Volume 327, number 1, 49–53 FEBS 12719 July 1993

Additional GPI-anchored glycoproteins on human platelets that are absent or deficient in paroxysmal nocturnal haemoglobinuria

János Polgár[a], Jeannine M. Clemetson[a], Daniela Gengenbacher[b] and Kenneth J. Clemetson[a]

[a]*Theodor Kocher Institute, University of Berne, CH-3012 Berne, Switzerland* and [b]*Department of Internal Medicine, Kantonsspital Basel, CH-4031 Basle, Switzerland*

Received 7 April 1993: revised version received 10 May 1993

In order to detect novel glycophosphatidylinositol (GPI)-anchored platelet proteins, human platelets were incubated with PI-specific phospholipase C (PI-PLC) and the supernatant was analysed by PAGE and silver-staining for additional protein bands. PI-PLC treatment resulted in the appearance of at least two additional novel GPI-linked glycoproteins (GP), GP500 and GP175, in the supernatant. Their presence on the platelet plasma membrane surface was demonstrated by periodate/[^{3}H]borohydride surface-labelling. Activation of platelets did not enhance the amount of GP500 and GP175 that could be cleaved by PI-PLC. In Triton X-114 phase partitioning of platelet membranes the membrane form of GP175, mfGP175, was in the Triton phase while mfGP500 was found in the water phase. Neither GP500 nor GP175 were present in the supernatant of surface-labelled platelets treated with PI-PLC from 4 patients, diagnosed as having paroxysmal nocturnal haemoglobinuria (PNH), but the supernatant from platelets from healthy volunteers treated the same way contained both.

Platelet; Glycoprotein; GPI-anchor; PI-PLC; PNH

1. INTRODUCTION

The attachment of proteins to the cell membrane through a glycosylphosphatidylinositol (GPI) moiety as an alternative anchor has been described for a large number of proteins [1–3]. Such anchors can be cleaved by phosphatidylinositol-specific phospholipases e.g. by phospholipase C (PI-PLC) [4].

All of the proteins deficient on affected blood cells in paroxysmal nocturnal haemoglobinuria (PNH) are GPI-anchored proteins [5] and, in most cases, the disorder is caused by defects in the synthesis of the anchor [6,7].

Although three known GPI-anchored proteins have been demonstrated on the surface of platelets as well as other cells [8–11] no systematic study has been carried out. We have analysed glycoproteins released from platelets by PI-PLC and have examined their presence on platelets from PNH patients.

2. MATERIALS AND METHODS

2.1. *Materials*

Kodak X-OMAT film, leupeptin, Triton X-114, thrombin, wheat germ agglutinin and prestained molecular weight standard mixture were from Sigma (St. Louis, MO), purified PI-PLC from Boehringer (Mannheim, Germany), phospholipase C (10% PI-specific activity), *N*-acetylglucosamine and *N*-ethylmaleimide were from Fluka (Buchs, Switzerland), [^{3}H]$NaBH_4$ from Amersham International (UK). Benzyloxy-carbonyl-Leu-Leu-Tyr-diazomethyl ketone (ZLLY) [12] was kindly provided by Dr Elliot Shaw, Friedrich Miescher Institute, Basle and anti decay accelerating factor (DAF) MoAb by Dr David J. Anstee, South Western Regional Blood Transfusion Centre, Bristol, UK.

Correspondence address. K.J. Clemetson, Theodor Kocher Institute, University of Berne, Freiestrasse 1, CH-3012 Berne, Switzerland. Fax: (41) (31) 65 37 99.

