Biotinylation of glycan chains in β_2 glycoprotein I induces dimerization of the molecule and its detection by the human autoimmune anti-cardiolipin antibody EY2C9

Arnaud DUPUY D'ANGEAC^{*1}, Ilias STEFAS[†], Hubert GRAAFLAND[‡], Frédéric DE LAMOTTE[§], Marcel RUCHETON[†], Caroline PALAIS[†], Anna-Karin ERIKSSON[†], Priscille BOSCH[†], Caroline ROSÉ[†] and Robert CHICHEPORTICHE^{*}

^{*}Laboratoire de Chimie Biomoléculaire, UM II, 8 rue de l'Ecole Normale, Montpellier 34216, Cedex 5, France, [†]ApoH-Technologies, IRD 911 avenue Agropolis, Montpellier 34032, France, [‡]Laboratoire d'Hématologie Générale, CHU Montpellier, 80 avenue Augustin Fliche, Montpellier 34295, Cedex 5, France, and [§]UMR 1096, INRA, 2 Place Viala, Montpellier 34060, Cedex 01, France

Binding of β_2 GPI (β_2 glycoprotein I), a human plasma protein, to AnPLs (anionic phospholipids) plays a key role in the formation of antiphospholipid antibodies involved in autoimmune diseases like antiphospholipid syndrome or systemic lupus erythematosus. We recently showed that binding of β_2 GPI to AnPLs was enhanced by biotinylation of its glycan chains with biotin-hydrazide. In the present study, we investigated why this chemical modification of β_2 GPI increased both its affinity for AnPLs and its recognition by anti-cardiolipin antibodies. Electrophoretic analysis showed that: (i) high molecular mass β_2 GPI (dimers and other oligomers) covalently coupled by imine bonds, were present in variable amounts in oxidized β_2 GPI and in β_2 GPI-bh (β_2 GPI-biotin-hydrazide), but were absent in native β_2 GPI; (ii) binding of β_2 GPI-bh to phosphatidylserine-coated microtitre plates generated high molecular mass polymers in a time-dependent manner. Native β_2 GPI did not polymerize in these conditions.

These polymers did not bind more strongly to AnPLs than the monomer β_2 GPI. However, in solution at 1 μ M β_2 GPI-bh essentially appeared as a dimer as revealed by light-scattering analysis. SPR (surface plasmon resonance) analysis showed that the increased affinity of β_2 GPI-bh for AnPL monolayers was due to a lower dissociation rate constant compared with native β_2 GPI. Finally, the monoclonal human aCL (auto-immune anti-cardiolipin antibody) EY2C9 bound to β_2 GPI-bh but did not bind to monomeric native and oxidized β_2 GPI. It is likely that the dimeric quaternary structure of β_2 GPI-bh is in fact responsible for the appearance of the epitopes targeted by the EY2C9 antibody.

Key words: anionic phospholipid (AnPL), autoimmune anti-cardiolipin antibody (aCL), β_2 glycoprotein I (β_2 GPI), fluorimetry, light-scattering, surface plasmon resonance (SPR).

INTRODUCTION

The β_2 GPI (β_2 glycoprotein I), also designated apolipoprotein H, is a 50 kDa (326 amino acids) serum glycoprotein [1–3] containing 11 disulphide bridges and four N-linked glycosylation sites. β_2 GPI contains four SCRs (short consensus repeats) from the CCP (complement control protein)-type module also known as 'Sushi' domains and a fifth C-terminal domain. CCP are modules of 60 amino acid residues and they contain two disulphide bridges [4]. The fifth domain of β_2 GPI contains 84 amino acids and three disulphide bridges. It is involved in β_2 GPI binding to AnPLs (anionic phospholipids) such as phosphatidylserine and cardiolipin [5–7]. It contains a very electropositive sequence of amino acids (Lys²⁸²-Asn-Lys-Glu-Lys-Lys²⁸⁷) which are responsible for binding negatively charged molecules such as DNA, heparin [8] or AnPL. The 313–316 sequence and particularly Trp-316 are essential for cardiolipin binding [9]. Amino-acid residues 311–317 allow the insertion of β_2 GPI into lipid membranes [10]. Many studies suggest that it may be a natural anti-coagulant. *In vitro* β_2 GPI inhibits intrinsic pathway activation [11], tenase [12] and prothrombinase [13] activities on the surface of activated

platelets, and synthetic phospholipid vesicles. β_2 GPI also binds to proteins such as calmodulin [14] or hepatitis B virus surface antigen [15,16], the membrane component of the hepatitis B virus.

Adhesion to AnPLs on low-density lipoproteins, platelets or apoptotic cells is believed to be the key property of β_2 GPI responsible for its physiological and pathological effects. The binding characteristics of β_2 GPI to artificial membranes expressing AnPL have been extensively studied [17–20]. In the presence of autoimmune antibodies with anti-cardiolipin activity (aCL), the affinity and maximal binding of β_2 GPI to AnPL monolayers [20] or solid state AnPL are greatly increased [21]. aCL either induces a conformational change of the binding site [22–26] or provokes dimerization of the β_2 GPI molecule [20,27,28]. Associations of β_2 GPI and AnPL are considered to act as antigens for aCLs associated with diseases such as systemic lupus erythematosus or anti-phospholipid syndrome. Several research groups have shown that AnPL-binding proteins such as β_2 GPI or prothrombin are the main target of aCLs [29–31]. It is not clear whether aCLs react with the interface between AnPL and β_2 GPI, using β_2 GPI itself after a conformation change [31–34] or with adducts of oxidized AnPL and β_2 GPI such as 1-palmitoyl-2-(5-oxovaleroyl)

Abbreviations used: aCL, autoimmune anti-cardiolipin antibody; AnPL, anionic phospholipids; β_2 GPI, β_2 glycoprotein I; β_2 GPI-bh, β_2 GPI-biotin-hydrazide; CCP, complement control protein; ECL, enhanced chemiluminescence; GAHIG, goat anti-(human IgM); GAMIG, goat anti-(mouse IgG); HPA, hydrophobic association; HRP, horseradish peroxidase; mAb, monoclonal antibody; 2ME, 2-mercaptoethanol; MW, molecular mass weight-average; NIH, National Institutes of Health; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; PS, phosphatidylserine; SAV-HRP, streptavidin-HRP; SCR, short consensus repeat; SPR, surface plasmon resonance; TBST, TBS-Tween 20; TMB, 3,3',5,5', tetramethylbenzidine.

¹ To whom correspondence should be addressed (email dangeac@univ-montp2.fr).

phosphatidyl choline [35], or the oxLig-1 [36]. In addition, some patients with antiphospholipid syndrome or systemic lupus erythematosus have antibodies that react with β_2 GPI layered on oxygenated polystyrene microtitre plates [37]. These anti- β_2 GPI antibodies presumably recognize a cryptic epitope of the β_2 GPI molecule exposed by its fixation to the microtitre plate. However, binding to AnPL is supposed to occur via domain V of β_2 GPI [7,17] whereas auto-immune anti- β_2 GPI reacts against epitopes located in domain IV or domain I of β_2 GPI [38,39]. Thus there is no direct relationship between the affinity for AnPL and antigenicity for aCLs, even though it was shown that β_2 GPI deprived from domain V by plasmin treatment was not able to bind aCLs [40].

