that this cross-talk between the two main platelet integrins is dependent on PLCγ2 activity and is regulated by the small GTPase Rap1b. Here we have shown that both PI3Kβ and Pyk2 are required for efficient stimulation of Rap1b. How PI3Kβ regulates binding of GTP to Rap1b is not clear. Activation of Rap1b by integrin α2β1 is completely dependent on the action of CalDAG-GEFI, and therefore it is likely that PI3Kβ can signal on CalDAG-GEFI. Although Ca²⁺ can directly stimulate CalDAG-GEFI, PI3Kβ signaling may be required as
well. It is interesting to note that PI3Kβ mediates Rap1b activation also downstream of the P2Y12 ADP receptor.4,44 Although P2Y12 receptor is unable to increase intracellular Ca\(^{2+}\), activation of Rap1b by ADP is still dependent on CalDAG-GEFI.45 Our results actually support the model of a strict cooperation between Ca\(^{2+}\) and PI3Kβ for maximal activation of CalDAG-GEFI leading to GTP-Rap1b accumulation. Whatever the mechanism, it is clear that the small GTPase Rap1b integrates many signaling pathways initiated by PLC\(γ\)2 in integrin \(α2β1\)-adherent platelets, and convey them to the inside-out activation of integrin \(αIIbβ3\). A general scheme illustrating the role of PI3Kβ and Pyk2 the signaling pathway linking integrin \(α2β1\) and integrin \(αIIbβ3\) is depicted in supplemental figure 1.

In conclusion, our results identify a novel mechanism for PI3Kβ activation in integrin \(α2β1\) outside-in signaling that depends on PLC\(γ\)2 and on the Ca\(^{2+}\)-sensitive tyrosine kinase Pyk2, and recognize an important role for this pathway in the inside-out activation of integrin \(αIIbβ3\).

ACKNOWLEDGEMENTS

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Contribution of Authors
Alessandra Consonni: designed and performed experiments, analysed data
Lina Cipolla: designed and performed experiments, analysed data
Gianni Guidetti: performed experiments, analysed data
Ilaria Canobbio: performed experiments, analysed data
Elisa Ciraolo: provided vital reagents, performed experiments
Emilio Hirsch: contributed vital new reagents, edited the manuscript
Marco Falasca: contributed vital new reagents, edited the manuscript
Mitsuhiko Okigaki: contributed vital new reagents, edited the manuscript
Cesare Balduini: analysed data, edited the manuscript
Mauro Torti: designed research, analysed data, wrote the manuscript, overall direction
REFERENCES


FIGURE LEGENDS

Figure 1. Activation of PI3Kβ by integrin α2β1 engagement.
A. Human platelets were allowed to adhere to immobilized monomeric type I collagen (i) or GFOGER peptide (ii) for 10, 30 or 60 minutes. Adherent cells, as well as non adherent cells (NA) from the 60 minutes samples, were collected and lysed. Akt phosphorylation was analysed by immunoblotting with anti-phospho-Akt(Ser473) antibody (upper panels). Equal loading of the samples was verified by subsequent immunoblotting with anti-tubulin (lower panels).
B. Human platelets were preincubated with DMSO, wortmannin (100 nM, 15 minutes), TGX-221 (0.5 μM, 10 minutes), AS252424 (0.5 μM, 10 minutes), or PIK-75 (0.5 μM, 10 minutes), and Akt phosphorylation (upper panel) was evaluated by immunoblotting in platelets adherent to monomeric type I collagen for 60 minutes. Subsequent analysis of pleckstrin levels (lower panel) was performed to control loading of the samples.
C. Wild type murine platelets (WT), as well as platelets from PI3KγKD or PI3KβKD mice were allowed to adhere to monomeric collagen through integrin α2β1 for 30 or 60 minutes, as indicated on the top. Non-adherent platelets (NA) were also collected after 60 minutes. The upper panel shows Akt phosphorylation in adherent and non-adherent platelets, while the lower panel shows the comparable expression of tubulin in all the samples.

Figure 2. Characterization of integrin α2β1-induced PI3Kβ activation.
A. Role of secreted ADP, TxA₂, and integrin αIIbβ3 in integrin α2β1-triggered Akt phosphorylation. Platelets were incubated with 0.5 mM aspirin (ASA) for 15 minutes, with 2 U/ml apyrase, 0.5 mM RGDS, or a mixture of 100 μM MRS2179 and 0.5 μM AR-C69931MX, for 2 minutes, and then allowed to adhere to monomeric collagen for 60 minutes. Non-adherent platelets from untreated samples (NA) were also collected. In (i), the upper panel shows a typical immunoblot with anti-phopho-Akt(Ser 473) antibody, and the lower panel shows the level of tubulin in the different samples. In (ii), a quantitative evaluation of Akt phosphorylation performed by densitometric analysis of immunoblots is reported. Data are the mean ± S.D. of three different experiments.
B. Analysis of Akt phosphorylation in platelets after 60 minutes of adhesion upon treatment with BAPTA-AM (20 μM, 30 minutes), RO318220 (10 μM, 5 minutes), PP2 (20 μM, 15 minutes), or 2-APB (100 μM, 10 minutes), as indicated. As a negative control, non adherent
platelets (NA) from untreated samples (none) were also analysed. Subsequent immunoblotting with anti-tubulin (lower panel) was performed as control for equal loading.