2.2 *Cells*

Platelets from healthy volunteers were isolated from buffy coats, less than 12 h after blood collection, obtained from the Central Laboratory of the Swiss Red Cross Blood Transfusion Service. Platelets from four patients diagnosed as having PNH (current platelet counts were 11, 24, 140 and 167×10^9 platelets/l) and from controls were isolated from EDTA-anticoagulated blood samples, less than 4 h after blood collection. The platelets were isolated by centrifugation and washed twice with 12 mM sodium citrate, 30 mM glucose, 120 mM NaCl, 10 mM EDTA pH 6.5 (buffer 1) and once with 4 mM glucose, 120 mM NaCl, 5 mM EDTA and 10 mM Tris-HCl pH 7.4. (buffer 2) (As judged by light microscopy, contamination with leukocytes and erythrocytes were less than $1:10^5$ and $1:10^4$ platelets, respectively.) Human erythrocytes were isolated from buffy coats by centrifugation and washed twice with buffer 1 and once with buffer 2. A crude preparation of peripheral blood leukocytes (about 80% leukocytes with platelets and erythrocytes) was obtained from buffy coats. After centrifugation at $1{,}000 \times g$ for 5 min, the interface containing the nucleated white cells was incubated with 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA pH 7.4 on ice for 10 min. The cells were centrifuged and washed twice with buffer 1 and once with buffer 2 by centrifuging at $600 \times g$ for 5 min. The supernatants and the top layer of the pellets were discarded.

2.3. *Surface-labelling by the periodate/[^{3}H]$NaBH_4$ method*

Washed platelets were surface-labelled by the periodate/[^{3}H]$NaBH_4$ method [13] as optimized for platelets by Steiner et al. [14].

2.4. *Treatment of washed platelets by PI-PLC*

Samples containing $5–20 \times 10^9$ platelets/ml were incubated with 0.2 U/ml PI-PLC, or 2 U/ml phospholipase C containing 10% PI-specific

activity of total, in the presence of 2 mM *N*-ethylmaleimide in buffer 2 at 37°C for 30 min. After incubation the samples were centrifuged at 10,000 × *g* and 4°C for 10 min and the supernatants were centrifuged again at 100,000 × *g* and 4°C for 1 h. Because of the limited sample volume the last centrifugation was omitted in the experiments with samples from PNH patients and the related control samples The supernatants were analysed by SDS-PAGE/silver staining or by SDS-PAGE/fluorography. Samples treated on the same way but without any PI-PLC were used as negative controls.

2.5. *Activation of platelets*

For some experiments platelets (1×10^9/ml) were activated with 0.5, 1 or 2 U/ml thrombin at 37°C for 5 min before treatment with PI-PLC.

2.6. *Triton X-114 phase partition*

Platelet plasma membranes were isolated [15] and Triton X-114 phase separation was performed as described by Clemetson et al. [16].

2.7. *Affinity chromatography with wheat germ agglutinin (WGA)*

The water phase and the Triton phase (diluted 10 times with 20 mM Tris-HCl, pH 7.4) of the Triton X-114 phase partition experiment and the supernatant from PI-PLC treated and control platelets were loaded individually on WGA columns The bound material was eluted with 2.5% *N*-acetylglucosamine and analysed by SDS-PAGE/silver-staining.

2.8 *SDS-PAGE*

SDS-PAGE was performed according to Laemmli [17]. The gels were silver-stained by the method of Morrissey [18] or were prepared for fluorography [19].

3. RESULTS

In order to find novel GPI-linked platelet proteins, washed human platelets were incubated with PI-PLC and the supernatant was analysed for additional protein bands compared to an untreated control in a silver-stained gel. In preliminary experiments, the concentration of platelets and PI-PLC, the type and concentration of protease inhibitor and the time of incubation were optimized. Since platelets incubated at 37°C release proteases (mostly calpains) it was necessary to add inhibitors that do not affectPI- PLC activity. ZLLY, a specific calpain inhibitor, leupeptin and *N*-ethylmaleimide were examined as protease inhibitors. ZLLY was particularly effective at low concentrations in inhibiting cleavage of GPIba and GPV from the platelet membranes, however, it was only available in small amounts. Incubation of platelets with PI-PLC resulted in the appearance of two protein bands, GP500 and GP175, present only in the supernatant of PI-PLC treated sample (Fig. 1, lane B) and absent in the supernatant of the negative control (Fig. 1, lane A). Any of the three inhibitors, mentioned before, were sufficient to inhibit proteolysis of GP500 and GP175. In the experiment shown in Fig. 1, *N*-ethylmaleimide was used as an effective inhibitor of GP500 and GP175 proteolysis although some cleavage of GPIb and GPV still occurred. Highly purified PI-PLC and a phospholipase C preparation containing 10% PI-specific activity of total were equally effective in causing GP500 and GP175 to appear in the supernatant. When the supernatant from PI-PLC