The results reported in the literature on the importance of glycan chains in the β_2 GPI molecule are contradictory. On one hand, Brighton et al. [21] showed that different β_2 GPI preparations had different affinities for AnPL and attributed these differences to differences in glycosylation of the β_2 GPI molecule. On the other hand, Willems et al. [20] observed no difference in the dissociation constant of native β_2 GPI and its isoforms obtained by fast protein liquid chromatography in PC (phosphatidylcholine)/PS (phosphatidylserine) or PC/cardiolipin monolayer assays. In addition, the affinity of various recombinant β_2 GPI preparations produced in baculovirus infected sf9 cells appears to be similar to that of the native molecule [18,41–43], although they are less glycosylated than native β_2 GPI.

We recently showed that oxidation and biotinylation of β_2 GPI glycan chains induces an increase in its affinity for AnPL similar to that obtained by the addition of anti- β_2 GPI or aCL antibodies [43]. Dimerization of β_2 GPI is supposed to be the reason for such an increase in affinity for AnPL [20,44]. In addition, dimerization of β_2 GPI may facilitate binding of aCLs by increasing the density of epitopes [45]. Sheng et al. [27] observed that anti- β_2 GPI bind with high affinity to a mutant form of β_2 GPI which spontaneously forms dimers. In the present study we analysed the reasons for the high affinity of β_2 GPI-bh (β_2 GPI-biotin-hydrazide). Native, oxidized β_2 GPI and β_2 GPI-bh were investigated for the presence of dimeric or polymeric forms using SDS/PAGE and light-scattering. Fluorimetry was used to study the environment of Trp-316 close to the AnPL-binding site. Binding of native, oxidized β_2 GPI and β_2 GPI-bh to PC/PS monolayers was studied by SPR (surface plasmon resonance) and their kinetic constants were analysed. Finally, binding of the aCL EY2C9 to native, oxidized β_2 GPI and β_2 GPI-bh was assessed using Western and slot-dot blotting.

MATERIAL AND METHODS

Products

β_2 GPI, prepared from a pool of control donors by affinity chromatography was kindly given by ApoH-Technologies (Montpellier, France). The murine anti- β_2 GPI monoclonal antibody 9G1 (isotype IgG1) directed against the domain 1 of β_2 GPI [46] was kindly given by Dr J. Arvieux (Brest, France) and the aCL EY2C9 (isotype IgM) [47] was kindly given by Dr K. Ichikawa (Sapporo, Japan). PS and PC, NaBH₃CN, SAV-HRP (streptavidin horseradish peroxidase) and TMB (3,3',5,5'-tetramethylbenzidine) were purchased from Sigma (St Louis, MO, U.S.A.). Goat anti-(human IgM) labelled with HRP and directed against the Fc fragment of the IgM molecule (GAHIG-HRP), goat anti-(mouse IgG) labelled with HRP (GAMIG-HRP) were purchased from Immunotech (Marseilles, France). PBS was purchased from Eurobio (les Ulis, France).

Labelling of β_2 GPI

Biotinylation of β_2 GPI using EZ-link™ biotin-hydrazide (Pierce Chemicals, IL, U.S.A.) was performed as described previously [43]. Briefly, β_2 GPI (0.5 mg/ml) and sodium meta-periodate solution (10 mM) were allowed to react in 0.1 M sodium acetate buffer (pH 5.5) for 30 min at 4 °C in the dark. Glycerol (15 mM final concentration) was added for 5 min at 0 °C to stop the reaction and the sample was dialysed overnight against 0.1 M sodium acetate buffer (pH 5.5). Then EZ-link™ biotin-hydrazide (5 mM final concentration) was allowed to react with agitation for 2 h at room temperature. Finally, samples were dialysed overnight against PBS buffer. The final product was called β_2 GPI-bh. The number of biotins incorporated in our β_2 GPI-bh preparations was measured by displacing 2-(4'-hydroxyazobenzene) benzoic acid (HABA) bound to avidin (Pierce Chemicals, IL, U.S.A.). This number ranged from 3–10 without a significant change in affinity of β_2 GPI-bh for AnPL (results not shown). The protein concentration was measured using the Micro BCA Protein Assay (Pierce Chemicals, IL, U.S.A.).

Analysis of β_2 GPI bound to PS-coated microtitre plates by immunoblotting

In order to bind a detectable amount of β_2 GPI, microtitre plates (Nunc, MaxiSorb) were coated with 15 µg/well PS in 30 µl of ethanol and the plates were wrapped in foil and the liquid allowed to evaporate overnight at 4 °C. Then 100 µl of native and β_2 GPI-bh, at 3×10^{-7} and 10^{-6} M in PBS were added to the PS-coated microtitre plates. The plates were incubated for 1 h at 4 °C to allow binding and prevent rapid dissociation, since we observed that decreasing the temperature to 4 °C decreased the dissociation rate constant of native β_2 GPI and β_2 GPI-bh to solid-state PS [43]. Subsequently the plates were incubated for 1 h 30 min at room temperature in the presence or absence of 10 mM NaBH₃CN in PBS to reduce imine bonds. Finally, the plates were incubated for 1 h at 4 °C to achieve association and prevent dissociation of bound β_2 GPI. In other experiments native and β_2 GPI-bh at 3×10^{-7} M were added to the PS-coated microtitre plates and the plates were incubated for a period ranging from 0 to 18 h at room temperature. Next, the supernatant was removed and the plates were washed three times with PBS at 4 °C. The adsorbed proteins were collected by the addition of 40 µl of SDS/PAGE sample buffer in each well, submitted to SDS/8 % PAGE and transferred to nitrocellulose membranes. Then the membrane was cut into 1.5 mm wide strips and each strip was individually labelled with the anti- β_2 GPI mAb 9G1, directed against the first domain of β_2 GPI in association with GAMIG-HRP. As a control, strips were labelled with GAMIG-HRP alone. After the addition of Western blotting detection reagents (Amersham), Hyperfilm-ECL® (enhanced chemiluminescence) (Amersham) was used to detect luminescence. As the concentration of native, oxidized or β_2 GPI-bh increased from 3×10^{-7} to 10^{-6} M, increasing amounts of proteins were retained on microtitre plates and almost no β_2 GPI was retained when no PS was coated on the plates (results not shown).

Nitrocellulose slot-dot blotting

Various β_2 GPIs at 1 µM were layered as a slot on the dry nitrocellulose membrane. The membrane was immersed in TBST (TBS-Tween 20) [25 mM Tris/HCl, 500 mM NaCl, 0.005 % (v/v) Tween 20, pH 7.4] for 10 min, cut into slices as described above and each slice was incubated with various concentrations of the human IgM EY2C9 for 1 h 30 min, washed with TBST four times and incubated for 1 h with GAHIG-HRP directed against the Fc fragment of the IgM molecule. Next, it was washed four times with TBST. As a control for slot deposition, strips were visualized

with the murine anti- β_2 GPI mAb 9G1 in association with GAMIG-HRP. Strips were also labelled with GAHIG-HRP and GAMIG-HRP alone. After the addition of Western blotting detection reagents (Amersham), Hyperfilm-ECL[®] (Amersham) was used to detect luminescence.

