Loss of PIP5KIβ demonstrates that PIP5KI isoform-specific PIP_2 synthesis is required for IP_3 formation

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The three isoforms of PIP5KI (α, β, and γ) synthesize $PI4,5P_2$ (PIP_2) by phosphorylating PI4P. Therefore, it is not clear why platelets, like all eukaryotic cells, have more than one isoform. To test the hypothesis that PIP5KI isoforms have nonoverlapping functions, we generated a murine line containing a null mutation of PIP5KIβ and analyzed the effect on platelet signaling. PIP5KIβ-null mice had normal platelet counts. In contrast to platelets lacking PIP5KIα, platelets lacking PIP5KIβ exhibited impaired aggregation accompanied by disaggregation. Although platelets lacking PIP5KIβ had only a moderate deficiency of PIP_2 under basal conditions, they had a striking deficiency in PIP_2 synthesis and IP_3 formation after thrombin stimulation. We have also observed that platelets lacking both PIP5KIα and PIP5KIβ have a complete loss of thrombin-induced IP_3 synthesis even though they still contain PIP5KIγ, the predominant PIP5KI isoform in platelets. These results demonstrate that PIP5KIβ, like PIP5KIα, contributes to the rapid synthesis of a pool of PIP_2 that is required for second-messenger formation, whereas the pool of PIP_2 synthesized by PIP5KIγ does not contribute to this process. Additionally, we found that PIP5KIβ-null platelets failed to form arterial thrombi properly *in vivo*. Together, these data demonstrate that PIP5KIβ is required for rapid PIP_2 synthesis, second-messenger production, and stable platelet adhesion under shear *in vivo*. These results also demonstrate that after stimulation of a G protein-coupled receptor, IP_3 is completely derived from a rapidly synthesized discrete pool of PIP_2 synthesized by PIP5KIα and PIP5KIβ.

phosphatidylinositol 4,5-bisphosphate | second messenger | platelet | thrombosis | inositol triphosphate

Phosphatidylinositol 4,5-bisphosphate, also known as PIP_2, plays an important role in several cellular events, including focal adhesion formation and actin reorganization. PIP_2 contributes to these events by two distinct signaling pathways. First, PIP_2 is a substrate of enzymes such as phospholipase C or PI3-kinase, which form second messengers such as IP_3, diacylglycerol (DAG), and phosphatidylinositol 3,4,5-trisphosphate (PIP_3). These second messengers then activate a variety of signaling pathways that affect actin dynamics. Additionally, PIP_2 can directly associate with and thereby regulate the activity of several actin-binding proteins, including talin, vinculin, and filamin (1, 2). Therefore, PIP_2 is a major regulator of cytoskeletal dynamics in all eukaryotic cells.

PIP_2 is synthesized by either the phosphorylation of phosphatidylinositol 4-phosphate (PI4P) by the lipid kinase phosphatidylinositol 4-phosphate 5-kinase type I (PIP5KI) or by the phosphorylation of PI5P by phosphatidylinositol 5-phosphate 4-kinase type II (PIP5KII or PIP4K). Radiolabeled phosphate pulse–chase experiments that analyzed the relative labeling rate of the D-4 and D-5 positions of the inositol ring suggest that PIP5KI-mediated phosphorylation of PI4P is the predominant pathway for PIP_2 synthesis (3, 4). Independent murine genes encode the three isoforms (α, β, and γ) of PIP5KI.** These isoenzymes are all capable of phosphorylating the same substrate, PI4P, at the 5-position of the inositol ring to generate the same product, PIP_2 (5–7). In addition, all three isoforms can be stimulated by small GTPases (Rho, Rac, Cdc42, and Arf6) and by phosphatidic acid. Despite this apparent biochemical redundancy, curiously, all cells contain more than one isoform. Notably, the three isoforms of PIP5KI have significantly dissimilar primary structures and different expression levels in different tissues, and they appear to localize within different compartments within some cells (3, 8–12). For example, PIP5KIα localizes in membrane ruffles (8), whereas PIP5KIβ localizes near endosomes (9), and PIP5KIγ is targeted to focal adhesions and nerve terminals (10–12).

Work by Majerus *et al*. (13) demonstrated that thrombin stimulation induces PIP_2 synthesis in platelets. Grondin *et al.* (14) subsequently linked the increase in PIP_2 synthesis after thrombin stimulation of platelets to the increase in PIP5KI activity at the cytoskeleton. PIP5KIα was also shown to alter its localization to the cell membrane upon stimulation of the thrombin receptor, PAR1 in COS-7 cells and in platelets (15, 16). Studies by Hartwig *et al*. (17) and Carpenter and coworkers (18) demonstrated that PIP5KIα stimulation led to actin filament assembly in response to thrombin and Rac1 in permeabilized platelets. In contrast, Gratacap *et al.* (19) showed that stimulation of the thromboxane A_2 stimulated PIP5K by the sequential activation of Rho and Rho kinase. The critical role of Rho and its effector Rho kinase on PIP5KI activation in intact human platelets was subsequently confirmed (16). Together, these results show that in platelets, Rho and its effector Rho kinase are the predominant regulators of platelet PIP5KI and PIP_2 synthesis.

