Aggregation of β_2-Glycoprotein I Induced by Sodium Lauryl Sulfate and Lysophospholipids†

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ABSTRACT: β_2-Glycoprotein I (β_2-GPI) is a plasma protein that binds to negatively charged substances such as DNA, heparin, and anionic phospholipids. The interaction of β_2-GPI with anionic phospholipids is intriguing in the context of the autoimmune disease antiphospholipid syndrome. To extend understanding of the binding mechanism to phospholipids, the interactions of β_2-GPI with amphiphiles, i.e., sodium lauryl sulfate and lysophospholipids, were examined. These amphiphiles induced the aggregation of β_2-GPI below the critical micelle concentration, indicating that the interaction of β_2-GPI with monodispersed amphiphiles is unstable, resulting in the formation of large aggregates. However, highly soluble monocaproylphosphatidic acid did not induce aggregation, suggesting that the hydrophobicity of the acyl chain is also an important factor for aggregate formation in addition to negative charges in the headgroup. A series of experiments using deletion mutants and a peptide showed that the fifth domain of β_2-GPI (domain V) is responsible for formation of aggregates observed for intact full-length β_2-GPI. In addition, the flexible loop (F307−C326) in the C-terminal of domain V, which consists of hydrophobic and positively charged residues, was identified as the important region for aggregation. These results indicate that β_2-GPI binds to the amphiphiles through the flexible loop of domain V, resulting in formation of large aggregates where both electrostatic and hydrophobic interactions are involved.

β_2-Glycoprotein (β_2-GPI[1]) is a glycosylated plasma protein consisting of a single polypeptide chain (326 amino acids) with a molecular mass of about 50 kDa (*1*, *2*). This protein is composed of five repeated domains. With the exception of the fifth domain (domain V), each domain shares a common motif named the complement control protein module, short consensus repeat, or Sushi domain. The primary structure of domain V deviates from the typical Sushi domain with respect to the additional 20 amino acid residues in its C-terminal (from F307 to C326). This region is highly mobile and is called the flexible loop (*3*−*5*). β_2-GPI has been shown to bind negatively charged substances such as DNA, heparin, and phospholipids. Especially, the interaction of β_2-GPI with anionic phospholipids is intriguing in the context of an autoimmune disease, antiphospholipid syndrome. This disease is characterized by the presence of antiphospholipid antibody (aPL) associated with thrombosis, fetal loss, and thrombocytopenia. aPL has been shown to require protein cofactors for effective binding to anionic phospholipids. Among these, β_2-GPI is the most common cofactor in recognition of the antigen by aPL in patients (*6*−*8*).

It has been proposed that aPL is an intrinsically weak antibody against β_2-GPI. This idea has been supported by data from several groups. Matsuura et al. (*9*) found that aPL binds to β_2-GPI immobilized on irradiated microtiter plates. Roubey et al. (*10*) showed that preincubation of aPL with β_2-GPI adsorbed free aPL, resulting in a decrease in the affinity to the β_2-GPI−lipid complex. Also, recombinant dimerized β_2-GPI or chemically cross-linked β_2-GPI has a strong affinity for aPL, suggesting that high antigen density is required for aPL binding (*11*, *12*). The Fab′ fragment of aPL has much lower affinity than the intact antibody, indicating that bivalent interactions of the antibody and β_2-GPI are essential for high-affinity binding (*10*, *11*, *13*). These findings suggest that binding of β_2-GPI to phospholipids increases the effective concentration of epitope so that aPL binds cooperatively to them. However, it is still unclear how β_2-GPI forms clusters in the presence of phospholipids to show high epitope density against aPL.

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[1] Abbreviations: aPL, antiphospholipid antibody; β_2-GPI, β_2-glycoprotein I; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; CMC, critical micelle concentration; MOPS, 1-oleoyl-2-hydroxy-*sn*-glycero-3-[phospho-L-serine]; MOPC, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; MOPG, 1-oleoyl-2-hydroxy-*sn*-glycero-3-[phospho-*RAC*-(1-glycerol)]; MOPA, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate; MCPA, 1-caproyl-2-hydroxy-*sn*-glycero-3-phosphate; MMPG, 1-myristoyl-2-hydroxy-*sn*-glycero-3-[phospho-*RAC*-(1-glycerol)]; MMPA, 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphate; MPPA, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate; loopless domain IV−V, domain IV−V of β_2-GPI, which lacks the peptide from His310 to Cys326 and has Cys288 substituted by Ala; NMR, nuclear magnetic resonance; NPN, *N*-phenyl-1-naphthylamine; PAGE, polyacrylamide gel electrophoresis; SDS, sodium lauryl sulfate.

