Biotinylation of glycan chains in β₂ glycoprotein I induces dimerization of the molecule and its detection by the human autoimmune anti-cardiolipin antibody EY2C9

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INTRODUCTION

The β₂ GPI (β₂ 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. β₂ 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 β₂ GPI contains 84 amino acids and three disulphide bridges. It is involved in β₂ GPI binding to AnPLs (anionic phospholipids) such as phosphatidylserine and cardiolipin [5–7]. It contains a very electropositive sequence containing 11 disulphide bridges and four N-linked glycosylation sites. This sequence and particularly Trp313–316 are essential for cardiolipin binding [9]. Amino-acid residues 311–317 allow the insertion of β₂ GPI into lipid membranes [10]. Many studies suggest that it may be a natural anti-coagulant. In vitro β₂ GPI inhibits intrinsic pathway activation [11], tenase [12] and prothrombinase [13] activities on the surface of activated platelets, and synthetic phospholipid vesicles. β₂ 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 β₂ GPI responsible for its physiological and pathological effects. The binding characteristics of β₂ 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 β₂ 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 β₂ GPI molecule [20,27,28]. Associations of β₂ GPI and AnPL are considered to act as antigens for aCLs associated with diseases such as systemic lupus erythematosus or anti-cardiolipin syndrome. Several research groups have shown that AnPL-binding proteins such as β₂ GPI or prothrombin are the main target of aCLs [29–31]. It is not clear whether aCLs react with the interface between AnPL and β₂ GPI, using β₂ GPI itself after a conformation change [31–34] or with adducts of oxidized AnPL and β₂ GPI such as 1-palmitoyl-2-(5-oxovaleroyl)
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 a cλ 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 antι-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, NaBH$_3$CN, SA V-HRP (streptavidin horseradish peroxidase) and TMB (3,3′,5′,7-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$^{-2}$ and 10$^{-4}$ 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 at 30 min at room temperature in the presence or absence of 10 mM NaBH$_3$CN 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$^{-5}$ 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-β₂GPI 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 × 10 mm quartz fluorescence cuvette. For proteins containing tyrosine and tryptophan, tryptophan can be excited selectively at 290–295 nm. β₂GPI solutions at 1 μM 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 solutions at 1° was used to detect luminescence.

**SPR studies of various β₂GPs**

A BIAcore 2000 instrument (BIAcore AB, Uppsala, Sweden) was used to study the interactions of various β₂GPs with PC/PS or PC monolayers. The BIAcore HPA (hydrophobic association) sensor chip was washed with 20 μl of octyl β-D-glucopyranoside. The liposome preparation, sonicated extemporaneously, was fused to the surface of the sensor flow cell at 1 μl/min. Aliquots (5 μl) 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 β₂GPI 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 μl/min with 50 mM Tris/HCl, 120 mM NaCl at pH 7.4. No significant binding of native, oxidized β₂GPI or β₂GPI-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:

\[ A + B \xrightarrow{K_{on}} AB \]  

Where \( A \) is the β₂GPI in solution and \( B \) the lipids. \( AB \) represents the β₂GPI–lipid complex. Concentrations at \( t = 0 \) are: \( A = \beta₂GPI \), \( B = R_{max} \) where \( R_{max} \) is the amount of available β₂GPI-binding sites on the lipid monolayer. \( AB = 0 \). The rate equations at time \( t \) are:

\[ \frac{dB}{dt} = -(k_{on} \times A \times B - k_{off} \times AB) \]  
\[ \frac{dAB}{dt} = (k_{on} \times A \times B - k_{off} \times AB) \]

The equilibrium constant, \( K_D \) is calculated from rate constant:

\[ K_D = \frac{k_{off}}{K_{on}} \]  

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

\[ A + B \xrightarrow{k_{on}} AB \xrightarrow{k_{off}} AB^* \]