That all cells possess more than one isoform of PIP5KI suggests that these isoforms do not have completely identical functions. Supporting this hypothesis, we have found that megakaryocytes lacking PIP5KIγ fail to anchor their cell membrane to the underlying cytoskeleton (20). In addition, Sasaki *et al*. (21) have shown that a murine line lacking PIP5KIα has a selective signaling defect in mast cells. These murine models suggest that individual PIP5KI isoforms are fulfilling specific niches within discrete domains of the

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**It should be noted that the nomenclatures used for the murine and human isoforms are not consistent. Murine PIP5KIβ is the ortholog of human PIP5KIα, and murine PIP5KIα is the ortholog of human PIP5KIβ. In this manuscript, only the murine nomenclature is used.

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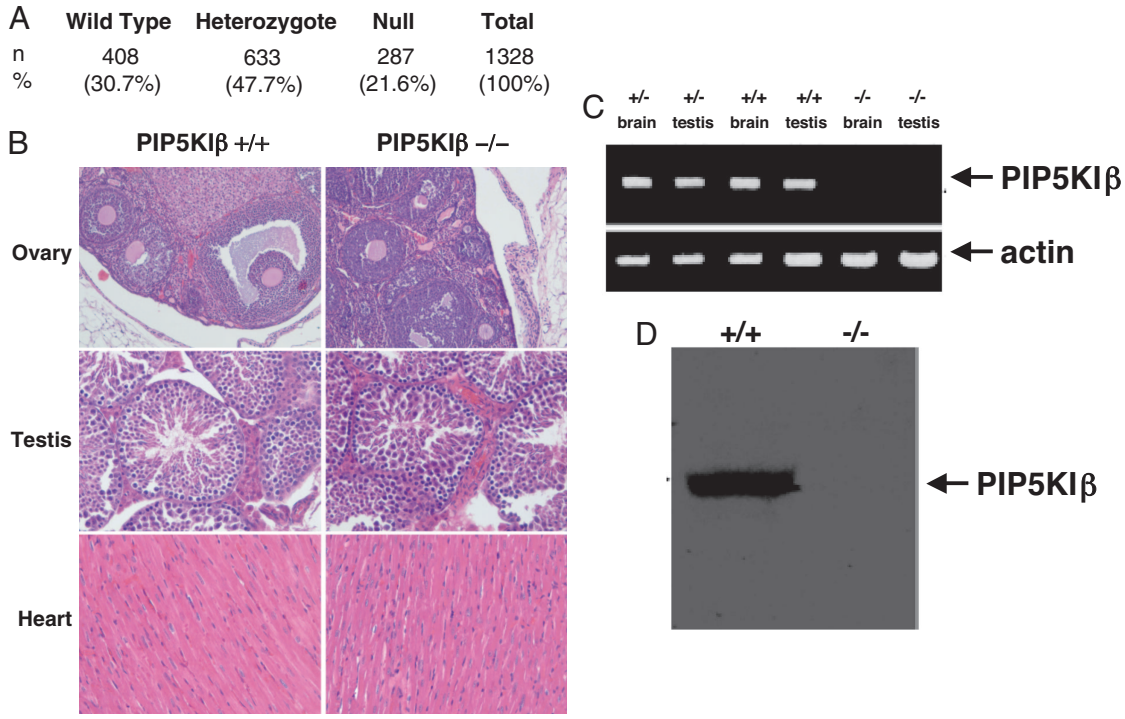


Fig. 1. Targeting of PIP5KIβ produces complete null muation. (*A*) Genotypes of offspring derived from matings of PIP5KI$\beta^{+/-}$ mice. Compared with the number of wild-type embryos generated, the PIP5KI$\beta^{-/-}$ mice were born at slightly less than the expected frequency. (*B*) Micrographs demonstrating the normal morphology of organs lacking PIP5KIβ. H&E stain was used on all organs. (Magnification: ovary, ×100; testis, ×200; heart, ×200.) (*C*) RT-PCR using a sense primer from exon 1 and an antisense primer from exon 3. The absence of the PCR product demonstrates the loss of PIP5KIβ message beyond the gene trap. In contrast, using primers specific for β-actin demonstrated that targeting of PIP5KIβ had no effect on expression of an unrelated mRNA. (*D*) Anti-PIP5KIβ immunoblot shows complete loss of protein derived from PIP5KI$\beta^{-/-}$ murine muscle.

cell. Of the three PIP5KI isoforms, PIP5KIα and PIP5KIβ have the most homologous primary sequence. This finding may imply that these two isoforms have functional redundancy, leaving PIP5KIγ to fulfill other specialized functions.