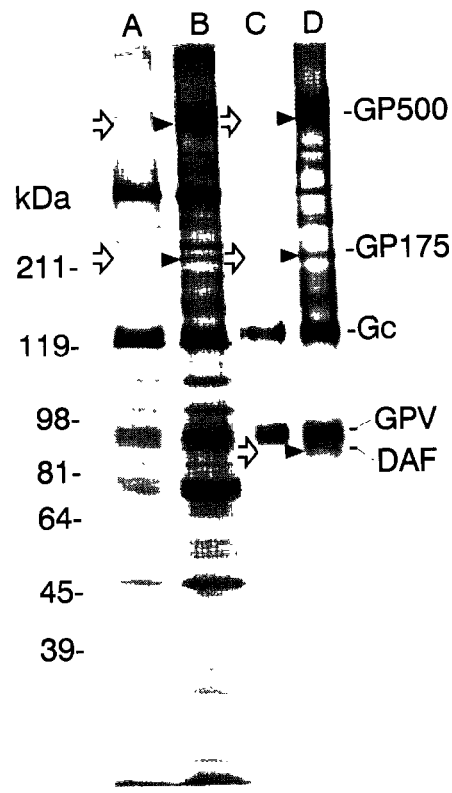

Fig. 1. Electrophoretic analysis of platelet supernatants and eluates from WGA column. (A) Supernatant of platelets incubated without PI-PLC (B) Supernatant of platelets incubated with PI-PLC. A and B were loaded on a WGA column and the bound material was eluted with GlcNAc. (C) The eluate from WGA using A. (D) The eluate from WGA using B. The samples were denatured under reducing conditions and separated on a 4 5–15% gradient polyacrylamide gel that was then silver-stained GPV, glycoprotein V; Gc, glycocalicin (the major proteolytic fragment of GPIb).

treated platelets was loaded on a WGA column both GP500 and GP175 were bound and could be eluted with *N*-acetylglucosamine showing that they are glycoproteins (Fig. 1, lane D). The positions of prestained marker proteins correlated poorly with the positions of glycoproteins of known mass in the gel. When the two novel glycoproteins were compared to glycoproteins of known mass in gradient polyacrylamide gels under non-reducing conditions, GP500 was above the position of thrombospondin trimer (420 kDa [20]) and GP175 was just above GPIb (170 kDa [21]) so that their masses are about 500 kDa and 175 kDa, respectively. Comparison of the migration of GP500 and GP175 under nonreducing and reducing conditions showed that both migrated slightly more slowly under reducing conditions (not shown). This indicates the presence of intramolecular disulphide bonds.

In order to rule out a leukocyte or erythrocyte origin of these novel glycoproteins, platelets (2×10^{10}/ml), leukocytes (3×10^7/ml) and erythrocytes (3×10^8/ml) were incubated with PI-PLC and the supernatants were analysed for the presence of GP500 and GP175 by SDS-

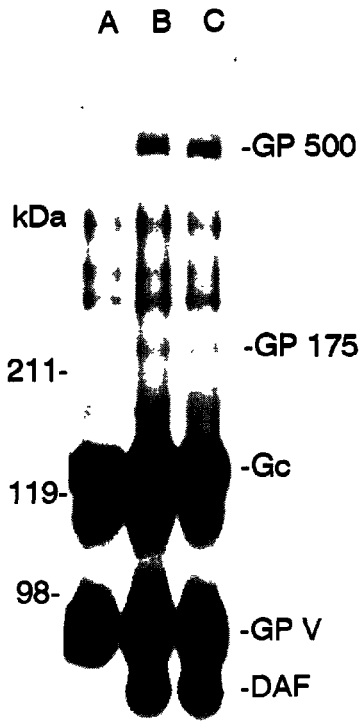

Fig. 2. Fluorogram of platelet supernatants. Supernatants of PI-PLC treated and untreated surface-labelled platelets were separated by SDS-PAGE and the gel was prepared for fluorography. (A) Supernatant of untreated platelets. (B,C) Supernatants of PI-PLC treated platelets. (B) purified PI-PLC. (C) Phospholipase C preparation with PI-specific activity 10% of total. In order to demonstrate GP500, GP175 and DAF, in the same gel, a 4.5–10% gradient gel was used and the run was stopped only when the 81 kDa prestained marker reached the bottom of the gel. Under these conditions glycoprotein V (GPV) and DAF were clearly separated. Gc: glycocalicin (the major proteolytic fragment of GPIb).