Where \( A \) is the β₂GPI in solution and \( B \) the lipids. \( AB \) represents the β₂GPI–lipid complex and \( AB^* \) a secondary β₂GPI–lipid complex. The concentrations at \( t = 0 \) are: \( A = \beta₂GPI \), \( B = R_{max} \) where \( R_{max} \) is the amount of available β₂GPI-binding sites on the lipid monolayer. \( AB = 0 \) and \( AB^* = 0 \). The rate equations at time \( t \) are:

\[ \frac{dB}{dt} = -(k_{on,1} \times A \times B - k_{off,1} \times AB) \]  
\[ \frac{dAB}{dt} = (k_{on,1} \times A \times B - k_{off,1} \times AB) \]  
\[ \frac{dAB^*}{dt} = -(k_{on,2} \times A \times B - k_{off,2} \times AB^*) \]  
\[ \frac{dAB^*}{dt} = (k_{on,2} \times A \times B - k_{off,2} \times AB^*) \]

\[ K_D = \frac{1}{\left( \frac{k_{on,1}}{k_{off,1}} \times \left( 1 + \frac{k_{on,2}}{k_{off,2}} \right) \right)} \]

**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 20,000 g before analysis to remove large aggregates. The various β₂GPI preparations were tested at concentrations ranging from 1–10 μM in PBS. All measurements were performed in Hellma QS 3 mm quartz cuvettes (Hellma GmbH & Co, Germany) in a volume of 45 μl. 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:

\[ \frac{KC}{R_g} = \frac{1}{M} + 2A_C \]
NaBH₃CN 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 β₂GPI and β₂GPI-bh adsorbed on to PS-coated microtitre plates and that their intensity increased after treatment with NaBH₃CN. Conversely, native β₂GPI was present as a single band at 50 kDa and NaBH₃CN treatment had almost no effect (Figure 2A). Several bands with a MW greater than 50 kDa were also present in the supernatant of oxidized β₂GPI and β₂GPI-bh, and their intensity was increased after NaBH₃CN treatment. By contrast, native β₂GPI was present as a single band at 50 kDa in the presence or absence of NaBH₃CN treatment. Imine bonds, formed by reaction of the aldehyde function on oxidized glycan chains with amine groups on the β₂GPI polypeptide chain, were thus responsible for polymer formation. The percentage of polymers in β₂GPI-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 β₂GPI. Thus it is likely that binding to AnPL induced oxidized β₂GPI and β₂GPI-bh polymer formation.

The same analysis was performed by Western blotting with the murine anti-β₂GPI mAb 9G1 as label. Several bands were present in the adsorbed fraction of β₂GPI-bh (Figure 2B), i.e. one major band at around 50 kDa and several minor bands at 90 and 130 kDa (Figure 2B). Native β₂GPI showed only one band at 50 kDa (Figure 2B). Oxidized β₂GPI showed a major band at 50 kDa and minor bands at 90, 130, 180 kDa (Figure 2B). When native, oxidized β₂GPI and β₂GPI-bh were treated with NaBH₃CN during adsorption to PS, the number and size of bands with a MW greater than 50 kDa increased in β₂GPI-bh and oxidized β₂GPI, whereas only a faint band was observed in native β₂GPI (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 β₂GPI-bh in the PS-bound fraction was smaller than in the supernatant (10% versus 24% for β₂GPI-bh) (Table 1). If the ability of high MW β₂GPI-bh to bind to PS is greater or similar to that of 50 kDa β₂GPI-bh, the percentage of high MW β₂GPI-bh bound to PS should be greater or similar to that present in the supernatant. The binding ability of the imine-bound high MW β₂GPI-bh is thus weaker than that of 50 kDa β₂GPI-bh. We also observed that there are greater amounts of high MW polymeric forms in β₂GPI-bh than in oxidized β₂GPI (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 β₂GPI-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 NaBH₃CN, we analysed the kinetics of the emergence of high MW polymers. β₂GPI-bh was layered at $3 \times 10^{-7} \text{M}$ 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 β₂GPI or SAV-HRP to detect the biotin groups bound to β₂GPI. The results in Figure 3(A) show that at $t = 0$ only β₂GPI-bh with a MW of 50 kDa was adsorbed. At 30 min and 1 h 30 min, β₂GPI with a higher MW was present and its percentage increased (Figure 3A). At 3 h and 24 h, trimeric and polymeric β₂GPI were present in high amounts. Dimeric β₂GPI-bh was present in the supernatant and its concentration remained almost constant during the incubation time (results not shown). When native monomeric β₂GPI was analysed with 9G1

