Microheterogeneity of beta-2 glycoprotein I: implications for binding to anionic phospholipids

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Considerable interest is currently focused on the interactions of beta-2 glycoprotein I (β_2 GPI) and anti-phospholipid antibodies with anionic phospholipids in an attempt to understand the association between these antibodies and clinical diseases such as thrombosis. The interactions of β_2 GPI and anionic phospholipids have only been characterized partially, and the physiological role of this glycoprotein remains uncertain. In this study we have explored in detail the physical and phospholipid-binding characteristics of a number of β_2 GPI preparations. We have found (i) that perchloric acid-purification methods are damaging to β_2 GPI during purification, (ii) that the dissociation constants of the various preparations for phosphatidylserine vary between 0.1–2 μ M and are considerably weaker than previously reported, (iii) that considerable differences in affinity of the various β_2 GPI

preparations for anionic phospholipids are obtained when comparing anionic phospholipids immobilized to a solid-phase versus phospholipid assembled in unilamellar vesicles, (iv) that the integrity of the fifth domain of β_2 GPI is important for binding immobilized anionic phospholipid but not especially important in binding vesicular anionic phospholipid, and (v) that β_2 GPI preparations with differing isoelectric species content bind anionic phospholipids differently, suggesting that varying glycosylation and/or protein polymorphisms impact upon phospholipid binding. These results highlight the importance of assessing the determinants of the interaction of β_2 GPI with anionic phospholipids assembled in unilamellar vesicles.

Key words: heparin, protein binding, protein structure, vesicles.

INTRODUCTION

Beta-2 glycoprotein I (β_2 GPI), also known as apolipoprotein H, is a lipid-binding glycoprotein that was identified in 1961 [1]. The primary structure, sequenced originally by Lozier et al. [2], consists of 326 amino acids that are arranged into five repeating domains termed short consensus repeats, complement consensus repeats or 'sushi' domains. Complete nucleotide sequencing by cDNA cloning has been reported for human β_2 GPI [3–6]. The protein sequence predicted by cDNA differs significantly from that of Lozier et al. [2]. First, single amino acid substitutions were described at residues 102 (Ser → Cys), 169 (Cys → Asp) and 247 (Leu → Val). The predicted changes at position 102 and 169 were more consistent with the invariant short-consensus-repeat structure. The substitution of leucine for valine at position 247 is a polymorphism of no structural importance [7]. Secondly, the disulphide-bond linkages in the fifth domain were determined to be between cysteine residues at positions 245 and 296 (Cys 1 → 4), 281 and 306 (Cys 2 → 5) and 288 and 326 (Cys 3 → 6). Lastly, cDNA sequencing predicted only four asparagine-linked glycosylation sites [3,5] based on the Asn-Xaa-Ser/Thr sequences at positions 143, 164, 174 and 234 in domains III and IV. Carbohydrate contributes almost 20% of the apparent molecular mass of 50 kDa, and studies have shown human β_2 GPI to be partially sialylated and galactosylated with bi-antennary and tri-antennary glycans [8]. More recently, a proportion of sialic acid and galactose was also found to be O-linked, probably to serine or threonine, at unknown locations within the β_2 GPI molecule [9]. Isoelectric-focusing studies [10,11] reveal β_2 GPI to be a heterogeneous molecule with five major and up to five minor

isoforms. The structural basis of this microheterogeneity is controversial. Variation in glycosylation, specifically sialylation, has been proposed as the structural difference between isoelectric species [10,11]. In contrast, more recent reports suggest that specific polypeptide polymorphisms account for this heterogeneity [12].

Whereas the physiological role of β_2 GPI remains unclear, many studies suggest that it may be a natural anti-coagulant. β_2 GPI has been demonstrated *in vitro* to inhibit intrinsic pathway activation [13], and to inhibit tenase [14] and prothrombinase [15] activities upon the surface of activated platelets and synthetic phospholipid vesicles. Plasma levels of β_2 GPI have been shown to fall in disseminated intravascular coagulation [13,16], consistent with consumption of β_2 GPI in this thrombotic process. The anti-coagulant action most probably resides in the ability of β_2 GPI to bind negatively charged surfaces including anionic phospholipids (AnPL) [17]. The lipid-binding properties of β_2 GPI have, however, only been characterized partially. A putative lipid-binding site in the fifth repeating domain of β_2 GPI has been proposed [18]. This site consists of a highly positively charged six-amino acid sequence Lys-Asn-Lys-Glu-Lys-Lys (residues 282–287) located in the fifth domain of β_2 GPI. Computer modelling of β_2 GPI, based on sequence homology with complement consensus repeat 13 of human Factor H, suggests that this sequence is surface exposed and thus suited to be a binding site [18]. Cleavage within the fifth domain completely abrogated binding of β_2 GPI to AnPL bound to a solid phase [19]. Studies with various synthetic peptides spanning the fifth domain of β_2 GPI confirm that a binding site for solid-phase AnPL is contained within the sequence Cys-281–Cys-288 [20]. Site-

Abbreviations used: β_2 GPI, beta-2 glycoprotein I; AnPL, anionic phospholipid(s); AS, ammonium sulphate; PA, perchloric acid; CL, cardiolipin.

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directed-mutagenesis studies have also demonstrated the importance of this region in binding to AnPL bound to a solid phase [21,22]. More recently, two polymorphisms in the fifth domain (Cys-306 → Gly-306 and Trp-316 → Ser-316) have been shown to influence binding to AnPL coated to a solid phase [23]. These are currently the only known structural determinants of β_2 GPI that influence its binding to AnPL. Additionally, most studies have looked exclusively at binding to AnPL coated to a solid phase.