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Only negatively charged phospholipids, such as cardiolipin and phosphatidylserine, can bind to β_2-GPI (*14*, *15*). In addition, the affinity of this interaction depends on the ionic strength. Analysis of the crystal structure of β_2-GPI revealed the presence of a highly positively charged cluster in domain V (*3*, *5*). The presence of highly positively charged peptide, corresponding to Cys281 to Cys288 of domain V, can inhibit the binding of β_2-GPI to phospholipid (*16*). The importance of this sequence was confirmed using site-directed mutagenesis (*17*). These observations suggest that the electrostatic interaction contributes to the binding of β_2-GPI to anionic phospholipids.

On the other hand, the C-terminal flexible loop in domain V has been shown to play a major role in the interaction with phospholipids. Plasmin specifically cleaves the flexible loop between Lys317 and Thr318, resulting in a nicked form of β_2-GPI (*18*). It has been shown that the nicked β_2-GPI fails to interact with acidic phospholipids (*4*, *15*, *18*−*20*). The naturally occurring mutants C306G and W316S were reported to be unable to bind to phospholipids (*21*). Mehdi et al. (*22*) made mutants L313G, A314S, and F315S and further confirmed the importance of the flexible loop. Interestingly, the interaction site in the flexible loop consists of hydrophobic and positively charged residues. Indeed, hydrophobic substances such as 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS) and retinol bound to the flexible loop, suggesting that the hydrophobic interaction is also important for binding to phospholipid (*23*).

Recently, our group solved the solution structure of domain V using nuclear magnetic resonance (NMR) and showed that the anionic surfactant sodium lauryl sulfate (SDS) bound to the flexible loop and the surrounding positively charged residues (*4*). As SDS has a negative charge and acyl chain, SDS is considered to be a good model for anionic phospholipids. This confirmed that electrostatic and hydrophobic interactions are important for the binding of β_2-GPI to anionic phospholipids. We searched for optimal experimental conditions for NMR experiments by changing the ratio of SDS to β_2-GPI, and found that β_2-GPI precipitated in the presence of an excess amount of SDS. Lysophospholipids closely resemble biological phospholipids but can exist in monodispersed form at relatively high concentrations (>10 μM) because of the single acyl chain. In this paper, to further understand the mechanism of the interaction of β_2-GPI with phospholipids and subsequent recognition by aPL, we examined the aggregation of β_2-GPI and its deletion mutants induced by lysophospholipids.

MATERIALS AND METHODS

Materials. Lysophospholipids were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Human intact β_2-GPI, bovine domain I, and recombinant domain V were prepared as described previously (*15*, *20*).

Preparation of Domain IV−*V and Loopless Domain IV*−*V.* Wild-type domain IV−V expressed in *Pichia pastoris* was not homogeneous because of glycosylation. To overcome this problem, the pUC118-β_2-GPI plasmid containing the full-length human β_2-GPI was first modified using PCR-based site-directed mutagenesis to introduce a mutation of Asn234 to Ala. This mutation disrupted the consensus sequence for addition of a carbohydrate chain in domain IV. The region from Arg182 to Cys326 was amplified by PCR using the primers 5′-cctctcgagaaaagagaagtaaaatgcccattcccatcaag and 5′-aaagaattcaccttagcatggctttacatcgg (*24*). For loopless domain IV−V, Cys288 was substituted by Ala using the oligo-DNA 5′-cctctgtatagctagccttcttttcc. The resulting pUC118-β_2-GPI (C288A) was modified by the same method as used for domain IV−V to introduce the mutation N234A. Then, the region from Arg182 to Glu309 was amplified using primers 5′-cctctcgagaaaagagaagtaaaatgcccattcccatcaag and 5′-tttccaaaaagcggccgcactttattccttgaagc. The modified DNAs were digested by *Xho*I and *Eco*RI and then ligated into the *P. pastoris* expression vector pPIC 9 (Invitrogen, Carlsbad, CA), resulting in pNPD45 and pNPD45 loopless. DNAs from the expression plasmids were integrated into host cells, GS115 (Invitrogen), by transforming his4 *P. pastoris* cells with plasmids digested with *Sal*I. Expressed proteins were purified by the same method as described for domain V (*20*). The molecular weights of purified proteins were confirmed by mass spectrometry, and the obtained values were identical to the calculated values within experimental error. We confirmed that domain IV−V was properly folded at neutral pH by circular dichroism and differential scanning calorimetry measurements (data not shown).

Preparation of P35 Loop Peptide. P35 was purchased from Peptide Institute Inc. (Osaka, Japan). Briefly, two peptides, FFCKNKEKKCSYTE (P14) and CFKEHSSLAFWKTDASDVKPC (P21), were chemically synthesized and purified separately. Cys10 in P14 was blocked by a nitropyridinesulfenyl group. In addition, Cys3 in P14 and Cys1 in P21 were blocked by acetamidomethyl groups. First, two peptides were incubated at pH 8 to form a disulfide bond between Cys10 in P14 and Cys21 in P21. The cross-linked heterodimer was purified by reverse-phased HPLC, and acetamidomethyl groups were removed in 1 mM I_2/MeOH. A second oxidation between Cys3 in P14 and Cys1 in P21 was carried out by air oxidation. The obtained oxidized peptide was purified by reversed-phase HPLC, and the purity was greater than 95%.

