Interaction of β2-Glycoprotein 1 with Phosphatidylserine-Containing Membranes: Ligand-Dependent Conformational Alterations Initiate Bivalent Binding†

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ABSTRACT: β2-Glycoprotein 1 (β2GP1), a 50 kDa serum glycoprotein that binds anionic phospholipid-containing membranes, plays a regulatory role in physiology and pathology. The protein is a member of the short consensus repeat (SCR) superfamily containing four typical repeating domains and an aberrant fifth domain constructed into an SCR-like core at the C-terminus. To investigate the contribution of the individual domains to the binding of β2GP1, a series of sequential domain-deleted recombinant protein fragments were generated and assessed for their interaction with PS-containing vesicles. Spectral analyses of lipid binding-dependent alterations in tryptophan emission spectra revealed that the (single) tryptophan residues of the individual domains underwent binding-dependent conformational alterations. Depending on the ionic strength, some domains moved from polar to nonpolar environments, while others moved from less polar to more polar environments. Analysis of a series of acrylamide quenching and resonance energy transfer experiments indicated that the binding of N-terminal domain 1 to PS membranes exists in two, ionic strength-dependent, conformations. At low ionic strengths, domain 1 bound to the vesicles and induced their precipitation and/or aggregation. At physiologic ionic strengths, domain 1 detached from the membrane surface while the remaining domains maintained their association with the membrane. Under these conditions, membrane-bound conformationally altered domain 1 projects away from the membrane surface, enabling it to interact with other proteins and/or cell surface ligands or receptors.

β2-Glycoprotein 1 (β2GP1)[1] is a major lipid binding protein in plasma that has been shown to play a role in autoimmunity (*1*−*3*), coagulation (*4*), and the clearance of apoptotic and senescent cells (*5*−*8*). It is composed of 326 amino acids and is a member of the short consensus repeat (SCR) superfamily containing four typical repeating domains and an aberrant fifth domain constructed onto an SCR-like core at the C-terminus (*9*−*11*). While both domains 1 and 5 have been shown to have lipid binding properties, domain 5 is the principal lipid binding domain (*12*−*14*). Binding of lipid to domain 5 occurs through a series of positively charged lysine residues (*15*, *16*) that likely results in the insertion of a partially hydrophobic loop into the lipid bilayer (*17*). From its crystal structure, Bouma et al. (*18*) suggested that domains 1−4 remain in solution, leaving the N-terminus available for other membrane interactions. Conceivably, this secondary reaction could involve the binding of domain 1 to other negatively charged lipids or to specific cell surface receptors.

Binding of β2GP1 to small particles such as negatively charged lipid vesicles or mitochondria results in their agglutination and subsequent precipitation (*19*, *20*). However, the mechanism responsible for the precipitation of membranes is largely unknown. Conceivably, precipitation could be due to simple aggregation as a result of bivalent (or multivalent) protein−lipid interactions or due to direct alterations in the hydrophobicity of the target membrane (*21*). Indeed, observations by Hagihara et al. (*12*) suggested that charge−charge interactions between β2GP1 and anionic lipids shield the charge repulsion that impedes protein−protein and anionic amphiphile−anionic amphiphile interactions, resulting in increased hydrophobicity. In principle, this could provide the driving force for the formation of large aggregates as a result of decreased solubility in aqueous media.

To further investigate and better characterize the interaction between β2GP1 and phospholipid membranes, we carried out a series of precipitation and intrinsic fluorescence measurements using sequential domain-deleted recombinant fragments of β2GP1 and phosphatidylserine-containing membranes. Our results indicate that membrane aggregation in low-ionic strength buffers is dependent on binding of ligand to domain 5 and subsequent binding to domain 1. This likely occurs through ligand-induced conformational alterations to domain 5 that propagate through the protein to domain 1. Domain 1 can then bind to PS on the same or adjacent membranes and induce vesicle aggregation, which results in precipitation of the target membranes, or at physiologic ionic strength, to cellular receptors that recognize and bind (target membrane-bound) β2GP1.

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[1] Abbreviations: β2GP1, β2-glycoprotein 1; D, domain; PC, phosphatidylcholine; PS, phosphatidylserine; Rho-PE, *N*-(lissamine rhodamine B sulfonyl)-1,2-diacyl-L-α-phosphatidylethanolamine; Tris buffer, 10 mM Tris and 0.1 mM EDTA (pH 7.0); SUV, small unilamellar vesicles; PE, phosphatidylethanolamine; dansyl-PE, *N*-[(5-dimethylamino)-1-naphthalenesulfonyl]-PE; RET, resonance energy transfer.