In this study we have investigated the affinity of various preparations of β_2 GPI for AnPL assembled in unilamellar vesicles. The affinity for AnPL in this more physiological form is compared with AnPL coated to a solid phase. Substantial differences were found between solid-phase and bilayer phospholipid-binding assays. We confirm that proteolysis in the fifth domain is an important determinant of binding to solid-phase AnPL but not as important for binding bilayer AnPL. Our results suggest that differing isoelectric species of β_2 GPI, some probably induced during purification, have variable affinities for AnPL. These results highlight the importance of assessing the binding by β_2 GPI of AnPL in physiological binding assays for determining a physiological role for this glycoprotein.

MATERIALS AND MATERIALS

Purification of β_2 GPI

Several preparations of human β_2 GPI were made, including β_2 GPI from perchloric acid (PA) precipitation of plasma (PA peaks 1, 2 and 3), β_2 GPI from ammonium sulphate (AS) precipitation of plasma in the presence of proteolytic inhibitors (AS peaks 1 and 2), and a β_2 GPI purified from plasma by affinity chromatography on a cardiolipin (CL) solid phase (CL-1). The PA-derived β_2 GPIs were made as described previously [16]. Briefly, 10 l of normal human plasma from several donors was first dialysed against 0.15 M NaCl/0.01 M Tris/HCl (pH 7.0; dialysis buffer) overnight. To this dialysed plasma 70% PA was added dropwise while stirring at room temperature to a final concentration of 3–5%. After 15 min the preparation was centrifuged, and the supernatant containing β_2 GPI was then pH-adjusted to 7.3–7.6 with saturated Na_2CO_3 solution and then dialysed overnight against the dialysis buffer. The dialysed supernatant was then applied to a heparin–Sephacryl CL-4B column (Pharmacia, Uppsala, Sweden) equilibrated in dialysis buffer, and bound protein eluted with 0.5 M NaCl/0.01 M Tris/HCl (pH 7.0). The β_2 GPI-containing fractions, as assessed by SDS/PAGE and β_2 GPI ELISA, were pooled, concentrated over a PM-10 Amicon membrane (Amicon Division, W. R. Grace & Co., Beverly, MA, U.S.A), and then subjected to size-exclusion chromatography using Sephacryl S-200 (Pharmacia) in the same buffer. Fractions containing β_2 GPI were pooled and dialysed against 0.05 M sodium acetate/0.05 M NaCl (pH 4.8). This preparation was then applied to an S-Sepharose fast-flow (Pharmacia) column which was developed with a linear gradient with 0.05 M sodium acetate/0.5 M NaCl (pH 5.2). Six β_2 GPI-containing peaks were resolved and each peak was pooled separately, dialysed against distilled water and concentrated in an Amicon concentrator (Amicon Division) with a PM-10 membrane to a final concentration between 600–1500 $\mu\text{g}/\text{ml}$. In order of elution the maximum absorbance at 280 nm of the peaks were 0.078, 0.398 (PA-1), 0.142, 0.297, 1.055 (PA-2) and 0.07 (PA-3).

Because PA is potentially damaging to polypeptide and carbohydrate moieties, an AS purification method of β_2 GPI was developed. Additionally, because of proteolysis in the fifth domain of β_2 GPI influencing its phospholipid binding, purifi-

cation was performed in the presence of a cocktail of proteolytic inhibitors. In preliminary experiments it was found that > 90% of plasma β_2 GPI was precipitated in two separate portions between AS saturations of 30–50% (containing 38% of total plasma β_2 GPI) and 60–70% (containing 57% of total plasma β_2 GPI). As it was not known if these two portions were identical they were purified separately. Normal human serum from healthy blood donors was first pooled and then cooled to 4 °C. Proteolytic inhibitors that were added included benzamide hydrochloride (20 μM , Sigma), aprotonin (5 μM , Trasylol®, 500 000 kallikrein-inactivating units/50 ml, Bayer Australia), PMSF (2 mM, Sigma) and disodium EDTA (10 mM). AS was added to achieve stepwise final concentrations of 30, 50 and 70%. After each addition the plasma was stirred continuously for 30 min at 4 °C. The precipitate was then harvested by centrifugation in 250-ml aliquots in a Beckman refrigerated centrifuge at 10 800 g, and the supernatant was returned to 4 °C for further precipitation steps as detailed. The collected precipitates for 30–50% and 50–70% (labelled AS-1 and AS-2 respectively) were then extensively dialysed separately against distilled water and then against 0.15 M NaCl/0.01 M Tris/HCl (pH 7.3). The dialysed preparations were then applied to heparin–Sephacryl, size-exclusion chromatography and, finally, anion-exchange chromatography as described previously. At anion-exchange, single peaks of β_2 GPI were eluted from the column. Finally the purified β_2 GPI was dialysed against distilled water and concentrated in an Amicon concentrator (Amicon Division) with a PM-10 membrane to a final concentration of 1500–6000 $\mu\text{g}/\text{ml}$. The final preparations were aliquoted and stored at –80 °C until use.

The final preparation of β_2 GPI (CL-1 or CL β_2 GPI) was copurified with anti-CL antibodies from plasma as described previously [24]. This plasma was obtained from a single patient with venous thrombosis, and the native plasma was positive for both lupus anti-coagulant and anti-CL antibodies. The β_2 GPI and anti-CL antibody containing eluate from the CL-affinity column was dialysed against PBS (Dulbecco 'A', Oxoid, Unipath, Hampshire, U.K.) and applied to anti-human IgG–agarose (Sigma Immunochemicals, A-3543). Fall-through fractions were collected and dialysed against 0.05 M sodium acetate/0.05 M NaCl (pH 4.8). This preparation was then chromatographed on S-Sepharose fast-flow (Pharmacia) as described previously. A number of elution peaks were obtained, with a major protein peak that eluted at 0.53 M NaCl. This peak was collected and dialysed against distilled water and concentrated over a PM-10 membrane in an Amicon concentrator to a final concentration of 600 $\mu\text{g}/\text{ml}$. The final preparations were aliquoted and stored at –80 °C until use.