Acrylamide quenching of Trp fluorescence

Fluorescence experiments were carried out on an Hitachi fluorescence spectrophotometer F-2500 (Hitachi High Technologies America Inc, CA, U.S.A.) using a 10 mm $\times$ 10 mm quartz fluorescence cuvette. For proteins containing tyrosine and tryptophan, tryptophan can be excited selectively at 290–295 nm. β_2 GPI solutions at 1 μ M in 20 mM Tris/HCl (pH 7.2) were excited at 290 nm with a slit width of 5 mm and an integration time of 0.05 s. The spectra were taken as the mean of 10 scans. The background of a solution without β_2 GPI was subtracted before determination of fluorescence intensities. Aliquots of stock solution of 5 M acrylamide in Tris/HCl were added to a 1 μ M solution of β_2 GPI and its concentration was varied in a 20 mM to 0.6 M range. The data were analysed using the Stern–Volmer equation.

$$\frac{I_0}{I} = 1 + K_a [Q] \quad (1)$$

where $[Q]$ is the acrylamide concentration in the sample, I_0 and I are the observed fluorescence intensities at 340 nm in the absence and the presence of quencher molecules. K_a , the quenching constant is determined graphically.

Liposomes

Mixtures of PC/PS (80:20), PC/CL (80:20) or PC at a final concentration of 10^{-6} M were evaporated and desiccated overnight. Lipids were dissolved in 2 ml of a solution containing 50 mM Tris/HCl, 120 mM NaCl, 3 mM CaCl_2 under agitation for 2 h at 40 °C, sonicated for 4 $\times$ 30 s with a Vibra-Cell (Sonics, CT, U.S.A.) and analysed by dynamic light-scattering on a Zetasizer nanoseries (Malvern, U.K.). The Z-average of the resulting liposomes was usually between 40 and 100 nm.

SPR studies of various β_2 GPIs

A BIAcore 2000 instrument (BIAcore AB, Uppsala, Sweden) was used to study the interactions of various β_2 GPIs with PC/PS or PC monolayers. The BIAcore HPA (hydrophobic association) sensor chip was washed with 20 μ l of octyl β -D-glucopyranoside. The liposome preparation, sonicated extemporaneously, was fused to the surface of the sensor flow cell at 1 μ l/min. Aliquots (5 μ l) of 10 mM NaOH were injected to remove incompletely fused liposomes. Each flow cell was prepared individually to obtain PC and PC/PS (80:20) monolayers in different flow cells of the same HPA sensor chip. All prepared surfaces provide a stable baseline for determination of various β_2 GPI binding specificities and kinetics. The lipid monolayers showed 2–3000 resonance unit values (results not shown). All studies were carried out at 20 °C at a flow rate of 5 μ l/min with 50 mM Tris/HCl, 120 mM NaCl at pH 7.4. No significant binding of native, oxidized β_2 GPI or β_2 GPI-bh was observed on PC monolayers. Sensorgrams obtained on PC monolayers were subtracted from sensorgrams obtained on PC/PS 80:20 monolayers. Association and dissociation rate constants were calculated by non-linear fitting of the primary sensorgram data using the BIA evaluation 4.1 software and the two models:

(i) The 1:1 Langmuir binding model described by the reaction scheme:

Where A is the β_2 GPI in solution and B the lipids. AB represents the β_2 GPI–lipid complex. Concentrations at $t = 0$ are: $A = [\beta_2\text{GPI}]$, $B = R_{\text{max}}$ where R_{max} is the amount of available β_2 GPI-binding sites on the lipid monolayer. $AB = 0$. The rate equations at time t are:

$$\frac{dB}{dt} = -(k_{\text{on}} \times A \times B - k_{\text{off}} \times AB) \quad (3)$$

$$\frac{dAB}{dt} = (k_{\text{on}} \times A \times B - k_{\text{off}} \times AB) \quad (4)$$

The equilibrium constant, K_D is calculated from rate constant:

$$K_D = \frac{k_{\text{off}}}{k_{\text{on}}} = \frac{1}{K_A} \quad (5)$$

(ii) We also used the two-state reaction model (conformational change) described by the reaction scheme:

Where A is the β_2 GPI in solution and B the lipids. AB represents the β_2 GPI–lipid complex and AB^* a secondary β_2 GPI–lipid complex. The concentrations at $t = 0$ are: $A = [\beta_2\text{GPI}]$, $B = R_{\text{max}}$ where R_{max} is the amount of available β_2 GPI binding sites on the lipid monolayer. $AB = 0$ and $AB^* = 0$. The rate equations at time t are:

$$\frac{dB}{dt} = -(k_{\text{on},1} \times A \times B - k_{\text{off},1} \times AB) \quad (7)$$

$$\frac{dAB}{dt} = (k_{\text{on},1} \times A \times B - k_{\text{off},1} \times AB) - (k_{\text{on},2} \times AB - k_{\text{off},2} \times AB^*) \quad (8)$$

$$\frac{dAB^*}{dt} = (k_{\text{on},2} \times AB - k_{\text{off},2} \times AB^*) \quad (9)$$

K_D was calculated from rate constants:

$$K_D = \frac{1}{\left(\frac{k_{\text{on},1}}{k_{\text{off},1}}\right) \times \left(1 + \frac{k_{\text{on},2}}{k_{\text{off},2}}\right)} \quad (10)$$

Light-scattering measurements

Dynamic and static light-scattering measurements were performed using a Zetasizer nanoseries from Malvern Instruments (Malvern, U.K.). The laser excitation was at 502 nm. Measurements were made at 20 °C at a 173° angle. Samples were spun for 30 min at 20000 g before analysis to remove large aggregates. The various β_2 GPI preparations were tested at concentrations ranging from 1–10 μ M in PBS. All measurements were performed in Hellma QS 3 mm quartz cuvettes (Hellma GmbH & Co, Germany) in a volume of 45 μ l. Size experiments were performed by dynamic light-scattering. The resulting auto-correlation function data were analysed by the cumulant method. Intensity size-distributions provided protein mean apparent diameters (nm). When the distribution was not monomodal, the peak with the smaller mean diameter was used. Fusion curves were obtained by increasing the temperature from 20 °C to 65 °C. The MW (weight-average) was calculated by static light-scattering using the equation:

$$\frac{KC}{R_0} = \frac{1}{M} + 2A_2C \quad (11)$$

Figure 1 Oxidation and biotinylation of β_2 GPI induces the formation of high MW polymers

Native, oxidized β_2 GPI and β_2 GPI-bh were labelled by AgNO_3 after SDS/8% PAGE in the absence (A) or presence (B) of 3×10^{-5} M 2ME. The data were scanned and analysed using the ImageJ software package NIH (National Institutes of Health, Bethesda, MD, U.S.A.). The percentage of each band was deduced from its position and intensity. The intensity of all bands is represented by 100%. Dimers represented 5% of oxidized β_2 GPI and 10% of β_2 GPI-bh in the absence of 2ME and 2% of oxidized β_2 GPI and 4% of β_2 GPI-bh in the presence of 2ME. No dimers appeared with native β_2 GPI.

where R_0 , the Rayleigh ratio of the analyte intensity to the incident intensity is measured using toluene as standard, K is an optical constant, M is the weight-average molecular mass of analyte, C is the concentration of analyte in g/ml and A_2 is the second virial coefficient. The molecular mass is calculated from the Debye plot of KC/R_0 versus C . No shape correction model was used. The refractive index increment (dn/dC) was 0.185 ml/g.