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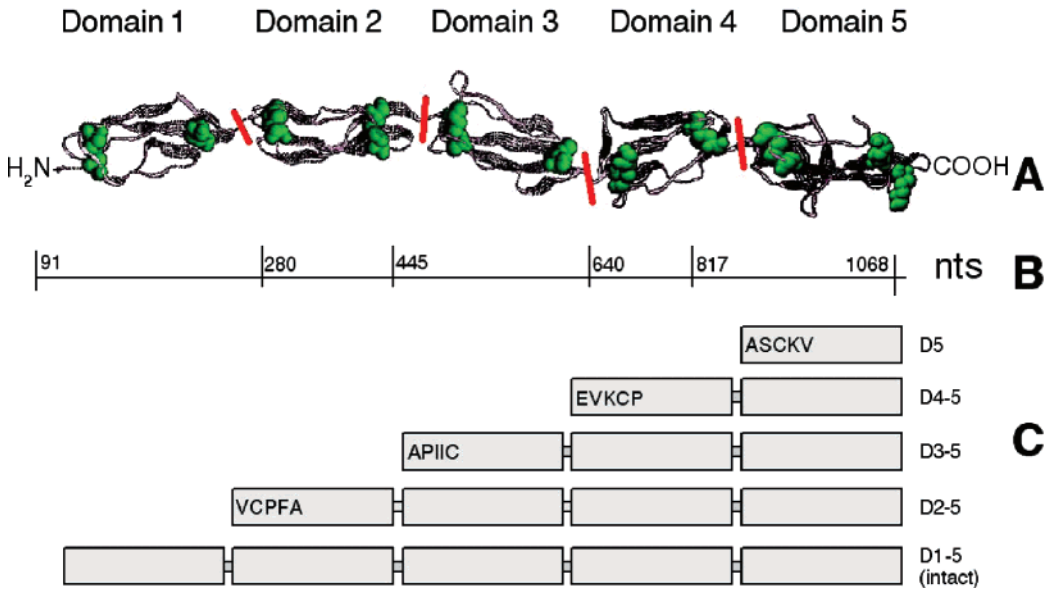

FIGURE 1: Construction of β2GP1 domain-deleted mutants. Deletion constructs were initiated from the linker region between the disulfide-linked domains (green), thereby keeping distal domains intact. The approximate locations of cut sites (red) are illustrated in panel A. Panel B shows the schematic diagram of the primer start site of the respective PCR amplification product. Panel C maps the domains present in the different constructs with their respective N-terminal amino acid sequence. Constructs were based on human liver cDNA (EMBL accession number X57847).

EXPERIMENTAL PROCEDURES

Materials. Dioleoylphosphatidylcholine (PC), bovine brain PS, N-Rho-PE, and dansyl-PE were from Avanti Polar Lipids (Alabaster, AL). All other reagents were obtained from Sigma (St. Louis, MO). Fluorescence was measured with a Perkin-Elmer LS50B spectrophotometer. Small unilamellar vesicles (SUV) were prepared by drying lipid under N_2 and sonicating in Tris buffer [10 mM Tris and 0.1 mM EDTA (pH 7.0)]. Large vesicles and titanium fragments were removed by centrifugation. Dansyl-PE-labeled vesicles were prepared from the sonicated preparation via solid phase exchange from glass. Briefly, an appropriate amount of dansyl-PE was dried under N_2. Vesicles were then added and vortexed. Gel filtration analysis revealed that virtually all of the dansyl-PE was incorporated into the vesicle membrane. Nucleotide sequence analysis and amino acid sequence analysis were performed by the University of Texas M. D. Anderson Cancer Center and Baylor College of Medicine Sequencing Core Facilities, respectively.

Construction and Expression of Full-Length and Domain-Deleted β2GP1. The pPIC6αA yeast shuttle expression vector (Invitrogen) and the Mut^+X-33 host strain (Invitrogen) were used. This expression vector contains the 5′ promoter and the 3′ transcription termination sequences of the methanol oxidase gene (*AOX1*). A yeast α mating factor signal sequence downstream of the *AOX1* promoter allows fusion of foreign cDNA for the secretion of the recombinant heterologous protein into the culture medium.

Five expression constructs were made on the basis of the structure of β2GP1 shown in Figure 1: (1) the entire coding region of β2GP1 cDNA without its cognate signal peptide (domains 1−5; 5′ primer used, 5′ GGAATTCGGACGGACCTGTCCCAAGC 3′), (2) domain 1 deleted (domains 2−5; 5′ primer used, 5′ GGAATTCGTATGTCCTTTTGC 3′), (3) domains 1 and 2 deleted (domains 3−5; 5′ primer used, 5′ GGAATTCGCTCCCATCATCTGC 3′), (4) domains 1, 2, and 3 deleted (domains 4−5; 5′ primer used, 5′ GGAATTCGTAAAATGCCCATTC 3′), and (5) domain 5 (5′ primer used, 5′ GGAATTCGCATCTTGTAAAGTAC 3′). A common 3′ primer (5′ TTCTAGATTAGCATGGCTTTAC 3′) was used for PCR (polymerase chain reaction) for all of the fragments. The forward primer (5′ GGAATTCGGACGGACCTGTCC 3′) and the reverse primer (5′ GCTCTAGATTATTTACAACTTGGC 3′) were used to clone the domain V-deleted (domains 1−4) fragment of β2GP1. PCR-amplified fragments were inserted in-frame between the *Eco*R1 and *Xba*I restriction sites of the pPICαA directly downstream from the α mating factor signal sequence. A stop codon was introduced at the end of each of these fragments to prevent fusion of the recombinant protein with the *c-myc* epitope and the His tag at the C-terminus. Recombinant constructs were propagated in *Escherichia coli* in the presence of 100 μg/mL blasticidin. The plasmid constructs were verified for authenticity by restriction analysis and nucleotide sequencing.

Transformation and Screening of Expression Clones. The recombinant plasmids were linearized with restriction enzyme *Sac*I and purified; 10 μg of the linearized recombinant plasmids was then used to transform host strain X-33 according to the manufacturer's protocol (Invitrogen). The transformants were selected on YPD (yeast extract peptone dextrose medium) plates containing 400 μg/mL blasticidin. Several clones for each of these constructs were restreaked on YPD plates with 400 μg/mL blasticidin to determine the true integrants. Ten clones for each of these constructs were then streaked on minimal dextrose (MD) and minimal methanol (MM) plates. Five clones for each construct growing equally well on both MD and MM plates were then grown in liquid MD and MM medium for 24, 48, 72, 96, and 120 h. At different time points, the supernatants and pellets for each clone were analyzed by Western blotting with rabbit antibody to human β2GP1. Clones showing the highest protein expression levels were used in large-scale preparations.