Characterization of β_2 GPI preparations

The various preparations of β_2 GPI were characterized by SDS/PAGE under reducing and non-reducing conditions, and were subjected to cation exchange, chromatofocusing and N-terminal sequencing. Samples for SDS/PAGE were run on 10% resolving and 4% stacking gels with 2-mercaptoethanol as reducing agent and stained with Coomassie Brilliant Blue. Cation exchange was performed using an FPLC system (Pharmacia) and a Mono-S HR 5/5 1-ml column (Pharmacia). Fractions containing 2 mg of each β_2 GPI preparation were dialysed against 0.05 M sodium acetate/0.05 M NaCl (pH 4.8). These preparations were then applied at 1 ml/min and resolved with a linear gradient with 0.05 M sodium acetate/1.0 M NaCl (pH 5.2). Each preparation resolved as a single peak and the salt concentration at resolution was recorded. Chromatofocusing was performed using an FPLC system (Pharmacia) and a Mono-P HR 5/5 1 ml column

(Pharmacia). The column was cleaned and equilibrated according to the manufacturer's instructions. The β_2 GPI preparations were dialysed against 0.25 M diethanolamine (pH 9.5), and a minimum of 1 mg of each preparation in a final volume of 500 μ l was loaded at a flow rate of 0.5 ml/min. After washing with a minimum of 30 ml of loading buffer, bound protein was allowed to elute sequentially with 25 ml of buffer A [5 ml of Polybuffer 96 (Pharmacia) in 100 ml of distilled water (pH 6.0)] and then 20 ml of buffer B [10 ml of Polybuffer 74 (Pharmacia) in 100 ml of distilled water (pH 4.0)]. This combination of buffers allowed chromatofocusing to be performed over a pH range from 9.5 to 4.0. Analysis of the original UV chromatographs was performed by accompanying FPLCdirector[™] software. Additionally, on each 0.5 ml fraction the protein concentration was determined by spectral absorbance at 280 nm, and the pH was measured to three decimal places with a Micro 2 pH/Ion Meter (EDT Instruments, CHK Engineering, Ermington, Sydney, Australia) with a Sensorex Epoxy Sealed Reference pH probe (CHK Engineering) and a temperature probe. The N-terminal sequence of each preparation was determined kindly by Dr. R. Simpson and colleagues (Ludwig Institute for Cancer Research, Melbourne, Australia) after preparative HPLC on a Nova-Pak C₁₈ column equilibrated with 0.1% trifluoroacetic acid and eluted with 0.1% trifluoroacetic acid/70% acetonitrile. The extent of cleavage in the fifth domain for each preparation of β_2 GPI was estimated from this analysis.

Binding of β_2 GPI to solid-phase phospholipids

The binding of the various β_2 GPI preparations to phospholipids coated to a solid phase was performed. Aliquots of the various β_2 GPI preparations were iodinated using iodobeads (Pierce, Rockford, IL, U.S.A.) as per the manufacturer's instructions. In each iodination, 50 μ g of β_2 GPI was combined with one iodobead and 5 μ l of Na¹²⁵I in a total reaction volume of 150 μ l. After 15 min of incubation the iodinated protein was separated from free ¹²⁵I on a 10 ml Bio-Rad desalting column (Bio-Rad PDG6) equilibrated with 0.1 M phosphate buffer (pH 7.0)/5 mg/ml BSA. Microtitre plates (Nunc High-Binding Break Apart) were coated with 30 μ l per well of 30 μ g/ml solution of either bovine phosphatidylserine or bovine phosphatidylcholine (Sigma) in ethanol. The plates were wrapped in foil and the liquid allowed to evaporate overnight at 4 °C. The plates were blocked with 200 μ l of 1% milk powder/0.5% BSA in PBS for 1 h at room temperature and then washed three times with 0.5% BSA in PBS. The various ¹²⁵I-labelled β_2 GPI preparations diluted in PBS were then added with and without unlabelled β_2 GPI in a total reaction volume of 100 μ l. After incubation at room temperature on a plate shaker for 60 min the supernatant was drained and the plate washed gently, once, with PBS. Total and bound radioactivity was determined by counting individual wells in a Packard Crystal II γ -radiation counter. All binding experiments were performed in duplicate.

The dissociation constants for the interaction of ¹²⁵I- β_2 GPI (K_H) and unlabelled β_2 GPI (K_C) with immobilized phospholipid are described by eqn. (1).

$$K_H = [H][PL]/[H \cdot PL]$$

$$K_C = [C][PL]/[C \cdot PL]$$

$$[PL]_T = [PL] + [H \cdot PL] + [C \cdot PL] \quad (1)$$

[H], [C] and [PL] are the concentrations of ¹²⁵I- β_2 GPI, unlabelled β_2 GPI and free immobilized phospholipid respectively, and [H · PL] and [C · PL] are the concentrations of ¹²⁵I-labelled and

unlabelled β_2 GPI-immobilized phospholipid complexes respectively. The subscript T denotes total reactant concentration. If the total concentration of unlabelled β_2 GPI is at least 10 times that of unlabelled- β_2 GPI-immobilized phospholipid complex, $[C]_T$ [C · PL], then $[C]_T \approx [C]$ and an expression for [H · PL] in terms of the constants K_H , K_C , $[H]_T$, $[C]_T$ and $[PL]_T$ can be derived from eqn. (1). This approach and a similar derivation have been described by Olson et al. [25]. The resulting equation is a quadratic in terms of [H · PL] as described by eqn (2).