**RESULTS**

Oxidation of glycan chains of β₂GPI induces covalent polymer formation

Figure 1 shows the SDS/PAGE profiles of native, oxidized β₂GPI and β₂GPI-bh preparations in solution as revealed by AgNO₃. Native β₂GPI appears as a single band at 50 kDa in the presence or absence of $3 \times 10^{-7}$ M 2ME (2-mercaptoethanol) (Figures 1A and 1B). Dimers of approx. 90 kDa were present in oxidized β₂GPI and in β₂GPI-bh (Figure 1A). Dimers represented 5% of oxidized β₂GPI and 10% of β₂GPI-bh. When SDS/PAGE was performed in the presence of $3 \times 10^{-6}$ M 2ME, the dimer concentration was decreased (2% and 4% for oxidized β₂GPI and β₂GPI-bh respectively, Figure 1B). However, polymeric β₂GPI 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 β₂GPI-bh to bind to PS-coated microtitre plates is not greater than that of 50 kDa β₂GPI-bh

Native, oxidized β₂GPI and β₂GPI-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 AgNO₃ 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 β₂GPI but an artifact due to AgNO₃ labelling. Since imine bonds can be hydrolysed during sample preparation for electrophoresis,
Figure 2  High MW polymeric β2GPI-bh are linked by imine bonds

Native β2GPI, oxidized β2GPI or β2GPI-bh were layered in each well of microtitre plates coated with 15 µg/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 µl 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 x 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-β2GPI mAb 9G1 at 5 µg/ml final concentration and GAMIG-HRP and revealed by ECL® (Amersham).

Table 1  SDS/PAGE analysis of adsorbed and non-adsorbed fractions of various β2GPI after binding on PS-coated microtitre plates

<table>
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<th>Protein</th>
<th>% Monomeric β2GPI + NaBH₃CN</th>
<th>% Dimeric β2GPI + NaBH₃CN</th>
<th>% Polymeric β2GPI + NaBH₃CN</th>
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<tr>
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<td>N.D.</td>
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<td>5</td>
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<td></td>
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<td>61</td>
<td>10</td>
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<tr>
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<td>96</td>
<td>3.5</td>
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<tr>
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9G1 recognizes high MW $\beta_2$-GPI-bh better than 50 kDa $\beta_2$-GPI

The percentage of monomers and polymers was always greater when $\beta_2$-GPI-bh was labelled with anti-$\beta_2$-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 $\beta_2$-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 $\beta_2$-GPI-bh layered on PS-coated microtitre plates at 5 x 10$^{-4}$ M whereas 9G1 + GAMIG-HRP was able to detect monomeric $\beta_2$-GPI-bh only when it was layered at 3 x 10$^{-7}$ M. However, 9G1 + GAMIG-HRP was able to detect dimeric $\beta_2$-GPI-bh at 5 x 10$^{-4}$ M whereas SAV-HRP was not (Figure 3B, see arrows). The percentage of dimeric $\beta_2$-GPI-bh was always greater when $\beta_2$-GPI-bh was revealed with 9G1 + GAMIG-HRP than with SAV-HRP (40 versus 21 at 5 x 10$^{-8}$ M and 36 versus 15 at 3 x 10$^{-7}$ M respectively). Thus 9G1 had a weaker apparent affinity for monomeric $\beta_2$-GPI-bh than for dimeric $\beta_2$-GPI-bh dimers and recognized monomeric $\beta_2$-GPI less efficiently than SAV.