PAGE/silver staining. No protein band corresponding to GP500 visible in platelet supernatant was found in supernatants from leukocytes and erythrocytes while a weak band with an intensity about one fifth of the GP175 band in platelet supernatant was detected in the supernatant of leukocytes but not of erythrocytes.

To show that GP500 and GP175 were on the platelet surface, before cleavage with the enzyme, surface-labelled platelets were treated with PI-PLC and the supernatant was analysed by gel electrophoresis and fluorography. Both GP500 and GP175 were labelled (Fig. 2). In Fig. 2 another labelled glycoprotein is visible only in the PI-PLC-supernatant (lanes B and C) and absent in the supernatant of the negative control (lane A). This labelled glycoprotein was shown by Western blotting to be DAF, a known GPI-linked glycoprotein on platelets [8,9] (data not shown).

Triton X-114 phase separation of platelet plasma membrane was performed and glycoproteins from the water phase and Triton phase were isolated by affinity chromatography on wheat germ agglutinin and the samples were analysed by SDS-PAGE/silver-staining for the presence of the membrane form of GP500 and GP175 (mfGP500, mfGP175). Because of the hydrophobic character of the GPI-anchor both glycoproteins were expected to be present in the Triton phase similar to other GPI-anchored proteins. Surprisingly, only mfGP175 but not mfGP500 was in the Triton phase. mfGP500 was predominantly in the water phase (data not shown).

The amount of GP500 and GP175 that could be released with PI-PLC from resting and activated platelets were compared. No difference was found in the intensity of silver stained bands of GP500 and GP175 of PI-PLC treated supernatants from resting compared with thrombin-activated platelets. Thrombin activation itself did not result in the release of GP500 and GP175.

It was expected that GP500 and GP175, as other GPI-anchored proteins, would be absent or deficient in PNH. Surface-labelled platelets from 4 PNH patients were treated with PI-PLC and the supernatants were analysed by SDS-PAGE/fluorography. In all cases platelets from healthy volunteers were used as positive (PI-PLC treated) and negative (no PI-PLC) controls. No labelled protein band in the position of either GP500 or GP175 could be identified in any of the 4 PNH patients (Fig. 3).

4. DISCUSSION

The proteins attached to the platelet plasma membrane by GPI-anchors described till now are: CD55 (decay accelerating factor or DAF) a glycoprotein with a mass of about 70 kDa [8,9], the 20 kDa CD59 molecule (which has a large number of alternative names e.g. membrane inhibitor of reactive lysis or homologous restriction factor 20) [10] and C8 binding protein which has a mass of 50 kDa [11]. Besides these complement regulatory proteins which are generally accepted to be GPI-linked, Dhar and Shukla [22] have reported an additional GPI-anchored platelet membrane glycoprotein in the higher molecular weight region which they suggested is GPIb. However, GPIb is a transmembrane glycoprotein with a known transmembrane domain linked to the cytoskeleton and does not contain consensus sequences for GPI attachment [23]. In our experiments we could not detect any cleavage of GPIb by PI-PLC. It is easily cleaved to a water-soluble fragment, glycocalicin, by several proteases including calpain. It seems likely that glycocalicin released by proteases such as calpain was responsible for the GPIb that they reported. We also could detect glycocalicin in the supernatant of PI-PLC treated platelets but the same amount of glycocalicin was also present in the supernatant of platelets that were incubated without PI-PLC. In Fig. 3 but not Fig. 2, GPIb is visible in all of the samples, including the supernatants. In the case of the PNH patients and related controls the final centrifugation was omitted and the microvesicles, formed as a result of