RESULTS

Oxidation of glycan chains of β_2 GPI induces covalent polymer formation

Figure 1 shows the SDS/PAGE profiles of native, oxidized β_2 GPI and β_2 GPI-bh preparations in solution as revealed by AgNO_3 . Native β_2 GPI appears as a single band at 50 kDa in the presence or absence of 3×10^{-5} M 2ME (2-mercaptoethanol) (Figures 1A and 1B). Dimers of approx. 90 kDa were present in oxidized β_2 GPI and in β_2 GPI-bh (Figure 1A). Dimers represented 5% of oxidized β_2 GPI and 10% of β_2 GPI-bh. When SDS/PAGE was performed in the presence of 3×10^{-5} M 2ME, the dimer concentration was decreased (2% and 4% for oxidized β_2 GPI and β_2 GPI-bh respectively, Figure 1B). However, polymeric β_2 GPI was still present, indicating that disulphide bonds may be also involved in polymer formation, in addition to imine bonds, or that part of the imine bonds are disrupted by 2ME.

The ability of high MW β_2 GPI-bh to bind to PS-coated microtitre plates is not greater than that of 50 kDa β_2 GPI-bh

Native, oxidized β_2 GPI and β_2 GPI-bh were adsorbed on to PS-coated microtitre plates and their monomeric and polymeric fractions, in the adsorbed fraction and in the supernatant, were analysed after SDS/PAGE and AgNO_3 labelling, as described in the Materials and methods section. A thin band was present at around 60–65 kDa (Figure 2A, see arrows) which was not β_2 GPI but an artifact due to AgNO_3 labelling. Since imine bonds can be hydrolysed during sample preparation for electrophoresis,

NaBH_3CN was used to reduce and stabilize the imine bonds. The results in Figure 2(A) show that several bands with an MW greater than 50 kDa were present in oxidized β_2 GPI and β_2 GPI-bh adsorbed on to PS-coated microtitre plates and that their intensity increased after treatment with NaBH_3CN . Conversely, native β_2 GPI was present as a single band at 50 kDa and NaBH_3CN treatment had almost no effect (Figure 2A). Several bands with a MW greater than 50 kDa were also present in the supernatant of oxidized β_2 GPI and β_2 GPI-bh, and their intensity was increased after NaBH_3CN treatment. By contrast, native β_2 GPI was present as a single band at 50 kDa in the presence or absence of NaBH_3CN treatment. Imine bonds, formed by reaction of the aldehyde function on oxidized glycan chains with amine groups on the β_2 GPI polypeptide chain, were thus responsible for polymer formation. The percentage of polymers in β_2 GPI-bh was greater after binding to PS-coated microtitre plates than before (10% and 24% in the adsorbed and non-adsorbed fraction respectively versus 10%) in solution (Table 1). Similar results were obtained using oxidized β_2 GPI. Thus it is likely that binding to AnPL induced oxidized β_2 GPI and β_2 GPI-bh polymer formation.

The same analysis was performed by Western blotting with the murine anti- β_2 GPI mAb 9G1 as label. Several bands were present in the adsorbed fraction of β_2 GPI-bh (Figure 2B), i.e. one major band at around 50 kDa and several minor bands at 90 and 130 kDa (Figure 2B). Native β_2 GPI showed only one band at 50 kDa (Figure 2B). Oxidized β_2 GPI showed a major band at 50 kDa and minor bands at 90, 130, 180 kDa (Figure 2B). When native, oxidized β_2 GPI and β_2 GPI-bh were treated with NaBH_3CN during adsorption to PS, the number and size of bands with a MW greater than 50 kDa increased in β_2 GPI-bh and oxidized β_2 GPI, whereas only a faint band was observed in native β_2 GPI (Figure 2B, Table 1). These results thus confirmed that oxidation of glycan chains is needed to induce the emergence of these high MW polymers.

The percentage of high MW β_2 GPI-bh in the PS-bound fraction was smaller than in the supernatant (10% versus 24% for β_2 GPI-bh) (Table 1). If the ability of high MW β_2 GPI-bh to bind to PS is greater or similar to that of 50 kDa β_2 GPI-bh, the percentage of high MW β_2 GPI-bh bound to PS should be greater or similar to that present in the supernatant. The binding ability of the imine-bound high MW β_2 GPI-bh is thus weaker than that of 50 kDa β_2 GPI-bh. We also observed that there are greater amounts of high MW polymeric forms in β_2 GPI-bh than in oxidized β_2 GPI (10% versus 1% in the adsorbed fraction and 24% versus 15% in the non-adsorbed fraction) (Table 1), suggesting that biotin-hydrazide groups play some role in polymer formation.

At $t = 0$, only 50 kDa β_2 GPI-bh binds to PS-coated microtitre plates

Since our PS-coated microtitre-plate-binding experiments included a 1 h 30 min incubation step, at room temperature in the presence or absence of NaBH_3CN , we analysed the kinetics of the emergence of high MW polymers. β_2 GPI-bh was layered at 3×10^{-7} M on PS-coated microtitre plates for a period ranging from 0 to 18 h and adsorbed and non-adsorbed fractions were analysed by Western blot labelling with 9G1 + GAMIG-HRP to detect the epitopes of β_2 GPI or SAV-HRP to detect the biotin groups bound to β_2 GPI. The results in Figure 3(A) show that at $t = 0$ only β_2 GPI-bh with a MW of 50 kDa was adsorbed. At 30 min and 1 h 30 min, β_2 GPI with a higher MW was present and its percentage increased (Figure 3A). At 3 h and 24 h, trimeric and polymeric β_2 GPI were present in high amounts. Dimeric β_2 GPI-bh was present in the supernatant and its concentration remained almost constant during the incubation time (results not shown). When native monomeric β_2 GPI was analysed with 9G1

Figure 2 High MW polymeric β_2 GPI-bh are linked by imine bonds

Native β_2 GPI, oxidized β_2 GPI or β_2 GPI-bh were layered in each well of microtitre plates coated with 15 μ g/well of PS. They were incubated for 1 h at 4 °C, 1 h 30 at 20 °C in the presence or absence of NaBH₃CN, then for 1 h at 4 °C. The supernatant was removed and the plates were washed three times with PBS at 4 °C. Adsorbed proteins were collected by the addition of 40 μ l of SDS/PAGE sample buffer in each well. **(A)** Adsorbed and non-adsorbed proteins layered at 10⁻⁶ M, in the presence or absence of NaBH₃CN, were submitted to SDS/8 % PAGE and labelled with AgNO₃. A thin artefactual band is indicated by an arrow. **(B)** Adsorbed proteins layered at 3 $\times$ 10⁻⁷ and 10⁻⁶ M, in the presence or absence of NaBH₃CN, were submitted to SDS/8 % PAGE, transferred to nitrocellulose membranes, labelled with the anti- β_2 GPI mAb 9G1 at 5 μ g/ml final concentration and GAMIG-HRP and revealed by ECL® (Amersham).

