

Hélène Peyro-Saint-Paul
 Medical Affairs Department, Ipsogen SA,
 Luminy Biotech Entreprises,
 Marseille, France

Fabienne Hermitte
 Research and Development Department, Ipsogen SA,
 Luminy Biotech Entreprises,
 Marseille, France

Contribution: H.P.-S.-P. and F.H. contributed equally to writing of the article.

Conflict-of-interest disclosure: H.P.-S.-P. and F.H. are employees of Ipsogen SA.

Correspondence: Hélène Peyro-Saint-Paul, MD, Ipsogen SA, Luminy Entreprises, Case 923, 163 Ave de Luminy, 13288 Marseille cedex 9, France; e-mail: peyro-saint-paul@ipsogen.com.

References

1. Warshawsky I, Mularo F, Hren C, Jakubowski M. Failure of the Ipsogen MutaScreen kit to detect the JAK2 617V>F mutation in samples with additional rare exon 14 mutations: implications for clinical testing and report of a novel 618C>F mutation in addition to 617V>F [letter]. *Blood*. 2010;115(15):3175-3176.
2. Murugesan G, Aboudola S, Szpurka H, et al. Identification of the JAK2 V617F mutation in chronic myeloproliferative disorders using FRET probes and melting curve analysis. *Am J Clin Pathol*. 2006;125(4):625-633.
3. Biglia O, le Coedic J, Hermouet S, Hermitte F, Maroc N. Development and validation of new molecular diagnostic assays for the JAK2 V617F screening and quantification [abstract]. Association for Molecular Pathology 2007 Annual Meeting Abstracts. *J Mol Diagn*. 2007;9:669. Abstract H633.
4. Cankovic M, Whiteley L, Hawley RC, Zarbo RJ, Chitale D. Clinical performance of JAK2 V617F mutation detection assays in a molecular diagnostics laboratory: evaluation of screening and quantitation methods. *Am J Clin Pathol*. 2009;132(5):713-721.
5. Ma W, Kantarjian H, Zhang X, et al. Mutation profile of JAK2 transcripts in patients with chronic myeloproliferative neoplasias. *J Mol Diagn*. 2009;11(1):49-53.
6. Tefferi A, Vardiman JW. Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia*. 2008;22(1):14-22.
7. Ipsogen. JAK2 MutaScreen Kit for the detection of JAK2 V617F mutation: kit for the detection of JAK2 V617F/G1849T mutation on human genomic DNA. Instructions for use Version 05, November 2008. http://www.ipsogen.com/uploads/tx_ipsolkdata/IFU_MS_K_mK_JAK2.pdf. Accessed June 3, 2010.

To the editor:

Beta 2 glycoprotein I is a substrate of thiol oxidoreductases

Beta 2 glycoprotein I (β 2GPI) is an abundant plasma protein recognized as the major autoantigen in the antiphospholipid syndrome. Although the crystal structure of β 2GPI has been resolved,^{1,2} its normal function remains unknown. We have been intrigued by the presence of a C-terminal cysteine (Cys326), which forms a loop-back disulfide link in the fifth domain of β 2GPI. In the current study we examined β 2GPI's potential to participate in thiol exchange reactions with the thiol oxidoreductases thioredoxin-1 (TRX-1) and protein disulfide isomerase (PDI).

The incorporation of free thiols into β 2GPI after reaction with TRX-1 or PDI was shown by labeling the products of this reaction with the selective sulfhydryl probe N^a-(3-maleimidylpropionyl) biocytin (MPB). The biotinylated proteins were visualized by Western blotting with streptavidin-horseradish peroxidase. Because β 2GPI does not contain unpaired cysteines, no labeling was observed after incubation with MPB (Figure 1A). Free thiols could not be introduced into β 2GPI by incubation with the reducing agent dithiothreitol (DTT) alone. However, free thiols could be introduced into β 2GPI after incubation with the reduced forms of the thiol oxidoreductases TRX-1 and PDI, identifying β 2GPI as a thiol oxidoreductase substrate (Figure 1A-C). An interesting effect caused by the reduction of β 2GPI by TRX-1 was a marked decrease in the affinity of anti- β 2GPI monoclonal and polyclonal antibodies as noted on the immunoblots.

