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Protein phosphatase 1 γ modulates steady state BAD phosphorylation and murine platelet survival

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Platelets play a critical role in hemostasis, thrombosis, immunity and tumor metastasis with a limited life span (7-10 days in humans [1] and 4-5 days in mice [2]). Understanding biochemical mechanisms that prolong platelet survival has implications in transfusion medicine.

Platelet senescence is tightly coupled to pro and anti-apoptotic pathways. In steady state platelets, anti-apoptotic protein B-cell lymphoma 2 (BCL-X_L) continually engage the pro-apoptotic BCL-2 antagonist killer (BAK) and restrain BAK activity. Over time, apoptosis ensues in part due to the degradation of anti-apoptotic BCL-X_L relative to BAK protein [3]. Furthermore, BCL-2 antagonist of cell death (BAD) protein can disrupt anti-apoptotic BCL-X_L signal and enable pro-apoptotic BAK to homo-oligomerize into pores on mitochondrial membrane and release apoptotic mediators that activate initiator caspase 9 [4]. Indeed, BAD knockout mice exhibit a modest but significant extension of platelet lifespan [5]. Importantly, serine (Ser) phosphorylation of BAD on amino acids 112, 136, 155 and 170 by serine/threonine (Ser/Thr) protein kinases A (PKA), PKB and PKC attenuate the apoptotic activity of BAD [6,7,8]. Steady state BAD phosphorylation is likely maintained by a concerted action of protein kinases and phosphatases. However, whether Ser/Thr protein phosphatases modulate BAD phosphorylation and platelet survival remains unknown.

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Since members of BCL-2 family possess consensus binding motifs for the catalytic subunit of protein phosphatase 1 (PP1c) [9], and BAD interacts with PP1cγ in lung epithelial cells [10], we investigated PP1cγ–BAD axis in platelets. To examine if PP1cγ can interact with BAD, we expressed PP1cγ as a glutathione S-transferase (GST) fusion protein in E.coli [11] (Fig. 1A) and performed pull down assay. BAD from resting mouse (Figs. 1B & C) and human (Figs. 1D & E) platelet lysate interacted with PP1cγ-GST protein but not with GST, respectively. Due to the unavailability of a PP1cγ isoform specific pharmacological inhibitor, further studies were conducted only in mouse using a genetic approach. To study if platelet PP1cγ can modulate BAD phosphorylation, we used platelets from wild type (WT) and PP1cγ $^{-/-}$ mice [12]. Compared to the resting WT platelets, phosphorylation of BAD at Ser 112 was enhanced in PP1cγ$^{-/-}$ platelets (Figs. 1F & G). BAD phosphorylation on Ser 136 and Ser 155 was comparable in resting WT and PP1cγ $^{-/-}$ platelets (not shown). These studies suggest that PP1cγ can engage BAD and regulate steady state Ser 112 BAD phosphorylation.

Ser 112 phosphorylation on BAD promotes the binding of BAD with 14-3-3 protein, sequesters BAD in the cytoplasm and prevents the heterodimerization of BAD with BCL-X_L protein thus quenching the death promoting activity of BAD [6]. Next, we studied BAD-14-3-3 interaction by co-immunoprecipitation assays. Lysate from resting WT platelets was immunoprecipitated with either anti-BAD or control IgG antibody and the immunoprecipitate was blotted with anti-14-3-3 antibody. Immunoblots of 14-3-3 immunoprecipitate but not IgG detected BAD, suggesting that BAD can interact with 14-3-3 in platelets (Figs. 1H & I). Importantly, compared to the resting WT platelets, we observed an increased interaction of BAD with 14-3-3 protein in PP1cγ $^{-/-}$ platelets (Figs. 1J & K). Enhanced engagement of BAD with 14-3-3 protein in resting PP1cγ $^{-/-}$ platelets can dampen apoptosis by precluding the binding of BAD to anti-apoptotic Bcl-xL.

