LETTER TO THE EDITOR

Specific O-glycans in the mechanosensory domain of glycoprotein Ibα are important for its stability and function

Yingchun Wang and Renhao Li

Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA, USA

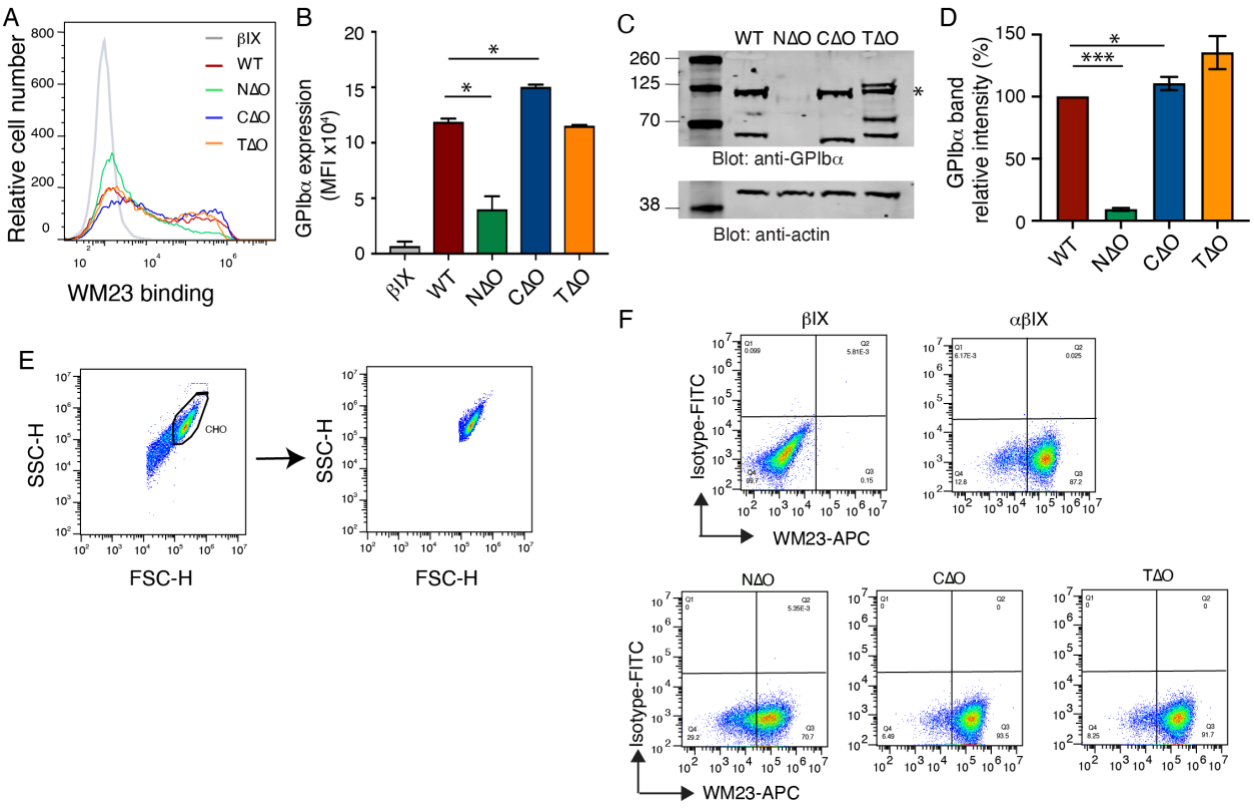
Correspondence:
R. LI - renhao.li@emory.edu

https://doi.org/10.3324/haematol.2022.281979

Supplemental information for:

Specific O-glycans in the mechanosensory domain of glycoprotein Ibα are important for its stability and function