To determine the cysteine residue(s) in the β 2GPI molecule involved in thiol exchange reactions, β 2GPI treated with the TRX-1/TRX-1 reductase/NADPH system and labeled with MPB was resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Figure 1B). Gel bands were excised, digested, and analyzed by liquid chromatography-tandem mass spectrometry. Mass spectral data were searched using Mascot (Version 2.2; Matrix Science) or converted to MzXML file format using ReAdW (Version 4.0.2)³ and submitted to the database search

program X!Tandem (Release 2008.12.01).⁴ The analysis revealed Cys326 to be predominantly labeled with biotin (F.H.P., S.R., M.Q., M.J.R., J.W.H.W., K.T., Y.I., J.Y.Z., R.G., J.C.Q., B.G., W.E.H., P.J.H., S.A.K., manuscript submitted). The structural features of the disulfide bond containing Cys326 and all disulfide bonds of the 2 structures of β 2GPI (PDB 1C1Z and 1QUB) were determined using the disulfide bond analysis tool available at www.cancerresearch.unsw.edu.au/CRCWeb.nsf/page/Disulfide+Bond+Analysis.⁵ The analysis showed that the Cys288-Cys326 disulfide is a $-/+$ right-handed hook ($-/+$ RHHook) configuration in both crystal structures of the protein.^{1,2} Although there is no other structural similarity with β 2GPI, the active site disulfides of oxidoreductases like TRX-1 or PDI are $+/-$ RHHooks. Of the 22 Cys residues in β 2GPI, Cys326 stands out as being exposed to solvent. The solvent accessibility values for Cys326 are 117 (PDB ID 1C1Z) and 103 (PDB ID 1QUB) Å² for the 2 structures. This high solvent exposure is consistent with reduction of the Cys288-Cys326 disulfide bond by thiol oxidoreductases.

Thiol oxidoreductases are becoming increasingly recognized as important mediators of platelet function.⁶ The prototype member, PDI, has been implicated in the activation of the fibrinogen receptor α IIB β 3⁷ and tissue factor.⁸ Several novel members of the thiol isomerase family have been recently shown to translocate to the platelet surface after platelet activation.⁹ In the current issue of *Blood*, Ioannou et al have developed a sensitive and specific streptavidin-capture enzyme-linked immunosorbent assay (ELISA) to detect reduced β 2GPI in plasma.¹⁰ With the same methodology β 2GPI is shown to be reduced after incubation with platelets, which can be attributed partially to the TRX-1 system (Figure 1D).

This study is the first to show the potential of β 2GPI to participate in thiol exchange reactions. Our finding suggests that β 2GPI may participate in redox processes in vascular biology.

