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High Affinity Binding of β_2-Glycoprotein I to Human Endothelial Cells Is Mediated by Annexin II*

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β_2-Glycoprotein I (β_2GPI) is an abundant plasma phospholipid-binding protein and an autoantigen in the antiphospholipid antibody syndrome. Binding of β_2GPI to endothelial cells targets them for activation by anti-β_2GPI antibodies, which circulate and are associated with thrombosis in patients with the antiphospholipid antibody syndrome. However, the binding of β_2GPI to endothelial cells has not been characterized and is assumed to result from association of β_2GPI with membrane phospholipid. Here, we characterize the binding of β_2GPI to endothelial cells and identify the β_2GPI binding site. ^{125}I-β_2GPI bound with high affinity (K_d ~18 nM) to human umbilical vein endothelial cells (HUVECs). Using affinity purification, we isolated β_2GPI-binding proteins of ~78 and ~36 kDa from HUVECs and EAHY.926 cells. Amino acid sequences of tryptic peptides from each of these were identical to sequences within annexin II. A role for annexin II in binding of β_2GPI to cells was confirmed by the observations that annexin II-transfected HEK 293 cells bound ~10-fold more ^{125}I-β_2GPI than control cells and that anti-annexin II antibodies inhibited the binding of ^{125}I-β_2GPI to HUVECs by ~90%. Finally, surface plasmon resonance studies revealed high affinity binding between annexin II and β_2GPI. These results demonstrate that annexin II mediates the binding of β_2GPI to endothelial cells.

β_2-Glycoprotein I (β_2GPI)[1] is an abundant plasma glycoprotein that consists of five homologous domains of approximately 60 amino acids each (1, 2). The first four of these are classical short consensus repeat domains, with extensive homology to those found in the complement-type repeats of Factor H (3). Each of these contains four conserved cysteines with a characteristic C_{1-3}, C_{2-4} disulfide bonding pattern, whereas domain 5 contains six cysteines, with C_{1-4}, C_{2-5}, and C_{3-6} disulfide linkages (4, 5). Domain 5 is also unique in its high content of lysine residues (5, 6). In the recently solved crystal structure of β_2GPI, these have been shown to contribute to the formation of a positively charged phospholipid binding region in domain 5 (7, 8).

The physiological function(s) of β_2GPI is uncertain. A role in lipid metabolism is suggested by the observations that 30% of plasma β_2GPI circulates in complex with lipoproteins (2, 9) and that β_2GPI accelerates triglyceride clearance in mice (10). β_2GPI also binds with high affinity to the atherogenic lipoprotein, Lp(a) (11). A role for β_2GPI as a naturally occurring anticoagulant is suggested by reports that it inhibits the prothrombinase (12) and Factor X activating complexes (13), although the physiologic importance of these effects is uncertain. β_2GPI may also promote the clearance of senescent cells (14, 15) and regulate the uptake of lipoproteins by macrophages (16).

Recently, β_2GPI has been found to be an important autoantigen in the antiphospholipid antibody syndrome (17–19), a disorder characterized by thrombosis and recurrent fetal loss in patients with circulating "antiphospholipid" antibodies (20, 21). It is now generally accepted that most antiphospholipid antibodies associated with the antiphospholipid antibody syndrome recognize β_2GPI bound to the cardiolipin-coated microplates used in clinical "anticardiolipin" assays (19, 22–27). Binding of β_2GPI to cardiolipin or another appropriate surface results in either a conformational change in the protein, exposing antigenic neoepitopes (28–30), or concentration of β_2GPI to an antigenic density at which it is more avidly bound by low affinity anti-β_2GPI antibodies (31). Antiphospholipid antibodies may also recognize epitopes occurring as a consequence of the formation of adducts between β_2GPI and oxidized cardiolipin (32, 33).

It has been suggested that β_2GPI may contribute to the pathogenesis of aPS-associated thrombosis by binding to platelets or endothelial cells and targeting them for anti-β_2GPI antibody-dependent activation. Binding of β_2GPI to these cells has been assumed to result from its interaction with membrane phospholipid. However, although β_2GPI binds with high affinity to purified anionic phospholipids (34, 35), its affinity for phospholipid preparations with a composition resembling that of cell membranes is low (36, 37). Consistent with this observation, β_2GPI binds with only micromolar affinity (38, 39), if at all (40), to activated platelets. In addition, human anti-β_2GPI

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[1] The abbreviations used are: β_2GPI, β_2-glycoprotein I; BS^3, bis(sulfosuccinimidyl)suberate; HUVEC, human umbilical vein endothelial cell; mAb, monoclonal antibody; t-PA, tissue-type plasminogen activator; PAGE, polyacrylamide gel electrophoresis; RU, response unit(s).

antibodies have not been convincingly shown to induce platelet activation (41).

