T-ANTIGEN (Gal β 3 GalNAc α -) CONTAINING GLYCOPROTEINS OF HUMAN RFACE

Madhan, M.,¹ Venkataraman, M.,² Zachariah Bobby,³ and Ananthanarayanan, P.H.⁴

Department of Biochemistry, Jawaharlal Institute of Postgraduate Medical Education and Research,
Pondicherry, India - 605 006.

ABSTRACT

Desialation of cell surface glycoconjugates due to bacterial or viral infection can expose epitopes like T-antigenic structure which can also occur during oncological transformations. Human platelet plasma membrane glycoproteins were isolated by jacalin affinity chromatography. Potential T-antigen containing glycoproteins which were not reported before could be identified on the Western blot using peanut agglutinin - horse radish peroxidase (PNA-HRP) after neuraminidase treatment. Alpha-galactosyl epitopes recognized by anti-gal were found to be absent in human platelet plasma membrane glycoproteins. Under the experimental conditions employed, the GP IIb α was identified most rich in T-antigenic structures. Probable role of exposed T-antigenic structures and α -galactosyl epitopes in pathological conditions is discussed. The identity of major glycoprotein bands was confirmed by differential lectin-binding studies with Concanavalin A on the Western blot. The higher binding affinity of jacalin for T-antigenic structures when compared to PNA enabled the isolation and detection of the antigen containing platelet surface glycoproteins which were not reported before.

KEY WORDS : α -Galactosyl epitope, T-antigen, Thrombocytopenia, Preleukemia, Jacalin.

INTRODUCTION

A general phenomenon following the loss of terminal sialic acid residues of cell surface glycoconjugates can be the exposure of

autoimmuno antigens. Such a desialation is a possible complication of viral or bacterial infections. Binding of immunoglobulins and / or complement can follow such structural modification of membrane glycoproteins and lead to lysis and

Author for correspondence :

Dr. Zachariah Bobby at above address.

¹. Present Address: International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi - 110 067.

². Present Address: Rajiv Gandhi Centre for Biotechnology, Jagathy, Trivandrum - 695 014.

⁴. Present Address: All India Institute of Public Health and Hygiene, Calcutta.

Abbreviations: PMSF, Phenyl methane sulfonyl fluoride; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; HRP, Horseradish peroxidase; BSA, Bovine serum albumin; Antigal, Anti- α -galactoside antibody; PNA, Peanut agglutinin; Con A, Concanavalin A; α -MG, Methyl- α -D-glucopyranoside; ITP Immune thrombocytopenic purpura; JSA, Jack fruit seed agglutinin (jacalin); WB, Western Blot; CBB, Coomassie brilliant blue.

recognition of platelets as foreign, by elements of the reticuloendothelial system. Exposure of novel antigens for autoantibody reaction could also occur following platelet activation due to the redistribution of internal glycoprotein pools on the cell surface (1). Platelet activation induced by auto antibodies can lead to secretion of factors like platelet derived growth factor which is a potent mitogen.

The present study was undertaken to identify the potential α -galactosyl (Gal α 3 Gal β 4 GlcNAc β -) and Thomsen-Friedenreich antigen (T-antigen) containing human platelet plasma membrane glycoproteins which can serve as autoimmuno antigens when exposed. The presence of abundant naturally occurring antibodies against these antigens in human circulation further emphasizes their possible role in the development of thrombocytopenia (2). Alpha-galactosyl epitope and T-antigen are known to appear on the surface of tumor cells (3). Assessment of the quantitative changes of these antigen containing glycoproteins and their corresponding antibodies will be helpful in the elucidation of the basis of thrombocytopenia and diagnosis of preleukemia. The first step towards this goal is the identification of potential α -galactosyl and T-antigen containing glycoproteins on the surface of human platelets.