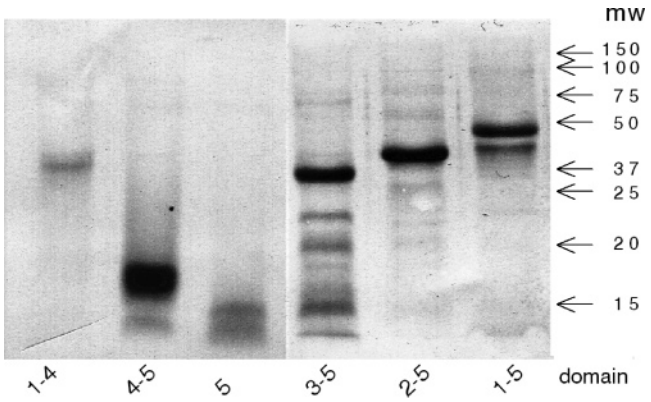

FIGURE 2: SDS−PAGE analysis of recombinant, domain-deleted β2GP1. Purified domain-deleted recombinant β2GP1 (~5 μg) was loaded onto SDS−PAGE gels and stained with Coomassie blue. Purity was estimated by laser scanning densitometry.

Purification of the Recombinant Proteins. Recombinant proteins were produced using culture conditions recommended by Invitrogen. Each clone was cultured in 5 mL of buffered minimal glycerol complex medium (BMGY) at 30 °C overnight with vigorous shaking. The cells were collected and inoculated to 25 mL of BMGY and grown for an additional 2 days. Cells from 25 mL of culture were used to inoculate 1 L of buffered minimal methanol complex (BMMY) medium (1.0% methanol). To maintain protein expression, the culture was continued for 4 days, adding methanol (1% final concentration) every 24 h. The culture medium was then clarified by centrifugation (4000*g* for 15 min), and the supernatant was dialyzed for 2 days at 4 °C against 50 mM Tris buffer. The solution was then applied to a DEAE-Sephacel column equilibrated in the same buffer. The flow-through was collected and applied to a heparin-Sepharose column. The flow-through from the heparin and the bound protein was eluted with 1 M NaCl. The proteins were analyzed by Western blotting and N-terminal sequencing to confirm the authenticity and cleavage of the α-factor leader sequence. The protein yield varied from 10 to 25 mg/L. To facilitate accurate molecular weight determinations, aliquots of protein were deglycosylated with *N*-glycosidase F. The deglycosylated proteins revealed molecular masses of 46.8, 35.6, 30.9, 17.1, 10.2, and 33.5 kDa for fragments 1−5, 2−5, 3−5, 4−5, 5, and 1−4, respectively (see Figure 2).

Gel Diffusion Assay. To assess the ability of β2GP1 to precipitate PS vesicles, a modified Octherlony gel diffusion test, commonly used to test the specificity of antigen/antibody reactions, was employed (*6*). In this assay, β2GP1 and PS vesicles diffuse from small opposing circular wells cut in agarose gels. Analogous to antibody/antigen reactions, a typical precipitin line is formed upon binding of β2GP1 to PS-containing vesicles. Microscope slides were coated with 0.9% agarose in Tris buffer to yield a punch hole volume of ~20 μL. The center holes were filled with the indicated vesicles and the surrounding wells with the recombinant proteins. The plates were developed overnight at 20 °C. Unbound protein and lipid were removed by washing for 24 h in large volumes of the same buffer. The gels were then dried, and the protein-containing precipitates were detected by staining with Coomassie Blue.

Fluorescence Measurements. The interaction of the five recombinant β2GP1 constructs with SUV was assessed by monitoring the fluorescence emission spectra of tryptophan (λ_{ex} = 278 nm). Briefly, 5 μL of recombinant protein solutions was added to 495 μL of Tris buffer in a cuvette, and emission was scanned from 300 to 400 nm upon addition of sequential aliquots of SUV (5 μL from 1 mM lipid). The experiments were then duplicated in buffers containing NaCl (150 mM). Resonance energy transfer (RET) experiments were carried out to monitor the interactions between the tryptophans of full-length β2GP1 and domain 5 with dansyl-PE-labeled, PS-containing vesicles. Acceptor vesicles consisted of PS, PC, and dansyl-PE (70/28/2). Emission spectra were recorded between 300 and 500 nm (λ_{ex} = 278 nm) before and after the addition of increasing amounts of acceptor vesicles. RET was assessed as a function of decreased tryptophan fluorescence (at 350 nm). Because the intrinsic tryptophan fluorescence of β2GP1 significantly increases upon lipid binding (see Figures 4 and 5), control curves were generated from the same batch of vesicles that did not contain dansyl-PE. RET was calculated using

$$1 - (I_2 - I_0)/(I_1 - I_0) \quad (1)$$

where I_0 is the relative fluorescence intensity of the tryptophan peak before the addition of acceptor vesicles and I_1 and I_2 are the relative fluorescence intensities of the tryptophan emission peak in the presence of identical concentrations of unlabeled and dansyl-PE-labeled vesicles, respectively.