Quaternary structure of $\beta_2$-GPI-bh at 1 $\mu$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 $\beta_2$-GPI and $\beta_2$-GPI-bh in PBS solution. From the Debye plot shown in Figure 4(A), we calculated a MW of 47 kDa for native $\beta_2$-GPI, 43 kDa for oxidized $\beta_2$-GPI and 80 kDa for $\beta_2$-GPI-bh. Thus $\beta_2$-GPI-bh at 1 $\mu$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 $\beta_2$-GPI at 1 $\mu$M and 20°C in PBS. The diameter of native $\beta_2$-GPI was approx. 10 nm. Small angle X-ray scattering measurements of $\beta_2$-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 $\beta_2$-GPI-bh was 21 nm (Table 2), which was correlated with an increased molecular mass. Native and oxidized $\beta_2$-GPI were present in monomeric form and $\beta_2$-GPI-bh was in polymeric form. BSA had a diameter of 10 nm as expected (Table 2).

Fusion curves of native $\beta_2$-GPI and oxidized $\beta_2$-GPI (representative of the monomeric form) and $\beta_2$-GPI-bh (representative of the dimeric forms) are presented in Figure 4(B). Between 20°C and 65°C, the diameter of native $\beta_2$-GPI and oxidized $\beta_2$-GPI did not change, whereas fusion occurred between 35°C and 50°C for $\beta_2$-GPI-bh in solution at 1 $\mu$M in PBS. It thus seemed that a large part of $\beta_2$-GPI-bh consisted of dimers linked by non-covalent bonds or readily hydrolysable bonds.

Tryptophan environment is similar in native $\beta_2$-GPI and $\beta_2$-GPI-bh

It was shown that among all $\beta_2$-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 $K_q$ was determined according to equation (1) as shown in Figure 5. We found similar $K_q$ values for native and $\beta_2$-GPI-bh ($K_q = 0.0061 \pm 0.0002$ M$^{-1}$ versus $K_q = 0.0064 \pm 0.0002$ M$^{-1}$). These results show that the accessibility of Trp residues and especially the Trp-316 to their aqueous environment was similar in native $\beta_2$-GPI and $\beta_2$-GPI-bh. This suggests that there was no conformational change in the site of binding of the domain V to AnPL.
The refractive index increment $dn/dC$ (ml/g) was 0.185. No shape correction model was used. (11). BSA was used as a control. The Rayleigh ratio was measured using toluene as standard. The refractive index increment was calculated by static light-scattering using equation (11). The Rayleigh ratio was measured by linear regression analysis, from static light-scattering data, using equation $\beta (dn/dC)$ was 0.185 ml/g.

Dynamic light-scattering measurements were performed on $\beta_2$GPI, oxidized $\beta_2$GPI and $\beta_2$GPI-bh at 1 $\mu$M in PBS at 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. The refractive index increment was calculated by static light-scattering data by increasing the temperature from 20°C to 65°C.

Table 2 Apparent diameter and molecular mass of various $\beta_2$GPIs in PBS

Dynamic light-scattering measurements were performed on $\beta_2$GPI, oxidized $\beta_2$GPI and $\beta_2$GPI-bh at 1 $\mu$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.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Diameter (nm)</th>
<th>MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native $\beta_2$GPI</td>
<td>9.65 ± 2.76</td>
<td>47 ± 1.1</td>
</tr>
<tr>
<td>Oxidized $\beta_2$GPI</td>
<td>10.24 ± 1.45</td>
<td>43 ± 3.7</td>
</tr>
<tr>
<td>$\beta_2$GPI-bh</td>
<td>21.15 ± 1.01</td>
<td>80 ± 2.5</td>
</tr>
<tr>
<td>BSA</td>
<td>10.62 ± 1.49</td>
<td>73 ± 2.1</td>
</tr>
</tbody>
</table>