In the nonactivated state, several anticoagulant moieties that play a central role in the maintenance of blood fluidity are expressed on the endothelial cell (42, 43). These include, among others, heparan sulfate proteoglycans, which bind and activate antithrombin (42), and thrombomodulin, which redirects the proteolytic activity of thrombin toward the activation of the natural anticoagulant, protein C (44). However, a variety of stimuli may induce endothelial cell activation, a process in which numerous transcriptional and posttranscriptional events occur that lead to the expression of adhesion molecules and procoagulant activity on the endothelial surface (44–46). These changes are associated with an increased risk of thrombosis in several clinical settings (46). That endothelial activation contributes to the pathogenesis of antiphospholipid antibody-associated thrombosis is suggested by the presence of increased levels of endothelial-derived proteins and microparticles in the plasma of patients with antiphospholipid antibodies (47–49). Furthermore, in contrast to platelets, anti-β_2GPI antibodies have been convincingly shown to activate endothelial cells in a β_2GPI-dependent manner (50, 51), although the mechanisms by which they do so remain undefined.

We hypothesized that endothelial cell activation mediated through this β_2GPI-dependent pathway would require a high affinity interaction between β_2GPI and a specific endothelial cell receptor, with receptor cross-linking subsequently induced indirectly through binding of anti-β_2GPI antibodies to receptor-bound β_2GPI. As an initial step in evaluating this hypothesis, we have characterized the binding of β_2GPI to endothelial cells. β_2GPI bound to endothelial cells through a high affinity interaction with annexin II, an endothelial cell receptor for tissue-type plasminogen activator (t-PA) (52–55) and plasminogen (52, 55, 56). Preliminary studies also suggest a potential role for annexin II in mediating anti-β_2GPI antibody-mediated endothelial cell activation.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture medium and reagents were obtained from Mediatech (Herndon, VA), and 96-well microplates were from Dynax (Chantilly, VA). Fetal bovine serum was from Hyclone (Logan, UT). Endothelial cell growth supplement was purified from bovine hypothalamii (57). Heparin-superflow was purchased from Sterogene (Seattle, WA), and Source 15S and gelatin-Sepharose was purchased from Amersham Pharmacia Biotech. Bis(sulfosuccinimidyl)suberate (BS^3), sulfosuccinimidyl 6-(biotinamido) hexanoate, Aquasil, and Super signal chemiluminescence reagent were from Pierce. Affi-Gel-HZ was from Bio-Rad, and polyvinylidene difluoride membranes were from Millipore (New Bedford, MA). Rabbit anti-annexin II polyclonal antibodies were prepared as described (52). A monoclonal anti-annexin II antibody (mAb Z014) was purchased from Zymed Laboratories Inc. (South San Francisco, CA). Reflection autoradiographic film was from NEN Life Science Products. Streptavidin-peroxidase, *Escherichia coli* lipopolysaccharide, polymyxin agarose, and all other chemicals were obtained from Sigma.

Cells—Human umbilical vein endothelial cells (HUVECs) were isolated as described and cultured in medium 199 (M199) containing 10% fetal bovine serum, 75 μg/ml endothelial cell growth supplement, 2 mM glutamine, and penicillin-streptomycin (complete medium) (58). All cells used were of passage 3 or lower. EAHY.926 cells (a hybrid human cell line derived from fusion of HUVECs and A549 carcinoma cells (59)), HEK 293, and MDA-MB-231 cells were cultured in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM glutamine.

Proteins—Fibronectin (60) and β_2GPI (9, 18) were isolated from human plasma as described previously (9, 18, 60). Purified, nonreduced β_2GPI migrated as a single ~50-kDa band when analyzed by SDS-PAGE, displayed the characteristic increase in M_r to ~65,000 upon reduction, and was recognized on immunoblots by anti-β_2GPI antibodies. β_2GPI was radiolabeled using Iodogen, per the manufacturer's protocol. Anionic phospholipid binding activity of the radiolabeled protein was confirmed by its ability to bind specifically to cardiolipin-coated microplates (5).

Binding of ^{125}I-β_2GPI to HUVECs—In initial studies, we observed that β_2GPI bound "specifically" to polystyrene tissue culture plates. We therefore developed an assay to minimize such interactions. Briefly, 96-well Immulon II plates were pretreated with Aquasil (0.5 mg/ml) and then coated with 50 μl of a 20 μg/ml solution of fibronectin. Endothelial cells were plated in individual wells and cultured in complete medium until confluent. Prior to binding assays, cells were washed and incubated for 6 h with serum-free medium 199 containing 0.1% fatty acid-free bovine serum albumin. Binding was measured at 4 °C, by incubating quadruplicate wells for 2 h with increasing concentrations of ^{125}I-β_2GPI in the absence (to determine total binding) or presence (to determine nonspecific binding) of a 100-fold molar excess of unlabeled ligand. After washing, bound ^{125}I-β_2GPI was measured in cell lysates using a gamma counter (61). Specific binding was defined as the difference between total and nonspecific binding, and binding isotherms were analyzed by nonlinear curve fitting using the least squares method (Kaleidograph, Abelbeck Software, Reading PA), as well as by the method of Scatchard (62).

In selected experiments, the reversibility of ^{125}I-β_2GPI binding was determined by incubating cells with 40 nM ^{125}I-β_2GPI at 4 °C for 2 h. The supernatant, containing radiolabeled ligand, was then removed and replaced with 100 μl of fresh medium containing 4 μM unlabeled β_2GPI. The radioactivity in cell supernatants at selected time points was then determined.

To assess the role of annexin II as an endothelial cell β_2GPI receptor, the ability of monoclonal and polyclonal anti-annexin II antibodies to inhibit the binding of ^{125}I-β_2GPI to endothelial cells was measured. Cells were prepared for binding studies and then incubated for 2 h with 20 nM ^{125}I-β_2GPI either alone, in the presence of 2 μm unlabeled β_2GPI (to determine nonspecific binding), or with the specified concentration of antibody. After washing, cell-bound radioligand was determined.