Acrylamide Quenching. Acrylamide (4 M) in Tris buffer was used to test the accessibility of the tryptophan residues in each domain to the surrounding aqueous solvent in lipid-bound and unbound states. Five microliters of each of the β2GP1 constructs (brought to the same molarity) was mixed with 495 μL of Tris buffer, and the relative fluorescence at the tryptophan emission peak was measured after the addition of the indicated amounts of acrylamide. The data were analyzed according to the modified Stern−Volmer equation for collisional quenching (*22*):

$$I_0/(I_0 - I) = 1/f + 1/(fK_{sv}[Q]) \quad (2)$$

where I and I_0 are the fluorescence intensities in the presence and absence of acrylamide, respectively, [Q] is the acrylamide concentration, and f and K_{sv} are the fraction of accessible fluorophores and modified collision constant, respectively. The values of f and K_{sv} were determined by plotting the data of $I_0/(I_0 - I)$ versus 1/[Q].

RESULTS

Recombinant β2GP1. SDS−PAGE analyses of the recombinant protein fragments used in this study are shown in Figure 2. Molecular masses of the fragments were consistent with the expected ~8 kDa contribution for each domain in the fully deglycosolated protein. Molecular masses were found to be ~47, 36, 30, 17, 10, and 33 kDa for fragments 1−5, 2−5, 3−5, 4−5, 5, and 1−4, respectively. Purity, determined by scanning densitometry, was >95% with the exception of that of fragment 3−5, which was ~70%. Authenticities of all the fragments were verified by N-terminal sequence analysis.

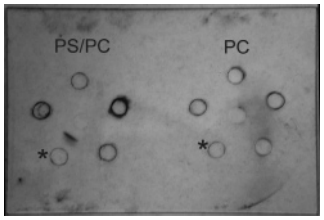

FIGURE 3: Precipitation of vesicles with domain-deleted β2GP1. Vesicles [20 μL from 1 mM PS/PC vesicles (75/25) or 100% PC] were loaded into the center wells of punched agarose (1% in Tris buffer) immediately followed by the addition of recombinant β2GP1 (20 μL from a 40 μM solution) to the surrounding wells. The plates were incubated overnight at 20 °C, washed with large volumes of Tris buffer to remove unbound protein, and stained with Coomassie blue. Clockwise from the asterisk: D1−5 (full-length), D2−5, D3−5, D4−5, and D5.

Binding of β_2GP1 to PS Vesicles. To determine the propensity of the domain-deleted β_2GP1 to bind PS-containing membranes, PS/PC vesicles were loaded into the center well of "Ochterlony" gel diffusion plates. The various β2GP1 fragments were then loaded into the five surrounding wells. After incubation at room temperature for 24 h, the plates were washed with large volumes of Tris buffer to remove nonprecipitated β2GP1 and stained with Coomassie blue. Figure 3 shows that the full-length recombinant protein interacted with the PS-containing membranes, resulting in precipitation of the complex. Precipitin lines did not form with any of the other constructs. Vesicles composed exclusively of PC were not precipitated. Because the principle lipid binding site of β2GP1 is located in domain 5, these results raise the possibility that both domain 1 and domain 5 are required for target membrane precipitation.

Although these results indicate that only the full-length protein can precipitate membranes, they do not rule out the possibility that the other domain-deleted proteins also bind to PS membranes, albeit without inducing precipitation. Since β2GP1 contains only five tryptophan residues, one in each disulfide-delineated domain, the relative contribution of each domain to binding can be estimated by monitoring the intrinsic tryptophan emission maxima and blue shift upon the addition of target membranes. Figure 4 shows that the sequential addition of target membranes to recombinant domains 1−5 (Figure 4A), 2−5, 3−5, and 4−5 resulted in a significant blue shift (Figure 4E) and enhancement in the intensity of the tryptophan fluorescence emission (Figure 4D). The addition of domain 5, on the other hand, resulted in an uncharacteristic decrease in fluorescence intensity (Figure 4D) with a very minor (~2 nm) blue peak shift (Figure 4E). Since the observed tryptophan emission spectra represent an average of all the tryptophans, it is difficult to predict the contribution of each domain to vesicle binding. Nonetheless, the dramatic difference in fluorescence enhancement between all the domains versus domain 5 and of domains 2−5 suggests that β2GP1 undergoes a conformational change upon binding to its target membrane. On the basis of the vesicle-dependent increase in fluorescence intensity, it appears that the tryptophans in domain 5 and domain 2 become more polar, while those tryptophans in domains 1, 3, and 4 become more hydrophobic upon lipid binding. The addition of control, neutral PC, vesicles did not alter the fluorescence emission maxima or the relative fluorescence intensity of the tryptophan residues (Figure 4B).

