Characterization of Phosphatidylserine-dependent 
\( \beta_2 \)-Glycoprotein I Macrophage Interactions

IMPLICATIONS FOR APOPTOTIC CELL CLEARANCE BY PHAGOCYTES*

(Received for publication, May 4, 1998, and in revised form, August 3, 1998)

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The binding and uptake of phosphatidylserine (PS)-expressing cells appears to involve multiple receptor-mediated systems that recognize the lipid either directly or indirectly through intermediate proteins that form a molecular bridge between the cells. Here we show that \( \beta_2 \)-glycoprotein I (\( \beta_2 \)GPI), a 50-kDa serum glycoprotein, binds PS-containing vesicles and serves as an intermediate for the interaction of these vesicles with macrophages. Chemical modification of lysines and cysteines abolished \( \beta_2 \)GPI-dependent PS uptake by inhibiting the binding of PS to \( \beta_2 \)GPI and the binding of PS-\( \beta_2 \)GPI complex to macrophages, respectively. Recognition was mediated by \( \beta_2 \)GPI and not by the lipid because antibodies to \( \beta_2 \)GPI inhibited binding of the complex to macrophages. These results indicate that human (THP-1-derived) macrophages bind \( \beta_2 \)GPI only after it is bound to its lipid ligand. Competition experiments with monosaccharides that inhibit lectin-dependent interactions, and PS-\( \beta_2 \)GPI binding experiments using deglycosylated \( \beta_2 \)GPI, suggested that carbohydrate residues were not required for macrophage recognition of the complex. Antibodies to putative macropage PS receptors (CD36, CD68, and CD14) did not inhibit uptake of the complex. These data suggest that \( \beta_2 \)GPI can bind cells that fail to maintain membrane lipid asymmetry and generate a specific bridging moiety that is recognized for clearance by a phagocyte receptor that is distinct from CD36, CD68, and CD14.

The emergence of phosphatidylserine (PS)† in the cells outer leaflet results in the expression of altered cell surface properties that regulates their recognition by phagocytes (1–3). Although PS recognition might include binding to specific PS receptors (1, 4, 5), class B scavenger receptors (6–8), and PS-\( \beta_2 \)GPI binding experiments using deglycosylated \( \beta_2 \)GPI, suggested that carbohydrate residues were not required for macrophage recognition of the complex. Antibodies to putative macropage PS receptors (CD36, CD68, and CD14) did not inhibit uptake of the complex. These data suggest that \( \beta_2 \)GPI can bind cells that fail to maintain membrane lipid asymmetry and generate a specific bridging moiety that is recognized for clearance by a phagocyte receptor that is distinct from CD36, CD68, and CD14.

* This work was supported in part by National Institutes of Health Grant DK 41714. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be advertised in accordance with 18 U.S.C. Section 1724 solely to indicate this fact.
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† The abbreviations used are: \( \beta_2 \)GPI, \( \beta_2 \)-glycoprotein I; PC, phosphatidylincholine; PS, phosphatidylserine; PS-\( \beta_2 \)GPI, \( \beta_2 \)GPI complexed to vesicles composed of PS/PC (1/1); TBS, Tris-buffered saline; NEM, N-ethylmaleimide.

The binding and uptake of phosphatidylserine (PS)-expressing cells appears to involve multiple receptor-mediated systems that recognize the lipid either directly or indirectly through intermediate proteins that form a molecular bridge between the cells. Here we show that \( \beta_2 \)-glycoprotein I (\( \beta_2 \)GPI), a 50-kDa serum glycoprotein, binds PS-containing vesicles and serves as an intermediate for the interaction of these vesicles with macrophages. Chemical modification of lysines and cysteines abolished \( \beta_2 \)GPI-dependent PS uptake by inhibiting the binding of PS to \( \beta_2 \)GPI and the binding of PS-\( \beta_2 \)GPI complex to macrophages, respectively. Recognition was mediated by \( \beta_2 \)GPI and not by the lipid because antibodies to \( \beta_2 \)GPI inhibited binding of the complex to macrophages. These results indicate that human (THP-1-derived) macrophages bind \( \beta_2 \)GPI only after it is bound to its lipid ligand. Competition experiments with monosaccharides that inhibit lectin-dependent interactions, and PS-\( \beta_2 \)GPI binding experiments using deglycosylated \( \beta_2 \)GPI, suggested that carbohydrate residues were not required for macrophage recognition of the complex. Antibodies to putative macropage PS receptors (CD36, CD68, and CD14) did not inhibit uptake of the complex. These data suggest that \( \beta_2 \)GPI can bind cells that fail to maintain membrane lipid asymmetry and generate a specific bridging moiety that is recognized for clearance by a phagocyte receptor that is distinct from CD36, CD68, and CD14.