C. Analysis of Akt phosphorylation in murine platelets from wild type (WT) and PLC\(\gamma\)2 knockout (PLC\(\gamma\)2 KO) mice. Adherent platelets were recovered after 30 and 60 minutes, as indicated on the top. Non-adherent cells were analysed after 60 minutes.

Figure 3. Analysis of Pyk2 phosphorylation induced by integrin \(\alpha_2\beta_1\). Adhesion-induced Pyk2 phosphorylation was evaluated on whole platelet lysates by immunoblotting with anti-phospho-Pyk2(Tyr402) antibody (upper panels). Subsequent staining with anti-tubulin or anti-pleckstrin antibody is reported in the lower panels, as control for equal loading.

A. Platelet adhesion to monomeric collagen (i) or GFOGER peptide (ii) was performed for the times indicated on the bottom. Samples of non-adherent platelets were collected after 60 minutes.

B. Analysis of Pyk2 phosphorylation in wild type, and PI3K\(\beta\)^KD murine platelets adherent to monomeric collagen for the 10, 30 or 60 minutes.

C. Effect of platelet incubation with BAPTA-AM (20 \(\mu\)M, 30 minutes), or 2-APB (100 \(\mu\)M, 10 minutes), on integrin \(\alpha_2\beta_1\)-induced Pyk2 phosphorylation. Platelet adhesion was performed for 60 minutes. None: control platelets, treated with DMSO; NA: non-adherent, untreated platelets.

D. Analysis of Pyk2 phosphorylation in platelets from wild type (WT) and PLC\(\gamma\)2 knockout (PLC\(\gamma\)2 KO) mice upon adhesion to monomeric collagen for 30 and 60 minutes.

Figure 4. Integrin \(\alpha_2\beta_1\)-induced PI3K\(\beta\) activation is impaired in Pyk2-deficient platelets. Comparative analysis of Akt phosphorylation in wild type and Pyk2 knockout platelets upon adhesion to monomeric collagen for 10, 30 and 60 minutes. A representative immunoblot is reported in (i). Quantification of Akt phosphorylation, performed by densitometric analysis of the immunoreactive bands, is reported in (ii). Black bars are wild type platelets, grey bars are Pyk2 KO platelets. Data are the mean ± SD of three different experiments (*, \(p<0.05\)).

Figure 5. Role of PI3K\(\beta\) and Pyk2 in platelet adhesion and spreading through integrin \(\alpha_2\beta_1\).

A. Wild type and PI3K\(\beta\)^KD platelets were allowed to adhere to immobilized monomeric collagen for 10 or 60 minutes. Adherent cells were permeabilised, stained with TRITC-
phalloidin, and adhesion (as number of cells/mm²) and spreading (as mean platelet area) were evaluated as described in Materials and Methods. (i) Representative image of adherent WT and PI3Kβ^KD^ platelet after 60 minutes (40X amplification). Quantification of adhesion and spreading is reported in (ii), and (iii), respectively. Data are the mean ± S.D. of three different experiments.

B. Integrin α2β1-mediated adhesion (i) and spreading (ii) of platelets preincubated with wortmannin (100 nM, 15 minutes), TGX-221 (0.5 μM, 10 minutes), or AS252424 (0.5 mM, 10 minutes), as indicated on the right, after 10 or 60 minutes, as indicated on the bottom. Results are expressed as mean ± S.D. of three different experiments.

C. Integrin α2β1-mediated adhesion (i) and spreading (ii) of platelets from wild type (WT) and Pyk2 knockout (Pyk2 KO) after 10, 30 or 60 minutes. Results are expressed as mean ± S.D. of three different experiments.

Figure 6. Role of PI3Kβ and Pyk2 in integrin α2β1-mediated Rap1b stimulation and integrin αIIbβ3 activation.