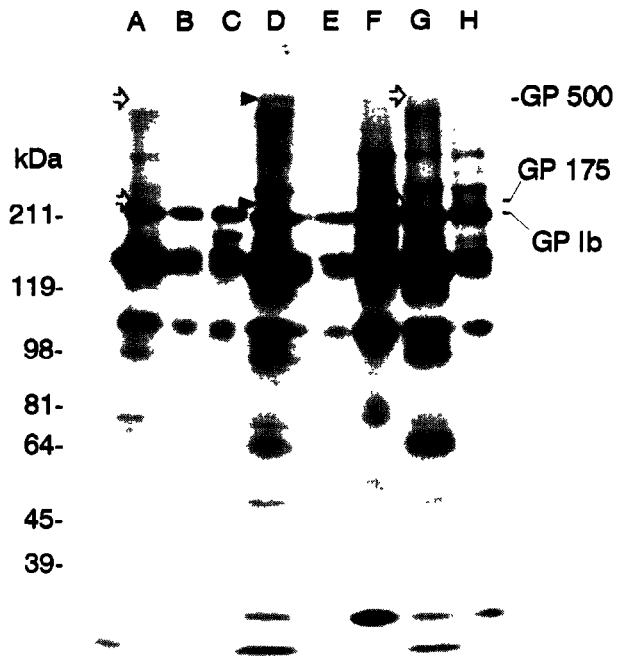

Fig. 3. Fluorogram of surface-labelled control and PNH platelet lysates, and supernatants and pellets from surface-labelled control and PNH platelets. (A,B,C) PNH patient, (A) PI-PLC supernatant, (B) pellet after PI-PLC treatment, (C) platelet lysate. (D–H) Control, (D) PI-PLC supernatant, (E) pellet after PI-PLC treatment, (F) platelet lysate, (G,H) supernatant and pellet from untreated platelets. Samples from a patient and a healthy volunteer were run in 4.5–15% gradient polyacrylamide gel and the gel was prepared for fluorography. Arrowheads indicate the positions of GP500 and GP175.

vesiculation from the platelet membranes during the incubation, were not removed. The GPIb seen is thus from vesicles. Its intensity is the same in the PI-PLC treated (lane D) and untreated (lane G) supernatants of control platelets clearly showing that its appearance is not PI-PLC dependent.

Incubation of platelets with PI-PLC resulted in the release of two additional GPI-linked platelet membrane glycoproteins, GP500 and GP175, in the supernatant showing that they are anchored to the plasma membrane by a GPI-anchor. The fact that no protein band in the position of GP500 and only a weak band in the position of GP175 (in case of leukocytes) could be detected in the supernatants of PI-PLC-treated leukocytes and erythrocytes, with concentrations several hundred times higher then the leukocyte and erythrocyte contamination of platelet samples used, confirms the platelet origin of these two novel glycoproteins described. Their location on the outer surface of the plasma membrane was further supported by surface-labelling of platelet samples. Fig. 2 shows that the two novel glycoproteins and DAF appeared simultaneously in the PI-PLC supernatant. Based on their sensitivity to PI-PLC two groups of DAF molecules can be distinguished on human blood cells. Only 10–15% of DAF is released from erythrocytes by PI-PLC compared with 60–80% from leukocytes [2]. Comparison of the amount of DAF in the supernatant and remaining pellet of PI-PLC treated platelets suggests that platelet DAF, like DAF on leukocytes, is readily released by PI-PLC. CD59 and C8 binding protein could not be directly identified under the conditions used here. There is a slight difference between the PI-PLC treated and untreated supernatants in the expected position of these proteins (Fig. 1, lanes A and B). Since they had already been identified on platelets we did not change our experimental conditions to characterise them better.

To date at least nine GPI-anchored proteins have been reported to be missing from blood cells in PNH [5]. The variety of proteins involved and the fact that mRNA of DAF from PNH cells is fully expressed [6] clearly show that the defect is post-translational. Recent evidence suggests that it is caused by the defective synthesis of the GPI anchor [7]. It is difficult to connect expression of GP500 and GP175 with the degree of thrombocytopenia since 2 patients had severe thrombocytopenia while 2 had platelet counts in the low normal range. GP500 and GP175 were undetectable in all of them. In addition to GP500 and GP175 there are some other bands only visible, or stronger in PI-PLC supernatants from controls compared to PI-PLC supernatants from PNH patients (Fig. 3, lanes D and A). The reason is not clear but decreased vesiculation from PNH platelets might possibly be a contributory factor [24].