Table 1 SDS/PAGE analysis of adsorbed and non-adsorbed fractions of various β_2 GPI after binding on PS-coated microtitre plates

Figure 2 was scanned and analysed using the ImageJ software package (NIH). The percentage of each band was deduced from its position and intensity. N.D., not detectable; β_2 GPI-ox, oxidized β_2 GPI.

	Protein	% Monomeric β_2 GPI + NaBH ₃ CN		% Dimeric β_2 GPI + NaBH ₃ CN		% Polymeric β_2 GPI + NaBH ₃ CN	
Adsorbed fraction AgNO ₃ labelling	β_2 GPI native	100	100	N.D.	N.D.	N.D.	N.D.
	β_2 GPI-ox	99	75	1	5	N.D.	20
	β_2 GPI-bh	90	61	10	24	N.D.	15
Non-adsorbed fraction AgNO ₃ labelling	β_2 GPI-native	96	96	3.5	3	N.D.	N.D.
	β_2 GPI-ox	88	80	12	18	3	2
	β_2 GPI-bh	76	68	20	23	4	9
Adsorbed fraction 9G1 + GAMIG labelling	β_2 GPI native	99	93	1	6	N.D.	N.D.
	β_2 GPI-ox	61	28	35	50	4	22
	β_2 GPI-bh	64	32	33	45	3	29

Figure 3 Binding to PS-coated microtitre plates induces the formation of high MW polymeric β_2 GPI-bh

(A) β_2 GPI-bh at 3×10^{-7} M were layered in each well of microtitre plates coated with $15 \mu\text{g}$ /well of PS. They were then incubated for 30 s ($t = 0$), 30 min, 1 h 30 min, 3 h and 18 h. The supernatant was removed and the plates were washed three times with PBS at 4°C . The adsorbed proteins were collected by the addition of $40 \mu\text{l}$ of SDS/PAGE sample buffer in each well, and adsorbed and non-adsorbed fractions were analysed by SDS/8% PAGE and transferred to nitrocellulose membrane. The membrane was cut into 1.5 mm wide strips and each strip was individually labelled with either the anti- β_2 GPI mAb 9G1 directed against the first domain of β_2 GPI at $5 \mu\text{g}/\text{ml}$ final concentration, in association with GAMIG-HRP or with SAV-HRP ($5 \mu\text{g}/\text{ml}$). As a control, strips were also labelled with GAMIG-HRP alone. Finally, strips were revealed by ECL[®] (Amersham). (B) β_2 GPI-bh at 5×10^{-8} M, 3×10^{-7} M and 10^{-6} M was layered in each well of microtitre plates coated with $15 \mu\text{g}/\text{well}$ of PS. Then they were incubated for 1 h at 4°C , 1 h 30 min at 20°C then 1 h at 4°C . The supernatant was removed and the plates were washed three times with PBS at 4°C . The adsorbed proteins were collected by the addition of $40 \mu\text{l}$ of SDS/PAGE sample buffer in each well, submitted to SDS/8% PAGE, transferred to nitrocellulose membrane and revealed with the anti- β_2 GPI mAb 9G1 at $5 \mu\text{g}/\text{ml}$ final concentration and GAMIG-HRP or with SAV-HRP ($5 \mu\text{g}/\text{ml}$).

and GAMIG-HRP, no dimers were observed during this period (results not shown).

These results confirm that the binding ability of the imine-bound high MW β_2 GPI-bh to PS microtitre plates is weaker than that of 50 kDa β_2 GPI-bh. It is not certain that these polymers even bind to AnPL since it is the 50 kDa β_2 GPI-bh and not the higher MW components present in the supernatant which binds at $t = 0$.

Moreover, it is likely that the increase in higher MW β_2 GPI-bh occurred by polymerization of β_2 GPI-bh bound to PS. In this case, the slight increase in high MW components in the supernatant may

have been due to the release of some newly formed polymers with low affinity for PS.

9G1 recognizes high MW β_2 GPI-bh better than 50 kDa β_2 GPI

The percentage of monomers and polymers was always greater when β_2 GPI-bh was labelled with anti- β_2 GPI mAb 9G1 than with SAV (63 % monomers, 35 % multimers versus 86 % monomers, 14 % multimers at 1 h 30 min, Figure 3A). Serial dilutions of β_2 GPI-bh were adsorbed on PS-coated microtitre plates and revealed by either 9G1 + GAMIG-HRP or SAV-HRP. The results in Figure 3(B) show that SAV-HRP was able to detect monomeric β_2 GPI-bh layered on PS-coated microtitre plates at 5×10^{-8} M whereas 9G1 + GAMIG-HRP was able to detect monomeric β_2 GPI-bh only when it was layered at 3×10^{-7} M. However, 9G1 + GAMIG-HRP was able to detect dimeric β_2 GPI-bh at 5×10^{-8} M whereas SAV-HRP was not (Figure 3B, see arrows). The percentage of dimeric β_2 GPI-bh was always greater when β_2 GPI-bh was revealed with 9G1 + GAMIG-HRP than with SAV-HRP (40 versus 21 at 5×10^{-8} M and 36 versus 15 at 3×10^{-7} M respectively). Thus 9G1 had a weaker apparent affinity for monomeric β_2 GPI-bh than for dimeric β_2 GPI-bh dimers and recognized monomeric β_2 GPI less efficiently than SAV.

Quaternary structure of β_2 GPI-bh at $1 \mu\text{M}$ in PBS is dimeric

Since biotin-hydrazide groups may be involved in polymer formation, static and dynamic light-scattering was used to analyse the quaternary structure of native, oxidized β_2 GPI and β_2 GPI-bh in PBS solution. From the Debye plot shown in Figure 4(A), we calculated a MW of 47 kDa for native β_2 GPI, 43 kDa for oxidized β_2 GPI and 80 kDa for β_2 GPI-bh. Thus β_2 GPI-bh at $1 \mu\text{M}$ in PBS is mostly dimeric. BSA, which was used as a control, gave a MW of 73 kDa (Table 2). The intensity size-distribution curve provided the mean apparent diameter of β_2 GPI at $1 \mu\text{M}$ and 20°C in PBS. The diameter of native β_2 GPI was approx. 10 nm. Small angle X-ray scattering measurements of β_2 GPI, performed by Hammel et al. [48] gave 12.7, 3 and 7 nm as the dimensions of the molecule which is in close agreement with our results. The diameter of β_2 GPI-bh was 21 nm (Table 2), which was correlated with an increased molecular mass. Native and oxidized β_2 GPI were present in monomeric form and β_2 GPI-bh was in polymeric form. BSA had a diameter of 10 nm as expected (Table 2).