Apoptosis begins with an activation of initiator caspase, caspase 9 [13], wherein, procaspase 9 (49 kDa) is cleaved into the active form (37 kDa). Compared to lysate from WT platelets, the intensity of cleaved caspase 9 (~37Kd) was reduced in PP1cγ $^{-/-}$ platelet lysate (Figs. 1L & M). These studies suggest that loss of PP1cγ could dampen the extent of caspase 9 activation in platelets. To test if PP1cγ impacts platelet clearance, tail veins of WT and PP1cγ $^{-/-}$ mice were injected with NHS-biotin to label platelets and their *in vivo* survival was tracked by flow cytometry using streptoavidin PE and anti-αIIb FITC antibodies [3]. Platelet half-life defined as time period in which ~50% of the biotinylated platelets disappear from circulation was modestly, but significantly increased in PP1cγ $^{-/-}$ mice (~53.32 hrs), compared to the WT mice (~45.34 hrs). (Fig. 1N). The delayed half-life of PP1cγ $^{-/-}$ platelets correlated moderately with increased platelet counts in PP1cγ $^{-/-}$ mice (Fig. 1O). These studies suggest that loss of PP1cγ can delay apoptosis and modestly prolong the basal life span of platelets. Indeed, PP1cγ promote apoptosis and necroptosis in part by dephosphorylating inhibitory phosphorylation sites on RIPK 1 [14].

A modest change in the *in vivo* survival study for PP1cγ $^{-/-}$ mice may be due to several factors: a) Potential compensation by additional Ser/Thr phosphatases such as PP1cα, PP2A, PP2B as there is precedence for these phosphatases to engage BAD or modulate BAD phosphorylation, [15, 16, 17] b) Modulation of BAD Ser112 phosphorylation by PP1cγ might

represent a minor subset of biochemical changes that impacts platelet life span. A previous study had shown that BAD Ser155 phosphorylation by PKA modulates platelet lifespan [18]. A limitation of the study is the use of global PP1cγ $^{-/-}$ mice may not allow to fully ascertain if the prolongation of PP1cγ $^{-/-}$ platelet life span is intrinsic to platelets. Nevertheless, our studies indicate that loss of PP1cγ led to the hyperphosphorylation of platelet BAD Ser112, which via an enhanced interaction with 14-3-3 delayed caspase mediated apoptosis and prolonged the basal life span of platelets (Fig. 1P).

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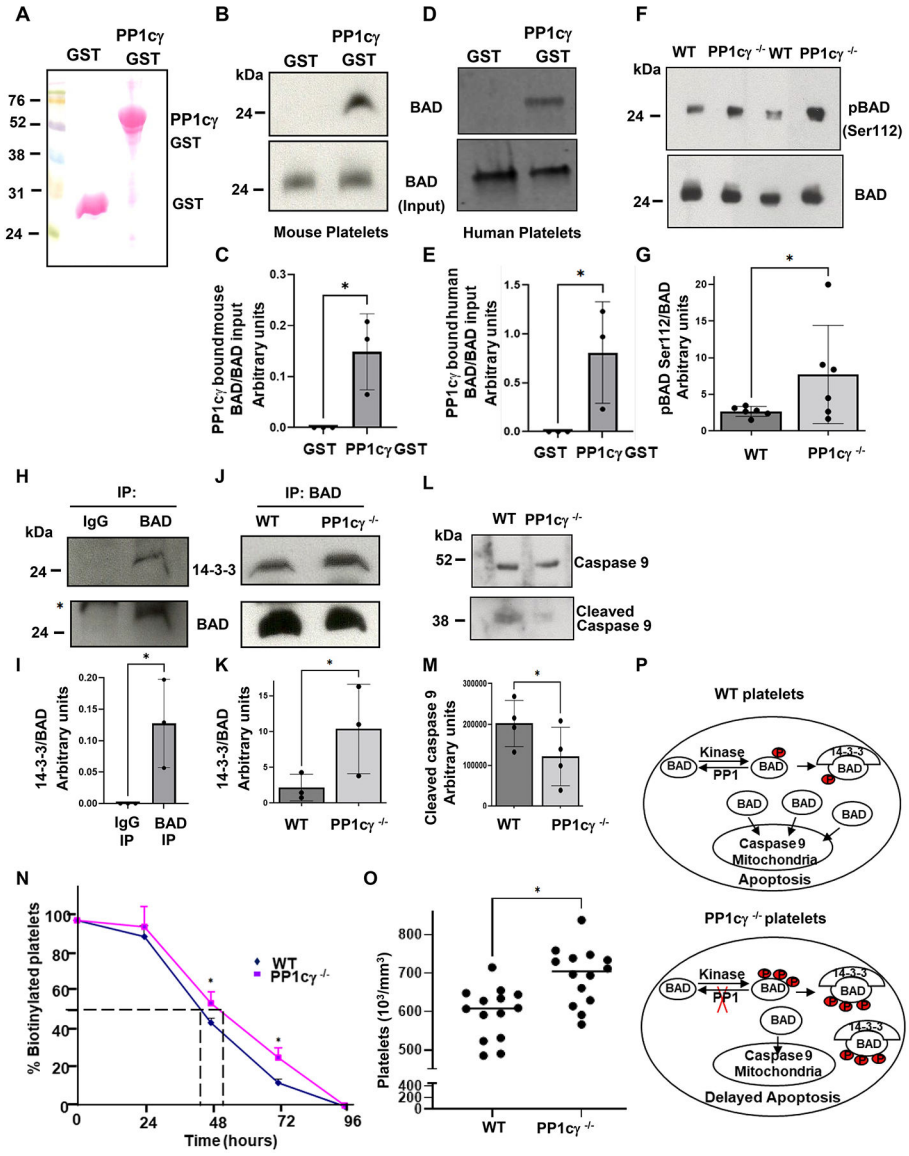