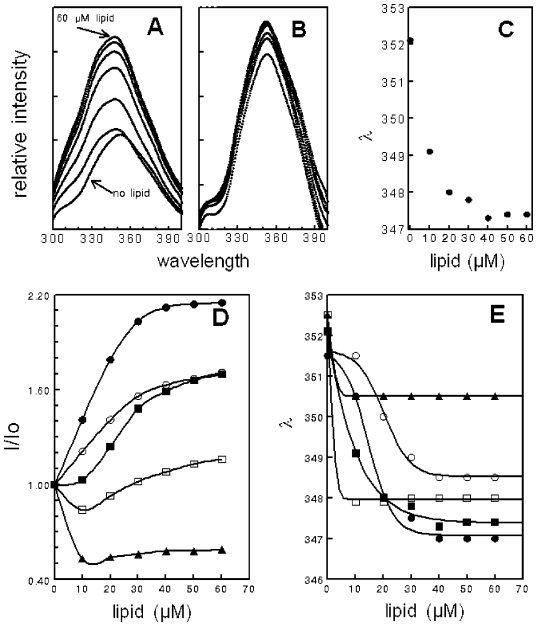

FIGURE 4: Fluorescence emission spectra of recombinant β2GP1 fragments incubated with vesicles. Increasing amounts of vesicles were added to full-length β2GP1 (0.4 μM), and the emission spectra were recorded: (A) PS/PC (75/25) vesicles and (B) PC vesicles. Panel C shows the spectral shifts calculated from the data shown in panel A. (D and E) Relative fluorescence intensity at the emission peaks and blue shift of the different fragments: (■) D1−5, (□) D2−5, (●) D3−5, (○) D4−5, and (▲) D5. I_0 is the peak intensity of protein alone; I is the peak intensity in the presence of vesicles.

In an attempt to dissociate the electrostatic interactions that bind β2GP1 to negatively charged membranes, tryptophan fluorescence was also measured in the presence of NaCl. Figure 5 shows that the progressive increases in the fluorescence intensity of domains 1−5 and 2−5 were abrogated in the presence of salt. Intensities of domains 3−5 and domains 4−5 were moderately affected, and that of domain 5 remained unchanged. By comparing the relative fluorescence intensities in the absence and presence of NaCl (Figure 4D vs Figure 5), we conclude that the interaction of domains 1 and 2 with target membranes is charge-dependent since the increase in tryptophan fluorescence was relieved by NaCl.

To directly assess the microenvironment of the protein's tryptophan residues and exposure to the aqueous phase, acrylamide quench curves were generated for all the recombinant proteins in the presence and absence of NaCl (150 mM). From data generated as described in the legend of Figure 6, quench constants were calculated from the slope and y-intercepts obtained from eq 2. With the exception of domains 1−4, the data presented in Table 1 show relatively consistent quenching constants for all the proteins in the absence of lipid. While all the proteins demonstrated a concomitant decrease in quench constants after binding vesicles in the absence of salt, domains 1−5 demonstrated a dramatic change in K_{sv} from 17.9 to 7.6 M^{-1}. The inclusion of 150 mM NaCl in the binding assay partially or totally reversed the binding constants to values consistent for the protein in the absence of vesicles. Consistent with the data showing a concomitant increase in fluorescence intensity upon binding of lipid to the full-length protein (domains 1−5), the decrease in K_{sv} observed for the protein in the presence of lipid suggests that the tryptophan in domain 1

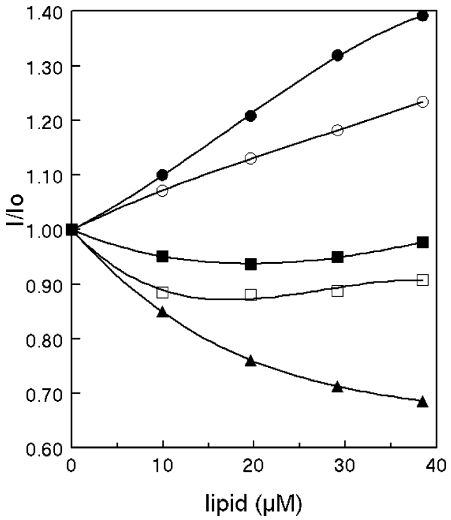

FIGURE 5: Fluorescence emission spectra of recombinant β2GP1 fragments incubated with PS/PC vesicles in the presence of 0.15 M NaCl. Increasing amounts of vesicles were added to the different β2GP1 fragments, and the fluorescence intensities at the emission peaks were recorded: (■) D1−5, (□) D2−5, (●) D3−5, (○) D4−5, and (▲) D5. I_0 is the peak intensity of protein alone; I is the peak intensity in the presence of vesicles.

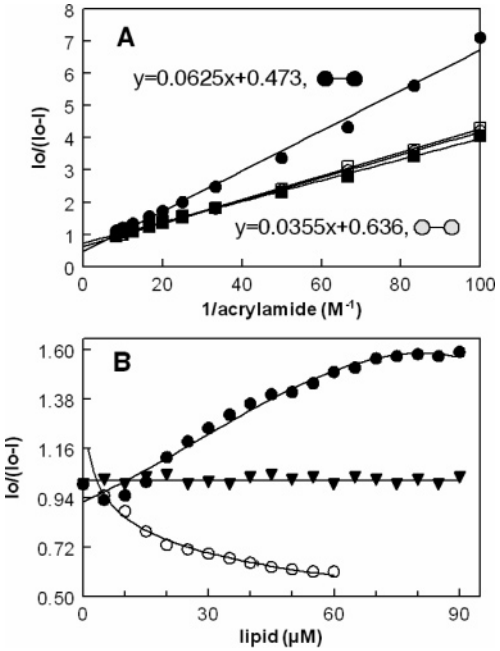

FIGURE 6: Acrylamide quenching of β2GP1 tryptophan fluorescence. (A) Modified Stern−Volmer plots of trytptophan fluorescence (λ_{ex} = 280 nm; λ_{em} = 350 nm) quenching of β2GP1 by acrylamide in Tris buffer. Protein and lipid concentrations were 0.4 and 30 μM, respectively: (empty symbols) β2GP1 alone, (filled symbols) β2GP1 with PS/PC vesicles, (circles) 10 mM Tris buffer, and (squares) 10 mM Tris buffer with 150 mM NaCl. (B) Relative fluorescence intensity as a function of lipid concentration: (●) D1−5, (○) D5, and (▼) D1−4.