$$[H \cdot PL]^2 K_C - [H \cdot PL] \{K_C([PL]_T + [H]_T + K_H) + K_H[C]_T\} + [PL]_T[H]_T K_C = 0 \quad (2)$$

The characterization of the binding was performed by two series of experiments. First, the affinity of ¹²⁵I- β_2 GPI to immobilized phospholipid (K_H) was estimated from experiments that measured the binding of a range of ¹²⁵I- β_2 GPI concentrations to a fixed concentration of immobilized phospholipid. Typically 5–10% of the total ¹²⁵I- β_2 GPI added bound to the immobilized phospholipid. Secondly, the affinity of unlabelled β_2 GPI for immobilized phospholipid (K_C) was estimated from experiments measuring the binding of a fixed concentration of ¹²⁵I- β_2 GPI (usually 100 000 c.p.m. added) to immobilized phospholipid as a function of unlabelled β_2 GPI concentration. Data were fitted to eqns. (1) and (2) by non-linear least-squares regression (Scientist software; Micromath, Salt Lake City, UT, U.S.A.).

Binding of β_2 GPI to phospholipid vesicles

Preparation of synthetic phospholipid vesicles

Large unilamellar vesicles were prepared as described by Mimms et al. [26], with minor modification. A total of 20 mg of bovine phosphatidylserine and bovine phosphatidylcholine (Sigma) were combined in a molar ratio of 1:3 and dried to a thin film on a borosilicate glass tube under a stream of nitrogen and then vacuum dried for a further 2 h. The dried lipid was then dissolved by vortexing in 0.5 ml of 1.1 M n-octyl- β -D-glucopyranoside (Sigma), which was then removed by dialysis at room temperature on a magnetic stirrer against 1 litre of 50 mM Hepes/0.125 M NaCl/0.02% sodium azide (pH 7.4; binding buffer) per day for 4 days. The supernatant was mixed periodically by pipetting, and after 4 days it became quite turbid. The resulting vesicles were isolated by gel filtration on a Sepharose CL-2B column (Pharmacia) equilibrated with the binding buffer and fractions were collected after measuring absorbance at 450 nm. The phospholipid concentration was determined by an assay for inorganic phosphate [27]. Flow-cytometric analysis on Becton FacStar confirmed the average diameter of the vesicle preparation to be approx. 250 nm compared with 2 μ m standard microspheres.

Competitive binding model

A competitive model of binding was modelled on that described by Olson et al. [25] using the technique of quantitative affinity chromatography. In this model the partitioning under equilibrium conditions of the ligand β_2 GPI was determined by its affinities for binding sites on the affinity matrix heparin-Sepharose or on a soluble competitor (e.g. phospholipid vesicles or soluble heparin). Initial experiments were performed to characterize the affinity (K_p) of the ligand β_2 GPI, L, for the affinity matrix heparin-Sepharose, P. Experiments were then repeated in the presence of a soluble competitor, C, and an estimate of the affinity of β_2 GPI for the soluble competitor (K_C) could be derived. The interactions of β_2 GPI with the affinity

matrix heparin–Sephacrose and the soluble competitor are described by:

$$K_p = [P]\chi[L]/[P \cdot \chi L] \quad (3)$$

$$K_c = [C]\eta[L]/[C \cdot \eta L] \quad (4)$$

where [P], [L] and [C] are the concentrations of the free affinity matrix heparin–Sephacrose, the ligand β_2 GPI and the soluble competitor respectively, χ is the number of binding sites for β_2 GPI on heparin–Sephacrose, η is the number of binding sites for β_2 GPI on the soluble competitor, and $[P \cdot \chi L]$ and $[C \cdot \eta L]$ are the concentrations of β_2 GPI–heparin–Sephacrose and β_2 GPI–competitor complexes respectively. The binding parameters for eqn. (3) are derived experimentally, and the competitive model can be solved for K_c as a cubic expression in terms of either known or experimentally derived parameters using total rather than free concentrations of competitor [see eqn. (5)].

$$D_1 \cdot [PL]^3 + D_2 \cdot [PL]^2 - D_3 \cdot [PL] + D_4 = 0 \quad (5)$$

where

$$D_1 = (K_p - K_c)$$

$$D_2 = \{K_c(L_T + 2\chi P_T + K_p) + K_p(\eta L_T \cdot \chi P_T - L_T - \chi P_T - K_p)\}$$

$$D_3 = \{K_c(2\chi P_T \cdot L_T + P_T^2 + K_p \cdot \chi P_T) + K_p(\eta C_T \cdot \chi P_T - L_T \cdot \chi P_T)\}$$

$$D_4 = K_c \cdot \chi P_T^2 \cdot L_T$$

and where P_T is the derived maximum number of binding sites for β_2 GPI on heparin–Sephacrose, L_T is the total concentration of β_2 GPI and C_T is the total concentration of competitor. The model assumes (i) that the interactions of β_2 GPI with the affinity matrix and competitor are strictly competitive, (ii) that β_2 GPI binds either the affinity matrix or the soluble competitor but not both simultaneously, and (iii) that β_2 GPI has only one binding site for either the affinity matrix or the soluble competitor. Data were fitted to eqns. (3) and (5) by non-linear least-squares regression (Scientist software, Micromath).