MATERIALS AND METHODS

Fresh Human Blood was collected in acid-citrate-dextrose in poly- allomer tubes and processed immediately.

a) Preparation of platelet plasma membrane-enriched fraction : The procedure was a modification of the method by Jennings and Philips

(4). Human platelet rich plasma was prepared and one-tenth volume of 100mM EDTA was added to prevent platelet activation. The preparation was first centrifuged at 1000 RPM for 10min at 25°C to remove the contaminant erythrocytes. A platelet pellet was obtained by subsequent centrifugation at 3500 RPM for 15min. at 25°C. It was suspended in 1ml of isotonic buffer (10mM Tris-HCl, 150mM NaCl, 5mM EDTA, pH 7.4) and subjected to glycerol step gradient centrifugation at 25°C to obtain a glycerol-loaded platelet pellet. The platelets were lysed in 2 ml of a cold hypotonic buffer (10mM Tris-HCl, 10mM EDTA, 1mM PMSF, 10mM benzamidine HCl, 15mM NaN_3 , pH 7.4). The lysate was centrifuged at 10,000g for 10min. at 4°C to obtain the plasma membrane enriched fraction.

b) Extraction of membrane proteins: The membrane preparation was suspended in EDTA-Tris [ET] buffer (20mM Tris-HCl, pH 7.4, 5mM EDTA, 1mM PMSF, 10mM benzamidine HCl, 15mM NaN_3) containing 4% Triton-X-100 and stirred gently at 4°C for 30 min. The extract was diluted 4 times with the ET buffer and centrifuged at 20,000g for 20 min. at 4°C. The supernatant was used for the purification of glycoproteins.

c) Lectin-Sepharose chromatography: Jacalin was coupled to Sepharose 4B at a concentration of 4mg/ml of gel according to the method of Kohn and Wilchek (5). The column (2x10 cm) was equilibrated with the ET buffer containing 0.1% Triton-X-100. The total extracted protein from platelets present in 60ml of blood was loaded into the column. After loading, the column was washed with 10 bed volumes of the equilibration buffer to ensure the complete removal of contaminant non-specific proteins. The bound glycoproteins were

eluted in bulk with 2 bed volumes of equilibration buffer containing 100mM galactose.

d) Characterisation: The jacalin-binding glycoproteins were subjected to SDS-PAGE on 8% and 10% gels under reducing conditions and stained with coomassie brilliant blue (6). The apparent molecular weights were determined using the markers BSA, IgG (heavy chain and light chain) and ovalbumin. The proteins were also transferred to nitrocellulose after the SDS-PAGE according to the method of Towbin et. al. (7). The Western blot was stained separately by the different methods described in Table 1. Colour development for the HRP conjugates on the Western blot was carried out with 4-chloro-1-naphthol for 30 min. at 25°C and the intensity of colour developed was visually estimated.

Neuraminidase treatment of the glycoproteins on the Western blot (about 20 µg initially subjected to SDS-PAGE) was carried out with the enzyme from *Clostridium perfringens* V (Sigma N 2876). The incubation was carried out with 100mU of the enzyme in 5ml of 0.2M acetate buffer, pH 5.0, containing 10mg BSA at 37°C for 20h.

e) Preparation of lectins and Anti-gal: Jacalin was purified according to the method of Suresh Kumar et. al. (8). Concanavalin A was purified from *Canavalia gladiata* using Sephadex G-50 as the affinity matrix according to the method of Surolia et. al. (9). Peanut agglutinin was prepared from *Arachis hypogea* using cross linked guar gum as the affinity matrix. Extraction of the lectin was carried out according to Lotan et. al. (10). Isolation of human plasma anti-α-galactoside antibody was carried out using sugar-specific binding to

galactomanan (11). The lectins and antibody were coupled to HRP by the glutaraldehyde method. In the case of staining with Con A, the Western blot was first exposed to the lectin, followed by washing and incubation with HRP.