Assay for the Formation of Aggregates with SDS. SDS and proteins were mixed and sonicated for 30 min, and then the mixtures were incubated for 30 min at 37 °C. After centrifugation at 20000*g* for 30 min, the amount of protein in the supernatant was assayed by the method of Bradford (*25*).

CMC Measurement by N-Phenyl-1-naphthylamine Fluorescence. The CMCs of SDS and lysophospholipids were determined using the fluorescence probe *N*-phenyl-1-naphthylamine (NPN) (*26*). The desired amount of lysophospholipids in chloroform was dried under vacuum and then dissolved in the same buffer, 20 mM MES at pH 6. Stock SDS solutions were diluted with the buffer. These solutions were sonicated for 30 min at 37 °C. A working solution of 1 μM NPN was prepared by dilution of a solution of NPN (1 mM) in 99% ethanol into the aqueous buffer shortly before use. After addition of NPN, the solutions were sonicated again for 30 min at 37 °C. NPN fluorescence was measured with a Shimadzu RF-5300PC fluorometer (Kyoto, Japan) using a 1 cm square quartz fluorescence cuvette at 37 °C. For determination of the CMC, the excitation wavelength was 356 nm with a slit width of 5 nm, and the emission intensity was recorded at 440 nm with a slit width of 3 nm. The fluorescence intensity was plotted against the concentra-

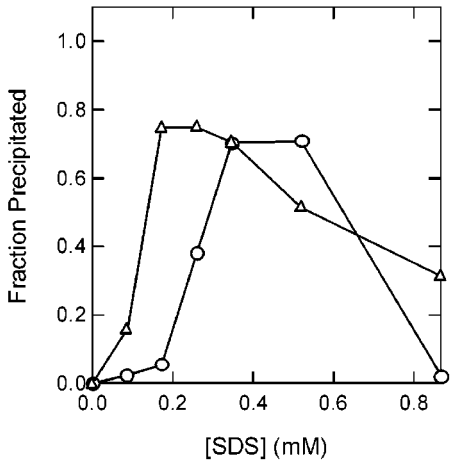

FIGURE 1: SDS-induced aggregation of intact β_2-GPI (○) and domain V (△) in 20 mM MES (pH 6) at 37 °C. The concentration of β_2-GPI and domain V was 5 μM. The amount of protein precipitated by centrifugation was plotted against the concentration of SDS.

tion of amphiphiles. In these plots, two straight lines defining the NPN emission intensity in an essentially aqueous environment and in the micellar environment could be traced. We used the point of intersection of these straight lines to define the CMC.

Assay for the Formation of Aggregates with Lysophospholipids. Each aliquot containing the desired amount of phospholipids in chloroform solution was dried under vacuum for 15 min. Then, 20 mM MES buffer at pH 6 was added to each tube. The solutions were sonicated for 30 min and centrifuged at 20000*g* for 30 min. The carefully removed supernatants were used as lipid solutions. Then, 35 μL of protein solution (80 μM) was added to 515 μL of lipid solution. These mixtures were incubated at 37 °C for 1 h and centrifuged at 20000*g* for 30 min. The resulting precipitates were subjected to SDS−polyacrylamide gel electrophoresis (PAGE) and phosphorus analysis (Wako Pure Chemical Industries, Osaka). The amount of protein in the precipitate was also calculated from the results of protein assay of the supernatant. In the case of P35, the supernatant was subjected to reversed-phase HPLC and the amount of peptide was estimated from the peak intensity. All measurements were repeated at least twice. The concentration of protein or peptide in the stock solution was determined by absorbance at 280 nm (*27*).

RESULTS

SDS-Induced Aggregation of β_2-GPI. Intact β_2-GPI and recombinant domain V were incubated with SDS, and whether they form aggregates was examined (Figure 1). The amount of aggregated protein was estimated by protein assay of the supernatant after centrifugation at 20000*g*. β_2-GPI aggregated with SDS, and the amount of aggregates reached a maximum at around 0.4 mM SDS. On the other hand, domain V formed aggregates at lower concentrations of SDS. This indicates the higher aggregation tendency of domain V than that of the full-length protein. In the absence of SDS, both intact β_2-GPI and domain V were soluble and did not precipitate after centrifugation. Intriguingly, the aggregates were dissolved with increases in the concentration of SDS. A biphasic feature in the aggregation induced by SDS was reported for melittin and myelin basic protein (*28*−*30*), where