Competitive binding experiments

All binding experiments were performed in 1.5-ml microcentrifuge tubes (Eppendorf, Amrad, Australia) at room temperature on a mixing wheel. Heparin–Sephacrose slurry was obtained from Pharmacia. The centrifuge tubes were precoated overnight at 4 °C with 10 mg/ml of poly(ethylene glycol) 20000 (Sigma). Preliminary experiments were performed to determine the time to equilibrium binding to heparin–Sephacrose, and the amount of heparin–Sephacrose required for substantial β_2 GPI binding. Binding was 90% complete within 10 min (results not shown) and, for all experiments, 20-min incubations were selected. The amount of heparin–Sephacrose was 30 μ l of a well-mixed 1-in-5 slurry of matrix in binding buffer unless otherwise stated. Total reaction volume was 500 μ l and all experiments were performed in duplicate. For binding experiments in the absence of competitor, 30 μ l of either buffer (control) or heparin–Sephacrose slurry was added to 470 μ l of binding buffer with a final β_2 GPI concentration of 30 μ g/ml. After 20 min of mixing, the microcentrifuge tubes were centrifuged for 10 s at 13000 *g* (Microfuge), and 20- μ l samples (in triplicate) of supernatant were added to the wells of a microtitre plate (Linbro flat-bottomed microtitre plate, ICN Pharmaceuticals). Free β_2 GPI concentration was measured by protein assay (BCA kit, Pierce). After sampling, known amounts of β_2 GPI were added in 60 μ l of buffer and the microcentrifuge tubes returned to the mixing wheel. As many as nine stepwise additions of β_2 GPI were performed. For binding experiments in the presence of competitor, a similar experimental design was used. In a total

reaction volume of 500 μ l and fixed total concentration of β_2 GPI, the concentration of a competitor was increased in a stepwise fashion. In some experiments the total concentration of β_2 GPI was maintained by addition of further β_2 GPI. Free ligand concentration was measured in triplicate as detailed previously. Controls performed with these experiments included the addition of the same volume of buffer in the place of competitor. In all experiments, bound ligand concentrations were obtained by subtraction of measured free concentrations from the known total concentrations. Experiments with and without competitor were also performed in the absence and presence of 2.0 mM CaCl_2 . In order to maintain constant ionic strength in these experiments the calcium was introduced by appropriate mixtures of the binding buffer and a buffer containing 41.7 mM CaCl_2 in 50 mM Hepes/0.02% sodium azide (pH 7.4).

Direct binding experiments

Further experiments were performed to assess directly the binding of the various β_2 GPI preparations to synthetic phospholipid vesicles using an ultracentrifuge method (Airfuge, Becton, Australia). Preliminary experiments were performed to determine the time required to sediment the synthetic phospholipid vesicles (results not shown). After 25 min of centrifugation, 95% of total phospholipid in the synthetic-vesicle preparation was sedimented. Wells of a 96-well microtitre plate were precoated with 10 mg/ml of poly(ethylene glycol) 20000 in binding buffer overnight at 4 °C. In a total reaction volume of 200 μ l/well, a range of concentrations of various β_2 GPI preparations were incubated with two different concentrations of phospholipid vesicles for 40 min at room temperature on a plate shaker. Experiments were performed in the absence and presence of 2.0 mM CaCl_2 as described previously. After incubation, 20- μ l aliquots (in triplicate) were added to a microtitre plate for determination of total β_2 GPI concentration, and the remaining mixture centrifuged in polyallomer tubes (Becton). After centrifugation, 20- μ l aliquots of the supernatant (in triplicate) were added to a microtitre plate for determination of free β_2 GPI concentration.

The dissociation constants for the interaction of β_2 GPI (K_D) with immobilized phospholipid are described by:

$$[\beta_2\text{GPI} \cdot \text{PL}] = \chi[\beta_2\text{GPI}]/(K_D + [\beta_2\text{GPI}]) \quad (6)$$

where $[\beta_2\text{GPI}]$, $[\text{PL}]$ and $[\beta_2\text{GPI} \cdot \text{PL}]$ are the concentrations of free β_2 GPI, free phospholipid and β_2 GPI–phospholipid complexes respectively, and χ is the number of binding sites for β_2 GPI on phospholipid. Phospholipid concentrations were expressed in terms of a derived concentration of surface-exposed phosphatidylserine, which was calculated to be 13.1% of the total phospholipid concentration. This calculation assumes a random distribution of phosphatidylserine to either inner or outer leaflets of the lipid bilayer, and is the multiplication of the molar ratio of phosphatidylserine (a quarter of the total phospholipid), and an estimate of the amount of phosphatidylserine surface exposed based on the geometry of the vesicle (52.46% for vesicles with an external diameter of 250 nm and a bilayer thickness of 6 nm). Data were fitted to eqn. (6) by non-linear least-squares regression (Scientist software, Micromath) to obtain the dissociation constant for PL (K_D).

RESULTS

Characterization of various β_2 GPI preparations

The physical characteristics of the various β_2 GPI preparations are summarized in Table 1. The preparations varied in molecular mass by approx. 6 kDa under both non-reducing and reducing

Table 1 Summary of physical characteristics of β_2 GPI preparations

The first column refers to the method of purification of β_2 GPI; PA-1, PA-2 and PA-3 were obtained from PA precipitation of plasma, AS-1 and AS-2 were from AS precipitation of plasma and CL-1 was from affinity chromatography of plasma (see the text for details). Molecular masses were determined by SDS/PAGE. Cleavage data were obtained from N-terminal sequencing.