RESULTS

By the method described above, sufficient jacalin-binding human platelet plasma membrane glycoproteins could be isolated to study their physicochemical characteristics. Sodium dodecyl sulfate polyacrylamide gel electrophoresis on 8% and 10% gels under reducing conditions revealed the presence of 18 protein bands (Fig.1). The bands were identified based on comparison between the estimated and reported molecular weights (12) as given in Table1. The results of staining the Western blot with various methods are given in Figs. 2-4. In all the cases, the relative intensity of staining was graded visually as given in Table 1.

DISCUSSION

The present study revealed the presence of 10 jacalin-binding glycoprotein subunits on the surface of human platelets. The jacalin-glycoprotein interaction was found to be sugar specific as it could be inhibited in the presence of 0.1M galactose. Investigations of jacalin's ligand specificity revealed its high affinity for α-galactopyranosides and particularly for the T-antigen with the structure, 1-β-D-galactopyranosyl-3-(α-2-acetamido-2-deoxygalactopyranoside) (13,14). Jacalin recognizes the T-antigenic structure on glycoproteins even when substituted with a terminal sialic acid residue (15). In agreement with this, in our study, the lectin recognized the platelet surface glyco-protein

Table 1. Physico Chemical Characteristics of Jacalin-Binding Human Platelet Surface Glycoproteins

Band No.	Mol.Wt. in KDa. 8% SDS-PAGE	Mol.Wt. in KDa. 10% SDS-PAGE	Staining intensity with CBB on SDS-PAGE	Staining intensity with amido black on WB	Staining intensity with JSA-HRP on WB	Staining intensity with Con A/HRP on WB	Staining intensity with Con A/HRP on WB in presence of 15mM α MG	Staining intensity with PNA-HRP on WB after Neuraminidase treatment	Possible identity of the band	Reported M.Wts. of glycoproteins in KDa
1	202 $\pm$ 2	ND	+	ND	ND	ND	ND	ND	-	-
2	185 $\pm$ 15	143	+	ND	ND	ND	ND	ND	-	-
3	170 $\pm$ 8	132	+	ND	ND	ND	ND	ND	GP Ia	167
4	143 $\pm$ 5	127	+	ND	ND	ND	ND	ND	GP Ib α	143
									Glycocalicin	145
5	112 $\pm$ 2	100 $\pm$ 2	+++++	++++	+++++	+	+	+++	GP IIb α	150
										110
										130
6	100 $\pm$ 2	92 $\pm$ 1	+++++	+++++	+	++++	++++	+++	GP IIIa	132
										105
										110
										114
7	88 $\pm$ 1	82 $\pm$ 1	++	++	+++	++++	++++	+++	GP IIb (GPV)	95
8	65 $\pm$ 4	70 $\pm$ 1	++	+++	++	++++	++++	+++	GP V	88
9	ND	66 $\pm$ 1	++	+	+++	+++	+++	+	GP VIa	82#
10	55 $\pm$ 1	58 $\pm$ 1	++	++	++++	++	ND	++	GP VIb	66
11	51	52	++	+++	++++	++	++	ND	GP 52	55-58
12	45 $\pm$ 1	46 $\pm$ 1	+++	+++	++++	+	ND	ND	GP VIII	52
13	41	38	++	ND	ND	+	ND	ND	-	46
14	35 $\pm$ 2	34	+	ND	++	+++	+++	+	-	-
15	28 $\pm$ 1	29	+	+	++	++++	++++	ND	GP Ib β	26,22
16	24	23 $\pm$ 2	+	+	ND	++	+	ND	GP IIb β	23
17	ND	23	++	ND	ND	+++	+++	ND	GP 17 (GP IX)	23.5, 27
18	17 $\pm$ 1	20 $\pm$ 1	++	ND	ND	++	ND	ND	-	17-22

ND - Not detectable; + - Very weak, ++ - Weak, +++ - Moderate, ++++ - Strong +++++ - Very strong

The proteolytic product of the GP-V by thrombin and thrombocytin has a M.Wt of 69 KDa and contains all the carbohydrate [24]

Fig. 1. SDS-PAGE of human platelet plasma membrane jacalin-binding glycoproteins on 10% acrylamide gel under reducing conditions stained with coomassie brilliant blue.