The result of experiments with thrombin-activated platelets showed that no additional GP500 and GP175 was expressed on the cell surface during platelet activation and the activation itself did not results in the release of these glycoproteins from the membrane surface.

Despite the presence of the hydrophobic GPI-anchor, mfGP500 was found in the water phase after Triton X-114 phase separation of platelet plasma membrane. This suggests that the protein part of the molecule is strongly hydrophilic, perhaps due to high glycosylation, which outweighs the hydrophobic character of the anchor.

The biological function of these two glycoproteins, GP500 and GP175, is not yet known, so that the question whether their lack might contribute to the symptoms of PNH remains open.

Acknowledgements. The work was supported by the Roche Research Foundation, the Swiss National Science Foundation (70UP-029647 and 31–32416.91) and the Central Laboratory, Swiss Red Cross Blood Transfusion Service, Berne (supply of buffy coats).

REFERENCES

[1] Ferguson, M.A.J. and Williams A.F. (1988) Annu. Rev. Biochem 57, 285–320.
[2] Low, M.G. (1987) Biochem. J. 244, 1–13
[3] Cross, G.A.M. (1990) Annu. Rev. Cell Biol. 6, 1–39.
[4] Ferguson, M.A J., Haldar, K. and Cross, G A.M. (1985) J. Biol. Chem. 260, 4963–4968.
[5] Rosse, W F. (1990) Blood 75, 1595–1601.

[6] Stafford, H.A., Tykocinski, M.L., Lublin, D.M., Holers, V.M., Rosse, W.F., Atkinson, J.P. and Medof, M.E. (1988) Proc. Natl. Acad. Sci. USA 85, 880–884.
[7] Doering, T.L , Masterson, W.J., Hart, G.W. and Englund, P.T. (1990) J. Biol. Chem. 265, 611–614.
[8] Nicholson-Weller, A., March, J.P., Rosen, C E., Spicer, D.B. and Austen, K.F. (1985) Blood 65, 1237–1244.
[9] Kinoshita, T., Medof, M.E., Silber, R. and Nussenzweig, V. (1985) J. Exp. Med. 162, 75–92.
[10] Morgan, B.P. (1992) Biochem. J. 282, 409–413.
[11] Blaas, P., Berger, B., Weber, S., Peter, H.H. and Hänsch, G.M (1988) J. Immunol. 140, 3045–3051.
[12] Anagli, J., Hagmann, J. and Shaw, E. (1993) Biochem. J. 289, 93–99.
[13] Gahmberg, C.G. and Andersson, L.C. (1977) J. Biol. Chem. 252, 5888–5894.
[14] Steiner, B., Clemetson, K.J and Lüscher, E.F. (1983) Thromb. Res. 29, 43–52.
[15] Clemetson, K.J., Capitano, A. and Lüscher, E.F. (1979) Biochim. Biophys. Acta 553, 11–24.
[16] Clemetson, K.J., Bienz, D., Zahno, M.L. and Lüscher, E.F. (1984) Biochim. Biophys. Acta 778, 463–469.
[17] Laemmli, U K. (1970) Nature 227, 680–685.
[18] Morrissey, J.H. (1981) Anal. Biochem. 117, 307–310.
[19] Laskey, R.A. and Mills, A.D. (1975) Eur. J. Biochem. 56, 335–341
[20] Lawler, J., Derick, L.H., Connolly, J.E., Chen, J.H. and Chao, F C. (1985) J. Biol. Chem. 260, 3762–3772.
[21] Wicki, A.N., Clemetson, J.M., Steiner, B., Schnippering, W. and Clemetson, K.J. (1992) in· Methods in Enzymology (Hawiger, J.J., Ed.) Platelets: Receptors, Adhesion, Secretion, Part B, Vol. 215, pp. 276–288, Academic Press, San Diego, CA.
[22] Dhar, A. and Shukla, S.D. (1991) Biochim. Biophys Acta 1091, 15–21.
[23] Lopez, J.A., Chung, D.W., Fujikawa, K., Hagen, F.S., Davie, E.W. and Roth, G.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2135–2139.
[24] Whitlow, M., Iida, K., Marshall, P., Silber, R. and Nussenzweig, V. (1993) Blood 81, 510–516.