Preparation of β_2 GPI	Molecular mass (kDa) (non-reduced/reduced)	Elution from Mono-S (M NaCl)	Cleavage (%)	
			at Lys-317/Thr-318	at Ala-314/Phe-315
PA				
PA-1	49/55	0.441	47.2	3.1
PA-2	46.5/53	0.535	0	9.2
PA-3	43/51	0.541	20	2.4
AS				
AS-1	48/56	0.528	0	2.6
AS-2	48/57	0.526	1.9	16.7
CL				
CL-1	46.5/55	0.530	No data	No data

Figure 1 SDS/PAGE of various β_2 GPIs under reducing conditions

Electrophoresis was performed in a 10% stacking and 4% resolving gel with 25–40 μ g of β_2 GPI per lane. Lanes 1 and 14, molecular-mass markers; lane 2, empty; lanes 3 and 13, PA-1; lane 4, PA-3; lane 5, PA-2; lane 6, AS-1 (batch 29/3/95); lane 7, AS-2 (29/3/95); lane 8, AS-1 (3/5/95); lane 9, AS-2 (3/5/95); lane 10, AS-1 (10/5/95); lane 11, AS-2 (10/5/95); and lane 12, CL-1. The purification methods are described in the text.

conditions with PA-1 the largest and PA-3 the smallest (Figure 1). Most of the β_2 GPI preparations eluted from the anion-exchange column between 0.52 and 0.54 M NaCl with the exception of PA-1, which eluted at 0.441 M NaCl. There was a significant inverse correlation between molecular mass under reducing conditions and elution from Mono S (Spearman's correlation, $r = -0.954$, $P = 0.019$).

N-terminal sequencing confirmed that all preparations were β_2 GPI and that there were varying degrees of proteolysis at Lys-317/Thr-318 or Ala-314/Phe-315 as described previously [21]. There was no correlation between size on SDS/PAGE and

Figure 2 Chromatofocusing of β_2 GPI preparations

Elution (absorbance at 280 nm) from Mono-P column according to pH (see the text for details). The various β_2 GPI preparations are PA-1 (a), PA-2 (b), PA-3 (c), AS-1 (d), AS-2 (e) and CL-1 (f).

degree of proteolysis in the fifth domain of β_2 GPI. PA-1, the largest by SDS/PAGE, was actually the most significantly cleaved preparation (about 50 % cleaved), whereas the other

Figure 3 Competition for binding solid-phase phosphatidylserine (PS) by ^{125}I - $\beta_2\text{GPI}$ and soluble unlabelled $\beta_2\text{GPI}$ using AS-2 preparation

The total concentration of ^{125}I - $\beta_2\text{GPI}$ (^{125}I -B2GPI) in the experiment was 80 nM and ■ represents the concentration of ^{125}I - $\beta_2\text{GPI}$ bound to immobilized PS. The solid line represents the best fit of the data to eqn. (2) with a dissociation constant, K_D , of 101 ± 23 nM.

preparations, which varied between 2.6 and 22.4% cleavage, were smaller in size.

Chromatofocusing experiments revealed significant differences in isoelectric species content between the various $\beta_2\text{GPI}$ preparations (Figure 2). Analysis of the original UV chromatographs revealed many more protein peaks than apparent in Figure 2. This was especially seen for the PA preparations, where there were often 20 or more small chromatographic peaks, suggesting complex modifications of the $\beta_2\text{GPI}$ preparations (results not shown). In general there was a correlation between decreasing size on SDS/PAGE (reducing or non-reducing conditions), elution at higher salt concentrations from anion exchange and isoelectric species of high pH.

Binding of $\beta_2\text{GPI}$ to AnPL

A representative displacement of radiolabelled $\beta_2\text{GPI}$ from solid-phase AnPL by unlabelled $\beta_2\text{GPI}$ is presented in Figure 3. The

dissociation constant K_D ($\pm$ S.D.) of AS-2 for immobilized AnPL was $101.1 (\pm 23.2)$ nM, and was similar to AS-1, PA-2 and PA-3 preparations (Table 2). No significant binding to solid-phase AnPL was detected for the most significantly cleaved preparation, PA-1. There was, in general, a correlation between the degree of proteolysis in the fifth domain of $\beta_2\text{GPI}$ and affinity for immobilized AnPL. Interestingly, whereas PA-1 was cleaved significantly, almost one-half of this preparation was not cleaved and yet no detectable binding was obtained. Under the experimental conditions in which 200 000 c.p.m. of each preparation were added (for comparison, approx. $0.1 \mu\text{M}$ for labelled $\beta_2\text{GPI}$), the amounts of PA-1, PA-2, PA-3, AS-1 and AS-2 bound were 0.08, 0.73, 1.33, 12.5 and 12.9% respectively.

Representative data for the binding characteristics of the $\beta_2\text{GPI}$ preparations from the solution-phase binding experiments are presented in Figures 4 and 5. The binding of AS-2 to heparin-Sepharose in the absence of competitors (Figure 4a), in the presence of soluble heparin (Figure 4b) and in the presence of synthetic phospholipid vesicles (Figure 4c) is illustrated. Binding of all $\beta_2\text{GPI}$ preparations to heparin-Sepharose in the absence of competitors was demonstrable. The binding isotherms were generally in accordance with eqn. (3), although in the absence of calcium the binding isotherm for PA-2 was slightly sigmoidal. The binding isotherms for the competition experiments with heparin fitted well with the mathematical model proposed for all preparations. The calculated affinities of the various $\beta_2\text{GPI}$ preparations for either the affinity matrix or soluble heparin were similar (Pearson's correlation, $r = 0.989$, $P = 0.007$). The elution from Mono-S was weakly inversely correlated with affinities of the various $\beta_2\text{GPI}$ preparations for heparin-Sepharose (Spearman's correlation, $r = -0.761$, $P = 0.089$). The affinity of PA-1 for heparin was an order of magnitude less than the other preparations of $\beta_2\text{GPI}$. In contrast, the binding isotherms of competition experiments with synthetic phospholipid vesicles with all the $\beta_2\text{GPI}$ preparations studied could not be fitted with the proposed model of binding (as illustrated in Figure 4c). The shapes of the curves suggested complex or co-operative binding. Nevertheless, all $\beta_2\text{GPI}$ preparations bound synthetic phospholipid vesicles to some degree, including PA-1.