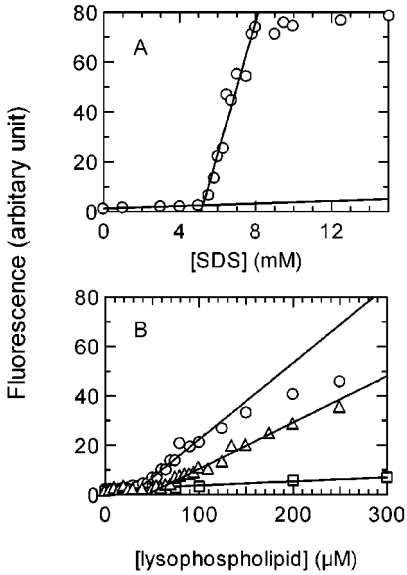

FIGURE 2: NPN fluorescence emission intensity as a function of amphiphile concentrations in 20 mM MES buffer (pH 6) at 37 °C: (A) SDS; (B) MOPS (○), MPPA (△), and MMPA (□). Two straight lines defining the NPN emission intensity in an essentially aqueous environment and in the micellar environment were traced. The points of intersection of these straight lines were used to define the CMC.

the dissolution at high SDS concentration was accompanied by global denaturation and α-helix formation. Although the aggregates were dissolved at SDS concentrations less than the CMC, we believe that this phenomenon was due to the global denaturation of β_2-GPI or domain V by SDS micelles. The CMC of SDS was measured using the fluorescent probe NPN under our experimental conditions, i.e., 20 mM MES, pH 6, at 37 °C, and was estimated to be 5.2 mM (Figure 2A). This was in good agreement with the reported CMC values of SDS, 2.6 mM in 10 mM phosphate buffer, pH 7.5, and 8.3 mM in pure water (*26*, *31*). This indicates that β_2-GPI binds to monodispersed substrates and forms aggregates.

Aggregation of Intact β_2-GPI with Lysophosphatidylserine. To determine whether phospholipid induces the aggregation of β_2-GPI as was the case for SDS, lysophosphatidylserine, 1-oleoyl-2-hydroxy-*sn*-glycero-3-[phospho-L-serine] (MOPS), was incubated with β_2-GPI. As lysophospholipids have only one acyl chain, they tend to form micelles rather than a lipid bilayer or vesicles. Aggregation was measured by SDS−PAGE of the precipitate after centrifugation at 20000*g* (Figure 3A). Without MOPS, only a negligible amount of β_2-GPI, probably formed from the nonspecific aggregates, was precipitated. With increasing concentration of MOPS, the amount of aggregates increased and reached a maximum at 50 μM MOPS. However, we observed no aggregation with 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (MOPC), which is thought not to interact with β_2-GPI (see below). Before incubation with protein, each concentration of lipid solution was centrifuged at the same speed, and no precipitate was observed. Thus, the observed precipitate was caused by the interaction between β_2-GPI and MOPS. We also estimated the amounts of protein in the supernatant by protein assay and of lysophospholipid in the precipitate by phosphorus assay (Figure 3B). Both assays for the amount of protein in the precipitate and supernatant gave identical results. The presence of MOPS in the precipitate was

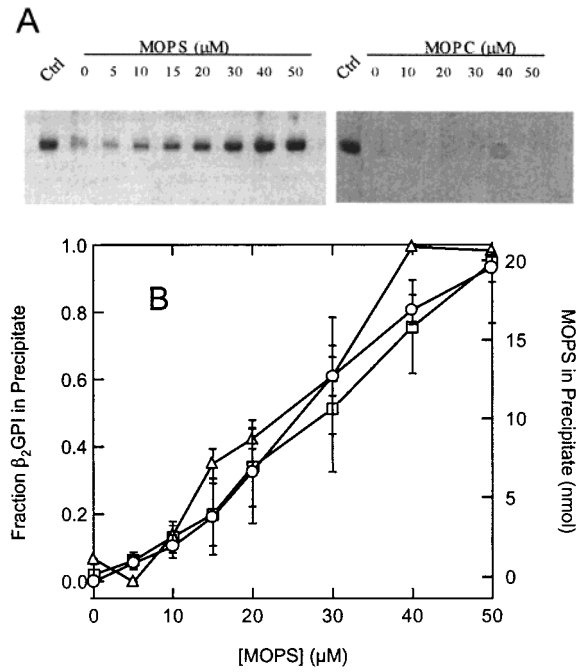

FIGURE 3: (A) Aggregation of intact β_2-GPI with MOPS monitored by SDS−PAGE in 20 mM MES buffer (pH 6) at 37 °C. The concentration of β_2-GPI was 5 μM. The precipitate after centrifugation was subjected to 15% SDS−PAGE. As a control, we also examined the effects of MOPC (right panel). The same amount of protein used in the reaction was subjected to the left lane as a control for quantification (first lanes in both panels). (B) MOPS-dependent aggregation of β_2-GPI measured by three different methods. The band density in panel A was quantified by an NIH image and plotted against the concentration of MOPS (○). The amount of precipitated protein was also calculated from the results of protein assay of the supernatant (△). The amount of precipitated MOPS measured by phosphorus analysis was also plotted (□). As the reaction volume was 550 μL, 27.5 nmol of MOPS was present in the reaction mixture at 50 μM lysophospholipid. Data obtained by all three methods were consistent with each other within experimental error.

confirmed by phosphorus assay. These results further confirmed that aggregation of β_2-GPI is induced by the interaction between β_2-GPI and lysophospholipids.