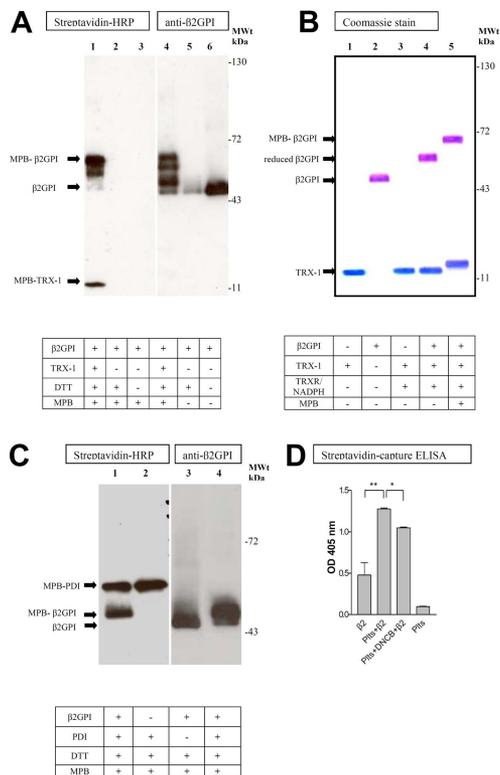


Figure 1. Free thiols introduced into β2GPI after reaction with TRX-1 and PDI; reduction of β2GPI on the platelet surface. Native or recombinant β2GPI was incubated with TRX-1 previously reduced by DTT (A) or TRX-R/NADPH (B) or with PDI reduced by DTT (C). Reduced TRX-1 was prepared by incubating 5 μM TRX-1 with 25 μM DTT or 10 nM TRX-R/200 μM NADPH for 1 hour at 37°C. Reduced PDI was prepared by incubating 500 nM PDI with 50 μM DTT. β2GPI (0.2 μM) was incubated with the TRX-1 or PDI mixtures for 1 hour at 37°C. To label free thiols, N^α-(3-maleimidylpropionyl) biocytin (MPB; 100 μM) was added to the β2GPI/TRX-1 or PDI solutions for 10 minutes at 37°C. The reaction was quenched with glutathione (200 μM) for 10 minutes at 37°C. All reactions were performed in 20 mM HEPES, 0.14 M NaCl buffer, pH 7.4. The reactions were resolved under nonreducing conditions on SDS-PAGE (4%-12%) and then transferred to polyvinylidene fluoride membranes. MPB-labeled β2GPI was detected with the streptavidin-horseradish peroxidase (1:1000). β2GPI was detected with the 4B2E7 monoclonal antibody 3.5 mg/mL or rabbit anti-β2GPI Ab 1.4 mg/mL. (A) Incorporation of MPB into β2GPI (at ~ 70 kDa) after treatment with TRX-1 (lane 1). MPB cannot be introduced into β2GPI after treatment with the reductant DTT alone defining β2GPI as a substrate of TRX-1 (lane 2). Lanes 4 and 5 show detection of the reduced β2GPI forms on SDS-PAGE and the decreased immunoreactivity of anti-β2GPI monoclonal antibody to reduced β2GPI (± MPB) compared with nonreduced β2GPI (lane 6). (B) Coomassie staining of β2GPI incubated with TRX-1/TRX-R/NADPH/MPB and separated on SDS-PAGE shows one β2GPI band at ~ 70 kDa in accordance with the MPB-labeled β2GPI band in the streptavidin-horseradish peroxidase blot (panel A lane 1) and the first β2GPI immunoreactive band on the anti-β2GPI blot (panel A lane 4). The remaining 3 β2GPI immunoreactive bands (between 50 and 70 kDa) apparent in the anti-β2GPI blot (panel A lane 4) are not detected on the Coomassie, showing that these β2GPI products have very low concentrations. (C) Incorporation of MPB into β2GPI after reaction with PDI. MPB-labeled β2GPI, after reduction with PDI, showed a minor shift in molecular size on the SDS PAGE (~ 50 kDa, panel C lane 1) in comparison to TRX-1 treated β2GPI (panel A lane 1), showing that TRX-1 had a greater effect than PDI on the biotin labeling and denaturing of β2GPI. Numbers indicate the molecular weight markers. (D) Detection of reduced β2GPI on the platelet surface by a β2GPI-specific streptavidin-capture ELISA. Platelets (in buffer or pretreated with the TRX-R inhibitor 1-chloro-2, 4-dinitrobenzene [DNCB]) were incubated with or without β2GPI. MPB was added to label any free thiols formed. After acetone precipitation to remove nonincorporated MPB, the precipitated MPB-labeled proteins were applied to a streptavidin plate, which captured MPB-labeled proteins including (reduced) β2GPI. β2GPI in 20 mM HEPES, 0.14 M NaCl buffer, pH 7.4 buffer alone plus MPB served as a negative control (first column). As a positive control β2GPI reduced by TRX-1/TRX-R/NADPH plus MPB was used. β2GPI incorporated MPB after incubation with platelets which was partially inhibited by DNCB. Platelets alone plus MPB gave a negligible signal on this ELISA. OD indicates optical density; β2, β2GPI; M, MPB; and TRN, TRX-1/TRX-R/NADPH. Data are expressed as mean ± SD, n = 3 in triplicate. *P < .02; **P < .01.

Freda H. Passam
 Department of Immunology, Allergy and Infectious Diseases,
 St George Hospital, Sydney, Australia

Soheila Rahgozar
 Department of Immunology, Allergy and Infectious Diseases,
 St George Hospital, Sydney, Australia

Miao Qi
 Department of Immunology, Allergy and Infectious Diseases,
 St George Hospital, Sydney, Australia

Mark J. Raftery
 Bioanalytical Mass Spectrometry Facility,
 University of New South Wales, Sydney, Australia

Jason W. H. Wong
 UNSW Cancer Research Centre,
 University of New South Wales, Sydney, Australia

Kumiko Tanaka
 Department of Immunology, Allergy and Infectious Diseases,
 St George Hospital, Sydney, Australia

Yiannis Ioannou
 Department of Immunology, Allergy and Infectious Diseases,
 St George Hospital, Sydney, Australia;
 and Centre for Rheumatology, Department of Medicine,
 University College London, London, United Kingdom