Direct binding assays of $\beta_2\text{GPI}$ to solution-phase phospholipid vesicles were performed for most of the preparations. Representative data for these experiments are illustrated in Figure 5 with preparation PA-3. The dissociation constants of the $\beta_2\text{GPI}$

Table 2 Comparison of cleavage in the fifth domain of $\beta_2\text{GPI}$ with the affinities for heparin, and immobilized and vesicular AnPL

The first column refers to the method of purification of $\beta_2\text{GPI}$; PA-1, PA-2 and PA-3 were obtained from PA precipitation of plasma, AS-1 and AS-2 were from AS precipitation of plasma and CL-1 was from affinity chromatography of plasma (see the text for details). Total percentage cleavage is the total cleavage (Lys-317/Thr-318 and Ala-314/Phe-315) in the fifth domain obtained from N-terminal sequencing. Affinity data are reported as $K_D \pm$ S.D. in μM (see the text for details). ND, not determined.

Preparation of $\beta_2\text{GPI}$	Total cleavage (%)	Affinity			
		Heparin-Sepharose	Soluble heparin	Vesicular AnPL	Immobilized AnPL
PA					
PA-1	50.3	49.6 ± 40.9	40.8 ± 7.1	1.69 ± 2.28	Nil binding
PA-2	9.2	4.04 ± 1.76	7.7 ± 1.09	0.46 ± 0.24	0.11
PA-3	22.4	2.36 ± 0.62	9.3 ± 1.76	0.42 ± 0.28	0.13
AS					
AS-1	2.6	6 ± 1.76	7.32 ± 0.39	1.13 ± 1.33	0.12
AS-2	18.6	4.92 ± 0.69	6.7 ± 0.45	1.13 ± 1.33	0.10
CL					
CL-1	ND	5.38 ± 1.32	6.18 ± 0.5	ND	ND

Figure 4 Binding of AS-2 β_2 GPI to heparin-Sepharose in the absence of competitors (a) or in the presence of soluble heparin (b) or phospholipid vesicles (c)

Binding performed in the absence (filled symbols) or presence (open symbols) of 2.0 mM CaCl_2 . The solid line in (a) represents the best fit of the data to eqn. (3) with a dissociation constant, K_D , of $4.04 \pm 1.76 \mu\text{M}$. In the competition experiments, $50 \mu\text{l}$ of a 1-in-5 slurry of heparin-Sepharose was used with the total concentration of AS-2 being $200 \mu\text{M}$. The solid line in (b) represents the best fit of the data to eqn. (5) with a dissociation constant, K_D , of $7.7 \pm 1.09 \mu\text{M}$ with or without CaCl_2 . The solid line in (c) represents the best fit of the data by third-order polynomial, as the data were not able to be fitted using eqn. (5) as described in the text. PS, phosphatidylserine.

preparations for solution-phase phospholipid vesicles were all similar and ranged between 0.4 and $2 \mu\text{M}$ (Table 2). Stoichiometry for the interaction of β_2 GPI and phosphatidylserine from direct binding assays was estimated as follows. The size of unilamellar vesicles averaged 250 nm by comparison with $2 \mu\text{M}$

Figure 5 Binding of PA-2 preparation of β_2 GPI to phospholipid vesicles

Experiments performed in the absence (●) or presence (○) of 2.0 mM CaCl_2 . The solid line represents the best fit of the data to eqn. (6), with dissociation constants, K_D , of $0.42 \pm 0.28 \mu\text{M}$ in the absence of Ca^{2+} and $0.55 \pm 0.27 \mu\text{M}$ in the presence of Ca^{2+} .

calibrated beads. Based on the experimentally derived maximal available binding sites, the phospholipid concentration, and the geometry of the unilamellar vesicle, one molecule of β_2 GPI occupied approximately 70 molecules of phosphatidylserine exposed in the outer leaflet of the vesicle. The affinity of PA-1 for vesicular phospholipid was an order of magnitude weaker than the other preparations. Affinity of the various β_2 GPI preparations for vesicular phospholipid was correlated weakly with elution from Mono-S (Spearman's correlation, $r = -0.984$, $P = 0.088$), molecular mass ($r = -0.996$, $P = 0.085$) and affinity to heparin-Sepharose ($r = 0.99$, $P = 0.162$), but not with the percentage of cleavage in the fifth domain ($r = 0.5$, $P = 0.5$).

DISCUSSION

In this study, a variety of β_2 GPI preparations were purified from plasma that differed significantly in their molecular mass, elution profiles from anion-exchange column and isoelectric-species content. The affinity of these preparations for AnPL vesicles was not well correlated with the degree of cleavage in the fifth domain, but instead correlated broadly with decreasing molecular mass, tighter binding to the anion-exchange column and more basic isoelectric-species content. Our results are consistent with varying degrees of glycosylation, most likely induced during purification, influencing affinity for AnPL. Additionally, the results of this study highlight two very important considerations in the study of β_2 GPI binding to AnPL, namely the presentation of AnPL and the structural integrity of β_2 GPI preparations used in the binding assays.