Isolation of Endothelial Cell Surface β_2GPI-binding Proteins—To determine whether β_2GPI might bind to an endothelial cell surface protein, we determined whether endothelial cell-bound ^{125}I-β_2GPI was incorporated into an SDS-stable complex following exposure of cells to the membrane-impermeable, bifunctional cross-linker, BS^3. HUVECs were incubated with 40 nM ^{125}I-β_2GPI for 2 h and then exposed to 4 mM BS^3 for 15 min. Detergent extracts were prepared and analyzed using 10% SDS-PAGE and autoradiography. The specificity of cross-linking was assessed by determining whether the amount of complex detected was reduced when studies were performed in the presence of a 100-fold molar excess of unlabeled β_2GPI and whether complexes of similar M_r were detected when studies were performed using human MDA-MB-231 breast carcinoma cells as a control.

To further assess whether β_2GPI bound specifically to an endothelial cell protein, biotinylated endothelial cell surface proteins were affinity-purified using immobilized β_2GPI. Cells were biotinylated using sulfosuccinimidyl 6-(biotinamido) hexanoate (63), and detergent extracts were prepared in a buffer containing 0.1 M Tris-HCl, pH 8.1, 1.0% Triton X-100, 2.5 mM EDTA, 10 μg/ml aprotinin, 50 μg/ml phenylmethylsulfonyl fluoride, 12.5 μg/ml leupeptin, 10 mM benzamidine and 10 μg/ml soybean trypsin inhibitor. β_2GPI-binding proteins were isolated by affinity chromatography using a 1-ml column of β_2GPI-conjugated to Affi-Gel-HZ (10 mg of β_2GPI/ml of gel). After washing the column with Tris-buffered saline containing 0.8 M NaCl, bound proteins were eluted using 0.1 M glycine-HCl, pH 3.0. Fractions of 0.5 ml were collected, and proteins within each separated by 10% SDS-PAGE (nonreducing conditions) and transferred to polyvinylidene difluoride. β_2GPI-binding proteins were detected by incubating the membrane for 30 min with streptavidin-peroxidase, followed by development using chemiluminescence. The binding of proteins within detergent extracts to a control column containing Affi-Gel-immobilized bovine serum albumin was determined in parallel, to assess the specificity of the affinity purification procedure.

Definitive identification of endothelial cell β_2GPI-binding proteins was pursued through larger scale affinity purification studies. Briefly, extracts from 6×10^9 EAHY.926 cells were subjected to affinity chromatography using a 5-ml column of β_2GPI-Affi-Gel HZ. After washing the column and eluting bound proteins, fractions were analyzed using 10% SDS-PAGE. After fixation, gels were stained using Coomassie Brilliant Blue, and protein bands were then excised, dehydrated in 100% acetonitrile, and stored at −80 °C. Sequences of tryptic peptides from the ~78-kDa band were determined by LC-mass spectrometry at Harvard MicroChem (Cambridge, MA). Parallel studies with the ~36- and ~60-kDa bands were performed at the William M. Keck Biomedical Mass Spectrometry Center, University of Virginia School of Medicine (Charlottesville, VA). Data base searches using nonredundant spectral information (SEQUEST) and partial peptide sequences (MS-Edman

TABLE I
Effect of microplate precoating on specific binding of ^{125}I-β_2GPI

Condition	^{125}I-β_2GPI specifically bound
	fmol
No treatment	140.0 ± 24.2[a]
Aquasil	17.6 ± 7.5
Aquasil + fibronectin	3.8 ± 1.1
Aquasil + HUVEC	7.2 ± 8.5
Aquasil + fibronectin + HUVEC	87.2 ± 2.7

[a] Data are expressed as mean of triplicate points ± S.D.

and BLAST) were performed to identify the tryptic peptides.

Effect of Transfection of HEK 293 Cells with Annexin II cDNA on β_2GPI Binding—Transient transfection of HEK 293 cells with annexin II cDNA was performed by incubating subconfluent cells either with 2 μl of an empty plasmid (pCMV5) or with the same plasmid containing the annexin II coding sequence, in the presence of Lipofectin (pCMV5-AII) (52). Three days after transfection, cells were trypsinized and replated in 96-well plates. The total, nonspecific, and specific binding of ^{125}I-β_2GPI to cells transfected with the empty and annexin II cDNA-containing vectors were then determined.

Measurement of the Binding of β_2GPI to Annexin II by Surface Plasmon Resonance—To determine whether β_2GPI bound to annexin II in a cell-free system, we assessed the interaction between these proteins by surface plasmon resonance using a Biacore 2000 (Biacore, Piscataway, NJ). β_2GPI was immobilized on a carboxymethyldextran (CM-5) biosensor chip using either amine or aldehyde coupling (64). For amine coupling, the chip was exposed to β_2GPI (3 μg/ml) in 10 mM sodium acetate, pH 6.0, at a flow rate of 5 μl/min for 2 min; this resulted in the immobilization of 198 response units (RU) of β_2GPI. For aldehyde coupling, carbohydrate residues on β_2GPI were oxidized by adding 20 μl $NaOI_4$ to 1 ml of a 1 mg/ml solution of β_2GPI in 100 mM sodium acetate, pH 5.5. After 15 min, the solution was desalted on a NAP-5 column (Amersham Pharmacia Biotech). The oxidized protein was immobilized by exposure to a CM-5 chip at a flow rate of 5 μl/min for 1 min, at which time the hydrazone bond was reduced by exposure to sodium cyanoborohydride. This resulted in the immobilization of 1062 RU of β_2GPI.

