Deletion of the p110β isoform of phosphoinositide 3-kinase in platelets reveals its central role in Akt activation and thrombus formation in vitro and in vivo

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Abstract

During platelet activation, phosphoinositide 3-kinases (PI3Ks) produce lipid second messengers participating in the regulation of functional responses. Here, we generated a megakaryocyte-restricted p110β null mouse model and demonstrated a critical role of PI3Kβ in platelet activation via an immunoreceptor tyrosine-based activation motif (ITAM), the GPVI-FcRγ complex, and its contribution in response to G-protein-coupled receptors (GPCR). Interestingly, the production of phosphatidylinositol 3,4,5-trisphosphate and the activation of PKB/Akt were strongly inhibited in p110β null platelets stimulated either via ITAM or GPCR. Functional studies showed an important delay in fibrin clot retraction and an almost complete inability of these platelets to adhere onto fibrinogen under flow condition suggesting that PI3Kβ is also acting downstream of αIIbβ3. In vivo studies showed that these mice have a normal bleeding time and are not protected from acute pulmonary thromboembolism but are resistant to thrombosis after FeCl₃ injury of the carotid suggesting that PI3Kβ is a potential target for antithrombotic drugs.
INTRODUCTION

Platelet activation is a highly regulated process involving various signalling pathways initiated by specific receptors coupled to heterotrimeric G proteins (GPCRs), integrins or immunoreceptor tyrosine-based activation motif (ITAM)-containing proteins. In all cases, key signalling enzymes such as phospholipases C (PLC) and phosphoinositide 3-kinase (PI3K) isoforms are activated. If the situation is clear for PLC (i.e. the PLC\(\beta\) isoforms are activated by heterotrimeric Gq proteins whereas the \(\gamma\) isoforms are stimulated via tyrosine phosphorylation and ITAM signalling), the implication of the different PI3K isoforms downstream of the major platelet receptors is still poorly known. Class Ia PI3Ks (\(\alpha,\beta,\delta\)), composed of a catalytic subunit (p110) and a regulatory subunit, are classically activated by their association with phosphotyrosine residues containing sequences via the SH2 domains of their regulatory subunit. \(^1\) However, the p110\(\beta\) isoform may not follow this rule since its activation has been proposed to involve both G\(\beta\gamma\) and phosphotyrosyl peptides. \(^2,^3,^4,^5\) Using a selective inhibitor of p110\(\beta\), Jackson et al. \(^6\) have proposed a role of p110\(\beta\) in the regulation of \(\alpha_{\text{IIb}}\beta_3\) integrin in a shear-dependent manner. Interestingly, this inhibitor prevented the formation of an occlusive thrombus generated \textit{in vivo}. To firmly establish the role of the PI3K\(\beta\) in platelet activation and evaluate its impact on haemostasis \textit{in vivo}, we created a mouse line in which this isoform has been inactivated by gene targeting selectively in the megakaryocyte lineage.
MATERIALS AND METHODS

Materials. Collagen was from Nycomed, U46619 from QBiogen Inc; integrilin from Glaxo Group Ltd; p110α, β, γ and δ antibodies from Santa Cruz Biotechnology; p85 antibody from Upstate; TGX-221 from Cayman Chemical and other reagents from Sigma-Aldrich.

Animals. Generation of the mice model is described in the supplemental methods. All mice experiments were approved by the IFR150-Toulouse Purpan institutional review board.

Preparation of murine platelets. Whole blood was drawn from the inferior vena cava of anesthetized mice into a syringe containing acid citrate dextrose (1 vol anticoagulant / 9 vol blood). Platelets were then prepared as previously described 7 and stimulated in the presence or absence of integrilin (40µg/ml). Aggregation was assessed using a Chrono-log dual channel aggregometer under stirring at 900 rev/minutes.

In vitro PI3K assay. Samples were immunoprecipitated as previously described, 8 and kinase activity assessed as previously described.9

Lipid extraction and analysis. Platelets were labelled with 0.4 mCi/ml [32P]orthophosphate, stimulated and their Phosphoinositides content was analysed as described previously.10

Clot retraction experiments. The platelet rich plasma (PRP) was obtained from pooled blood samples from several mice by centrifugation for 4 minutes at 250 g at 37°C. Clot retraction studies were performed as described.11

Platelets interaction on immobilized fibrinogen under flow conditions. This technique is described in the supplemental methods.
**Ferric chloride carotid artery injury model of thrombosis.** This technique is described in the supplemental methods.