Fusion curves of native β_2 GPI and oxidized β_2 GPI (representative of the monomeric form) and β_2 GPI-bh (representative of the dimeric forms) are presented in Figure 4(B). Between 20°C and 65°C , the diameter of native β_2 GPI and oxidized β_2 GPI did not change, whereas fusion occurred between 35°C and 50°C for β_2 GPI-bh in solution at $1 \mu\text{M}$ in PBS. It thus seemed that a large part of β_2 GPI-bh consisted of dimers linked by non-covalent bonds or readily hydrolysable bonds.

Tryptophan environment is similar in native β_2 GPI and β_2 GPI-bh

It was shown that among all β_2 GPI Trp residues only the Trp-316 of domain V is mainly detected by fluorescence [49]. To test whether the exposure of tryptophan residues to the aqueous environment was modified by biotinylation of the glycan chains or its dimeric quaternary structure, we used the water-soluble quencher acrylamide. The Stern-Volmer quenching constant K_a was determined according to equation (1) as shown in Figure 5. We found similar K_a values for native and β_2 GPI-bh ($K_a = 0.0061 \pm 0.0002 \text{ M}^{-1}$ versus $K_a = 0.0064 \pm 0.0002 \text{ M}^{-1}$). These results show that the accessibility of Trp residues and especially the Trp-316 to their aqueous environment was similar in native β_2 GPI and β_2 GPI-bh. This suggests that there was no conformational change in the site of binding of the domain V to AnPL.

Figure 4 Analysis of the quaternary structure of various β_2 GPI in solution by static and dynamic light-scattering

(A) Debye plot of native, oxidized β_2 GPI and β_2 GPI-bh at $1 \mu\text{M}$ in PBS and 25°C . MW were calculated by linear regression analysis, from static light-scattering data, using equation (11). BSA was used as a control. The Rayleigh ratio was measured using toluene as standard. The refractive index increment dn/dc (ml/g) was 0.185 . No shape correction model was used. (B) Melting curves of native, oxidized β_2 GPI and β_2 GPI-bh at $1 \mu\text{M}$ in PBS were calculated from dynamic light-scattering data by increasing the temperature from 20°C to 65°C .

Table 2 Apparent diameter and molecular mass of various β_2 GPIs in PBS

Dynamic light-scattering measurements were performed on β_2 GPI, oxidized β_2 GPI and β_2 GPI-bh at $1 \mu\text{M}$ concentration in PBS at 20°C . Results were analysed by the cumulant method. Intensity size-distributions provided protein mean apparent diameters (nm). The MW was calculated by static light-scattering using equation (11). The Rayleigh ratio was measured using toluene as standard. No shape correction model was used. The refractive index increment (dn/dc) was 0.185 ml/g .

Protein	Diameter (nm)	MW (kDa)
Native β_2 GPI	9.65 ± 2.76	47 ± 1.1
Oxidized β_2 GPI	10.24 ± 1.45	43 ± 3.7
β_2 GPI-bh	21.15 ± 1.01	80 ± 2.5
BSA	10.62 ± 1.49	73 ± 2.1

Binding constants of native, oxidized β_2 GPI and β_2 GPI-bh to PC/PS monolayers

Sensorgrams depicting binding of various preparations of β_2 GPI, for 500 s at concentrations ranging from 125 nM to $1 \mu\text{M}$, are

Figure 5 Acrylamide quenching of native and β_2 GPI-bh tryptophan fluorescence

Stern–Volmer plots for the fluorescence quenching of β_2 GPI and β_2 GPI-bh at $1 \mu\text{M}$ in PBS at 25°C . Acrylamide concentrations varied from 20 – 600 mM . K_a values were calculated by linear regression using the Graphpad software package. Solid lines represent the best fit of equations (1) to the data. K_a were $0.0061 \pm 0.0002 \text{ M}^{-1}$ and $0.0064 \pm 0.0002 \text{ M}^{-1}$ for native β_2 GPI and β_2 GPI-bh respectively.

shown in Figure 6. β_2 GPI-bh (Figure 6A) associated less rapidly than native or oxidized β_2 GPI (Figures 6B and 6C) and there was no plateau, and binding steadily increased. It dissociated slowly and incompletely (Figure 6A). The β_2 GPI-bh curves were better fitted when using a two-state reaction model (conformational change) than with the Langmuir 1:1 model (Figure 6A). By contrast, native β_2 GPI bound rapidly to PC/PS monolayers (Figure 6A, Table 3), reached a plateau and dissociated rapidly and totally from the bilayers in 150 s (Figure 6A, Table 3). Sensorgrams of oxidized β_2 GPI (Figure 6C) were similar to that of native β_2 GPI and both were best fitted using the Langmuir 1:1 model (Figure 6C). χ^2 values of 159 , 496 , 420 and 987 were obtained for native β_2 GPI at 250 , 500 , 1000 and 2000 nM using the Langmuir 1:1 model versus 3280 , 6370 , 173 and 285 using the two-state reaction model. Similar values were obtained for oxidized β_2 GPI (results not shown). At higher native or oxidized β_2 GPI concentrations, the two-state reaction model seems to better fit the data suggesting that the conformational change proposed in β_2 GPI-bh may also occur in native β_2 GPI but at much higher concentrations.

Table 4 shows the values of constants obtained for β_2 GPI-bh, using the two-state reaction model to fit the data at concentrations ranging from 125 nM to 500 nM . The mean K_D value of 16 nM was comparable with values we obtained by binding to PC/PS bound to microtitre plates [43]. $K_{on,1}$ was approx. 2-fold lower at 500 nM than 125 nM . Accessibility of negatively charged lipids may decrease as β_2 GPI-bh concentration increases. The second reaction step (the postulated conformational change) was faster at high, than low concentrations (Table 4) since $K_{on,2}$ and $K_{off,2}$ were 30-fold and 6-fold higher at 500 nM than 125 nM respectively (Table 4). A higher concentration may facilitate the conformational change responsible for β_2 GPI-bh auto-association. Our K_D values for native β_2 GPI ($K_D = 202 \text{ nM}$) agree with those described by Willems et al. on monolayers, Brighton et al. and Gamsjaeger et al. on bilayers [20,21,50]. They were weaker than that of β_2 GPI-bh ($K_D = 16 \text{ nM}$) and than those we previously reported on PC/PS bound to microtitre plates [43]. We thus confirmed that the affinity of β_2 GPI-bh for PC/PS is at least 12-fold higher than that of native β_2 GPI.

The aCL EY2C9 binds to β_2 GPI-bh

We tested whether the dimeric quaternary structure of β_2 GPI-bh could cause detection by aCLs. We layered native oxidized

Figure 6 Binding kinetics of various β_2 GPI by SPR

Sensorgrams showing binding of (A) β_2 GPI-bh; (B) native β_2 GPI; (C) oxidized β_2 GPI to HPA sensor chip surfaces fused with PC/PS (80:20 w/w) monolayers, after subtracting binding to surfaces fused with PC only monolayers, at 125 nM–1 μ M. Fits (dashed lines) were obtained by using either the Langmuir 1:1 model (B, C) or the two-state reaction model (A).