Jing Yun Zhang
 Department of Immunology, Allergy and Infectious Diseases,
 St George Hospital, Sydney, Australia

Rosalie Gemmill
 Department of Hematology,
 St George Hospital, Sydney, Australia

Jian Chen Qi
 Department of Immunology, Allergy and Infectious Diseases,
 St George Hospital, Sydney, Australia

Bill Giannakopoulos
 Department of Immunology, Allergy and Infectious Diseases,
 St George Hospital, Sydney, Australia

Will E. Hughes
 The Garvan Institute of Medical Research,
 Sydney, Australia

Philip J. Hogg
 UNSW Cancer Research Centre,
 University of New South Wales, Sydney, Australia

Steven A. Krilis
 Department of Immunology, Allergy and Infectious Diseases,
 St George Hospital, Sydney, Australia

*F.H.P. and S.R. contributed equally to the manuscript.

Acknowledgments: The authors thank Dr I. Schousboe, University of Copenhagen, for the kind donation of native β2GPI.

Subsidized access (of M.J.R.) to the Bioanalytical Mass Spectrometry Facility of the University of New South Wales with infrastructure provided by the New South Wales government coinvestment in the National Collaborative Research Infrastructure Scheme is gratefully acknowledged.

This work was supported by research grants from the Australian National Health and Medical Research Council (to S.A.K.), by a research grant from the Foundation of the Greek Society of Hematology (to F.H.P.) and by an Arthritis Research Campaign Clinician Scientist Fellowship, United Kingdom (grant 17821 to Y.I.).

Contribution: F.H.P., S.R., and S.A.K. designed research; F.H.P., S.R., M.Q., M.J.R., K.T., and J.C.Q. performed research; R.G., J.W.H.W., P.J.H., and W.E.H. contributed new analytic tools; and F.H.P., S.R., M.J.R., J.W.H.W., Y. I., J.Y.Z., B.G., P.J.H., and S.A.K. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Steven A. Krilis, Professor and Director, Department of Immunology, Allergy and Infectious Diseases, St George Hospital, University of New South Wales, 2 South St, Kogarah 2217, New South Wales, Australia; e-mail: s.krilis@unsw.edu.au.

References

1. Schwarzenbacher R, Zeth K, Diederichs K, et al. Crystal structure of human beta2-glycoprotein I: implications for phospholipid binding and the antiphospholipid syndrome. *EMBO J*. 1999;18(22):6228-6239.
2. Bouma B, de Groot PG, van den Elsen JM, et al. Adhesion mechanism of human beta(2)-glycoprotein I to phospholipids based on its crystal structure. *EMBO J*. 1999;18(19):5166-5174.
3. Keller A, Eng J, Zhang N, Li XJ, Aebersold R. A uniform proteomics MS/MS analysis platform utilizing open XML file formats. *Mol Syst Biol*. 2005;1:2005.0017.
4. Craig R, Beavis RC. TANDEM: matching proteins with tandem mass spectra. *Bioinformatics*. 2004;20(9):1466-1467.
5. Schmidt B, Ho L, Hogg PJ. Allosteric disulfide bonds. *Biochemistry*. 2006;45(24):7429-7433.
6. Lahav J, Wijnen EM, Hess O, et al. Enzymatically catalyzed disulfide exchange is required for platelet adhesion to collagen via integrin alpha2beta1. *Blood*. 2003;102(6):2085-2092.
7. Essex DW, Li M, Miller A, Feinman RD. Protein disulfide isomerase and sulfhydryl-dependent pathways in platelet activation. *Biochemistry*. 2001;40(20):6070-6075.
8. Chen VM, Ahamed J, Versteeg HH, Berndt MC, Ruf W, Hogg PJ. Evidence for activation of tissue factor by an allosteric disulfide bond. *Biochemistry*. 2006;45(39):12020-12028.
9. Holbrook LM, Watkins NA, Simmonds AD, Jones CI, Ouwehand WH, Gibbins JM. Platelets release novel thiol isomerase enzymes which are recruited to the cell surface following activation. *Br J Haematol*. 2010;148(4):627-637.
10. Ioannou Y, Zhang J-Y, Passam FH, et al. Naturally occurring free thiols within beta2-glycoprotein I in vivo: nitrosylation, redox modification by endothelial cells, and regulation of oxidative stress-induced cell injury. *Blood*. 2010;116(11):1961-1970.