Fig. 2. Western blot of jacalin-binding human platelet plasma membrane glycoproteins transferred from 10% SDS-PAGE gel. A, Stained with Amido Black; B, Stained with jacalin-HRP; C, Stained with jacalin-HRP in presence of 0.1M galactose.

Fig. 3. Western blot of jacalin-binding human platelet plasma membrane glycoproteins transferred from 10% SDS-PAGE gel stained with PNA-HRP. A, Without neuraminidase treatment; B, After neuraminidase treatment of the western blot.

Fig. 4. Western blot of jacalin-binding human platelet plasma membrane glycoproteins transferred from 10% SDS-PAGE gel stained with Con A / HRP. A, Without any hapten sugar; B, In presence of 15mM α MG; C, In presence of 500mM α MG.

subunits without prior desialation. This indicated the presence of potential T-antigen and/or α -linked galactosyl residues in the identified glycoproteins. Unlike jacalin, the peanut agglutinin recognizes only the exposed T-antigenic structures of O-linked oligo-saccharide chains (16). In the present study of platelet plasma membrane glycoproteins, the PNA-HRP identified 7 protein bands only after neuraminidase treatment on the Western blot. Previous studies by electrophoresis of whole platelets after reduction followed by fluorography of neuraminidase/galactose oxidase/ [^3H] NaBH₄ labeling identified Ib α , IIb α and IIIb (17). This is the first time jacalin-affinity chromatography has been employed to isolate the T-antigen containing platelet surface glycoproteins. Additional T-antigen containing glycoprotein subunits could be identified by the present method. The presence of T-antigenic structures in the platelet surface glycoproteins, GP IIb α , GP IIIa, GP IIIb, GP V and GP VI was not conclusively reported before. Lectin affinity chromatography is a dynamic process in the sense, relative affinity plays a greater role and the glycoproteins with higher affinity bind preferentially. In this regard the higher binding affinity of jacalin enabled isolation of the above mentioned glycoproteins by affinity chromatography and their interaction with PNA-HRP on Western blot proved the presence of T-antigenic epitopes in them. Another significant observation is the absence of α -galactosyl residues in normal human platelet surface glycoproteins. Anti-gal recognizes, through its variable region, terminal galactose moieties in $\alpha(1\rightarrow3)$ linkage, and to a lesser extent, those in $\alpha(1\rightarrow2)$ and $\alpha(1\rightarrow6)$ linkages (2,18). The lack of interaction between anti-gal HRP with any of the glycoproteins even after neuraminidase treatment indicated the absence of α -galactosyl

epitopes in them. On the Western blot, in addition to the glycoproteins detected by peanut agglutinin, jacalin interacted with GP 52, GP VIII and GP Ib β . This observation points to the probable presence of T-antigenic structures in them as they do not contain α -galactosyl residues. The superior detection power of jacalin could be attributed to its higher affinity towards the T-antigenic structures. Hortin and coworkers have demonstrated the occurrence of a single T-antigenic structure (even when sialated) as adequate in mediating the binding of a glycoprotein to jacalin (15).

The relative intensity of staining between the glycoprotein bands varied significantly with the different methods of staining. The most intensely staining bands with CBB and amidoblack were those of GP IIb α and GP IIIa, which contain a considerable proportion of the protein of the platelet surface and are less glycosylated than most other membrane glycoproteins. The intensity of staining for GP IIb α with jacalin-HRP was very strong, but it was very weak for GP IIIa. However with Con A/HRP, the intensity of staining for GP IIb α was very weak and it was strong with GP IIIa. The differential Con A binding illustrates the presence of predominant N-linked branched mannose structures without fucose substitution in GP IIIa and its scarcity in GP IIb α as reported earlier (19). The above observations further confirmed the identity of glycoprotein bands. The higher intensity of jacalin-binding with GP IIb α reveals the presence of abundant T-antigen containing O-linked oligosaccharide chains in the glyco-protein compared to GP IIIa. In most of the platelet surface glycoproteins like GP IIIa, GP IIIb, GP V, GP VIa, GP Ib β and GP 17, the predominant N-linked