Binding constants of native, oxidized $\beta_2$GPI and $\beta_2$GPI-bh to PC/PS monolayers

Sensorgrams depicting binding of various preparations of $\beta_2$GPI, for 500 s at concentrations ranging from 125 nM to 1 $\mu$M, are shown in Figure 6. $\beta_2$GPI-bh (Figure 6A) associated less rapidly than native or oxidized $\beta_2$GPI (Figures 6B and 6C) and there was no plateau, and binding steadily increased. It dissociated slowly and incompletely (Figure 6A). The $\beta_2$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 $\beta_2$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 $\beta_2$GPI (Figure 6C) were similar to that of native $\beta_2$GPI and both were best fitted using the Langmuir 1:1 model (Figure 6C). $\chi^2$ values of 159, 496, 420 and 987 were obtained for native $\beta_2$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 $\beta_2$GPI (results not shown). At higher native or oxidized $\beta_2$GPI concentrations, the two-state reaction model seems to better fit the data suggesting that the conformational change proposed in $\beta_2$GPI-bh may also occur in native $\beta_2$GPI but at much higher concentrations.

Table 4 shows the values of constants obtained for $\beta_2$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]. $K_{\text{on}}$ was approx. 2-fold lower at 500 nM than 125 nM. Accessibility of negatively charged lipids may decrease as $\beta_2$GPI-bh concentration increases. The second reaction step (the postulated conformational change) was faster at high, than low concentrations (Table 4) since $K_{\text{off}}$ was 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 $\beta_2$GPI-bh auto-association. Our $K_D$ values for native $\beta_2$GPI ($K_D = 202$ 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 $\beta_2$GPI-bh ($K_D = 16$ nM) and than those we previously reported on PC/PS bound to microtitre plates [43]. We thus confirmed that the affinity of $\beta_2$GPI-bh for PC/PS is at least 12-fold higher than that of native $\beta_2$GPI.

The aCL EY2C9 binds to $\beta_2$GPI-bh

We tested whether the dimeric quaternary structure of $\beta_2$GPI-bh could cause detection by aCLs. We layered native oxidized...
by using either the Langmuir 1:1 model (equations 2, 3, 4) 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.

**DISCUSSION**

Our results confirm that the affinity of β₂GPI-bh for AnPL was greater than that of native β₂GPI. They also demonstrate the presence of polymeric forms in oxidized β₂GPI and β₂GPI-bh and their absence in native β₂GPI. These polymeric forms were analysed for their ability to bind to AnPL and detection by anti-β₂GPI and αCL antibodies. In solution, by static and dynamic light-scattering, β₂GPI-bh was dimeric at 1 μM in PBS, whereas native and oxidized β₂GPI were monomeric. The dimeric structure of β₂GPI-bh in solution could explain its greater affinity for AnPL compared with that of monomeric native or oxidized β₂GPI. On the other hand, electrophoretic analysis of β₂GPI-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 β₂GPI-bh, were also present. Our fusion experiments showed that in solution, dimeric β₂GPI-bh dissociated in monomers by heating. Thus after the addition of SDS and heating at 100°C, dimeric β₂GPI-bh may dissociate and migrate like a 50 kDa monomer. However, high MW components were also present in our preparations of oxidized β₂GPI after electrophoresis. Oxidized β₂GPI 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 β₂GPI and β₂GPI-bh and their amounts were increased after the reduction of imine bonds by NaBH₃CN. 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 3** Binding constants of native and oxidized β₂GPI to PC/PS monolayers