Aggregation of Intact β_2-GPI with Various Lysophospholipids. β_2-GPI is known to bind liposomes containing anionic phospholipids. However, because of the lack of electrostatic interaction between protein and lipid, this protein does not bind to liposomes composed of only neutral phospholipid. To investigate the headgroup dependence of this aggregation, MOPC, 1-oleoyl-2-hydroxy-*sn*-glycero-3-[phospho-*RAC*-(1-glycerol)] (MOPG), and 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (MOPA) were mixed with β_2-GPI, and formation of aggregates was measured (Figure 4). MOPG and MOPA have an anionic headgroup as MOPS and induced aggregation of β_2-GPI. However, neutral MOPC did not show any aggregation even at a high concentration of MOPC (see also the right panel in Figure 3A). This observation suggests that the aggregation observed here is also governed by the electrostatic interaction.

The effects of the length of the acyl chains in aggregation were examined using 1-caproyl-2-hydroxy-*sn*-glycero-3-phosphate (MCPA), 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphate (MMPA), and MOPS (Figure 5). CMC values of MOPS, MOPG, and MOPA have not been reported. Although the CMC of MOPS estimated by NPN fluorescence

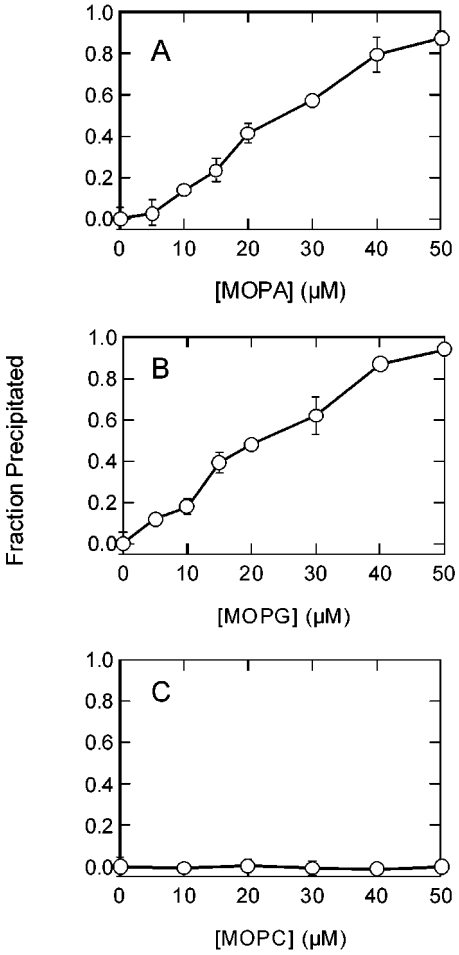

FIGURE 4: Aggregation of β_2-GPI with various lysophospholipids. A 5 μM concentration of intact β_2-GPI was mixed with MOPA (A), MOPG (B), and MOPC (C) in 20 mM MES buffer (pH 6) at 37 °C. The amount of precipitated protein was calculated from the results of protein assay of the supernatants after centrifugation.

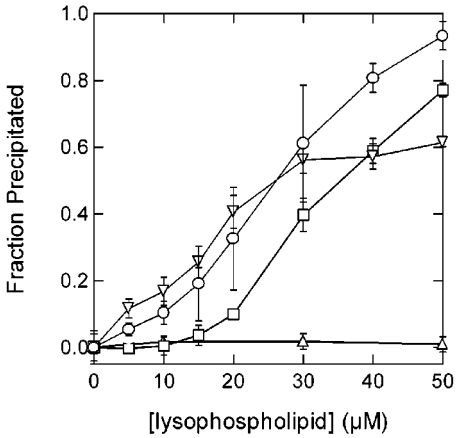

FIGURE 5: Aggregation of β_2-GPI with MOPS (○), MPPA (▽), MMPA (□), and MCPA (△) in 20 mM MES buffer (pH 6) at 37 °C. The lengths of the acyl groups of MOPS, MPPA, MMPA, and MCPA were 18, 16, 14, and 6, respectively. The oleoyl group is not saturated, and the other two groups have saturated acyl chains. The fraction precipitated was calculated from the results of protein assay of the supernatants.

under our experimental conditions was 35 μM (Figure 2B), aggregation was observed at lower concentrations. To further clarify whether the monodispersed lysolipids can induce aggregation, we examined the aggregation of β_2-GPI with