Figure 1.

1A. Ponceau S staining of the purified GST proteins. **1B**. Purified GST and PP1cγ-GST proteins coupled to glutathione sepharose beads was mixed with mouse platelet lysate. Beads were washed and PP1cγ interacting proteins separated by SDS-PAGE and immunoblotted with anti-BAD antibody (upper panel). Lysate used for pull down assay has BAD and shown as input (lower panel). **1C**. Densitometry quantification of PP1cγ bound BAD/BAD in lysate. Data is mean +/− SD n=3,*t-test $p<0.05$. **1D**. Studies identical to **1C**, except human platelet lysate was used. **1E**. Densitometry quantification of PP1cγ bound human BAD. Mean +/− SD, n=3, *$p<0.05$. **1F**. Lysate from washed wild type (WT) and PP1cγ $^{-/-}$ platelets was immunoblotted with anti-pBAD Ser112 antibody (upper panel) and anti-BAD antibody (lower panel). **1G**. Densitometry quantification of the ratio of pBAD/BAD. Mean +/− SD; n=6 * $p<0.05$. **1H**. Immunoprecipitation (IP) of WT platelet lysate with anti-IgG and anti-BAD antibodies followed by immunoblotting with anti-14–3-3 antibody

(upper panel) and anti-BAD antibody (lower panel). *Denotes cross reaction of secondary HRP antibody to the rabbit light chain antibody used for IP. **1I**. Densitometry of the ratio of 14–3-3/BAD. Mean +/− SD, n=3, $p<0.05$. **1J** Lysate from WT and PP1cγ$^{-/-}$ platelets was immunoprecipitated with anti-BAD antibody and immunoblotted with anti-14–3-3 antibody (upper panel) and anti-BAD antibody (lower panel). **1K**. Densitometry of the ratio of 14–3-3/BAD. Mean +/− SD; n=3 *$p<0.05$.**1L**. Lysate from WT and PP1cγ$^{-/-}$ mice was immunoblotted with anti-caspase 9 antibodies that detects cleaved (lower panel) and total caspase 9 (upper panel). **1M**. Densitometry quantification of cleaved caspase. Mean +/− SD; n=4 *$p<0.05$. **1N.** 6–12 weeks old WT and PP1cγ $^{-/-}$ mice on Balb/C background were intravenously injected with biotin and two-color flow cytometry analysis of blood performed every 24 hrs. Biotinylated platelets at the first blood draw was set at 100%. Data is mean +/− SD of 7–8 mice. *$p<0.05$. All animal studies were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine. **1O**. Whole blood from WT and PP1cγ $^{-/-}$ mice was studied using automated Scil Vet ABC analyzer for platelet counts. n=11–14 *$p<0.05$. **1P**. Proposed model for the delayed platelet apoptosis in PP1cγ $^{-/-}$ mice.