Table 3 Binding constants of native and oxidized β_2 GPI to PC/PS monolayers

Kinetic constants (K_{on} and K_{off}) and equilibrium constants (K_D) were calculated from SPR data by non-linear fitting of the primary sensorgram data of Figures 6(B) and 6(C) using the BIAcore evaluation software package 4.1 and a Langmuir 1:1 model according to equations (2), (3), (4) and (5). The values are the means for three to four different concentrations.

Proteins	PC/PS (80:20, w/w)		
	K_{on} (1/Ms)	K_{off} (1/s)	K_D (nM)
Native β_2 GPI	$(1.42 \pm 0.19) \times 10^5$	$(2.85 \pm 0.46) \times 10^{-2}$	202 ± 60
Oxidized β_2 GPI	$(1.66 \pm 0.29) \times 10^5$	$(4.33 \pm 0.11) \times 10^{-2}$	257 ± 31

β_2 GPI and β_2 GPI-bh at 1 μ M on to nitrocellulose membrane, as described in the Materials and methods section. Slots were cut into slices and each slice was incubated with various concentrations of the human IgM EY2C9 and murine anti- β_2 GPI 9G1 (Figure 7A). There were no marked differences in the amounts of the various deposits on the membrane, since labelling with 9G1 was similar for native oxidized β_2 GPI and β_2 GPI-bh. Although EY2C9 bound strongly to β_2 GPI-bh at a concentration as low as 3×10^{-8} M, native and oxidized β_2 GPI were not recognized by EY2C9 even at 10^{-7} M. The fact that α 1-glycoprotein, biotinylated on its glycan chains similarly to β_2 GPI-bh, was not recognized by EY2C9 (results not shown) means that the EY2C9 target was not biotin-hydrazide.

To test whether EY2C9, similarly to 9G1, would bind more strongly to high MW than 50 kDa β_2 GPI, native β_2 GPI and β_2 GPI-bh were analysed by SDS/8% PAGE and transferred to nitrocellulose membrane. The membrane was cut into 1.5 mm wide slices and each slice was revealed with EY2C9 antibody at 10^{-7} M, 3×10^{-8} M and 10^{-8} M in association with GAHIG-HRP. Slices were also revealed with 9G1 in association with GAMIG-HRP and with SAV-HRP. As a control, slices were revealed with GAHIG HRP and GAMIG-HRP alone. The results in Figure 7(B) show that EY2C9 recognized 50 kDa and 90 kDa β_2 GPI-bh but did not react with native β_2 GPI. The relative importance of the dimeric form versus total forms were much higher when labelled with EY2C9 than with 9G1 + GAMIG-HRP or SAV-HRP (40%, 25% and 4% respectively). β_2 GPI-bh (90 kDa) was thus detected more easily by EY2C9 than 50 kDa β_2 GPI-bh.

DISCUSSION

Our results confirm that the affinity of β_2 GPI-bh for AnPL was greater than that of native β_2 GPI. They also demonstrate the presence of polymeric forms in oxidized β_2 GPI and β_2 GPI-bh and their absence in native β_2 GPI. These polymeric forms were analysed for their ability to bind to AnPL and detection by anti- β_2 GPI and aCL antibodies. In solution, by static and dynamic light-scattering, β_2 GPI-bh was dimeric at 1 μ M in PBS, whereas native and oxidized β_2 GPI were monomeric. The dimeric structure of β_2 GPI-bh in solution could explain its greater affinity for AnPL compared with that of monomeric native or oxidized β_2 GPI. On the other hand, electrophoretic analysis of β_2 GPI-bh in solution demonstrated that it was present mostly as a 50 kDa monomer even though small amounts of high MW components (90 kDa and 130 kDa), corresponding to dimeric and trimeric β_2 GPI-bh, were also present. Our fusion experiments showed that in solution, dimeric β_2 GPI-bh dissociated in monomers by heating. Thus after the addition of SDS and heating at 100°C, dimeric β_2 GPI-bh may dissociate and migrate like a 50 kDa monomer. However, high MW components were also present in our preparations of oxidized β_2 GPI after electrophoresis. Oxidized β_2 GPI in solution is monomeric and should thus migrate in SDS/PAGE similarly to a 50 kDa monomer. It is likely that these high MW polymers, which arise by imine formation between free aldehyde groups of oxidized glycan chains and amino groups on the polypeptide chain, are formed when the molecule is heated at 100°C for 10 min before electrophoresis. Binding to PS-coated microtitre plates also greatly facilitated the formation of high MW polymers in oxidized β_2 GPI and β_2 GPI-bh and their amounts were increased after the reduction of imine bonds by NaBH_3CN . It is likely that polymer formation depends on the number of free aldehyde groups on glycan chains and the accessibility of amino groups on the polypeptide chain. Moreover, imine bond formation requires the amino groups on the polypeptide chain to

Table 4 Binding constants of β_2 GPI-bh to PC/PS (80:20, w/w) monolayers

K_{on} , K_{off} and K_D constants were calculated from SPR data of Figure 6(A), by non-linear fitting of the sensorgram data using the BIAcore evaluation software package 4.1 and a two-state reaction model according to equations (6), (7), (8), (9), and (10). The results are shown at different β_2 GPI-bh concentrations (125 nM, 250 nM and 500 nM).

Protein	$[\beta_2\text{GPI-bh}]$ (nM)	$K_{on,1}$ (1/Ms)	$K_{off,1}$ (1/s)	$K_{on,2}$ (1/s)	$K_{off,2}$ (1/s)	K_D (nM)
β_2 GPI-bh	125	1.96×10^4	2.88×10^{-4}	6.69×10^{-6}	1.01×10^{-5}	8.8
	250	1.37×10^4	5.49×10^{-4}	1.32×10^{-5}	3.28×10^{-5}	28.6
	500	1.08×10^4	6.38×10^{-4}	2.22×10^{-4}	5.89×10^{-5}	12.4

Figure 7 EY2C9 binding to various β_2 GPI

(A) Native β_2 GPI, oxidized β_2 GPI and β_2 GPI-bh at 1 μ M were layered as separate slots on nitrocellulose membrane, dried and washed in TBST for 15 min. The membrane was cut into 1.5 mm wide strips and each strip was individually labelled with EY2C9 IgM at various concentrations (10^{-7} M and 3×10^{-8} M) for 1 h 30 min. GAHIG-HRP was added for 1 h. Strips were also labelled with the murine anti- β_2 GPI mAb 9G1 at 5 μ g/ml final concentration in association with GAMIG-HRP. For control, strips were labelled with GAHIG-HRP and GAMIG-HRP alone. Hyperfilms ECL[®] (Amersham) were used to detect luminescence. (B) Native β_2 GPI and β_2 GPI-bh were analysed by SDS/8% PAGE and transferred to nitrocellulose membrane. The membrane was then cut into 1.5 mm wide strips and each strip was individually labelled with EY2C9 at various concentrations (10^{-7} M, 3×10^{-8} and 10^{-9} M) in association with GAMIG-HRP. Strips were also labelled with the murine anti- β_2 GPI mAb 9G1 at 5 μ g/ml final concentration in association with GAMIG-HRP or with SAV-HRP (5 μ g/ml). For control, strips were labelled with GAHIG-HRP or GAMIG-HRP alone. Hyperfilms ECL[®] (Amersham) were used to detect luminescence.

be in the vicinity of the oxidized glycan chains. This is favoured by the binding of β_2 GPI to PS plates. It is not clear that these high MW polymers bind to AnPL at all, since our binding experiments on PS-coated microtitre plates showed that it was the 50 kDa β_2 GPI-bh and not the higher MW components present in the supernatant which bound at $t = 0$. In addition, they seemed to

occur by polymerization of β_2 GPI already bound to PS. These covalent polymers, which appear after binding to AnPL, thus differ from the dissociable dimers that are present in solution before binding to AnPL.