MMPA. The CMC value of 1-myristoyl-2-hydroxy-*sn*-glycero-3-[phospho-*RAC*-(1-glycerol)] (MMPG) was reported to be 3 mM in pure water, 288 μM in 10 mM phosphate (pH 7), and 160 μM in 0.1M Tris•HCl (pH 7.5) (*32*, *33*). MMPA has the same acyl chain as MMPG and is considered to have a similar CMC value. Indeed, fluorescence of NPN did not change between 0 and 300 μM MMPA under our conditions (Figure 2B). Aggregation with MMPA started at 20 μM. This value was far below the CMC of MMPA, indicating that β_2-GPI interacted with monodispersed lysolipids and gathered them to form large aggregates. However, β_2-GPI was not precipitated with MCPA at all. MCPA has only six carbons in the acyl group. Thus, MCPA is highly soluble and considered not to form micelles. In addition, a higher concentration of MMPA was required for aggregation than in the case of MOPS. These observations suggest that the strong hydrophobicity of lipids is also necessary for aggregation. The oleoyl group is not saturated, and the other two groups have saturated acyl chains. To examine the effect of the degree of unsaturation of acyl chains, we measured the aggregation of β_2-GPI with MPPA, which has a saturated acyl chain (Figure 5). The CMC of MPPA was determined to be 60 μM by the NPN fluorescence method (Figure 2). The titration curve of MPPA was similar to that of MPPA, although it exhibited less tendency of aggregation than MOPS at high concentrations. These suggests that the aggregation tendency is not affected significantly by a slight change in the degree of unsaturation.

Aggregation of Deletion Mutants and Peptide. To identify the region of β_2-GPI responsible for aggregation with lysophospholipid, we made a series of deletion mutants and peptide (Figure 6). As the expression yield of domain V lacking the flexible loop (Cys306−Cys326) was low, we made domain IV−V lacking this loop (loopless domain IV−V) instead of domain V. The aggregation of each deletion mutant with MOPS was examined by protein assay of the supernatant after centrifugation.

Domain V showed a nearly identical ability to aggregate in comparison with intact full-length β_2-GPI (Figure 7A). This showed that domain V has most of the information required for aggregation with lysophospholipid. The flexible loop region of domain V is important for the interaction with phospholipids. Domain I is known to bind anionic phospholipid with weaker affinity than intact β_2-GPI or domain V (*15*). Sequence identity between bovine and human domains I is 83%, and the number of positive charges is the same. Thus, we used bovine domain I, which was prepared by cyanogen bromide digestion of bovine β_2-GPI, to study the role of the N-terminal region of β_2-GPI on the aggregation with lysophospholipids. About 40% of domain I was precipitated with MOPS at moderate lipid concentration (Figure 7A). It is unclear why the precipitates disappeared at higher concentrations of MOPS. It may have been due to the detergent effects of MOPS of high concentrations. The loopless domain IV−V lacking this loop showed about 50% less aggregation than domain IV−V (Figure 7B). This indicates the importance of the flexible loop region for aggregation. However, aggregation was not completely abolished, suggesting the contribution of other parts of domain V to aggregation. The peptide from 281 to 288 in domain V, CKNKEKKC, was proposed to be one of the phospholipid binding sites (*16*). This region in loopless domain IV−V may also be important for aggregation. Two peptides corresponding to the flexible loop and phospholipid binding region were synthesized and oxidized, resulting in a cross-linked peptide (denoted as P35). P35 did not show any aggregation seen in other proteins (Figure 7C). Although the proposed phospholipid binding elements are included in its sequence, P35 lacks the information for aggregation.

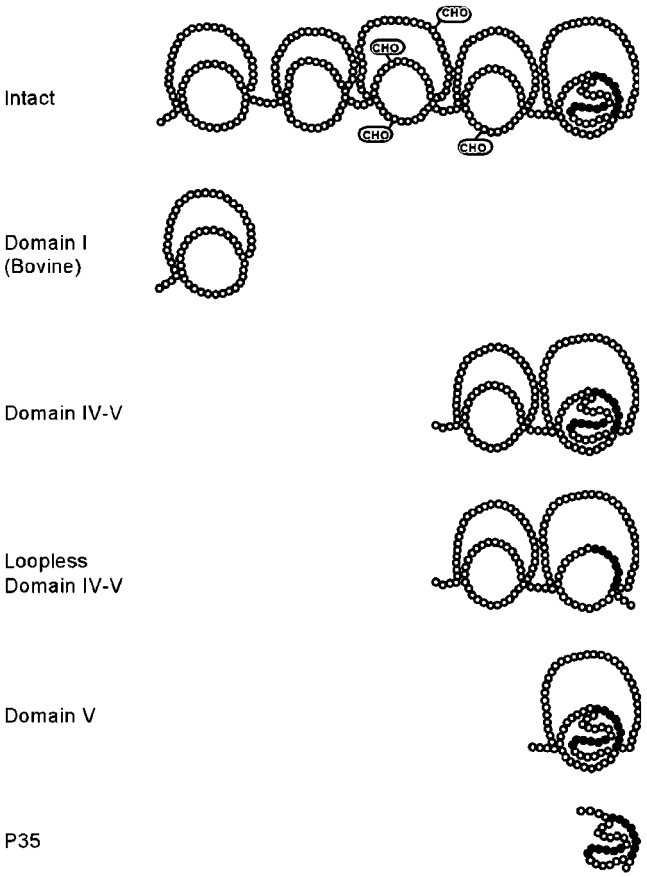

FIGURE 6: Schematic representation of intact full-length β_2-GPI, deletion mutants, and the peptide P35. Two strong lipid binding sites are shown with filled circles. CHOs indicate putative glycosylation sites.