oligosaccharide chains were of the high mannose and or hybrid types. The absence of any inhibition for Con A binding with 15mM α MG revealed the absence of biantennary complex type oligosaccharide side chains in these glycoproteins. However biantennary complex type is the major N-linked oligosaccharide chains of GP VIb and GP VIII whereas GP IIb β contains all the three types. The biantennary complex type oligosaccharide chains are known to bind only weakly to Con A and can be eluted with 15mM α MG. High mannose and hybrid type oligosaccharide chains establish strong interaction with Con A and require 500mM α MG for elution (20).

The present study revealed the presence of several potential T-antigen containing glycoproteins on the surface of human platelets. Desialation of these glycoproteins during bacterial or viral infection can expose the cryptic antigenic structures leading to autoimmune response against the platelet surface. In many cases of ITP, autoimmune antibody is directed against auto antigenic determinants of a specific platelet membrane GP IIb-GP IIIa or Ib-IX complex (21). The pathogenesis of ITP in children most often presents acutely following a viral infection (22). The expression of either T-antigen or α -galactosyl residues in pathological

conditions is expected to parallel with a depression in the plasma levels of corresponding antibodies (23). However, owing to the nonspecificity of such antibody assays, the antigen specific assays are stated to be more useful in making a diagnosis of ITP.

In human tissues, the terminal α -galactosyl epitopes recognized by anti-gal are suppressed which maintains the immunological balance. However, this suppression is not absolute. The presence of cryptic terminal α -galactosyl residues in human RBC as well as availability of exposed terminal α -galactosyl residues on senescent human RBC had been noted (2). The present study proved the absence of either cryptic or exposed α -galactosyl residues on the normal human platelet surface glycoproteins.

Acknowledgement

The authors gratefully acknowledge the grant from DST, India-Young Scientist Award Project. We also gratefully acknowledge Dr. P.S. Appukuttan, Department of Biochemistry, Sree Chitra Tiruna! Institute of Medical Sciences and Technology, Trivandrum, India for his critical evaluation of the manuscript.

REFERENCES

1. Woods, V.L., Wolff, L.E. and Keller, D.M. (1986) Resting platelets contain a substantial centrally located pool of glycoprotein IIb-IIIa complex which may be accessible to some but not other extracellular proteins. *J. Biol. Chem.* 261(32), 15242-15251.
2. Galili, U., Rachmilewitz, E.A., Peleg, A. and Flechner, I. (1984) A unique natural human IgG antibody with anti-alpha-galactosyl specificity. *J. Exp. Med.*, 160(5), 1519-1531.

3. Elices, M.J. and Goldstein, I.J. (1989) Biosynthesis of bi-, tri- and tetraantennary oligosaccharides containing alpha-D-galactosyl residues at their nonreducing termini. Branch specificity of the Ehrlich tumor cell alpha (1,3) -galactosyltransferase. J. Biol. Chem. 264 (3), 1375-1380.
4. Jennings, L.K. and Philips, D.R. (1982) Purification of glycoproteins IIb and III from human platelet plasma membranes and characterization of a calcium-dependent glycoprotein IIb-III complex. J. Biol. Chem. 257(17), 10458-10466.
5. Kohn, J. and Wilchek, M. (1984). The use of cyanogen bromide and other novel cyanylating agents for the activation of polysaccharide resins. Appl. Biochem. Biotech. 9, 285-305.
6. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(259), 680-685.
7. Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A 76 (9), 4350-4354.
8. Suresh Kumar, G., Appukuttan, P.S. and Basu, D. (1982) Alpha-D-Galactose- specific lectin from jack fruit (*Artocarpus integra*) seed. J. Biosci. 4, 257-261.
9. Surolia, A., Prakash, N., Bishayee, S. and Bachhawat, B.K. (1973) Isolation and comparative physico-chemical studies of Concanavalin A from *Canavalia ensiformis* and *Canavalia gladiata*. Ind. J. Biochem. Biophys. 10 (3), 145-148.
10. Lotan, R. Skutelsky, E., Danon, D. and Sharon, N. (1975) The purification, composition, and specificity of the anti-T lectin from peanut (*Arachis hypogaea*). J. Biol. Chem. 250 (21), 8518-8523.
11. Jaison, P.L. and Appukuttan, P.S. (1992) Rapid isolation of human plasma anti-alpha-galactoside antibody using sugar-specific binding to sugar galactomannan or agarose. Ind. J. Biochem. Biophys. 29(3), 266-270.
12. Hack, N. and Crawford, N. (1984) Two-dimensional polyacrylamide- gel electrophoresis of the proteins and glycoproteins of purified human platelet surface and intracellular membranes. Biochem J. 222 (1), 235-246.
13. Sastry, M.V., and Surolia, A. (1986) Intrinsic fluorescence studies on saccharide binding to *Artocarpus integrifolia* lectin. Biosci. Reports 6 (10), 853-860.