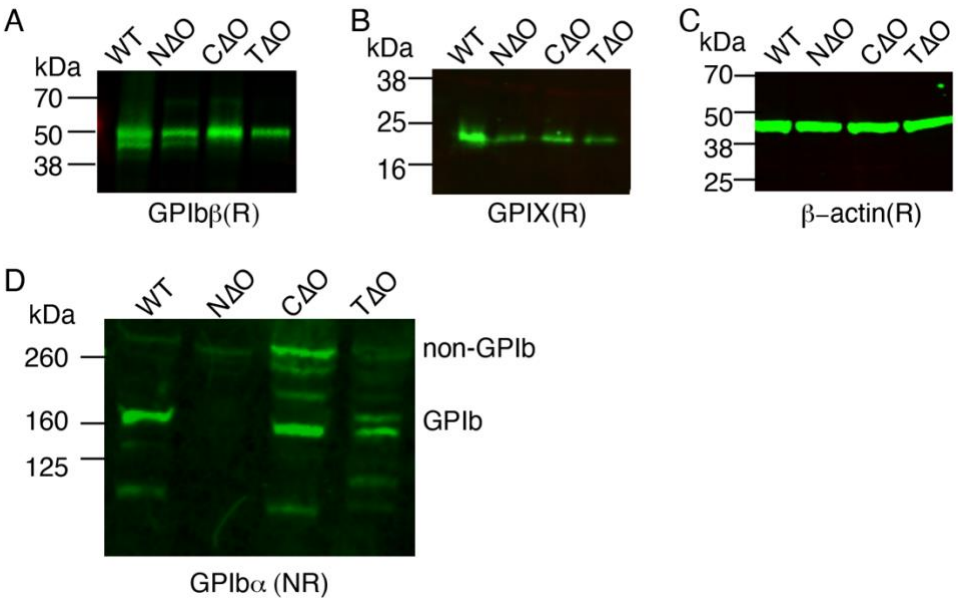
Yingchun Wang, Renhao Li



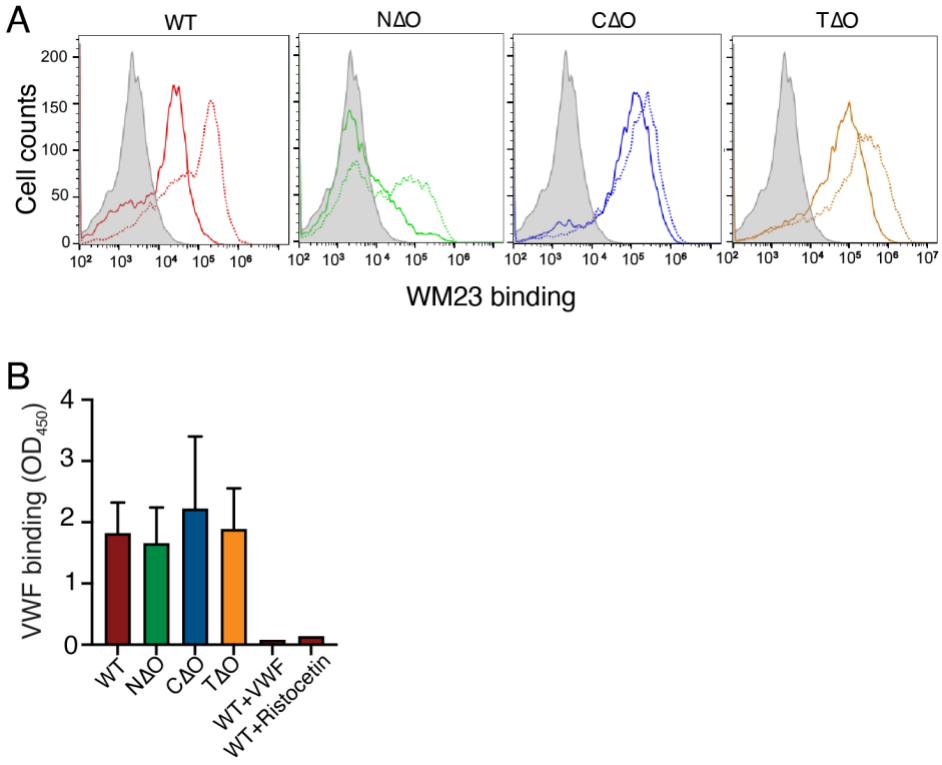
Supplemental Figure 1. Effects of removing O-glycosylation in the MSD on expression of GPIbα in transfected CHO cells. (A) Overlaid flow cytometry histograms showing surface expression of GPIbα variants in transiently transfected CHO cells that expressed wild-type GPIbβ and GPIX. The mammalian expression vector pcDNA3.1/Hygro containing the wild-type GPIbα cDNA[1, 2] was used as the template for mutagenesis. In each variant, mutations of the target putative O-glycan site of MSD domain, serine or threonine residues were substituted by alanine in three distinct regions of GPIbα MSD (Fig. 1A) using a Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA). The NΔO variant contains T418A, S419A, T421A, S425A, T427A, S428A, T431A, S434A, T435A, T438A, T439A and T440A mutations. The CΔO variant contains S444A, S448A, T449A, and T452A mutations. The TΔO variant contains S472A and S473A mutations. pcDNA3.1/Hygro vector containing the WT and mutated GPIbα cDNAs were transiently transfected into CHO K1 cells stably expressing GPIbβ and GPIX using Lipofectamine