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Kₐ (1/Ms)</th>
<th>Kₑ (1/s)</th>
<th>Kᵦ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native β₂GPI</td>
<td>(1.42 ± 0.19) × 10⁵</td>
<td>(2.85 ± 0.46) × 10⁻²</td>
<td>202 ± 60</td>
</tr>
<tr>
<td>Oxidized β₂GPI</td>
<td>(1.66 ± 0.29) × 10⁵</td>
<td>(4.33 ± 0.11) × 10⁻²</td>
<td>257 ± 31</td>
</tr>
</tbody>
</table>

β₂GPI and β₂GPI-bh at 1 μM 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-β₂GPI 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 β₂GPI and β₂GPI-bh. Although EY2C9 bound strongly to β₂GPI-bh at a concentration as low as 3 × 10⁻⁸ M, native and oxidized β₂GPI were not recognized by EY2C9 even at 10⁻⁷ M. The fact that α₁-glycoprotein, biotinylated on its glycan chains similarly to β₂GPI-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 β₂GPI, native β₂GPI and β₂GPI-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 10⁻⁹ M in association with GAGHR-HRP. Slices were also revealed with 9G1 in association with GAGHR-HRP and with SAV-HRP. As a control, slices were revealed with GAGHR and GAGHR-HRP alone. The results in Figure 7(B) show that EY2C9 recognized 50 kDa and 90 kDa β₂GPI-bh but did not react with native β₂GPI. The relative importance of the dimeric form versus total forms were much higher when labelled with EY2C9 than with 9G1 + GAGHR-HRP or SAV-HRP (40 %, 25 % and 4 % respectively). β₂GPI-bh (90 kDa) was thus detected more easily by EY2C9 than 50 kDa β₂GPI-bh.

**Figure 6** Binding kinetics of various β₂GPI by SPR

Sensorgrams showing binding of (A) β₂GPI-bh; (B) native β₂GPI; (C) oxidized β₂GPI 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 μM. Fits (dashed lines) were obtained by using either the Langmuir 1:1 model, (B, C) or the two-state reaction model (A).
were used to detect luminescence. Strips were also labelled with the murine anti-

were used to detect luminescence. Strips were also labelled with the murine anti-

be in the vicinity of the oxidized glycan chains. This is favoured by the binding of \( \beta_2 \text{GPI} \) 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 \( \beta_2 \text{GPI-bh} \) and not the higher MW components present in the supernatant which bound at \( t = 0 \). In addition, they seemed to occur by polymerization of \( \beta_2 \text{GPI} \) 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 \( \beta_2 \text{GPI-bh} \) 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 \( AB^\ast \)) resulting from the reaction of \( \beta_2 \text{GPI-bh} \) with PS. \( AB \) is obtained after a rapid adsorption phase due to electrostatic interactions between PS and the AnPL binding site of the \( \beta_2 \text{GPI-bh} \) molecule. Then a second reaction step with lower kinetic constants leads to the formation of \( AB^\ast \). Our binding experiments on PS-coated microtitre plates, demonstrated that binding to AnPL induced polymerization of \( \beta_2 \text{GPI-bh} \) in a time-dependent manner. In addition, when we measured the binding of \( \beta_2 \text{GPI-bh} \) to apoptotic cells by flow cytometry, we did not observe any saturation and patches of \( \beta_2 \text{GPI-bh} \) were visible by confocal microscopy, suggesting that after binding to AnPL, \( \beta_2 \text{GPI-bh} \) auto-associates with neighbouring molecules [43]. A fast step of \( \beta_2 \text{GPI} \) binding to AnPL (\( AB \)), may thus be followed by a slower phase of auto-association of the molecule with neighbouring \( \beta_2 \text{GPI-bh} \) molecules on the monolayer surface (\( AB^\ast \)). Clustering of \( \beta_2 \text{GPI} \), 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 \( \beta_2 \text{GPI} \) were best fitted using the Langmuir 1:1 model. This is in line with the inability of native \( \beta_2 \text{GPI} \) 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 \( \beta_2 \text{GPI} \) may also occur but at higher concentrations than with \( \beta_2 \text{GPI-bh} \). Gamsjaeger et al. [50] used a two-state reaction model to fit their sensorgrams of native \( \beta_2 \text{GPI} \) on POPC/POPS (1-palmitoyl-2-oleoyl-sn-glycerol-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 \( \beta_2 \text{GPI} \) and \( \beta_2 \text{GPI-bh} \). 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 \( \beta_2 \text{GPI} \) molecules and be directly responsible for the dimeric quaternary structure. Since oxidized \( \beta_2 \text{GPI} \) has the same affinity for AnPL as native \( \beta_2 \text{GPI} \), interactions of biotin groups with AnPLs may be involved.