DISCUSSION

Amphiphile-Induced Aggregation of β_2-GPI. In the present study, we demonstrated that β_2-GPI interacts with monodispersed SDS and anionic lysophospholipids, resulting in the formation of large aggregates. Burnstein and Legmann reported that the precipitation of chylomicrons and VLDL by SDS requires β_2-GPI, although the mechanism of this reaction remains unclear (*34*). As it is known that β_2-GPI is present in all major lipoprotein density fractions, especially in chylomicrons, VLDL, and HDL (*35*), these lipoproteins were coprecipitated with β_2-GPI and SDS as aggregates. There have been several reports on the SDS-induced aggregation of peptides and proteins (*28*−*30*, *36*). In all cases, aggregation was observed at low concentrations of SDS (<1 mM), supposedly less than its CMC. At SDS concentrations higher than the CMC, the proteins and peptides were dissolved completely accompanied by denaturation of proteins, as observed under the conditions of SDS−PAGE. Upon tight interaction with micelles, proteins and peptides tend to adopt an α-helical conformation.

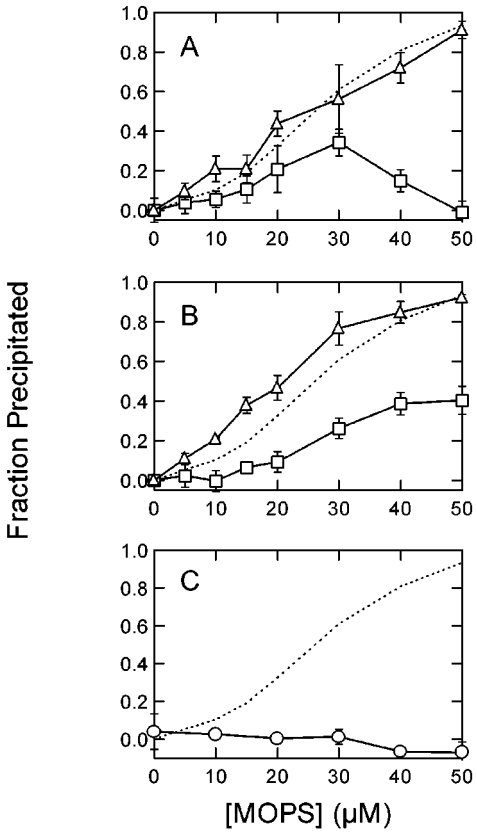

FIGURE 7: Aggregation of deletion mutants and the peptide with MOPS. Proteins or peptide at 5 μM was reacted with MOPS in 20 mM MES buffer (pH 6) at 37 °C. (A) Domain V (○) and bovine domain I (□). (B) Domain IV−V (△) and loopless domain IV−V (□). (C) P35 (○). For comparison, the aggregation curve of intact β_2-GPI is presented in each panel by a broken line.

On the other hand, there is only one reported example showing the interaction of monodispersed lysophospholipid with protein. Self-association of myelin basic protein was enhanced by lysophosphatidylcholine at submicellar concentrations (*29*). However, because this experiment used neutral lysophospholipid and myelin basic protein associates with itself even in the absence of lysolipid, this phenomenon is of limited relevance to our observations. We also examined aggregation of lysozyme (pI = 11), cytochrome *c* (pI = 10), and BSA (pI = 5) with MOPS (data not shown). Surprisingly, although BSA did not aggregate with MOPS, cytochrome *c* and lysozyme formed a precipitate in the presence of MOPS. Lysozyme and cytochrome *c* are known to bind anionic phospholipids (*37*, *38*). Thus, the aggregation by lysophospholipid at concentrations below the CMC is not specific to β_2-GPI but is considered to be a common feature of proteins that bind to the anionic phospholipid.