The affinities for AnPL in this study are weaker than found by Wurm [17] but consistent with several other investigators [20,21,28,29]. The variation in estimates of affinity of β_2 GPI for AnPL in these studies is possibly attributable to many factors but probably relates mostly to varying buffer conditions. The stoichiometry calculated for AnPL vesicles in this study (one β_2 GPI:70 phosphatidylserines) is similar to that reported by Willems et al. [30,31] of one β_2 GPI:75 lipid molecules.

That β_2 GPI in plasma is heterogeneous has been well described, although the structural basis for this is controversial. Early published data on the microheterogeneity of β_2 GPI suggest this to be the result of variation in carbohydrate content of the molecule [10,11]. However, large population studies [32,33] suggest that protein polymorphisms are the likely explanation of this microheterogeneity. On the basis of the banding patterns found by isoelectric-focusing gels performed on serum from hundreds of blood donors, four alleles (APO H*1–4) have been proposed to explain the inter-individual variation. Neuraminidase and endoglycosidase F treatment resulted only in a cathodal shift (more basic) of the banding patterns, which were otherwise maintained [32]. Consistent with this proposal are the recently described protein polymorphisms accounting for the APO H*3^w and APO *H1 alleles (Ser → Trp at residue 316, Asp → Ser at residue 88, respectively) [12]. A further protein polymorphism was also described with a substitution of glycine for cysteine at position 306 (originally reported as glycine for serine but later clarified) [23]. These polymorphisms, located close to the proposed lipid-binding site, have been shown to influence β_2 GPI ability to bind AnPL in solid-phase assays. In summary, whereas variation of glycosylation certainly can contribute to the microheterogeneity of β_2 GPI, the differing isoforms of β_2 GPI seen between individuals appear to be due to alterations of the polypeptide chain, many as-yet undescribed.

The exact differences between the various β_2 GPI preparations used in this study are not clear. The β_2 GPI preparations purified and studied in this report varied in molecular mass and isoelectric-species content. The decreasing molecular mass was not explained by proteolytic cleavage in the fifth domain of β_2 GPI. That disruption of the carbohydrate moieties occurred is supported indirectly by the presence of isoelectric species more basic than normally seen in the smaller β_2 GPI preparations. PA is a strong acid with oxidative properties, and damage to the β_2 GPI molecule during purification is likely. The differences of molecular mass could be consistent with loss of sialic acid molecules that contribute a total of 3 kDa. The numerous peaks (often > 15–20) seen on chromatofocusing, especially with the PA preparations, is also supportive of the conclusion that PA is damaging to the β_2 GPI molecule. We therefore propose that the varying molecular mass, isoelectric-species content and affinity for AnPL are thus due to the variation in glycosylation at least partly induced by the method of purification. Given that the starting plasma was from a large pool of donors, as yet undescribed polymorphisms could also explain some of the heterogeneity of our preparations. There is no other published evidence that glycosylation could influence β_2 GPI's binding to AnPL. The affinity for AnPL of various recombinant β_2 GPI preparations appears to be similar to native protein [21–23]. No effect of N-glycanase treatment upon β_2 GPI binding to CL coated to solid phase was found by several investigators [19,34,35]. All the above-mentioned studies have, however, used ELISA-type binding assays.

The results from the binding experiments reported from this study are important for another reason. There were considerable differences between the affinity of our β_2 GPI preparations for solid-phase AnPL and vesicular AnPL in solution. Most investigators studying the interaction of β_2 GPI, AnPL and anti-phospholipid antibodies use ELISA-based assays. These assays have led to considerable advances in this field including the description of the phospholipid-binding site in the fifth domain of β_2 GPI [19,20]. Our results confirm the importance of the integrity of the fifth domain containing this binding site in determining binding to solid-phase AnPL. Interestingly 50% of the PA-1 preparation had an intact fifth domain, yet binding was

not detectable by ELISA. Binding of this preparation to vesicular AnPL was detectable, although weaker than the less-cleaved preparations. In ELISA binding assays, AnPL are bound hydrophobically to the plastic wells of the ELISA plate. Under these conditions the phospholipids adopt a monolayer film and the density of monolayer lipids is low [36,37]. This is quite different to the bilayer presentation of AnPL in the unilamellar vesicles in this study and more importantly *in vivo*, such as in platelet membranes. In lipid bilayers, lipid density is higher and there is considerable movement of individual phospholipid molecules within the bilayer. In our vesicular AnPL binding assay cleavage near the phospholipid-binding site had little influence on affinity for AnPL. This difference presumably relates to the difference in AnPL presentation. This highlights the need for more studies of AnPL assembled in a more physiological form. Interestingly, we (T. A. Brighton, Y.-P. Dai, P. J. Hogg and C. N. Chesterman, unpublished work) and others [38] have not been able to demonstrate significant binding to activated platelet membranes or to platelet microparticles by β_2 GPI alone.

In conclusion, proteolysis in the fifth domain of β_2 GPI is an important determinant of binding to solid-phase AnPL but not to vesicular AnPL. Additionally, our results suggest that variation in carbohydrate content of the various β_2 GPI preparations also influences this interaction. Purification methods utilizing PA may significantly alter the functional properties of the molecule, and should be avoided. We feel a thorough re-examination of the structural determinants of β_2 GPI that influence binding to vesicular AnPL is required. This information may provide a better understanding of the nature and physiological significance of the interaction between β_2 GPI and physiological membranes such as platelets and platelet microparticles. These results have important implications also for the study of anti-phospholipid antibody-associated thrombosis.

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