Platelets, like other hematopoietic cells, undergo rapid bursts of both PIP_2 synthesis and actin cytoskeletal reorganization after agonist stimulation (16, 18). We have shown that platelets contain PIP5KIβ and PIP5KIγ but very little PIP5KIα (16). To begin to understand the unique contribution of PIP5KIβ to cell biology and development, we generated a murine line lacking this isoform of PIP5KI and studied the effect of this mutation on platelet biology. Loss of PIP5KIβ induced biochemical and cellular defects within platelets, demonstrating its unique function within these cells.

Results

Loss of PIP5KIβ Leads to Partial Intrauterine Loss. To elucidate the functions of PIP5KIβ, we used an ES cell line that contained a β-geo gene trap within the first intron of the PIP5KIβ gene [supporting information (SI) Fig. S1*A*] (22). The gene trap strategy used to create this ES cell line was designed to create an abnormal mRNA transcript from the trapped allele that would produce a fusion protein corresponding to the first 28 aa of PIP5KIβ fused to β-galactosidase. This ES cell line was used to create chimeric founders that gave rise to germ-line heterozygotes harboring the targeted PIP5KIβ-null mutation.

Heterozygous PIP5KI$\beta^{+/-}$ mice were intercrossed, and viable PIP5KIβ mice were identified. Genotyping offspring of PIP5KI$\beta^{+/-}$ crossings demonstrated that both PIP5KI$\beta^{-/-}$ mice and PIP5KI$\beta^{+/-}$ mice were born at slightly less than the anticipated frequency (Fig. 1*A*). Compared with the number of PIP5KI$\beta^{+/+}$ mice produced from these pairings, slightly 30% fewer PIP5KI$\beta^{-/-}$ mice and 22% fewer PIP5KI$\beta^{+/-}$ mice were born than anticipated by unbiased Mendelian Genetics. Mice lacking PIP5KIβ who survived to birth appeared normal and survived to adulthood. However, both PIP5KI$\beta^{+/-}$ and PIP5KI$\beta^{-/-}$ mice bred poorly and produced few offspring. This finding demonstrates that loss of PIP5KIβ impairs conception and leads to mild fetal loss. Histologic examination demonstrated that the PIP5KIβ-null mutation had no influence on the morphologic appearance of the gonads or the heart (Fig. 1*B*). The gross development of the brain, thymus, lung, liver, stomach, kidney, skeletal muscle, spleen, uterus, and bone marrow was also normal in mice lacking PIP5KIβ (data not shown). PIP5KIβ-null mice had complete blood counts, including platelet counts that were similar to their wild-type littermates. Furthermore, PIP5KIβ-null mice exhibited no spontaneous hemorrhage.

Sequence analysis of genomic DNA from targeted mice confirmed that the site of gene trap insertion was 5′ to exons 2–14, including the entire kinase domain (data not shown). Therefore, the predicted fusion protein lacks all phosphoinositol kinase activity. We confirmed that the gene trap insertion resulted in loss of wild-type PIP5KIβ expression by RT-PCR and by anti-PIP5KIβ immunoblotting (Fig. 1 *C* and *D*). These findings show that although a complete null mutation within the PIP5KIβ gene leads to partial embryonic lethality, some PIP5KIβ-null mice can survive to adulthood without this enzyme.

Platelets Lacking PIP5KIβ Exhibit Disaggregation. Because second messengers generated from PIP_2 by PLC and PI3K contribute to the signaling responsible for platelet aggregation (23–27), we analyzed whether platelets with defective PIP_2 production exhibited an *ex vivo* aggregation defect. We found small decreases in the extent of aggregation of platelets from PIP5KIβ-null mice that were stimulated by the PAR4 thrombin receptor agonist peptide (AYPGQV), the thromboxane A_2 receptor agonist (U46619), or ADP (Fig. 2). The impaired aggregation was most apparent at lower agonist concentrations and became less apparent as the concen-