reorients into a more hydrophobic environment, thereby becoming more shielded from the quencher. Although K_{sv} values reflect a decrease in the degree of exposure of tryptophan to a polar environment, they can also be due to decreased fluorescence lifetimes. It should be noted, however, that since lifetime is related to fluorescence intensity increase, the decrease in K_{sv} for the domains that showed increased intensity upon lipid binding (Figure 4) suggests that the decrease in K_{sv} indeed reflects protection of the tryptophans from the acrylamide quencher; e.g., they reorient into a more hydrophobic environment. Importantly, both the quench constants (Table 1) and the fluorescence intensity (Figure 6B) of domains 1−4 remained unchanged in the presence of vesicles. This suggests that binding of domain 5 to the target membrane is essential for initiation of a reaction that propagates a conformational change through the protein causing a dramatic alteration in domain 1 that facilitates concomitant binding to the target membrane.

Table 1: Stern−Volmer Quenching Constants[a]

	K_{sv} (M^{-1})		
	protein alone	PS/PC vesicles with Tris buffer	PS/PC vesicles with Tris and NaCl
D1−5	17.9	7.6	22.5
D2−5	19.4	16.0	17.2
D3−5	20.7	11.4	15.5
D4−5	19.6	13.1	21.2
D5	17.6	13.1	16.6
D1−4	12.9	13.5	−

[a] K_{sv} values for domain-deleted fragments of β2GP1 in the presence and absence of vesicles with and without NaCl (150 mM). K_{sv} values of protein alone in both Tris buffer (10 mM) and NaCl (150 mM) buffers yielded identical values. Experimental conditions are the same as those described in the legends of Figures 4 and 5.

Resonance Energy Transfer between β2GP1 and Target Membranes. Previous studies have raised the possibility that binding of the fifth domain of β2GP1 initiates the absorption of domains 1−4 parallel to the bilayer surface of the target membrane (*17*). To confirm our data suggesting that the tryptophans in domain 5 and domain 1 were excluded and included into the hydrophobic membrane environment of the target vesicles, respectively, resonance energy transfer experiments between the tryptophans of the full-length protein and domain 5 to dansyl-PE-containing vesicles (75/23/2 PS/PC/dansyl-PE) were carried out. Sequential emission scans were taken immediately upon the addition of increasing concentrations of control vesicles (without dansyl-PE) and dansyl-containing acceptor vesicles. RET was calculated from the difference in fluorescence intensity of the tryptophan emission peaks of control versus dansyl-PE-containing vesicles using eq 1. The data presented in Figure 7 show an increase in the RET of the full-length protein with increasing acceptor vesicle concentrations that plateaued at ~40% RET efficiency. The addition of NaCl (to 150 mM) resulted in an immediate decrease in the RET to ~20% efficiency. RET was not detected with domain 5. Taken together with the data presented above, these results suggest that the binding of β2GP1 to membranes can be separated into two distinct interactions: (1) primary binding of domain V that is insensitive to ionic strength and (2) a secondary binding reaction involving domain 1 that is ionic strength-dependent. It should be noted that while these binding activities appear to be distinct, both are PS-dependent since domain 1 does not interact with vesicles composed exclusively of PC (*21*).

β2GP1-Dependent Precipitation of Lipid Vesicles. These data raise the possibility that, in addition to domain 5, domain 1 is essential for vesicle aggregation that results in vesicle precipitation. To test this directly, PS/PC vesicles were labeled with trace amounts of Rho-PE and incubated with saturating amounts of all the β2GP1 fragments. The proteins

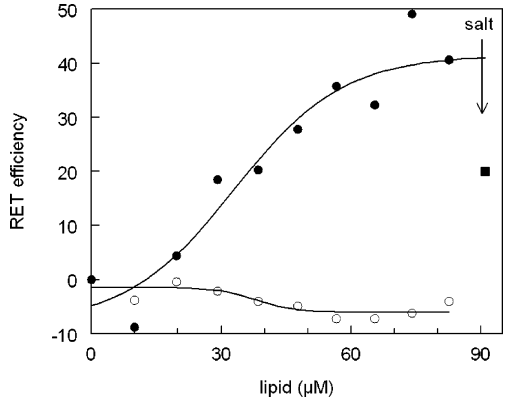

FIGURE 7: RET between tryptophan and dansyl-PE-labeled vesicles. Intrinsic tryptophan fluorescence (λ_{ex} = 278 nm) was used for RET to dansyl-PE by monitoring tryptophan emission maxima. Increasing concentrations of acceptor vesicles were added to full-length β2GP1 and D5 (0.4 μM), and complete emission spectra (from 300 to 525 nm) were recorded (100 nm/min). Once the RET plateau was reached, salt was added (to 150 mM) and the spectra were again recorded after 10 min at 20 °C. RET efficiency was calculated as described in Experimental Procedures: (●) D1−5 and (○) D5.