A. Analysis of Rap1b activation. Active GTP-bound Rap1b was precipitated from platelets that have been allowed to adhere to monomeric collagen for 60 minutes upon incubation with DMSO (none), wortmannin (100 nM, 15 minutes), TGX-221 (0.5 μM, 10 minutes), or AS252424 (0.5 mM, 10 minutes), as indicated on the bottom. A representative immunoblots is reported in (i), where the upper panel shows the active form of Rap1b, and the lower panel the level of total Rap1b present in the platelet lysates. Quantification of Rap1b activity was performed by densitometric analysis of the immunoblots, and the results are reported in (ii). The amount of active Rap1b in adherent DMSO-treated platelets was taken as 100%. Data are the mean ± S.D. of three different experiments (* = p<0.05).

B. Comparative analysis of Rap1b activation in wild type (WT) and Pyk2-deficient platelets (Pyk2 KO) after adhesion to monomeric collagen for 60 minutes. Both a representative immunoblot (i), and quantitative analysis (ii) of Rap1b activation are reported. Data in (ii) are the mean ± S.D. of three different experiments (* = p<0.05).

C. Analysis of specific binding of biotinylated fibrinogen to adherent platelets. The effect of preincubation of platelets with wortmannin (100 nM, 15 minutes), or TGX-221 (0.5 μM, 10 minutes) is reported in (i), where the binding of fibrinogen to DMSO-treated control platelets was taken as 100%. The comparative binding of fibrinogen to adherent platelets from wild
type (WT) or Pyk2 knockout (Pyk2 KO) mice is reported in (ii). In both cases, data are the mean ± S.D. of four different experiments (* = p<0.05, *** = p<0.001).

Figure 7. Defective thrombus formation in the absence of Pyk2 or catalytically active PI3Kβ. CSFE-labeled platelets in whole blood from wild type (WT), Pyk2 KO and PI3KβKD mice were perfused over immobilized monomeric collagen at a shear rate of 1,000 sec⁻¹ for 4 minutes. Images were taken after brief rinse of the coverslips with washing buffer (2 minutes) and are reported in the upper panels. Thrombus formation on the coverslips was evaluated by measuring the covered area in 10 different and randomly taken microscopic fields and results are reported in the histogram in the lower panel (black bars) as the mean ± SD of four different experiments. Coverslips were then further perfused with washing buffer for 10 minutes, and additional images were taken to evaluate thrombus stability. The remaining area covered by thrombi upon extensive washing is reported in the histogram in the lower panel (white bars) as mean ± SD of four different experiments.
Figure 2

A (i)

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P-Akt(Ser473)
tubulin

ASA
apyrase
MRS + AR-C
RGDS

- + - +
- - - -
- - + +
- - - -
- + - + +

(ii)

Akt phosphorylation %

none ASA ASA-apyrase MRS-AR-C RGDS RGDS-apyrase RGDS-MRS-AR-C

B

<table>
<thead>
<tr>
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P-Akt(Ser473)
tubulin

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P-Akt(Ser473)
tubulin

WT PLC/2 KO
WT PLC/2 KO
WT PLC/2 KO
Figure 3

A (i) monomeric collagen

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A (ii) GFOGER peptide

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WT  PI-3K<sup>+/+</sup>  WT  PI-3K<sup>+/+</sup>  WT  PI-3K<sup>+/+</sup>

pleckstrin

C

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none  BAPTA-AM  NA  none  2-APB

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WT  PLC<sup>2</sup> KO  WT  PLC<sup>2</sup> KO

tubulin
Figure 4

(i)

(ii)

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**WT**

**Pyk2 KO**

**P-Akt(Ser473)**

**tubulin**

AKT phosphorylation, %

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* Indicates significant difference.
Figure 5

A  (i)  WT
     (ii)  Adhesion
     (iii)  Spreading
PI-3KβKD

B  (i)  Adhesion
     (ii)  Spreading

C  (i)  Adhesion
     (ii)  Spreading
Figure 6

A (i) kDa

29- Rap1b-GTP
29- Rap1b TOT

none wortmannin TGX-221 AS-252424

(ii) Rap1b-GTP, %

None * *

Wortmannin TGX-221 AS-252424

B (i) kDa

29- Rap1b-GTP
29- Rap1b TOT

WT PyK2-KO

(ii) Rap1b-GTP, %

WT *

PyK2-KO

C (i)

(ii)

fäbringen binding, %

none wortmannin TGK-221

WT PyK2-KO

***

*
Figure 7

WT     Pyk2 KO    PI-3KβKD

Covered area, %

WT     Pyk2 KO    PI-3KβKD
Role and regulation of phosphatidylinositol 3-kinase \( \beta \) in platelet integrin \( \alpha 2\beta 1 \) signaling

Alessandra Consonni, Lina Cipolla, Gianni Guidetti, Ilaria Canobbio, Elisa Ciraolo, Emilio Hirsch, Marco Falasca, Mitsuhiro Okigaki, Cesare Balduini and Mauro Torti