**Statistical Analysis.** Statistical significance was analysed using unpaired Student’s test using Microsoft Excel 2007.
RESULTS AND DISCUSSION

Conditional genetic inactivation of p110β in the megakaryocytic lineage

To determine the stability in platelets of p110β protein which lacks the coding sequence of exons 21 and 22 of the kinase domain after Cre excision (p110Δ21,22 protein), PF4-Cre/p110βfloxflox and p110βfloxflox platelet lysates were analyzed by western blotting. A severe p110β deficiency was found in PF4-Cre/p110βfloxflox platelets (12.5 ± 6% of control) (Figure 1A) indicating that the protein is either not produced or unstable. As expected, this deficiency is restricted to platelets (Supplementary Figure 1) and has no impact on the expression of other class I PI3K catalytic subunits (p110α, p110γ and p110δ). A significant reduction (56 ± 7%) in the level of the regulatory subunit p85, probably due to an instability of the free p85 protein, was observed (Figure 1A). However, the PI3K activity associated to p85 was not significantly different (Figure 1B) indicating an efficient association with p110α and δ. Only a residual lipid-kinase activity was detected in p110β immunoprecipitates whereas the other Class I PI3K catalytic subunits were highly active (not shown). Thus, this mouse model is a conditional specific deletion of p110β in platelets.

PI3Kβ is critical for phosphatidylinositol(3,4,5)-trisphosphate (PIP3) production and PKB/Akt activation downstream of both GPCR and ITAM signalling

We then tested the impact of PI3Kβ on the production of PIP3 in response to agonists binding either GPCR, such as thrombin receptor, or receptors bearing ITAM motif, such as GPVI-Fc receptor γ-chain (FcRγ) complex. Collagen has two receptors, GPVI and α2β1 integrin. The GPVI-FcRγ complex, specifically stimulated by the snake venom toxin convulxin, activates cell signalling via tyrosine kinases. Surprisingly, the PIP3 production was inhibited in platelets stimulated either by thrombin or by convulxin showing that both GPCR and ITAM-bearing receptor signalling involve p110β to produce this lipid second messenger (Figure 1C). Accordingly, upon stimulation with thrombin, thromboxane A2 analogue (U46619), convulxin or
collagen, p110β-null platelets showed an almost complete inhibition of PKB/Akt phosphorylation compared to control platelets. This defect of activation of this PI3K effector persisted even with high doses of agonists (90 % ± 4; 83% ±11; 92.4 ± 6; 88% ± 2.5, respectively) (Figure 1D) and was not due to a shift in the time course of activation or a defect in aggregation (Figure 1E). Consistent with this, the selective PI3Kβ inhibitor, TGX-221, strongly inhibited PKB/Akt-phosphorylation in wild-type platelets stimulated with the different agonists (Figure 1F) showing that the catalytic function of p110β is responsible for PKB/Akt activation. These findings reveal a crucial involvement for PI3Kβ in PKB/Akt activation in response to the major physiological platelet agonists and that PI3Kγ, PI3Kα or PI3Kδ, which are present and catalytically active in these platelets, are unable to take over this function of p110β. The PI3K isoform activated by GPVI is mainly p110β since stimulation of both PKB/Akt and PLCγ (as shown by the decrease in phosphatidic acid production, Figure 1G) was nearly abolished in p110β-null platelets challenged by convulxin.

_Deletion of p110β affects functional platelet responses_

In contrast to control platelets, p110β-null platelets showed a slight defect in aggregation response to low concentration of thrombin and U46619. This defect was stronger in response to collagen (Figure 2A) and ADP (Supplementary Figure 2B). Raising agonist concentrations increased aggregation but did not restore a normal response. Remarkably, p110β-null platelets were unable to aggregate upon GPVI triggering by convulxin. Similar results were obtained with TGX-221-treated platelets (Supplementary Figure 2A). These data suggest that platelet aggregation in response to GPCR agonists may be partially independent of PI3Kβ-mediated Akt activation while PI3Kβ is mandatory for ITAM-mediated platelet activation. It is important to note that PI3Kβ is activated upstream of αIIbβ3-integrin engagement both in response to GPVI or GPCR triggering, since blockage of αIIbβ3 by integrilin did not affect PIP3 production (Figure
1C) and Akt phosphorylation (not shown). However, evidence is accumulating that PI3Kβ is also acting downstream of αIIbβ3. 20, 21

In agreement, we observed an important delay in fibrin clot retraction induced by p110β-null platelets suggesting a role of this PI3K in organizing an efficient αIIbβ3 mediated contractility (Figure 2B). To better investigate the role of PI3Kβ in integrin αIIbβ3-dependent platelet adhesion, we performed flow-based adhesion assays over a fibrinogen matrix under arterial flow conditions using whole blood. Integrin αIIbβ3 outside-in signals stabilize and sustain αIIbβ3 adhesive bonds necessary for the maintenance of firm adhesion contacts under shear conditions.19,20 Our results show a critical role for PI3Kβ in the regulation of this process (Figure 2C). A role of PIP3 has been suggested in platelet adhesion to immobilized fibrinogen under flow. 20 Here, we propose that the major PI3K involved in PIP3 production downstream of αIIbβ3 is the β isoform of Class IA.