The binding of increasing concentrations of annexin II, delivered at a flow rate of 30 μl/min, was then measured in real time. Binding data was analyzed by Global analysis using BiaEval 3.0 software (Biacore), in which the association and dissociation data for a series of annexin II concentrations is fit simultaneously (65). In parallel, association data was analyzed following linear transformation (66). The equation used in these studies was $dRU/dt = k_a[\text{annexin II}]RU_{max} - RU(k_a[\text{annexin II}] + k_d)$, where RU_{max} = the maximal binding response. The use of this equation to derive the K_d from real time surface plasmon resonance data has been described (66). This approach allows the definition of fast and slow components of association and thus reveals binding heterogeneity (66, 67).

RESULTS

Binding of ^{125}I-β_2GPI to HUVECs—Preliminary attempts to measure the binding of ^{125}I-β_2GPI to HUVECs plated in standard 96-well tissue culture plates were complicated by specific binding of the ligand to control wells that contained no cells. However, because ^{125}I-β_2GPI bound specifically to fluid-phase endothelial cells, we focused on developing an assay in which its binding to endothelial cell monolayers could be assessed. Pretreatment of Immulon II plates with Aquasil abolished the binding of ^{125}I-β_2GPI to the plates, which, however, could still be coated with sufficient fibronectin to support endothelial cell adhesion and growth. Using this system, we observed that the binding of ^{125}I-β_2GPI to wells treated with Aquasil and fibronectin was only ~5% of that to identically prepared wells in which confluent monolayers of HUVECs were present (Table I). Therefore, this assay allowed us to selectively measure the specific binding of ^{125}I-β_2GPI to endothelial cells.

^{125}I-β_2GPI bound to HUVECs specifically and in a time-dependent manner (Fig. 1*A*). Binding was reversible in the presence of excess unlabeled ligand (Fig. 1*B*). Analysis of saturation isotherms revealed saturable, high affinity binding (K_d ~18 nM, B_{max} ~645,000 sites/cell) (Fig. 2). Binding was not inhibited by heparin (1–10 units/ml) or by pretreatment of cells with heparinase. However, although ^{125}I-β_2GPI bound to endothelial cells with an affinity similar to that of annexin V (68), a phospholipid-binding protein that binds with high affinity (K_d ~7 nM) to anionic phospholipid exposed on the surface of activated platelets (69), it bound to 14-fold fewer sites (68). These results suggested a fundamental difference in the interactions of β_2GPI and annexin V with endothelial cells. Furthermore, the high affinity binding of ^{125}I-β_2GPI to HUVECs, when compared with its weak interaction with platelets (K_d = 0.5–1.0 μM) (38–40), suggested that the mechanisms of its binding to these two cell types differed.

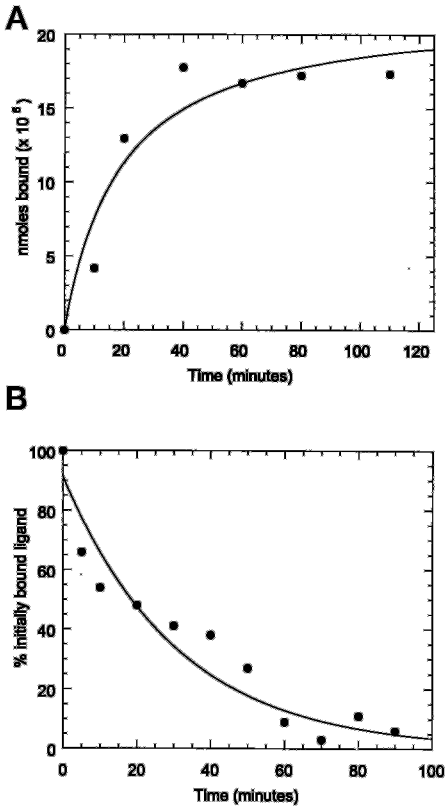

FIG. 1. **Time course and reversibility of β_2GPI binding to endothelial cells.** *A,* time course. HUVECs were prepared for binding assays as described under "Experimental Procedures" and then incubated at 4 °C with 40 nM ^{125}I-β_2GPI, in the absence or presence of 4 μM unlabeled ligand. At various times thereafter, the amount of ^{125}I-β_2GPI specifically bound to cells was determined. *B,* reversibility of binding. HUVECs were incubated with 40 nM ^{125}I-β_2GPI for 2 h at 4 °C. Supernatant containing the radiolabeled ligand was then replaced with cold PBS containing 4 μM unlabeled β_2GPI (time = 0 min). At various times thereafter, supernatants were removed, and the amount of ^{125}I-β_2GPI that remained bound to the cells was measured. All points were determined in quadruplicate. These experiments are representative of three so performed.