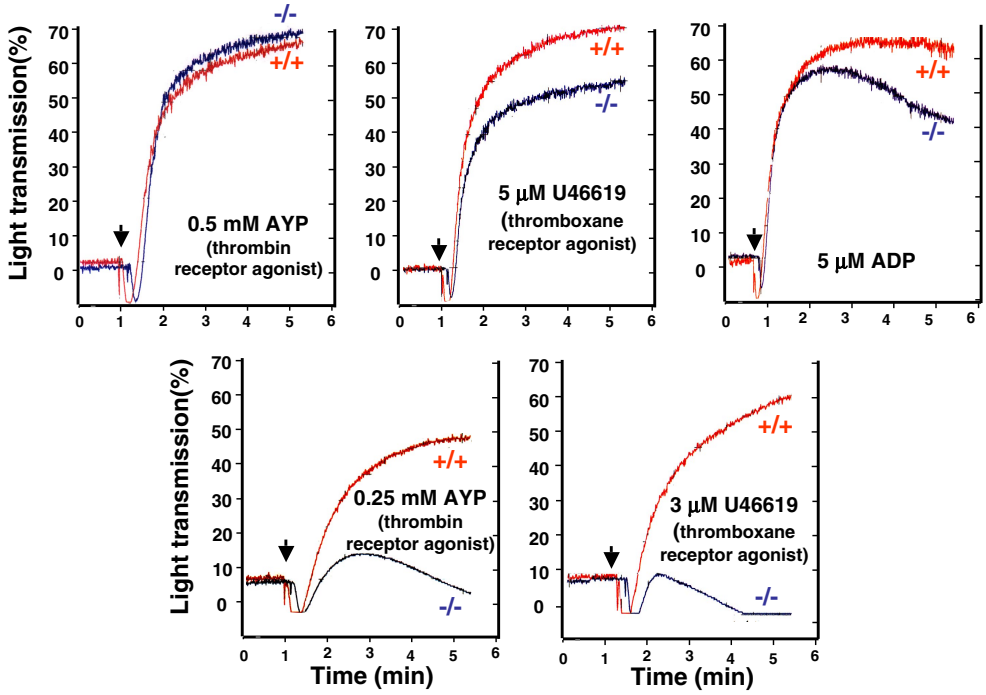


Fig. 2. Platelets lacking PIP5KIβ have an aggregation defect. Murine platelets lacking PIP5KIβ were analyzed after agonist stimulation in a Lumi-Dual aggregometer. Compared with platelets derived from wild-type littermates, platelets derived from PIP5KIβ-knockout mice have a defect in aggregation in response to low doses of all analyzed agonists and exhibit disaggregation. Higher doses of agonists are able to overcome this defect. Results are representative of six experiments.

tration of agonist was increased. When the aggregation tracings were observed over time, disaggregation was notable particularly when submaximal doses of agonists were used as the stimulant. These data show that PIP5KIβ-derived second messengers are required for maximal and stable platelet aggregation.

It is possible that PIP_2 synthesis by other PIP5KI isoforms compensates for the loss of PIP5KIβ in platelets. Quantitative PCR demonstrates that the expression of neither PIP5KIα nor PIP5KIγ mRNA is up-regulated in PIP5KIβ-null cells. We and others have recently reported the phenotype of a murine line lacking PIP5KIγ (28, 29). However, the early lethality caused by the lack of this PIP5KI isoform precludes studies of platelet aggregation. Mice lacking PIP5KIα have also been recently described, and these mice are viable (21). Even though platelets contain very little PIP5KIα, we analyzed whether PIP5KIα contributes to platelet aggregation. In cells lacking PIP5KIα, quantitative PCR indicates that there is no up-regulation of the mRNA of either PIP5KIβ or PIP5KIγ. As shown in Fig. S2, platelets lacking PIP5KIα had no defect in platelet aggregation at all analyzed concentrations of agonists. This finding confirms that the PIP5KIβ isoform but not the PIP5KIα isoform is required for *ex vivo* platelet activation.

Contribution of PIP5KIβ to the Synthesis of PIP_2. After thrombin stimulation, PIP5KI synthesizes PIP_2, and this product can be hydrolyzed by phospholipase C to generate second messengers such as IP_3. Therefore, we analyzed PIP_2 concentrations in platelets lacking PIP5KIβ-knockout platelets. Platelets were loaded with radioactive orthophosphate, and cells were stimulated, lysed, and fractionated by thin-layer chromatography as demonstrated by a typical experiment shown in Fig. 3*A*.

Results from pooled analysis of six experiments are shown in Fig. 3*B*. As reported, PIP_2 levels normally decline within seconds after agonist stimulation, presumably because PIP_2 is consumed during the formation of second messengers. However, the concentration of PIP_2 is rapidly restored within 1 min by PIP5KI-mediated phosphorylation of PI4P. Platelets lacking PIP5KIβ had only moderately decreased concentrations of PIP_2 before agonist stimulation. In contrast, after agonist stimulation, PIP5KIβ-null platelets had marked impaired PIP_2 synthesis. This impaired synthesis resulted in significantly decreased concentrations of PIP_2 within 45 s after exposure of the platelets to agonists, although there was enough synthesis to bring the concentration of PIP_2 up to basal levels. It is notable that the concentration of the PIP_2 precursor, PI4P, in the PIP5KIβ-null platelets was not significantly different from the concentration found in wild-type platelets. These data show that PIP5KIβ is required for the rapid synthesis of PIP_2 in platelets after stimulation of G protein-coupled receptors.

Both PIP5KIα and PIP5KIβ, but Not PIP5KIγ, Contribute to the Production of IP_3. After thrombin stimulation, PIP_2 synthesized by PIP5KI can be hydrolyzed by phospholipase C to generate DAG and IP_3. To test the hypothesis that the rapid production of PIP_2 by PIP5KI is required for production of IP_3, we analyzed the concentration of IP_3 in cells lacking specific PIP5KI isoforms. We observed that after thrombin stimulation of wild-type control platelets, the concentration of IP_3 had a predicted rapid rise followed by a quick decline toward baseline (Fig. 4). Platelets lacking PIP5KIα exhibited no overt defect in IP_3 formation after thrombin stimulation (Fig. 4 *Top*).

