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Is GPIbα really a master regulator of platelet activation?

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We read with great interest the recent publication from Yan et al in *Blood Advances* entitled "Essential role of glycoprotein Ibα in platelet activation".[1] The study presents findings showing that the deletion of 10 amino acids (aa) from the cytoplasmic tail of GPIbα in mouse platelets results in defective platelet responses downstream of the VWF-GPIbα interaction but also all other major platelet receptors including GPVI, thromboxane receptor (TR), P2Y12 and PAR-4. Unsurprisingly, these mice exhibited impaired haemostasis and reduced thrombus formation after laser- or $FeCl_3$-induced endothelial injury of arterioles and were protected in a model of pulmonary thromboembolism.[1] Mechanistically, this thromboprotective phenotype was linked to reduced PKC activity in $10aa^{-/-}$ platelets. Complementary to these findings, the authors also show that a myristoylated peptide of GPIbα (MPαC)[2] previously shown to prevent the binding of 14-3-3ζ to GPIbα is able to activate platelets via PKC in washed platelets but not in platelet rich plasma (PRP). While the authors should be commended for the amount of data generated in this article, there are several crucial issues with the study that requires further consideration.

Primarily, we would like to comment on the validity of the $10aa^{-/-}$ mouse model. Due to the difficulty of genetically modifying platelets and the limited availability of specific inhibitors of platelet receptors, the creation of novel mouse models is invaluable to provide insights into molecular mechanisms of platelet signalling/activation. There are many GPIbα mouse models nicely reviewed by Jerry Ware,[3] and particularly relevant in the context of the present $10aa^{-/-}$ model are the hTg^{Y605X} and *GpIbα*$^{\Delta sig/\Delta sig}$ mice which harbour a deletion of the last 6 or 24 aa of the intracellular tail of GPIbα, respectively.[4,5] The haemostatic responses in the $10aa^{-/-}$ mice are quite different from the ones observed in hTg^{Y605X} and *GpIbα*$^{\Delta sig/\Delta sig}$ mice.[4,5] Briefly, none of these two mice exhibited altered haemostasis. The increased in bleeding time observed in $10aa^{-/-}$ mice could perhaps have been influenced by a more severe challenge to the mouse haemostatic system (5mm tail transections versus 2mm for *GpIbα*$^{\Delta sig/\Delta sig}$ mice). CRP- as well as thrombin- and ADP-induced platelet aggregation was normal in *GpIbα*$^{\Delta sig/\Delta sig}$ mice as well as platelet accumulation and fibrin deposition after laser induced-thrombus formation.[5] However, a significant decrease in P-selectin and activated $\alpha_{IIb}\beta_3$ expression was observed in CRP-stimulated *GpIbα*$^{\Delta sig/\Delta sig}$ compared to wild-type platelets but not when platelets were stimulated with ADP or thrombin.[5] In light of the results from Yan et al showing a thromboprotective phenotype for $10aa^{-/-}$ mice in laser- or $FeCl_3$-induced thrombosis models of microvessels, it is also important to highlight that in a $FeCl_3$-induced carotid thrombosis model, hTg^{Y605X} mice were unable to form stable thrombi and exhibited increased embolization events, despite normal haemostasis and platelet counts.[6] It is unclear whether this defect in hTg^{Y605X} mice can be attributed to decreased signalling or lower affinity of human GPIbα for murine VWF.[7] While Yan et al do mention the differences observed in their mouse model compared to hTg^{Y605X} and *GpIbα*$^{\Delta sig/\Delta sig}$ mice, they have not considered why this might be.

There is very little information on the generation of the $10aa^{-/-}$ mice via CRISP-Cas9 technology. Data provided verifying that only the last 10 aa of the cytoplasmic tail of GPIbα have been deleted and no other changes is not entirely convincing. This now becomes

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important for interpreting the discrepancies between the different platelet response phenotypes among the different mouse models. The western-blot using an anti-GPIbα C-terminal antibody (Figure 4B) suggests that indeed a portion of the tail of GPIbα has been deleted, however it is also important to highlight no further information on this antibody is provided. Moreover, loss of binding does not confirm precise deletion of the last 10aa of GPIbα. It is therefore essential that the GPIbα genomic DNA flanking the site of modification is sequenced to ensure that the desired deletion and stop codon have been correctly generated, and at the right genomic location. Omitting such details limits readers' ability to establish how reliable the presented data is and its significance, given already generated models gave very different phenotypes.

Results using the MPαC peptide also seem unclear. MPαC is a GPIbα C-terminal sequence peptide with phosphorylated Ser^{609} and has been shown to inhibit VWF-induced platelet aggregation in PRP.[2] They also have previously shown that it diminished platelet aggregation triggered by low-dose thrombin in washed human platelets at 10µM concentration.[8] Although they have reproduced these results (Figures S2 & S9), the author's *in vitro* results demonstrate that 100µM MPαC induces activation of washed human and mouse platelets, but not when platelets are in plasma. Based on this, it is therefore difficult to reconcile how MPαC could exert an effect *in vivo* by rescuing the defects seen in $10aa^{-/-}$ washed platelets. The authors did not discussed the possible off-target impacts of MPαC , nor do they make consideration of the mechanism of entry for MPαC into platelets in plasma-free versus whole blood conditions. To add to the complexity of the MPαC mode of action, the authors have previously demonstrated that when injected into C57BL/6J mice, this peptide delayed occlusion time after $FeCl_3$ injury of the carotid,[9] which is in stark contradiction with the present findings *in vivo* where it shortens the occlusion time in $10aa^{-/-}$ mice while having no effect on WT animals.

Finally, the authors primarily focus on the role the GPIbα cytoplasmic tail has on moderating PKC activity via sequestering 14-3-3 isoforms, a concept they proposed 19 years ago, and conclude this is how the GPIbα cytoplasmic tail regulates platelet activation.[2] Here again it is puzzling that the authors have not even considered there could be additional mechanisms taking place; particularly as the GPIbα cytoplasmic tail has other binding partners it uses to transduce platelet signals, which the authors have not discussed. It is very difficult to understand how this 14-3-3-sequestration model works in different scenarios: normal and shorter GPIbα in the presence of the MPαC peptide with or without platelet agonists.

In summary, Yan et al assign an important role for the tail of GPIbα in platelet signalling and activation through its binding to 14-3-3 promoting PKC activation. Although this is an elegant concept, we suggest that these conclusions deserve additional thoughts based on the lack of key information related to the tools and models used and especially considering Bernard Soulier syndrome (BSS) patients generally have normal platelet aggregation profiles with agonists other than ristocetin.[10,11] Few case studies nevertheless report defective platelet aggregation with ADP or collagen, but here again, the difficulty of adequately compare aggregation profiles from BSS patients' platelets with healthy platelets due to their size and low counts should be acknowledged and perhaps deserved further investigation.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest to declare.

AUTHOR CONTRIBUTION

BEW and IIS-C developed, wrote, and proofread this commentary.

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