![Figure 7 EY2C9 binding to various \( \beta_2 \text{GPI} \)](image)

\( \beta_2 \text{GPI nat} \)
\( \beta_2 \text{GPI-ox} \)
\( \beta_2 \text{GPI-bh} \)

\( \beta_2 \text{GPI-bh} \) and \( \beta_2 \text{GPI} \) were best fitted using the Langmuir 1:1 model. This is in line with the inability of native \( \beta_2 \text{GPI} \) 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 \( \beta_2 \text{GPI} \) may also occur but at higher concentrations than with \( \beta_2 \text{GPI-bh} \). Gamsjaeger et al. [50] used a two-state reaction model to fit their sensorgrams of native \( \beta_2 \text{GPI} \) 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.

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in the decreased dissociation of bound β2-GPI-bh and thus in its increased affinity for AnPL.

YE2C9 antibody binds to dimeric β2-GPI-bh. Monomeric native β2-GPI and oxidized β2-GPI were not recognized by YE2C9. We observed that YE2C9 and 9G1 bound more strongly to polymeric than monomeric β2-GPI-bh. But 9G1 also bound more strongly to 90 kDa than 50 kDa oxidized β2-GPI (Figure 2B), whereas YE2C9 did not bind at all to oxidized β2-GPI (Figure 7A). There are differences between YE2C9 and 9G1. (i) The murine anti-β2-GPI mAb IgG 9G1 binds to β2-GPI in solution whereas YE2C9 binds to β2-GPI only when it is layered on oxygenated polystyrene plates. (ii) In addition, 9G1 is directed against domain I of β2-GPI whereas YE2C9 is directed against domain IV of the molecule.

Recently, Liu et al. observed binding of aCL YE2C9 to β2-GPI modified by the addition of a modified ketocholesterol, noted oxlig1 [36]. YE2C9 may bind to oxlig1 or to the β2-GPI oxlig1 interface. However, this adduct on the target was similar to that of native β2-GPI [44]. It is likely that 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 YE2C9 binding to oxidized β2-GPI, suggesting that dimerization itself does not create epitopes recognized by aCLs. In the experiments reported by Liu et al., aCL YE2C9 bound to β2-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. [45] proposed that aCL binds to β2-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.

Our experiments may have physiological implications. Oxidation alone was not sufficient to induce recognition by aCL YE2C9. Oxidized β2-GPI was not recognized by YE2C9 and its affinity for AnPL was similar to that of native β2-GPI (results not shown). Polymerization of the β2-GPI molecule was also not sufficient since imine-bound polymers were present in oxidized β2-GPI, which was not recognized by YE2C9. The addition of biotin groups to glycan chains of β2-GPI or adducts of modified ketocholesterol on the polypeptide chain of the β2-GPI molecule were essential for recognition of the molecule by aCL YE2C9.

We conclude that these changes could lead to auto-association of the β2-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 Rouby et al. [45] and observed by Sheng et al. [27], auto-association of β2-GPI will increase it; (iii) if the binding site is created by the union of at least two β2-GPI molecules, auto-association may create this site. These results suggest that the disaccobar dimeric structure of β2-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-β2-GPI and aCL antibodies.

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β2GPI detection by anti-cardiolipin antibody EY2C9


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