Consistant with our *ex vivo* data, during the reviewing of this study, a report highlighted the important role of PI3Kβ in mouse platelet regulation.21

Interestingly, our model of megakaryocyte/platelet-restricted p110β deficiency allowed to investigate the role of this PI3K *in vivo*. Deletion of p110β did not significantly modify the bleeding time (2.6 ± 0.43 min for Cre- versus 3.40 ± 0.59 min for PF4-Cre+) (supplementary Figure 3) and did not protect against acute thromboembolism induced by injection of a mixture of collagen and epinephrine (100% of mortality at 2 min for both Cre- and PF4-Cre+ upon 0.3mg/kg collagen and 60µg/kg epinephrine). To investigate the relevance of PI3Kβ in pathologic occlusive thrombus formation *in vivo*, FeCl3 injury was induced on carotid artery and time to occlusion and blood flow were determined. Animal in which blood flow stopped, but then resumed, were scored as having an unstable thrombus. Whereas 100% of control mice presented a complete occlusion of the vessel 9 ± 1.1 minutes after injury (mean occlusion time), 90% of megakaryocyte/platelets -p110β null mice partially occluded (17.95 ± 4.86 % occlusion 30 minutes after injury) and among them, 30% presented an unstable thrombus. Only 10% of
megakaryocyte/platelets -p110β null mice presented a complete occlusion (Figure 2D). These data suggest that PI3Kβ is critical for thrombus formation after arterial injury. This result is consistent with those from Jackson et al. showing that inhibition of p110β by TGX-221 abolishes arterial occlusive thrombus formation in a modified ‘Folts-type’ thrombosis model in rat.  

Altogether these data suggest that PI3Kβ is a potentially interesting new target for antithrombotic drugs.
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AUTHORSHIP CONTRIBUTION STATEMENT

V.M. and G.C. have designed and performed most experiments and analysed data; J.G-G., B. V., M-P.G. and V.M. have produced the mice; C.C. performed carotid artery thrombosis; M. J-P. has purified Cvx; M.P., B.P. and M-P.G. designed research, supervised the work, analyzed data and wrote the paper.

CONFLICT OF INTEREST DISCLOSURE

B.V. is an advisor to Intellikine, San Diego. The other authors have no conflict of interest to disclose.
REFERENCES


FIGURE LEGENDS

Figure 1

*Genetic inactivation of p110β catalytic subunit specifically in megakaryocytes and platelets.* (A) Western blot showing class I PI3K p110 isoforms and p85 expression in PF4-Cre-negative p110\(^{\text{flox/flox}}\) control (Cre-) or PF4-Cre-positive p110\(^{\text{flox/flox}}\) p110β-null (PF4-Cre+) platelet lysates. Solid bars, (Cre-) platelet lysates; open bars, (PF4-Cre+) platelet lysates. Average results are mean ± SEM for 4 experiments (right panel) (B) p110β and p85 subunits were immunoprecipitated with specific antibodies, and the associated lipid kinase activity was assayed with phosphatidylinositol as a substrate. Solid bars, (Cre-) platelet lysates; open bars, (PF4-Cre+) platelet lysates. Average results are mean ± SEM for 3 experiments (C) Platelets from control or PF4-Cre/p110β\(^{\text{flox/flox}}\) mice were stimulated under non aggregating conditions by indicated agonists and the radioactivity of PIP3 was determined as described under "Experimental Procedures." Results are mean ± SEM of 3 independent experiments. (D) Platelets were stimulated under aggregating conditions by thrombin, U46619, collagen or convulxin during 7 min at the indicated concentration. Lysates were submitted to immunoblotting with anti-Akt-Ser\((P)^{473}\) or total Akt antibodies, as indicated. Quantification by densitometric analysis of the western blots is shown (right panels) and data are expressed as % of P-Akt in response to thrombin (0.5 IU/ml), U46619 (1 µM), collagen (10 µg/ml), convulxin (10 nM), and are mean ± SEM of 3 independent experiments. (E) Platelets were stimulated under aggregating or non aggregating (integrilin) conditions by thrombin (0.5 IU/ml) during different times and analysed as in D. (F) Platelets were stimulated under aggregating conditions by thrombin, U46619, collagen or convulxin in absence or in presence of TGX-221 (0.5 µM) and analysed as in D. (G) Platelets from p110\(^{\text{flox/flox}}\) control (Cre-) or PF4-Cre/p110β\(^{\text{flox/flox}}\) (PF4-Cre+) mice were stimulated under non aggregating conditions with indicated agonists and the radioactivity of phosphatidic acid (PtdOH) was determined as described under "Experimental Procedures." Results are mean ± SEM of 3 independent experiments. Statistical analysis: (*, p<0.05; **, p<0.01; ***, p<0.005)
Figure 2