The emergence of phosphatidylserine (PS)† in the cells outer leaflet results in the expression of altered cell surface properties that regulates their recognition by phagocytes (1–3). Although PS recognition might include binding to specific PS receptors (1, 4, 5), class B scavenger receptors (6–8), the lipopolysaccharide receptor (2, 9–11) or thrombospondin-receptors (1, 4, 5), class B scavenger receptors (6–8), the lipid ligand.

These data suggest that PS binding by \( \beta_2 \)GPI induces a lipid-dependent conformational change that exposes a specific epitope that is recognized by a macrophage receptor that is distinct from other previously described lipid receptors.

EXPERIMENTAL PROCEDURES

Materials and Routine Procedures—Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Carrier-free N-[3-\( ^{125} \)I]-iodo-4-hydroxybenzylpropionyl dipalmitoylphosphatidylethanolamine (\( ^{125} \)I-PE) was synthesized as described previously (22). RPMI 1640, fetal calf serum, and penicillin/streptomycin were from Life Technologies, Inc. Phorbol 12-myristate 13-acetate and the \( \beta_2 \)GPI chemical modification reagents were from Sigma. Neuraminidase (Clostridium perfringens), N-glycosidase-F (recombinant, Escherichia coli) and O-glycosidase-F (Diplococcus pneumoniae) were from Boehringer Mannheim. \( \beta_2 \)GPI was purified from pooled human plasma (Gulf Coast Regional Blood Center, Houston, TX) by perchloric acid precipitation, ion exchange chromatography, and heparin affinity chromatography as described previously (15, 23). Antibodies to \( \beta_2 \)GPI were produced in rabbits and IgG was purified over protein A-Sepharose. F(ab’)_2 was produced from purified IgG by papain digestion followed by gel filtration and protein A-Sepharose chromatography to remove undigested IgG. Monoclonal antibodies against CD11b (clone 44), CD36 (clone SM0), and CD14 (UCHM-1) were from Sigma. Antibodies to CD14 (clone TUK4) and CD68 (clone KPI) were purchased from Dako. CD14 antibodies (clone 61D3), kindly provided by J. D. Capra (Oklahoma Medical Research Foundation), were purified from ascitis on protein G-Sepharose. Multilamellar vesicles and small lipid vesicles containing \( ^{125} \)I-PE (1 \( \mu \)Ci/ml) were prepared from the indicated lipids (1 mg/ml) by vortexing and sonication, respectively.

Chemical Modification of \( \beta_2 \)GPI—For N-ethylmaleimide (NEM), 30 nmol of \( \beta_2 \)GPI in TBS (20 mM Tris, 150 mM NaCl, pH 7.4) were treated with 4.8 \( \mu \)mol of NEM (80 mM stock in TBS) for 60 min at 20 °C. For diketene, 30 nmol of \( \beta_2 \)GPI in borate buffer (300 mM, pH 9.5) were treated with 2 \( \mu \)mol of diketene for 20 min at 20 °C (24). For phenacylbromide, 30 nmol of \( \beta_2 \)GPI in TBS were treated with 7.0 \( \mu \)mol of phenacylbromide (238 mM stock in Me2SO) for 60 min at 37 °C (25). For phenylglyoxal, 30 nmol of \( \beta_2 \)GPI in carbonate/hicarbonate buffer (125 mM, pH 7.5) were labeled with 22 \( \mu \)mol of phenylglyoxal (745 mM stock in Me2SO) for 90 min at 20 °C (26). For cyclohexanediene, 30 nmol \( \beta_2 \)GPI in borate buffer (0.1 M, pH 8.0) were labeled with 10 \( \mu \)mol of 1,2-cyclohexanediene (24). Excess reagents were removed by gel filtration or dialysis.

Deglycosylation of \( \beta_2 \)GPI—\( \beta_2 \)GPI was desialylated by treating 20 nmol of \( \beta_2 \)GPI in phosphate buffer (100 mM, pH 6.0) with neuraminidase (2 units) for 24 h at 37 °C according to the manufacturer's instructions. The product was purified by ion-exchange chromatography on
DEAE-Sepharose equilibrated with 50 mM Tris, 2 mM NaCl, pH 7.2. The eluate was pooled and concentrated by heparin-Sepharose affinity chromatography as described previously (15, 23). Complete deoxyribosylation of β₂GPI was achieved by incubating 30 nmol of β₂GPI in phosphate buffer (100 mM, pH 7.2) with neuraminidase (1 unit), N-glycosidase-F (10 units), and O-glycosidase-F (25 milliunits) for 48 h at 37 °C as described by the manufacturer. The sample was purified on DEAE-Sepharose/heparin affinity chromatography as described above.