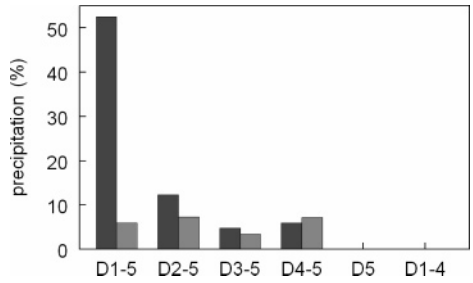

FIGURE 8: Precipitation of PS/PC vesicles with domain-deleted β2GP1. PS/PC vesicles (0.5 mM) were labeled with Rho-PE (~0.1%) and incubated with the indicated fragments (0.2 μM) in Tris buffer (dark gray bars) or Tris buffer containing 150 mM NaCl (light gray bars) for 16 h at 20 °C. Aliquots were removed before and after centrifugation (5000*g*), and the fraction of precipitated vesicles was calculated from the decrease in supernatant fluorescence after centrifugation.

were incubated in low- and physiologic-ionic strength buffers for 1 h at room temperature and centrifuged at 5000*g* for 10 min, and the residual fluorescence in the supernatant was determined. The data in Figure 8 show that the extent of vesicle precipitation dramatically decreased with a decrease in protein size. With the exception of the full-length protein, ionic strength did not affect binding.

DISCUSSION

The interaction between β2GP1 and various phospholipid-containing target membranes forms the basis for the purported physiologic and pathogenic functions of the protein. Physiologically, the protein can participate in the regulation of coagulation and fibrinolysis by binding to the PS-containing prothrombinase complex (*23*), factor XI (*24*, *25*), and plasminogen (*26*), respectively. It participates in the "immune" clearance of liposomes (*27*) and apoptotic cells and debris (*6*, *28*−*30*) that express negatively charged lipids. The protein also binds to the surface of endothelial cells (*31*, *32*) where it likely functions as a regulator of angiogenesis (*33*). Pathologically, it serves as the principal antigen in autoimmune "antiphospholipid" syndrome where autoantibodies bind to ligand-dependent conformationally altered β2GP1 that result in a significant increase in the risk of thromboembolisms (*34*−*36*).

One of the major issues regarding the interaction of β2GP1 with target membranes and/or cells is that there is an ~1000-fold difference in the dissociation constants of the protein in low- and high-ionic strength [$\sim10^{-9}$ and $\sim10^{-6}$ M, respectively (*20*, *37*)] buffers. Several studies have shown that while β2GP1 binds to target cells and membranes at physiologic salt concentrations (*19*, *20*, *27*, *31*), the binding to target membranes in low-ionic strength buffers results in their agglutination and precipitation (*20*, *21*). Conceivably, this can occur through high-affinity (low-salt)-dependent global alterations in the target membranes that result in increased hydrophobicity and subsequent precipitation from the aqueous phase (*21*) or because of low-salt-dependent multiple protein−membrane cross-linking reactions (*17*, *38*). The notion that precipitation of membranes in low-ionic strength buffers is the result of multiple protein−membrane interactions is supported by the observations of Hagihara et al. (*12*), who have shown that in addition to the relatively high-affinity binding of domain 5 to lipids, domain 1 also binds to target membranes, albeit with relatively low affinities. Moreover, the crystal structure of β2GP1 indicates that its five domains exist in the form of a fish hook, J-like structure, suggesting a functional partitioning of the protein over the C- and N-termini (*18*, *39*).

The interaction of β2GP1 with PS-containing vesicles was monitored by assessing the tryptophan fluorescence emission maxima and blue shift (an indication of an increase in the hydrophobicity of the environment of the tryptophans) of the various fragments upon binding to the target membranes. Figures 4 and 5 show that the fluorescence maxima and blue shift were dependent on the total number of domains present in the recombinant fragments. Several tentative conclusions can be drawn by comparing the data obtained in the presence and absence of salt. In low-ionic strength buffer, domain 5, in contrast to the other fragments, exhibited a decrease in fluorescence suggesting that, upon binding, its tryptophan reorients to a more polar environment. Domains 4−5 showed a dramatic increase in intensity that compensated for the decrease in the intensity of domain 5, suggesting that the domain 4 tryptophan becomes protected in the target membrane. An additional increase in fluorescence intensity was observed in domains 3−5, suggesting that domain 3 also embeds itself in the bilayer membrane. Domains 2−5, on the other hand, showed a slight decrease in intensity, suggesting that the domain 2 tryptophan became more polar. Fluorescence intensity was restored with the full-length protein (D1−5), suggesting that domain 1 resides in a hydrophobic environment. The pattern of relative fluorescence intensities between the various fragments was, in general, preserved in the presence and absence of salt with the exception of the full-length protein. In this case, the intensity decreased to levels that were slightly below and above the intensities of domains 2−5 in the absence and presence of salt, respectively. Taken together, these data indicate that the hydrophobic protection conferred by domain 1 in the full-length protein is relieved at physiologic ionic strength, resulting in tryptophan emissions consistent with that of domains 2−5.

Acrylamide quench curves were generated to directly assess the accessibility of the tryptophan residues to the