Although platelets derived from PIP5KIβ-null littermates had normal basal IP_3 levels, they had an $\approx$50% blunted increase in IP_3 that was apparent even 5 s after agonist stimulation (Fig. 4 *Middle*). These data indicate that PIP5KIβ synthesis of PIP_2 contributes to immediate IP_3 formation and that PIP5KIα is not required for the formation of this second messenger within thrombin-stimulated platelets. The data also show that loss of PIP5KIβ does not completely eliminate the production of IP_3 after thrombin stimulation.

Because PIP5KIα and PIP5KIβ have a high degree of homology, we had expected that both of these isoforms would have been capable of rapidly synthesizing the pool of PIP_2 that is used for IP_3 formation. Yet our data shown in Fig. 4 *Top* and *Middle*

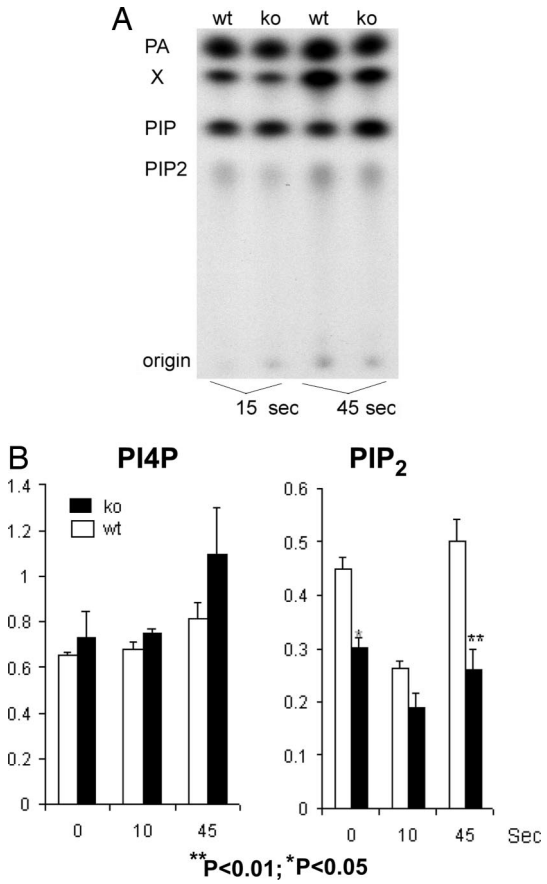


Fig. 3. Loss of PIP5KIβ induces a defect in polyphosphoinositide synthesis. Thin-layer chromatography of extracts of radiolabeled platelets derived from wild-type (wt) and PIP5KIβ-knockout (ko) mice is shown. (*A*) Results of a representative experiment show effects of thrombin stimulation. (*B*) Pooled analysis of six experiments show the effect of loss of PIP5KIβ mutation on PIP and PIP_2 concentrations after thrombin stimulation.

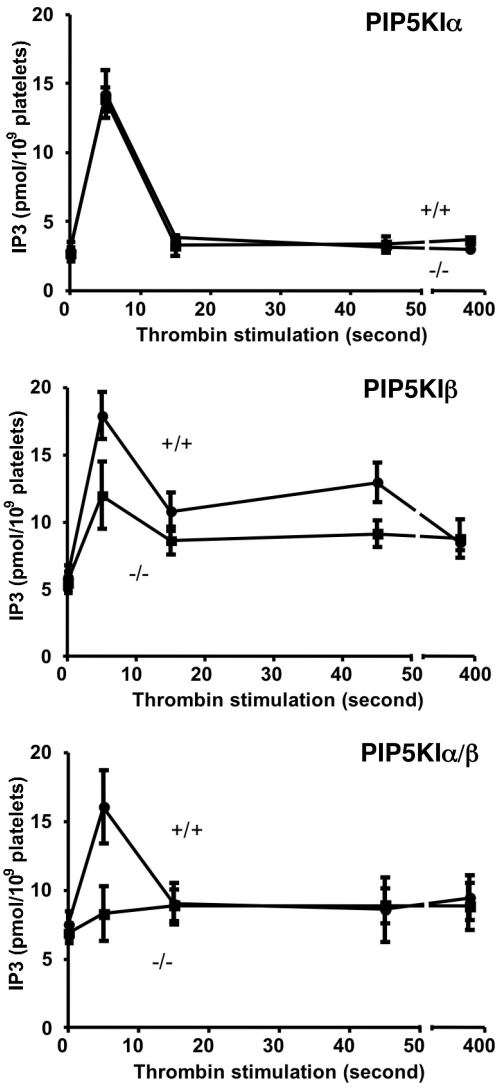
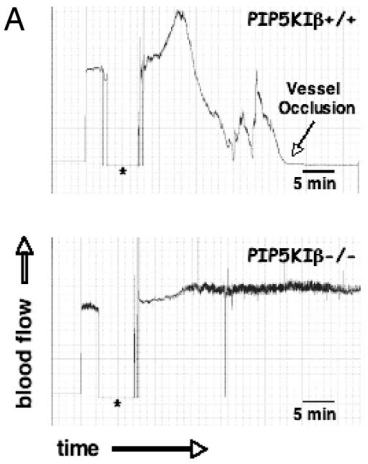