Binding of β₂GPI to PS—The binding of β₂GPI to phospholipids was monitored by lipoplotting and by gel diffusion using 125I-labeled small unilamellar vesicle. For the lipoplot, unmodified and chemically modified β₂GPI were electrophoresed on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane. The membrane was blocked with 1% ovalbumin in TBS and incubated for 60 min at 20 °C with 125I-labeled PS/PC (1/1) small unilamellar vesicles at 0.5 mg of lipid/ml in TBS. Unbound lipid was removed by washing in TBS. Binding of the labeled vesicles to β₂GPI was determined by autoradiography.

For the gel diffusion, microscope slides were coated with 0.9% agarose incubated at 37 °C for the indicated time, washed, and solubilized in RPMI 1640 medium (without serum) containing multilamellar vesicles with phorbol 12-myristate 13-acetate (0.1 nmol/2.5 ml). Adherent macrophages (106 cells) in 24-well Costar plates were washed with TBS and re suspended in 250 µl of RPMI 1640 medium (without serum) containing multilamellar vesicles—β₂GPI complexes (200 µg of lipid/100 µg of β₂GPI/ml). The cells were incubated at 37 °C for the indicated time, washed, and solubilized in 0.2% SDS. Lipid uptake was determined by scintillation counting.

RESULTS

β₂GPI-dependent PS Uptake by Macrophages—The effect of β₂GPI and PS on the uptake of 125I-labeled PS-containing vesicles was determined. Fig. LA shows the uptake of PS/PC (1/1) vesicles by macrophages as a function of β₂GPI concentration. Maximal uptake occurred at ~2 µM β₂GPI, which is approximately half the concentration found in normal human serum (13). Fig. 1B shows the effect of PS concentration on vesicle uptake. The inclusion of β₂GPI enhanced the uptake of 50 mol % PS >4-fold during the 1-h time course of the experiment. Interestingly, the degree of β₂GPI-dependent enhance-
macrophages were incubated with 1, 125I-PS/β2GPI complexes; 2, 125I-PS/PC vesicles and β2GPI added to the macrophages simultaneously; 3, β2GPI was added to the macrophages for 1 h followed by the addition of 125I-PS/PC vesicles; 4, same as 3 except that the cultures were washed before the addition of 125I-PS/PC vesicles. 5, 125I-PS/PC vesicles alone. *Inset, FITC-β2GPI antibody staining of macrophages incubated with (top), β2GPI alone and (bottom), PS/β2GPI complex. B, 1, 125I-PS-β2GPI complexes; 2, 125I-PS-diketene-treated β2GPI; 3, 125I-PS/PC alone.

Fig. 3. Differential uptake of β2GPI-bound PS vesicles. 125I-Labeled or unlabeled PC and PS/PC (1/1) vesicles were preincubated with β2GPI, and macrophage uptake was determined after 1 h at 37°C. Equal concentrations of lipid were mixed in the case of the combined vesicle experiments.

Modification of histidines (phenacylbromide) and arginines (cyclohexanediol and phenylglyoxal) did not inhibit uptake, whereas blocking of lysines (diketene) and cysteines (NEM) did. Although inhibition in the case of lysine modification was indirect (due to inhibition in ligand binding; see Fig. 2B), acylation of cysteines with NEM directly inhibited the binding of PS-β2GPI to macrophages. This was concluded from the results of lipoblot (Fig. 4C) and gel diffusion (Fig. 4D) experiments which showed that the NEM-treated protein, unlike diketene-treated protein, still bound 125I-PS/PC vesicles, and by the finding that the inhibition was bypassed by the addition of β2GPI antibodies (Fig. 4B). It should be noted that circumvention of inhibition by β2GPI antibodies was due to the binding of the PS-β2GPI-IgG complex to the Fc receptor of the cells. Similar experiments carried out with Fab fragments resulted in inhibition of PS-β2GPI uptake, suggesting that the antibodies bind to a β2GPI site that is critical to macrophage recognition (Fig. 5).