Evidence for a β_2GPI-binding Protein(s) on the Endothelial Cell Surface—To assess whether a cell surface protein might mediate the binding of β_2GPI to endothelial cells, we determined whether endothelial cell-bound ^{125}I-β_2GPI could be cross-linked to such a protein using the membrane impermeable, homobifunctional cross-linker, BS^3. SDS-PAGE analysis of detergent extracts from cells incubated with ^{125}I-β_2GPI followed by exposure to BS^3 revealed radioactive bands of ~50 and ~90 kDa, consistent with free ^{125}I-β_2GPI, and ^{125}I-β_2GPI cross-linked to a second protein of ~40 kDa (Fig. 3). The specificity of this interaction was demonstrated by the observations that formation of the ~90-kDa complex was inhibited by a 100-fold molar excess of unlabeled β_2GPI and that this complex

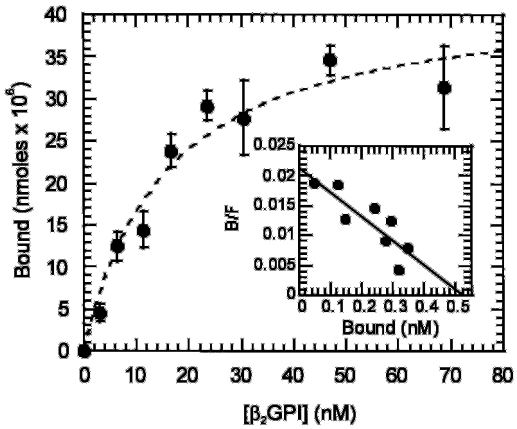

FIG. 2. **Specific binding of ^{125}I-β_2GPI to endothelial cells.** HUVECs were prepared for binding assays as described under "Experimental Procedures." Cells were then incubated for 2 h at 4 °C with increasing concentrations of ^{125}I-β_2GPI, in the absence or presence of a 100-fold molar excess of unlabeled ligand. Supernatants were then removed, and cells were quickly washed prior to determination of cell-bound ligand. All points were determined in triplicate. Specific binding in this experiment comprised 80–90% of total binding. Curve fitting was performed by the least squares method, using the Kaleidograph software program, as well as by the method of Scatchard (*inset*). This experiment is representative of four so performed.

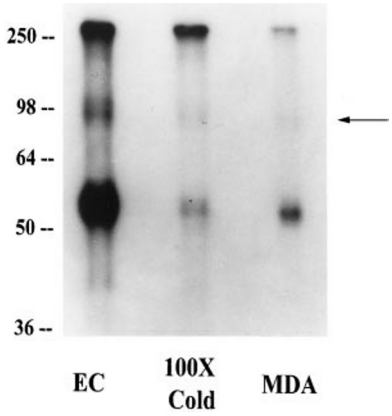

FIG. 3. **Cross-linking of ^{125}I-β_2GPI to a cell surface protein.** HUVECs (*left* and *middle lanes*) or MDA-MB-231 breast carcinoma cells (*right lane*) were incubated with 60 nM ^{125}I-β_2GPI, at 4 °C, for 2 h. Supernatant was then removed, and cells were incubated for an additional 15 min with 15 mM BS^3. Cells were then washed, and cell extracts prepared and analyzed by 10% SDS-PAGE and autoradiography. Autoradiograms were developed by exposure to Reflection® autoradiographic film for 48 h at 4 °C. A protein band of approximately 90 kDa (*left lane*, *arrow*), which was markedly reduced in intensity when studies were performed in the presence of 6 μM unlabeled β_2GPI (*middle lane*), was observed in extracts of HUVECs exposed to BS^3. This band was not observed in extracts of MDA-MB-231 cells treated in a manner identical to the cells in the *left lane*. *EC,* endothelial cells.

was not detected when identical experiments were performed using MDA-MB 231 breast cancer cells (Fig. 3).

To further examine the interaction of β_2GPI with endothelial cell proteins, we affinity-purified biotinylated endothelial cell surface proteins using a column of β_2GPI conjugated to Affi-Gel HZ. Analysis of biotinylated proteins purified from detergent extracts of 4×10^6 endothelial cells yielded primary bands of ~98 and ~78 kDa (Fig. 4, *left lane*). A less intense band of ~36 kDa was also observed, and a ~60-kDa band was present inconsistently. No endothelial cell proteins were isolated using control columns containing Affi-Gel-bovine serum albumin.

Identification of the Endothelial Cell β_2GPI-binding Protein—We next wished to identify the endothelial cell β_2GPI-binding proteins detected in the small-scale studies described above. However, due to the difficulty associated with culturing

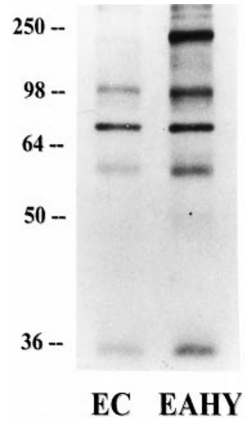

FIG. 4. **Affinity purification of β_2GPI-binding proteins from endothelial cells (*EC*) and EAHY.926 cells (*EAHY*).** HUVEC cell surface proteins were biotinylated using sulfosuccinimidyl 6-(biotinamide) hexanoate. Cells were then washed, and detergent extracts were prepared. Extracts were subjected to affinity chromatography on Affi-Gel HZ. Columns were washed with tris-buffered saline containing 0.8 M NaCl until the A_{280} of the effluent reached 0, and bound proteins were then eluted using 0.1 M glycine-HCl, pH 3.0. Proteins within each fraction were separated by 10% SDS-PAGE under nonreducing conditions and transferred to polyvinylidene difluoride membranes. Biotinylated proteins were detected by incubating membranes with streptavidin-peroxidase and developing the membranes using chemiluminescence. HUVEC extracts yielded bands of ~98, ~78, ~60 and ~36 kDa, whereas extracts of EAHY.926 cells contained these proteins, as well as an additional protein of >200 kDa. This experiment is representative of six so performed.