14. Mahanta, S.K., Sastry, M.V. and Surolia, A. (1990). Topography of the combining region of a Thomsen-Friedenreich-antigen-specific lectin jacalin (*Artocarpus integrifolia agglutinin*) - A thermodynamic and circular-dichroism spectroscopic study. *Biochem. J.* 265 (3), 831-8.
15. Hortin, G.L. and Trimpe, B.L. (1990) Lectin affinity chromatography of proteins bearing O-linked oligosaccharides: application of jacalin-agarose. *Anal. Biochem.* 188 (2), 271-277.
16. Pereira, M.D., Kabat, E.A., Lotan, R. and Sharon, N. (1976) Immunochemical studies on the specificity of the peanut (*Arachis hypogaea*) agglutinin. *Carbohydr. Res.* 51(1), 107-118.
17. McGregor, J.L., Clemetson, K.J., James, E., Greenland, T. and Dechavanne, M. (1979) Identification of human platelet glycoproteins in SDS-polyacrylamide gels using ¹²⁵I labelled lectins. *Thrombosis Res.* 16 (5-6), 825-831.
18. Wislander, J., Mansson, O., Kallin, E., Gabrielli, A., Nowack, H. and Timpl, R. (1990) Glycoconjugate J. 7, 85-100.
19. Clezardin, P., McGregor, J.L., James, E., Dechavanne, M. and Clemetson, K.J. (1984) In : Marker proteins in inflammation Eds. Arnaud, J. Bienvenu and P. Laurent, Walter de Gruyter, Berlin, New York. Vol. II, p. 601-604.
20. Osawa, T. and Tsuji, T. (1987) Fractionation and structural assessment of oligosaccharides and glycopeptides by use of immobilized lectins. *Ann. Rev. Biochem.* 56, 21-42.
21. Woods, V.L., Oh, E.H., Mason, D. and McMillan, R. (1984) Autoantibodies against the platelet glycoprotein IIb/IIIa complex in patients with chronic ITP. *Blood* 63(2), 368-375.
22. McCrae, K.R., Samuels, P. and Schreiber, A.D. (1992) Pregnancy associated thrombocytopenia: pathogenesis and management. *Blood* 80 (11), 2697-2714
23. Chen, Y., Jain R.K., Chandrasekharan, E.V. and Matta, K.L. (1995) Use of sialylated or sulfated derivatives and acrylamide copolymers of Galbeta 1, 3GalNAc alpha- and GalNAc alpha- to determine the specificities of blood group T- and Tn - specific lectins and the copolymers to measure anti-T and anti-Tn antibody levels in cancer patients. *Glycoconjugate J.* 12 (1), 55-62.
24. Berndt, M.C., Philips, D.R. (1981). Purification and preliminary physicochemical characterization of Human Platelet Membrane Glycoprotein V. *J. Biol. Chem.* 256, 59-65.