Fig. 4. PIP5KIα and PIP5KIβ are required for IP_3 formation. The effect on IP_3 production of loss-of-function mutations within the PIP5KIα and PIP5KIβ genes was analyzed. After stimulation by thrombin, the concentration of 1,4,5-IP_3 was determined by using methods similar to those described by Rittenhouse and Sasson (37). Although loss of PIP5KIα had no effect on thrombin-induced IP_3 production, loss of PIP5KIβ impaired production of IP_3 even at the earliest analyzed time point. Loss of both PIP5KIα and PIP5KIβ completely ablated thrombin-stimulated IP_3 production in platelets.

indicate that loss of PIP5KIα has no apparent effect on IP_3 synthesis, whereas the loss of PIP5KIβ does impair IP_3 formation. To account for these observations, we hypothesized that because PIP5KIα is much less abundant in platelets than PIP5KIβ, under normal circumstances, PIP5KIα only makes a minor contribution to platelet second-messenger formation that is not detectable by our assay system. We further hypothesized that in the absence of PIP5KIβ, the contribution of PIP5KIα to platelet second-messenger formation would become apparent.

To test these hypotheses, we initiated a breeding program to generate mice lacking both PIP5KIα together with PIP5KIβ (PIP5KI$\alpha^{-/-}$ PIP5KI$\beta^{-/-}$ mice). Pairing mice that were each homozygous for the null mutation within PIP5KIα and heterozygous for PIP5KIβ (PIP5KI$\alpha^{-/-}$ PIP5KI$\beta^{+/-}$ mice) has up to this point produced very few litters. Of these offspring, only five mice lacked both isoforms. The platelets from four of these five mice lacking both PIP5KIα together with PIP5KIβ have been analyzed for IP_3 formation. The results of pooled analysis of these studies is shown in Fig. 4 *Bottom*. This analysis demonstrates that platelets lacking both PIP5KIα together with PIP5KIβ have a total absence of IP_3 formation after the stimulation of a G protein-coupled receptor. It is notable that by mRNA analysis, PIP5KIγ is the most abundant isoform of PIP5KI in platelets and megakaryocytes (www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc = GSM160043) (16). Therefore, the data shown in Fig. 4 *Bottom* show that platelets lacking both PIP5KIα together with PIP5KIβ have an absence of IP_3 formation even though they still have PIP5KIγ. This result demonstrates that PIP5KIγ does not synthesize a pool of PIP_2 that is used for second-messenger formation after stimulation of the thrombin receptor.

Loss of PIP5KIβ Leads to an *in Vivo* Thrombosis Defect. To determine whether loss of PIP_2 synthesis and IP_3 formation resulted in a defect *in vivo*, we tested the ability of these mice to form stable occlusions by using a chemical-induced carotid injury model (30, 31). Ferric chloride was applied to an exposed carotid artery of anesthetized mice, and the formation of thrombi was monitored by using a Doppler flow meter. A representative experiment is shown in Fig. 5*A*. The Doppler tracing of a wild-type mouse shown in the *Top* demonstrates a completely occlusive thrombus in response to the chemical irritant. In contrast, the PIP5KIβ-null littermate failed to form any vessel occlusion from this injury (Fig. 5*A Bottom*). The table shown in Fig. 5*B* shows that loss of PIP5KIβ significantly reduced thrombosis occurrence compared



B

Genotype	Thrombus	No thrombus
PIP5KIβ+/+	3 (75%)	1 (25%)
PIP5KIβ-/-	1 (20%)	4 (80%)

Fig. 5. Lack of PIP5KIβ reduces ferric chloride-induced thrombus formation in the mouse carotid artery. Carotid arteries of PIP5KI$\beta^{+/+}$ and PIP5KI$\beta^{-/-}$ mice were subjected to ferric chloride-induced injury, and blood flow was monitored as a measure of thrombus formation. (*A*) Representative experiment showing the Doppler tracings from a PIP5KI$\beta^{+/+}$ mouse (*Upper*) and a PIP5KI$\beta^{-/-}$ littermate (*Lower*). The asterisk shows the time that the source of injury was removed from the blood vessel. After ≈1.5 min, an occlusion developed in the artery of the PIP5KI$\beta^{+/+}$ mouse (note the downward reflection of the Doppler tracing). In contrast, there was no disruption of blood flow in the carotid artery of the PIP5KI$\beta^{-/-}$ mouse. (*B*) The percentage of mice that formed thrombi that resulted in greater than a 50% reduction in blood flow is reported (PIP5KI$\beta^{+/+}$, $n = 4$; PIP5KI$\beta^{-/-}$, $n = 5$).

with the wild-type littermates. This indicates that PIP5KIβ plays a critical role in platelet adhesion *in vivo*, probably through regulating second-messenger production.