Role of Carbohydrate on Macrophage Uptake—To determine the role carbohydrates might play in the binding of PS-β2GPI to macrophages, PS-β2GPI was incubated with macrophages in the presence of various monosaccharides. The data presented in Fig. 6A show that these monosaccharides did not inhibit uptake, suggesting that macrophage PS-β2GPI interactions do not involve lectin-like carbohydrate binding moieties. Interestingly, deglycosylation of β2GPI enhanced uptake ~3-fold (Fig. 6B), possibly due to decreased charge repulsion by removal of sialic acid residues.

Effect of Charge on PS-β2GPI Interaction with Macrophages—To determine the nature of PS-β2GPI-macrophage interactions, uptake was assessed in the presence of amino acids and negatively charged groups. Table I shows that lysine and arginine inhibited uptake, whereas serine, leucine, and valine did not. Interestingly, while phosphoserine, phosphate, succinate, and butyrate inhibited uptake, aspartate and glutamate were without effect. Analysis of PS-β2GPI interaction by gel diffusion (18) in the presence of the inhibitors (data not shown) indicated that inhibition of binding to the macrophage surface was not because of dissociation of the PS-β2GPI complex. It should be noted that these inhibitors were specific for PS-β2GPI complexes because they did not significantly influence macrophage uptake of PS alone (Table I).

Effect of Antibodies to Phagocyte Receptors on PS-β2GPI Uptake—To determine whether putative PS recognition pathways are involved in the specific uptake of PS-β2GPI complexes by macrophages, uptake was determined in the presence of antibodies to CD36 (6–8), CD68 (4), and CD14 (2, 9, 11). As determined by fluorescence microscopy using fluorescein-conjugated secondary antibodies, all the monoclonals, with the exception of CD36, bound to the macrophage membrane (not shown). Unlike the significant inhibition (~50%) obtained with apoptotic cells (9, 11) and symmetric red blood cell ghosts (2), 61D3 antibodies (anti-CD14) did not significantly inhibit the uptake of PS-β2GPI (Table II). Antibodies against other macrophage surface antigens (CD11b, CD36, and CD68) and against other CD14 epitopes (monoclonals TUK4 and UCHM-1) were also without significant effect. Surprisingly, all of the monoclonals with the exception of 61D3 significantly enhanced the uptake of PS/PC vesicles in the absence of β2GPI. The reasons for this observation are not clear.

Discussion

β2GPI is a well characterized plasma glycoprotein that binds negatively charged phospholipids. This property is responsible for regulating thrombosis by competing with clotting factors for PS expressed on the surface of activated platelets (19). Similar PS binding activities have also been shown to occur with synthetic negatively charged phospholipid vesicles (15, 16) and apoptotic thymocytes (17, 18). Formation of these PS/β2GPI complexes has been shown to result in a major change in the proteins conformation (29, 30) that might result in the expression of a new epitope (31), which, in certain individuals, generates an autoimmune response. While the mechanism for the generation of these immune responses is not known, this new epitope could be responsible for the recognition and removal of PS-expressing apoptotic and senescent cells from the host.

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2 The reason for the ~7-fold increase in PS-β2GPI uptake in the case of phenylglyoxal-modified β2GPI is not known.
Using an *in vitro* model for macrophage binding, we showed that the uptake of PS liposomes by macrophages was greatly enhanced by the addition of \( \beta_2 \)GPI that saturated at \( 2 \mu M \). Since the average concentration of \( \beta_2 \)GPI in normal human plasma is about 4 \( \mu M \) (13), these data raise the possibility that this property could be the principal function of \( \beta_2 \)GPI *in vivo*. Interestingly, as shown in Fig. 2, ligand-free \( \beta_2 \)GPI did not bind macrophages. However, once \( \beta_2 \)GPI bound PS, the complex became rapidly associated with the macrophage membrane. The requirement for ligand binding was further shown by the inability of macrophages to bind diketene-treated (lysine-blocked) \( \beta_2 \)GPI even in the presence of PS.

PS-\( \beta_2 \)GPI complex binding to macrophages was shown to be inhibited by lysine and arginine as well as negatively charged monosaccharides and deglycosylation. Fig. 5 shows the effect of chemical modification on \( \beta_2 \)GPI-dependent uptake of PS/PC vesicles. Chemically modified \( \beta_2 \)GPI was preincubated with \(^{125}\text{I-PS/PC vesicles, and macrophage uptake was determined. A, in the absence and B, presence of } \beta_2 \text{GPI antibodies. Lipid binding of chemically modified } \beta_2 \text{GPI was determined by C, lipoblot and D, gel diffusion analysis. } \beta_2 \text{GPI treatments: 1, control (not treated); 2, diketene; 3, NEM; 4, phenacylbromide; 5, cyclohexanediol; 6, phenylglyoxal; and the negative controls; 7, PS/PC vesicles alone; and 8, PC vesicles alone. D, clockwise left, control } \beta_2 \text{GPI, phenacylbromide, NEM, cyclohexanediol, and diketene. Right, control, cyclohexanediol, and phenylglyoxal.}