sufficient HUVECs to allow preparative scale isolation of such protein(s), we sought a transformed cell line that expressed similar proteins. No β_2GPI-binding proteins could be affinity-purified from THP-1, CHO, HEK 293, or MDA MB-231 cells (not shown). However, protein bands with a mobility identical to that of the proteins isolated from HUVECs were detected in detergent extracts of EAHY.926 cells, which also expressed an additional β_2GPI-binding protein of higher M_r (Fig. 4, *right lane*). These studies suggested that EAHY.926 would be suitable for isolation of endothelial β_2GPI-binding protein(s).

Definitive identification of endothelial cell β_2GPI-binding proteins was pursued by affinity purification of extracts from 6×10^9 EAHY.926 cells, using a 5-ml β_2GPI-Affi-Gel HZ affinity column. Coomassie Blue-stained gels of fractions eluted from the affinity column revealed bands of ~78 and ~36 kDa (Fig. 5), corresponding to bands isolated from extracts of cell surface-biotinylated HUVECs. A faint band of ~60 kDa (not well visualized in Fig. 5) was also observed, although the ~98-kDa band isolated from the biotinylated cells was not. Mass spectrometric sequencing of two tryptic peptides from the ~78-kDa band and nine peptides from the ~36-kDa band revealed sequences corresponding to annexin II (Table II). These results suggested a binding interaction between annexin II and β_2GPI.

Evidence That Annexin II Serves as an Endothelial Cell Receptor for β_2GPI—To assess the role of annexin II as an endothelial cell β_2GPI receptor, we determined whether anti-annexin II antibodies inhibited the binding of ^{125}I-β_2GPI to endothelial cells. A 10-fold molar excess of a monoclonal anti-annexin II antibody (mAb Z014) inhibited the binding of 20 nM ^{125}I-β_2GPI to endothelial cells to a similar extent (90%) as a 100-fold excess of unlabeled β_2GPI. Binding was unaffected by a control, nonimmune murine IgG_1 (Fig. 6). Specificity of the monoclonal antibody was confirmed by the observation that it recognized only a single protein (~36 kDa) in detergent extracts of endothelial cells when assessed in immunoblot studies. A polyclonal anti-annexin II antibody also inhibited the

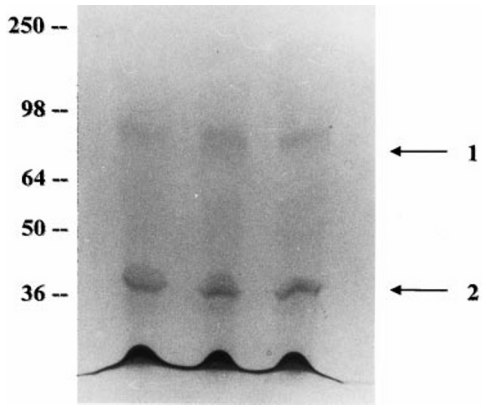

FIG. 5. **Large scale affinity purification of β_2GPI-binding proteins from EAHY.926 cells.** Detergent extracts were prepared from 6×10^9 EAHY.926 cells and subjected to affinity chromatography on β_2GPI-Affi-Gel HZ. The column was washed and eluted as described in the legend to Fig. 4. Proteins within each fraction were analyzed by 10% SDS-PAGE, and gels were stained using Coomassie Brilliant Blue. The majority of eluted protein was contained within fractions 4–6, corresponding to the lanes shown here (*left* to *right*). The ~78- and ~36-kDa bands (*arrows 1* and *2*, respectively) from each lane were excised for preparation of tryptic peptides, which were isolated and sequenced using LC-mass spectrometry.

TABLE II
Peptide sequences from tryptic digests of the ~78- and ~36-kDa proteins isolated from EAHY.926 cells

Peptide sequence	Corresponding region in annexin II
78-kDa band	
SLYYYIQQDTK	314–324
TNQELQEINR	126–135
36-kDa band	
YDAGVK	199–204
D*X*YDAGVKR[a]	197–205
W*X*S*X*MTER	213–220
W*XXX*MoTER[b]	213–220
QTVHE*XX*CK	2–10
DVPKW*X*SMTER	207–220
*X*S*X*EGHSTPPSAYGSVK	11–25
V*X*DYE*XX*DQDAR	185–196
EDGSV*X*DYE*XX*DQDAR	181–196

[a] *X* designates I or L.
[b] Mo designates oxidized methionine.