(A) Role of PI3Kβ in promoting platelet aggregation. Platelets from p110\textsuperscript{floxfloxflox} control (Cre-) or \textit{PF4-Cre/p110\textsuperscript{floxfloxflox}} (\textit{PF4-Cre+}) mice were stimulated with thrombin, U46619, collagen or convulxin and aggregation was assessed using a Chrono-log dual channel aggregometer under stirring at 900 rev/minutes for 7 minutes. The profiles shown are representative of five independent experiments. Quantifications of the maximum of aggregation at 6 min are shown and are mean ± SEM of 5 independent experiments (right panels). 

(B) Role of PI3Kβ in promoting fibrin clot retraction. Photographs show the extent of clot retraction in wild-type and p110β-null PRP samples treated with 10 IU/ml thrombin. Quantification of the volume of serum extruded from the clot is shown. Results are the mean ± SEM of 3 independent experiments.

(C) DIOC\textsubscript{6}-labeled platelets in whole blood from p110\textsuperscript{floxfloxflox} control (Cre-) or \textit{PF4-Cre/p110\textsuperscript{floxfloxflox}} (\textit{PF4-Cre+}) mice were perfused through a fibrinogen-coated Bioflux plates at a shear rate of 1500 seconds for 5 minutes. Representative images at 5 minutes are shown. Area covered by platelets was measured. Results shown are the mean ± SEM of 5 experiments. Statistical analysis: (*, p<0.05; **, p<0.01; ***, p<0.005).

(D) Thrombotic response of mice to ferric chloride injury of the carotid artery. Flow rates were measured in the carotid artery following exposure to 7% FeCl\textsubscript{3} during 3 minutes. The experiment was stopped after 30 minutes. (a) For each genotype the number of mice forming a stable occlusion are shown in black. The number of mice that formed an unstable occlusion that resolved is shown in gray. The number of mice that formed only a partial occlusion is shown in white. (b) Representative flow traces for each cases (stable occlusion, no occlusion and unstable occlusion).
A

B

C

Martin et al., Figure 1A-B-C
Figure 1G

Graphs showing the fold increase in PtdOH under different conditions for Cre- and PF4-Cre+ cells.

- Left panel: 
  - Resting: Cre- vs. PF4-Cre+.
  - Thrombin 0.5 U/ml: Cre- vs. PF4-Cre+.

- Right panel: 
  - Resting: Cre- vs. PF4-Cre+.
  - Convulxin 10 nM: Cre- vs. PF4-Cre+.
A

**Cre -**

- **Thrombin**
  - 0.5 IU/mL
  - 0.3 IU/mL
  - 0.2 IU/mL

- **U46619**
  - 1 μM
  - 0.5 μM
  - 0.25 μM

- **Collagen**
  - 10 μg/mL
  - 5 μg/mL
  - 2.5 μg/mL

- **Convupxin**
  - 15 nM
  - 10 nM

**PF4-Cre +**

- **Thrombin**
  - 0.5 IU/mL
  - 0.3 IU/mL
  - 0.2 IU/mL

- **U46619**
  - 1 μM
  - 0.5 μM
  - 0.25 μM

- **Collagen**
  - 10 μg/mL
  - 5 μg/mL
  - 2.5 μg/mL

- **Convupxin**
  - 15 nM
  - 10 nM

**Percent of maximal aggregation**

- **Thrombin (IU/mL)**
  - 0.5
  - 0.3
  - 0.2

- **U46619 (μM)**
  - 1
  - 0.5
  - 0.25

- **Collagen (μg/mL)**
  - 10
  - 5
  - 2.5

- **Convupxin (nM)**
  - 15
  - 10

*By Martin et al., Figure 2A*
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