Fig. 6 shows the effect of monosaccharides and deglycosylation on PS-\( \beta_2 \)GPI uptake. A, \(^{125}\text{I-PC and } ^{125}\text{I-PS/PC vesicles were preincubated with and without } \beta_2 \text{GPI. Macrophage uptake was then determined in the presence of the indicated monosaccharides at } 20 \text{ mM. 1, control; 2, N-acetylglucosamine; 3, N-acetylgalactosamine; 4, methyl-\( \alpha \)-D-glucopyranoside; 5, methyl-\( \alpha \)-D-mannopyranoside; 6, } \text{L(-)-fucose; 7, } \text{D(+)-galactose; 8, } \text{D(+)-glucose; 9, } \text{N-acetylneuramic acid; 10, } ^{125}\text{I-PS/PC vesicles alone; and 11, } ^{125}\text{I-PC vesicles alone. B, uptake of desialyated and deglycosylated PS-\( \beta_2 \)GPI complexes. 1, control; 2, deglycosylated } \beta_2 \text{GPI; 3, desialyated } \beta_2 \text{GPI; 4, } ^{125}\text{I-PS/PC vesicles alone; 5, } ^{125}\text{I-PC vesicles alone.}

Using an *in vitro* model for macrophage binding, we showed that the uptake of PS liposomes by macrophages was greatly enhanced by the addition of \( \beta_2 \)GPI that saturated at \(-2 \mu M\). Since the average concentration of \( \beta_2 \)GPI in normal human plasma is about 4 \( \mu M \) (13), these data raise the possibility that this property could be the principal function of \( \beta_2 \)GPI *in vivo*. Interestingly, as shown in Fig. 2, ligand-free \( \beta_2 \)GPI did not bind macrophages. However, once \( \beta_2 \)GPI bound PS, the complex became rapidly associated with the macrophage membrane. The requirement for ligand binding was further shown by the inability of macrophages to bind diketene-treated (lysine-blocked) \( \beta_2 \)GPI even in the presence of PS. PS-\( \beta_2 \)GPI complex binding to macrophages was shown to be inhibited by lysine and arginine as well as negatively charged monosaccharides and deglycosylation.
macrophage binding can be obtained from results which showed that removal of the carbohydrate moieties (33) resulted in >3-fold enhancement in β2GPI-dependent uptake (Fig. 6).

Several studies have suggested that the redistribution of PS from the cells inner to outer leaflet signals for removal of these cells by the reticuloendothelial system (1–3, 16). Although several mechanisms might be responsible for phagocyte recognition of PS expressing apoptotic cells, Price et al. (17) proposed that the interaction of circulating β2GPI with redistributed anionic phospholipid may, by itself, generate a novel ligand by which apoptotic cells are recognized. Indeed, other studies have indicated that β2GPI could play a central role in this recognition process (16). Combined with these previous studies, the data presented here provide evidence for the existence of a receptor on the macrophage membrane that specifically binds β2GPI in a ligand-dependent manner. Although the motif on the protein responsible for the macrophage membrane interaction is not known, it could result from a lipid-dependent conformational change (29, 30) that is specifically bound to a cell surface receptor, a process that can be inhibited with anti-β2GPI F(ab')2 (Fig. 5). Several reports have suggested the involvement of macrosialin (CD68) (4), scavenger receptor (CD36) (6–8), and the lipopolysaccharide receptor (CD14) (2, 9, 11) in the recognition of PS on apoptotic cells. Antibodies directed against these cell surface moieties, however, did not significantly inhibit β2GPI-dependent PS uptake. The inability to obtain more than 20% inhibition raises the possibility that either more than one cell surface component is involved in β2GPI-dependent recognition or that recognition is inhibited because of steric hindrance by antibody bound to an unrelated proximal site. Although further studies will be required to identify the putative PS-β2GPI-dependent macrophage receptor, the data presented here argue for the existence of such a receptor. Because of the relative abundance of β2GPI in plasma, it could play an important physiologic role by bridging PS-expressing cells to phagocytes for their ultimate disposal.

Acknowledgments—We thank Drs. Killion and Cookie for constructive criticism and Ash Leek for technical assistance.

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