Mechanism of Aggregation. As β_2-GPI binds to lysolipid through charge−charge interactions, positive charges in β_2-GPI shield charge repulsion between negatively charged headgroups of anionic amphiphiles. This shielding effect increases the apparent hydrophobicities of anionic amphiphiles. In addition, this effect also neutralizes the charge repulsion between β_2-GPI molecules and increases the apparent hydrophobicity. The observed aggregates were precipitated by low-speed centrifugation, indicating that they were much larger than the usual micelles. It is likely that β_2-GPI and amphiphiles attracted each other by electrostatic and hydrophobic interactions, forming large amorphous aggregates, where the hydrophobic regions of each molecule interacted nonspecifically. The major driving force to form aggregates comes from the hydrophobic acyl chain in lysophospholipid, because the least hydrophobic lipid, MCPA, was unable to aggregate with β_2-GPI. The recent finding that the flexible loop in domain V binds to hydrophobic substances suggests the importance of hydrophobic interaction for the binding to phospholipid (*23*). Thus, it is possible that the hydrophobicity of the acyl chain is necessary for binding to β_2-GPI. At concentrations below the CMC, amphiphiles cannot form a stable micelle structure, even though the hydrophobic interactions of the amphiphile−β_2-GPI complex favor further association of the amphiphiles. This may result in the formation of amorphous aggregates. When the concentration of amphiphiles becomes higher than the CMC, the aggregates are expected to be dissolved again by the formation of stable and soluble micelle−β_2-GPI complexes as observed for SDS (Figure 1).

Isolated domain V aggregated at a lower concentration of SDS than full-length β_2-GPI (Figure 1). In the case of MOPS, domain V and full-length β_2-GPI showed almost identical ability to aggregate (Figure 7A). It is difficult to understand this difference of two amphiphiles directly, because SDS is less hydrophobic than MOPS and the headgroups are largely different. In any case, the importance of domain V is obvious, suggesting that domain V has most of the information required for aggregation with amphiphiles. On the other hand, domain I formed less aggregates than domain V and intact β_2-GPI. Thus, we consider that domain I has only a minor contribution to the aggregation of full-length β_2-GPI. The flexible loop in the C-terminal of domain V has a critical role in the aggregation of β_2-GPI with anionic lysophospholipids. This region has been shown to be essential for binding to phospholipids. Interestingly, P35 containing all proposed phospholipid binding sites did not aggregate. The short peptide from position 281 to position 288, which was included in P35, was reported to inhibit the interaction between β_2-GPI and phospholipid (*16*). However, their data indicate that the affinity of this peptide to phospholipids was about 10-fold lower than that of intact β_2-GPI. We showed that P35 bound to the hydrophobic ligand bis-ANS with affinity lower than that of domain V (*23*). These observations suggest that aggregation with lysophospholipid requires strong interaction as seen in intact β_2-GPI or domain V. Circular dichroism data indicate that this peptide is unfolded under physiological conditions (*23*), suggesting that a flexible but unique backbone topology is important for strong interaction with phospholipids. It is also possible that, while the binding occurs in the flexible loop peptide, participation of other regions of β_2-GPI is required for aggregation.

Biological Relevance of Aggregation. The repulsive forces between the charged headgroup in anionic phospholipids should be unfavorable for cluster formation between β_2-GPI and anionic phospholipids on the membrane. The results presented here indicate that β_2-GPI shields the charge repulsion, allowing the association of anionic lysophospholipids with each other. This shielding effect probably makes it possible to form clusters of anionic phospholipids with β_2-GPI on the physiological membrane. Domain V is highly positively charged with an isoelectric point of around 10. Therefore, repulsive forces between positive charges in

domain V also prevent the bivalent interaction with aPL, which requires two β_2-GPI molecules to be in close proximity. The interaction with anionic phospholipids neutralizes this repulsive force. In addition, it has been reported that interaction of anionic lipids and proteins induces domain formation in a mixed-lipid bilayer (*39*−*41*). It is possible that the interaction between β_2-GPI and lysophospholipid is strong enough to form a cluster of antigens on the membrane without aPL. However, Takeya et al. (*42*) showed that aPL enhances the β_2-GPI binding to anionic phospholipid. Therefore, we considered that charge−charge interaction between phospholipid and β_2-GPI may work cooperatively with bivalent interaction of aPL, enabling the cluster formation of β_2-GPI on the physiological membrane.

It has been shown that there is a relatively large amount of lysophosphatidic acid in serum. Recent studies have shown that lysophosphatidic acid acts through G protein-coupled receptors to evoke a host of responses in numerous target cells (*43*, *44*). The concentrations of lysophosphatidic acid are 98 μM in human serum and 26 μM in plasma (*45*). At this concentration of anionic lysophospholipid, β_2-GPI likely forms aggregates with lysophospholipid (Figures 4 and 5). To calculate the protein:lipid molar ratio in the aggregates, the amounts of MOPA and MOPG in the precipitates were estimated by phosphorus analysis (data not shown; MOPS; see Figure 3B). The ratios of protein to MOPS, MOPA, and MOPG were about 1:7, protein:lipid molar ratio, indicating the significant density of β_2-GPI in the aggregated particles. Thus, it is tempting to speculate that aggregates of β_2-GPI with anionic lysophospholipids, especially lysophosphatidic acid, are among the physiological antigens for aPL.

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