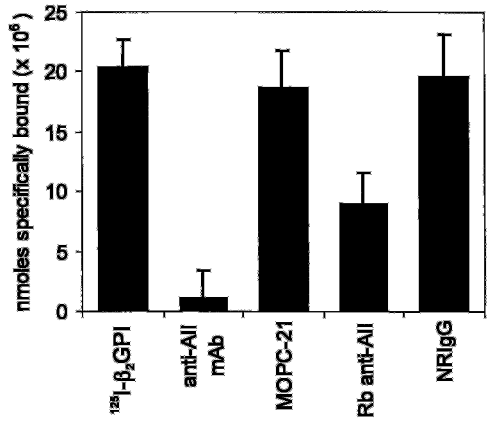

FIG. 6. **Effect of anti-annexin II antibodies on the binding of ^{125}I-β_2GPI to endothelial cells.** HUVECs were prepared for binding studies and then incubated for 2 h at 4 °C with 20 nM ^{125}I-β_2GPI in the presence of 2 μM unlabeled β_2GPI (to determine nonspecific binding), 200 nM anti-annexin II mAb Z014, 200 nM MOPC-21 (mouse IgG_1 control), 2 μM rabbit anti-annexin II antibody (*Rb anti-AII*), or 2 μM nonimmune rabbit IgG (*NRIgG*). This figure depicts the amount of ligand specifically bound (in cpm) in the presence of the various competitors. This experiment is representative of three so performed, with all points determined in quadruplicate.

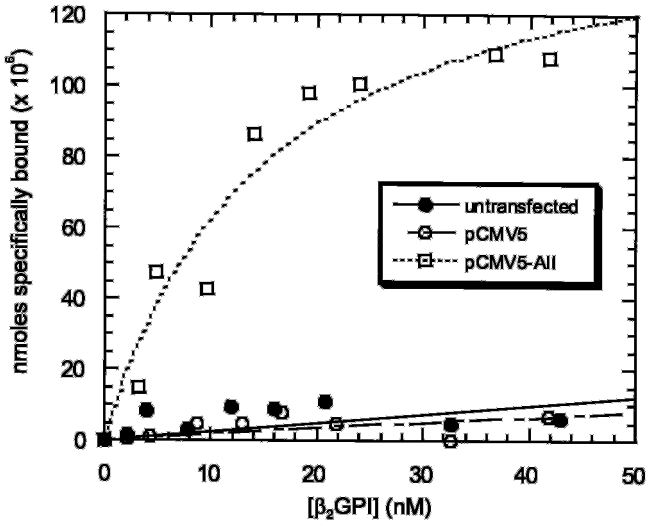

FIG. 7. **Effect of transfection of HEK 293 cells with annexin II cDNA on the binding of ^{125}I-β_2GPI.** HEK 293 cells were transfected with either pCMV5 containing annexin II cDNA (pCMV5-AII), or the empty pCMV5 vector (pCMV5). After 72 h, cells were released from tissue culture dishes and replated in 96-well plates for endothelial cell ^{125}I-β_2GPI binding assays. The specific binding of ^{125}I-β_2GPI to untransfected cells (●) or cells transfected with pCMV5 (○) or pCMV5-AII (□) was determined as described. Significantly more ^{125}I-β_2GPI bound specifically to cells transfected with pCMV5-AII, with a K_d of ~13 nM. This experiment is representative of two so performed, with all points determined in duplicate.

binding of β_2GPI to HUVECs, although somewhat less potently (Fig. 6). The extent of inhibition caused by this antibody was similar to that with which it inhibited the binding of t-PA to endothelial cells in a prior report (52).

To further assess the role of annexin II in β_2GPI binding, we measured the binding of ^{125}I-β_2GPI to HEK 293 cells transfected with either an empty vector (pCMV5) or the same vector containing an annexin II cDNA (pCMV5-AII). As previously reported, immunoblot analyses revealed only trace amounts of annexin II in detergent extracts of untransfected or pCMV5-transfected HEK 293 cells (52, 70). In contrast, cells transfected with pCMV5-AII expressed abundant annexin II (not shown). Furthermore, these cells bound 10-fold more ^{125}I-β_2GPI than either untransfected cells or cells transfected with the empty vector (Fig. 7), with an affinity identical to that with which ^{125}I-β_2GPI bound to HUVECs (K_d ~13 nM). These observations, together with the effects of anti-annexin II antibodies on β_2GPI binding, support the conclusion that annexin II represents an important endothelial cell β_2GPI binding site.

Measurement of the Binding of Annexin II to β_2GPI by Surface Plasmon Resonance—The studies described above demonstrate that annexin II mediates the binding of β_2GPI to endothelial cells but do not address the issue of whether phospholipid, glycosaminoglycans, or other cell surface proteins are also required for this interaction. To address this issue, we determined whether β_2GPI and annexin II bound to one another in a cell-free system, by measuring the binding of fluid-phase annexin II to β_2GPI immobilized on a carboxymethyldextran biosensor chip. Regardless of whether β_2GPI was immobilized by amine or aldehyde coupling, rapid and concentrationdependent binding of annexin II was observed (Fig. 8). The dissociation constant (K_d) for binding was determined using the association and dissociation rate constants, calculated using the Global analysis feature of BiaEval 3.0 (65), as well as by linear transformation of the association data (66). The former method yielded a dissociation constant 2–4-fold higher than that obtained when measuring the equilibrium binding of