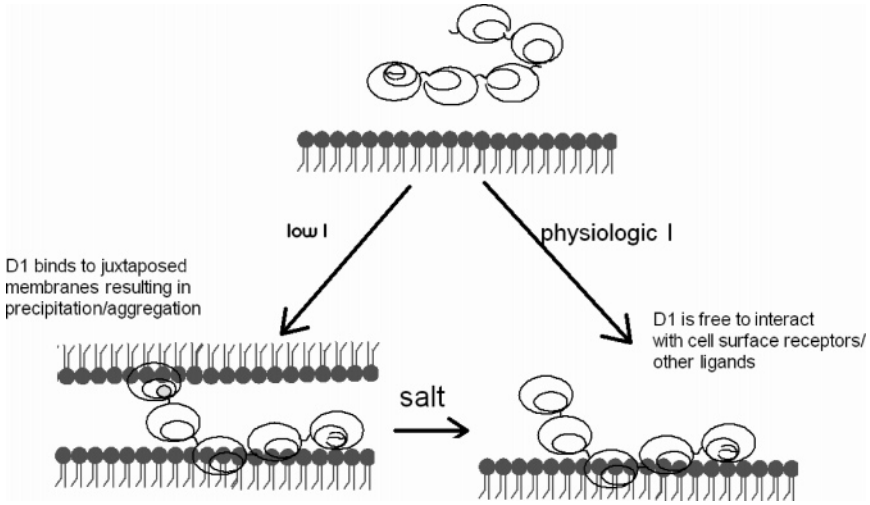

FIGURE 9: Interaction of β2GP1 with lipid membranes. Domain 5 resides in the aqueous phase upon binding to anionic membranes. The decreased fluorescence of domain 5 is compensated by domain 4 in D4−5, suggesting that domain 4 is membrane-bound. The inclusion of domain 3 (D3−5) resulted in a dramatic increase in fluorescence, suggesting that it becomes embedded in the membrane. The decrease in fluorescence with D2−5 suggests that domain 2 resides in the aqueous phase. The decrease in tryptophan fluorescence, RET, and precipitation indicate that domain 1 is released from the membrane under physiologic conditions. Since β_2GP1 binds multiple lipid residues through domain 5 and domain 1, it induces intervesicular aggregation and precipitation by binding juxtaposed membranes. *I* is the ionic strength.

aqueous environment. The data presented in Figure 6 calculated as described in eq 2 indicated that the quenching constant (K_{sv}) for the full-length protein in low-salt buffer decreased from 17.9 to 7.6 mol/L^{-1} in the presence of lipid. This dramatic decrease in K_{sv} was completely reversed when the experiment was carried out in the presence of 150 mM NaCl (Table 1).

Similar to the relative patterns of fluorescence intensities shown in Figures 4D and 5, the observed decrease in quenching constants for the other constructs followed a consistent pattern. For example, domains 3−5, which demonstrated the highest I/I_0 values (Figures 4 and 5), demonstrated a large alteration in lipid-bound quench constants followed by domains 4−5 and 2−5. Consistent with the notion that the binding of β2GP1 to membranes is dependent on binding through domain 5, major alterations in K_{sv} (Table 1) or increases in fluorescence intensities (Figure 6B) of domains 1−4 in the presence of PS vesicles were not observed. These data reinforce the notion that the interaction of β2GP1 with target membranes is indeed dependent on domain 5.

These data show that the individual β2GP1 domains exhibit ionic strength-dependent water-soluble and membrane-bound states. Taking all of the data together, we can estimate the orientation of the protein in its membrane-bound state(s). Under conditions of low ionic strength, domain 5 binds to the membrane through its multiple lysine residues which likely results in juxtaposition of its tryptophan to the charged PS headgroup. This would explain the lipid-dependent decrease in fluorescence intensity and protection from acrylamide. The fact that this tryptophan did not function as an energy donor in the RET experiments raises the possibility that the dansyl-PE was segregated from the tryptophan. The inclusion of domain 4 (D4−5) compensated for the decreased tryptophan fluorescence of domain 5, suggesting that domain 4 became associated with the membrane. The dramatic increase in fluorescence upon the inclusion of domain 3 (D3−5) indicates that domain 3 becomes tightly embedded in the bilayer membrane, providing a hydrophobic, acrylamide-protected environment for its tryptophan. The inclusion of domain 2 (D2−5) resulted in decreased tryptophan fluorescence and only a minor decrease in the extent of acrylamide quenching, suggesting that domain 2 resides in the aqueous phase. The only dramatic change in the relative tryptophan fluorescence intensity and extent of acrylamide quenching in high-ionic strength buffers occurred for the full-length protein (D1−5). This suggests that low-ionic strength-dependent bound domain 1 is released from its relatively hydrophobic, membrane-protected, environment in an ionic strength-dependent manner. This conclusion is reinforced by the RET (Figure 7) and precipitation data (Figure 8) which showed a dramatic decrease in RET efficiency and the absence of precipitation upon the addition of salt. A model consistent with these data depicting the various domain conformations acquired in the absence and presence of salt is shown in Figure 9.

Taken together, these data support the hypothesis that β2GP1 can adopt at least two conformations upon binding to PS-containing targets (*17*, *18*). In low-ionic strength buffers, the protein assumes a conformation in which both the C- and N-termini are oriented along the plane of the lipid bilayer with each domain acquiring its specific membrane interaction. In isotonic buffers, however, only the C-terminus is bound, leaving the N-terminus in solution. A domain 5 binding-dependent conformational change still occurs, albeit association of the N-terminus with the target membrane is precluded. Such a situation would provide a conformationally altered N-terminus that becomes available for physiologically important β2GP1 interactions. Indeed, the protein has been shown to be a target for autoimmune "anti-phospholipid" antibodies (*3*, *40*) and is known to bind to annexin II (*31*, *32*), apolipoprotein E receptor 2 (*41*), and megalin (*42*), suggesting that it can simultaneously bind multiple lipid and protein ligands on opposing cell surfaces.

In summary, this study examined the interactions of β2GP1 with PS-containing membranes by a number of techniques.

The results suggest that large-scale structural changes to β2GP1 are initiated upon the initial interaction of domain 5 with a target membrane. The physiologic significance of this is likely related to domain 1-dependent alterations that occur through conformational changes initiated upon domain 5 binding that facilitate the subsequent binding of domain 1-specific autoimmune antibodies (*43*, *44*) and domain 1 binding to PS-expressing target cells (e.g., apoptotic cells and their debris).

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