Discussion

Our results demonstrate that mice lacking PIP5KIβ have decreased fertility, and platelets lacking PIP5KIβ have defective aggregation, polyphosphoinositide production, and *in vivo* thrombosis formation. The infertility defect was present in mice lacking PIP5KIβ alone and accentuated in mice lacking both PIP5KIα together with PIP5KIβ. Histological examination failed to identify an anatomic defect in gonads derived from male and female PIP5KIβ-null mice (Fig. 1*B*). Previous studies have suggested that alterations in IP_3 signaling can impede ovulation in other organisms (32, 33). Recently, Lee and coworkers (34) showed that *Caenorhabditis elegans* lacking a PIP5K homolog, ppk-1, also have a fertility defect. Their work demonstrates that decreased ppk-1 expression does not affect oocyte maturation, but instead causes sterility by reducing contractility of the gonad sheath. Furthermore, they showed that this defect in contractility correlates with defective IP_3 formation. At this point, it is not clear whether a similar ovulation defect accounts for the fertility defect observed in PIP5KIβ-null mice.

This work demonstrates that continuous or rapid synthesis of PIP_2 is a critical component of second-messenger formation. Estimates of number of PIP_2 molecules within human platelets vary between 7.5×10^5 and 2.4×10^6 (13, 35–38). This is approximately the same number of molecules of total IP_3 (1,4,5-P3 plus 1,3,4-P3) synthesized within the first 15 s after thrombin stimulation (37). That PIP_2 levels only decrease by ≈25% after thrombin stimulation indicates that the majority of IP_3 is derived from a combination of preexisting and newly synthesized PIP_2. This result is consistent with our finding that PIP5KIβ-null platelets had markedly decreased IP_3 levels immediately after thrombin exposure, and platelets lacking both PIP5KIα and PIP5KIβ had a total absence of thrombin-induced IP_3 formation. Our data also suggest that early IP_3 production is at least partially derived from a small pool of labile PIP_2 that turns over rapidly in response to agonists.

We have recently generated a murine line lacking PIP5KIγ, the other dominant PIP5KI isoform found within platelets and megakaryocytes, and we found that that loss of PIP5KIγ leads to early mortality (28). Consequently, we were not able to study platelets lacking this enzyme. PIP5KIγ-null megakaryocytes derived from yolk sac hematopoietic progenitor cells bleb their cell membrane because of a defective connection between their cytoskeleton and the plasma membrane (20). This failure to anchor the cell membrane to the underlying cytoskeleton was not observed in megakaryocytes lacking either PIP5KIα or PIP5KIβ. In addition, we found that overexpression of PIP5KIβ in PIP5KIγ-null megakaryocytes failed to revert this phenotype. Although the complex biology of megakaryocytes and platelets likely differs, these observations of PIP5KIγ-null megakaryocytes are consistent with what we conclude in this current study of PIP5KIα- and PIP5KIβ-null platelets. Despite the ability of individual PIP5KI isoforms to all synthesize the same phospholipid product, we have found that the isoforms fulfill unique functions within individual cells. Additional analysis of cells lacking specific PIP5KI isoforms should further illuminate the individual and unique roles performed by distinct PIP5KI isoforms.

Immunoblotting of platelet lysates with PIP5KI isoform-specific antibodies (16) along with SAGE analysis (www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc = GSM160043) has demonstrated that PIP5KIγ is the most abundant PIP5KI isoform in murine platelets and megakaryocytes. Therefore, it is notable that platelets lacking both PIP5KIα and PIP5KIβ but still containing PIP5KIγ have no rise in IP_3 after thrombin stimulation. This observation suggests three alternative hypotheses as to why PIP5KIγ cannot compensate for the loss of PIP5KIα or PIP5KIβ. First, activation of specific PIP5KI isoforms may be regulated differently. Consistent with this first hypothesis is the observation that outside of the catalytic core domain, there is little amino acid sequence homology between PIP5KIγ and the other PIP5KI isoforms. Work by Yin and coworkers (39) has shown that inhibition of PIP5KIγ expression in HeLa cells by RNAi impairs IP_3 formation after stimulation of the histamine receptor. This finding suggests that in this tissue culture cell line, after a specific stimuli, PIP5KIγ can produce a pool of PIP_2 that contributes to second-messenger formation. A second hypothesis to explain why PIP5KIγ does not contribute to thrombin-induced second-messenger formation is that different PIP5KI isoforms are localized within different discrete microdomains near the cell membrane. In this situation, individual PIP5KI isoforms contribute to compartmentalized pools of PIP_2 that control different aspects of actin dynamics. Finally, it is also possible that in addition to synthesizing PIP_2, PIP5KIα and PIP5KIβ bind an unknown accessory protein that is critical for second-messenger formation. In this model, PIP5KIγ would be incapable of interacting with this regulatory protein and therefore unable to contribute to IP_3 formation. Regardless of which explanation is predominant, the available data suggest that PIP5KIα and PIP5KIβ synthesize a pool of PIP_2 that allows IP_3 formation, whereas PIP5KIγ generates PIP_2 that is required for the stable association of the membrane with the cytoskeleton (20).