2000 (Invitrogen) as previously described.[3, 4] After transfection for 48 hours, to measure the surface expression level of GPIbα, washed cells ($1x10^6$ cells/ml) were treated with anti-GPIbα WM23 antibody at room temperature for 20 minutes, washed and then incubated with APC-conjugated goat anti-mouse IgG for 20 minutes. Samples were fixed by 4% paraformaldehyde and analyzed by flow cytometry. The signal was quantitated by the mean fluorescence intensity (MFI) for the entire cell population (10,000 cells) as described.[2, 5, 6] In parallel, after transfection, cells were grown in Dulbecco's Modified Eagle's Medium with 10% fetal bovine and selected with 500 ug/ml hygromycin. Then cells were stained using WM23 and sorted on Sony cell sorter sh800z to generate stable cell lines. (B) The mean fluorescence intensity of each variant expression was quantified and plotted as bar plots. The data were presented as the mean ± standard deviation from 3 independent experiments (mean±SD, n=3). Comparison of result was performed by one-way ANOVA. *, $P< 0.05$; ***, $P< 0.001$. (C) Representative Western blots showing cellular expression of GPIbα in stably transfected CHO cells. $5x10^5$ cells of each denoted variant were lysed in the cell lysis buffer containing 1% Triton X-100, 5 mM $CaCl_2$, 5 mM N-ethylmaleimide, 58 mM sodium borate, pH 8.0, and 10% (v/v) protease inhibitor cocktail for mammalian tissues. The supernatant from the cell lysate was mixed with standard Tris-glycine SDS sample buffer, resolved in SDS gels (4-12% Bis-Tris NuPAGE gel wit MOPS running buffer) under reducing conditions, and transferred to the nitrocellulose membrane, which was blotted by WM23 or anti-β-actin antibody. The asterisk denotes the fully glycosylated GPIbα band. Molecular weight markers are labeled on the left. (D) Bar plot of relative levels of GPIbα in stably transfected CHO cells as detected by Western blot. The GPIbα band was quantitated by densitometry and normalized with the wild-type band being 100%. The level of TΔO represents the combined levels of both bands. (E) Representative flow cytometry plots showing the gate that was placed in the SSC vs. FSC plot

for CHO cells and used for detecting protein surface expression on stably transfected CHO cells. (F) Dot plots showing expression of GPIbα in noted transfected CHO cells, detected by WM23. The corresponding histograms are shown as Figure 1B.