The association and dissociation curves of β_2 GPI-bh to PC/PS monolayers were fitted using the two-state reaction model. This model, described by equation (6), assumes the existence of two different complexes (AB and AB*) resulting from the reaction of β_2 GPI-bh with PS. AB is obtained after a rapid adsorption phase due to electrostatic interactions between PS and the AnPL binding site of the β_2 GPI-bh molecule. Then a second reaction step with lower kinetic constants leads to the formation of AB*. Our binding experiments on PS-coated microtitre plates, demonstrated that binding to AnPL induced polymerization of β_2 GPI-bh in a time-dependent manner. In addition, when we measured the binding of β_2 GPI-bh to apoptotic cells by flow cytometry, we did not observe any saturation and patches of β_2 GPI-bh were visible by confocal microscopy, suggesting that after binding to AnPL, β_2 GPI-bh auto-associates with neighbouring molecules [43]. A fast step of β_2 GPI binding to AnPL (AB), may thus be followed by a slower phase of auto-association of the molecule with neighbouring β_2 GPI-bh molecules on the monolayer surface (AB*). Clustering of β_2 GPI, accompanied by protein-induced rigidification of the membrane was recently demonstrated by Gamsjaeger et al. using atomic force microscopy and electron paramagnetic resonance [50].

At a low concentration, sensorgrams of native and oxidized β_2 GPI were best fitted using the Langmuir 1:1 model. This is in line with the inability of native β_2 GPI to polymerize in binding experiments on PS-coated microtitre plates. At higher concentrations, the two-state reaction model seems to better fit the data, suggesting that auto-association of native β_2 GPI may also occur but at higher concentrations than with β_2 GPI-bh. Gamsjaeger et al. [50] used a two-state reaction model to fit their sensorgrams of native β_2 GPI on POPC/POPS (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine) 80:20 bilayers. However, they used an L1 sensorchip and POPC/POPS 80:20 bilayers whereas we used an HPA sensorchip and PC/PS 80:20 monolayers. Proteins and lipids can move more freely on bilayers than on monolayers which are more rigid. It may facilitate auto-association of neighbouring molecules.

SPR measurements also confirmed the importance of the modified glycan chains, which were responsible for the difference between oxidized β_2 GPI and β_2 GPI-bh. Biotin in its physiologically active form is covalently linked to the active site of enzymes known as carboxylases or decarboxylases and acts as a co-enzyme for carbon dioxide transfer. Biotin of glycan chains could thus interact with carboxyl groups of PS. It could also interact with carboxyl groups present on neighbouring β_2 GPI molecules and be directly responsible for the dimeric quaternary structure. Since oxidized β_2 GPI has the same affinity for AnPL as native β_2 GPI, interactions of biotin groups with AnPLs may be involved

in the decreased dissociation of bound β_2 GPI-bh and thus in its increased affinity for AnPL.

EY2C9 antibody binds to dimeric β_2 GPI-bh. Monomeric native β_2 GPI and oxidized β_2 GPI were not recognized by EY2C9. We observed that EY2C9 and 9G1 bound more strongly to polymeric than monomeric β_2 GPI-bh. But 9G1 also bound more strongly to 90 kDa than 50 kDa oxidized β_2 GPI (Figure 2B), whereas EY2C9 did not bind at all to oxidized β_2 GPI (Figure 7A). There are differences between EY2C9 and 9G1. (i) The murine anti- β_2 GPI mAb IgG 9G1 binds to β_2 GPI in solution whereas EY2C9 binds to β_2 GPI only when it is layered on oxygenated polystyrene plates. (ii) In addition, 9G1 is directed against domain I of β_2 GPI whereas EY2C9 is directed against domain IV of the molecule. Recently, Liu et al. observed binding of aCL EY2C9 to β_2 GPI modified by the addition of a modified ketocholesterol, noted oxlig1 [36]. EY2C9 may bind to oxlig1 or to the β_2 GPI oxlig1 interface. However, this adduct on the β_2 GPI molecule similarly to biotin-hydrazide modified glycan chains could, (i) induce the right conformational change of the β_2 GPI molecule and expose the cryptic epitope which is the target of EY2C9; (ii) the adduct could induce the dimerization of the molecule. Roubey et al. [45] proposed that aCL binds to β_2 GPI dimers because the epitope density is greater on dimers than on monomers. Another possibility is that the binding site is created by the union of two molecules. This union must be specific since the presence of imine-bound polymers did not improve EY2C9 binding to oxidized β_2 GPI, suggesting that dimerization itself does not create epitopes recognized by aCLs. In the experiments reported by Liu et al., aCL EY2C9 bound to β_2 GPI modified by the addition of a modified ketocholesterol, but did not bind after methylation of the carboxyl group of the modified ketocholesterol [36]. Lutters et al. have shown that some of their dimeric β_2 GPI constructions did not present an increased affinity for AnPL [44]. It is likely that the dimeric quaternary structure of β_2 GPI-bh is actually responsible for the appearance of epitopes targeted by EY2C9.

Our experiments may have physiological implications. Oxidation alone was not sufficient to induce recognition by aCL EY2C9. Oxidized β_2 GPI was not recognized by EY2C9 and its affinity for AnPL was similar to that of native β_2 GPI (results not shown). Polymerization of the β_2 GPI molecule was also not sufficient since imine-bound polymers were present in oxidized β_2 GPI, which was not recognized by EY2C9. The addition of biotin groups to glycan chains of β_2 GPI or adducts of modified ketocholesterol on the polypeptide chain of the β_2 GPI molecule were essential for recognition of the molecule by aCL EY2C9. We conclude that these changes could lead to auto-association of the β_2 GPI molecule. This auto-association could then induce: (i) the hypothesized conformational change that exposes the cryptic epitopes recognized by aCLs; (ii) if the epitope density is the key to aCL recognition, as postulated by Roubey et al. [45] and observed by Sheng et al. [27], auto-association of β_2 GPI will increase it; (iii) if the binding site is created by the union of at least two β_2 GPI molecules, auto-association may create this site. These results suggest that the dissociable dimeric structure of β_2 GPI, in solution, is responsible for its greater affinity for AnPL and its increased ability to form covalent polymers which are better targeted than monomers by anti- β_2 GPI and aCL antibodies.

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