Materials and Methods

Generation of Genetically Altered Mice. Berkeley Bay Genomics Group provided ES cell lines (XE248) containing disruption of one allele of the PIP5KIβ

genes by β-geo random insertion mutagenesis (22). Using a RT-PCR, Southern blotting, and a sequencing-based strategy, we identified the specific site of insertion of the β-geo cassette within the first intron of the gene. Location of the Southern blot probe and PCR primers are shown in Fig. S1. Generation of chimeric mice was performed at the Transgenic Core Facility at the University of Pennsylvania. Mice lacking PIP5KIα have been described in ref. 21.

Immunoblotting. Tissues derived from murine muscle were lysed in NuPAGE LDS sample buffer (Invitrogen) and then fractionated by electrophoresis on a 7–12% [bis(2-hydroxyethyl)amino]tris(hydroxymethyl) (Bistris) gel. After transfer to nitrocellulose membranes, blots were incubated with anti-mouse PIP5KIβ antibody according to the manufacturer's instructions (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-mouse IgG was used as the secondary antibody, and signals were detected by using ECL (Amersham Biosciences).

Platelet Aggregation and Secretion. Heparin-anticoagulated platelet-rich plasma (PRP) was isolated as described above. The concentration of platelets in the PRP was adjusted to 2.5×10^8 platelets per ml by using Hepes–Tyrode's buffer (pH 7.4) (134 mM NaCl, 3 mm KCl, 0.3 mM NaH_2PO_4, 12 mM $NaHCO_3$, 2 mM $MgCl_2$, 5 mM Hepes, 5 mM glucose, 0.35% BSA). Platelet aggregation was measured by the turbidometric method at 37°C in a Lumi-Dual aggregometer (Chrono-log).

Polyphosphoinositide Analysis. ***Quantitation of PIP_2 and PIP.*** Blood from the vena cava of anesthetized mice was anticoagulated with 15 units/ml heparin and centrifuged at 200 × *g* for 7 min at room temperature to obtain PRP and again at 800 × *g* for 10 min to isolate platelets. Platelets were washed once with modified Tyrode's buffer (pH 6.5) (134 mM NaCl, 3 mm KCl, 0.3 mM NaH_2PO_4, 12 mM $NaHCO_3$, 2 mM $MgC1_2$, 1 mM EGTA, 1 μM PGE_1, 5 mM Hepes, 5 mM glucose, 0.35% BSA). The density of platelets was adjusted to 1×10^9/ml by using modified Tyrode's buffer (pH 7.4) without PGE_1, EGTA, and NaH_2PO_4 but supplemented with 1 mM $CaC1_2$. Platelet suspensions (0.5 ml) were incubated (''phosphate-starved'') at 37°C for 30 min and then supplemented with 0.5 mCi of ^{32}P for 90 min. The incorporation of ^{32}P was halted by the addition of 50 μl of ice-cold hydrochloric acid and 1 volume of methanol:chloroform (1:1) on ice. Mixtures were vortexed for 1 min to extract phosphoinositides and then centrifuged at 2,000 × *g* for 3 min. The chloroform phase was isolated, and the remaining aqueous phase was extracted again with chloroform. The two extracted chloroform phases were combined and evaporated under N_2 flow. The dried residual was resuspended in 30 μl of ice-cold chloroform. Phosphoinositides in the chloroform solution were fractionated by TLC and analyzed as described in ref. 16.

Quantitation of IP_3. This procedure was performed by using modifications of the protocol described by Rittenhouse and Sasson (37). Briefly, washed mouse platelets were prepared as described above, and their density was adjusted to $7–10 \times 10^8$/ml before dividing into 0.2-ml aliquots. One unit/ml thrombin (Chrono-log) was added for indicated times before terminating the reaction by adding 0.2 vol of ice-cold 20% $HClO_4$. After a 20-min incubation on ice, the reaction was adjusted to pH 7.5 by the addition of 1.5 M KOH and then centrifuged at 2,000 × *g* for 15 min at 4°C. The supernatants were collected and stored at −80°C. Quantitation of IP_3 was performed by using a radioreceptor IP_3 kit (PerkinElmer) according to the manufacturer's instructions.

Carotid Artery Injury Induced by $FeCl_3$. Mice weighing 20–30 g at the age of 8 weeks were anesthetized with 80 mg/kg i.p. Nembutal (30, 31). A midline incision was made in the neck, and the right carotid artery was exposed by blunt dissection. A 1 × 1-mm patch of No. 1 Whatman filter paper, soaked in 10% $FeCl_3$, was applied to the exposed artery for 2 min. After removal of the filter paper, the artery was washed with PBS, and blood flow was recorded by using a small-animal blood-flow meter (model T106; Transonic Systems) for 30 min. The percentage of mice that formed thrombi that resulted in greater than a 50% occlusion of blood flow is reported.

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