Supplemental Figure 2. Western blots of GPIb-IX subunits in stably transfected CHO cells. Representative Western blots showing cellular expression of (A) GPIbβ, (B) GPIX, (C) β-actin in stably transfected CHO cells, following protocols described in Supplement Figure 1C. (D) Formation of GPIb complex, and non-GPIb complex,[7] was detected by western blot by WM23 under non-reducing conditions.



Supplemental Figure 3. Characterization of stably transfected CHO cells. (A) Overlaid flow cytometry histograms showing surface expression of GPIbα in noted stably transfected CHO cells, after treatment of vehicle (5% DMSO, solid trace) or 100 μM GM6001 (dotted trace) at 37˚C for 24 h. GPIbα expression was detected by flow cytometry using antibody WM23. Gray histogram: CHO-WT cells stained with only the goat anti-mouse antibody. (F) Bar plots of GPIbα expression level in each transfected CHO cell treated with vehicle (unfilled bar graph) or GM6001 (color-filled bar graph), quantified by mean fluorescence intensity. (B) Remove of O-glycosylation in the MSD does not affect VWF binding to GPIbα. 5μg/ml anti-GPIbα antibody, VM16d (Novus Biologicals) were coated to high-binding 96-well microtiter plates. Lysates of stable CHO cells expressing WT, NΔO, CΔO, and TΔO were diluted, and added to the wells for 1 hour at room temperature. The plate was washed six times with Tris-buffered saline with 0.1% Tween-20. Each sample was incubated with 300 ng human VWF and 1.25 mg/ml ristocetin. VWF binding was

detected using 1:2000 diluted HRP-conjugated anti-VWF polyclonal antibody (Dako). After binding and washing, 1-Step Ultra-TMB substrate (ThermoFisher) was added to each well and quenched with 2 M H_2SO_4. Absorbance at 450 nm was measured on a microtiter plate reader and the background absorbance was subtracted. Only ristocetin or VWF is as control Empty wells were used to subtract baseline absorbance. Comparison of the result was performed by one-way ANOVA. No statistical difference was detected among WT and three variants.

References:

1. Mo X, Nguyen NX, Mu FT, Yang W, Luo SZ, Fan H, et al. Transmembrane and trans-subunit regulation of ectodomain shedding of platelet glycoprotein Ibα. Journal of biological chemistry. 2010 Oct 15;285(42):32096-104.
2. Mo X, Liu L, Lopez JA, Li R. Transmembrane domains are critical to the interaction between platelet glycoprotein V and glycoprotein Ib-IX complex. J Thromb Haemost. 2012 Sep;10(9):1875-86.
3. Mo X, Luo S-Z, Munday AD, Sun W, Berndt MC, Lopez JA, et al. The membrane-proximal intermolecular disulfide bonds in glycoprotein Ib influence receptor binding to von Willebrand factor. J Thromb Haemost. 2008 Jul 19;6(10):1789-95.
4. Tao Y, Gan C, Zhang X, Liu L, Zakas PM, Doering CB, et al. Unaccompanied mechanosensory domain mediates low expression of glycoprotein Ibalpha: implications for Bernard-Soulier syndrome. J Thromb Haemost. 2020 Feb;18(2):510-7.
5. Mo X, Lu N, Padilla A, López JA, Li R. The transmembrane domain of glycoprotein Ibβ is critical to efficient expression of glycoprotein Ib-IX complex in the plasma membrane. J Biol Chem. 2006;281(32):23050-9.
6. Mo X, Nguyen NX, McEwan PA, Zheng X, Lopez JA, Emsley J, et al. Binding of platelet glycoprotein Ibβ through the convex surface of leucine-rich repeats domain of glycoprotein IX. J Thromb Haemost. 2009 Sep;7(9):1533-40.
7. Luo S-Z, Mo X, López JA, Li R. Role of the transmembrane domain of glycoprotein IX in assembly of the glycoprotein Ib-IX complex. J Thromb Haemost. 2007 Dec;5(12):2494-502.