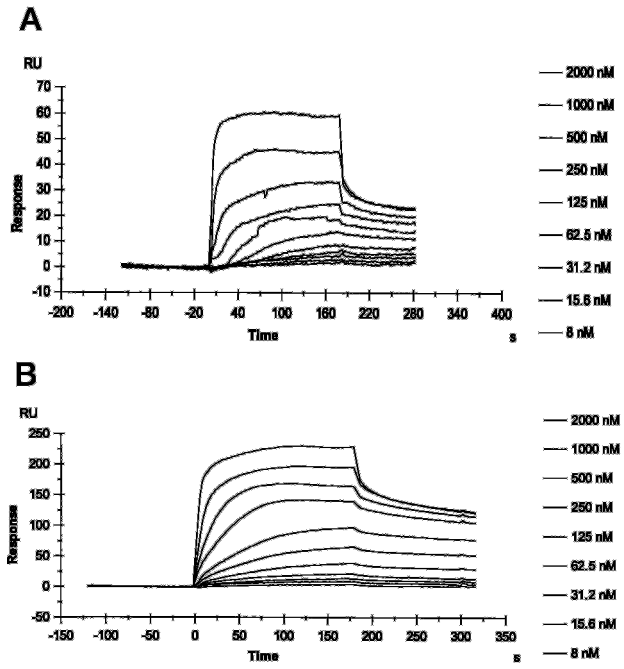

FIG. 8. **Binding of annexin II to immobilized β_2GPI-measurement by surface plasmon resonance.** β_2GPI was immobilized on a carboxymethyldextran biosensor chip using amine (*A*) or aldehyde (*B*) coupling. The immobilized ligand was then exposed to annexin II, and binding was measured in real time. Following the binding of annexin II, dissociation was measured in a similar manner. K_d values were determined from experimentally derived association and dissociation rate constants. The concentrations of annexin II used to obtain the binding curves are depicted on the *right*.

β_2GPI to HUVECs (Table III). The latter revealed fast and slow components of association, reflecting binding heterogeneity in this system (66, 67). Use of the more relevant fast component for calculation of the K_d, however, yielded values (5–12.75 nM) identical to those determined for the binding of β_2GPI to intact endothelial cells. Hence, although these results do not exclude the possibility that other cell surface components may stabilize or promote the β_2GPI-annexin II interaction, they demonstrate that such components are not essential for binding.

TABLE III
K_d values for the binding of annexin II to immobilized β_2GPI using surface plasmon resonance

	β_2GPI coupling method	
	Amine	Aldehyde
	nM	
K_d (Global Fit)	89.9	33.4
K_d (fast)[a]	12.75	5
K_d (slow)[a]	127.5	71

[a] Designates the use of the fast or slow association rate constant for calculation of the K_d.

DISCUSSION

These studies demonstrate that annexin II mediates the binding of β_2GPI to endothelial cells. This conclusion is supported by 1) the affinity purification of annexin II from endothelial cells using β_2GPI-Affi-Gel, 2) the near-complete inhibition of β_2GPI binding to endothelial cells by anti-annexin II antibodies, 3) enhanced binding of β_2GPI to annexin II-transfected HEK 293 cells, and 4) the direct demonstration of high affinity binding of annexin II to β_2GPI using surface plasmon resonance.

Identification of annexin II ($M_r \sim 36$) as the primary β_2GPI binding site on unactivated endothelial cells is consistent with the pattern of protein bands affinity-purified using β_2GPI-Affi-Gel. Mass spectrometric sequencing of tryptic peptides from the ~36- and ~78-kDa bands isolated in both the small and large-scale affinity purification procedures confirmed that each of these contained annexin II. The ~78-kDa band most likely represented an annexin II homodimer, because it migrated identically to a spontaneously forming homodimer present in preparations of recombinant annexin II. Mass spectrometric sequencing of tryptic peptides from the ~60-kDa band also yielded sequences identical to those within annexin II, as well as heat shock protein 27 ($M_r \sim 22.3$) (71) and human DNA-binding protein A ($M_r \sim 38.6$) (72). Hence, this band may represent a partially degraded annexin II homodimer or, more likely, a complex between annexin II and an additional, intracellular protein. Finally, the ~98-kDa band observed only in the small scale affinity purification procedure may represent an annexin II heterotetramer (AIIt) containing 2 molecules of annexin II and 2 molecules of p11 (11 kDa) (70, 73), although the inability to detect this band in the large scale procedure precluded its definitive identification.

An additional high molecular mass β_2GPI-binding protein was observed only in extracts of cell surface biotinylated EAHY.926 cells and thus was most likely derived from A549 carcinoma cells (the non-HUVEC fusion partner used to create the EAHY.926 cell line). We speculate that this protein may be megalin, a low density lipoprotein receptor family member recently shown to bind β_2GPI (74), or perhaps another low density lipoprotein receptor family member. Taken together with the endothelial cell studies, however, these results suggest that β_2GPI may interact with unique cell surface proteins differentially expressed on specific cell types. However, we would emphasize that our studies do not exclude a biologically important interaction of β_2GPI with cellular phospholipids under some circumstances (75, 76).

The annexins are a family of structurally related proteins, each of which consists of an N-terminal "tail" and C-terminal "core" domain (77). The core domains of different annexins share 40–70% homology (78) and consist of a series of 70 amino acid endonexin repeats (78). In contrast, the length and amino acid composition of the tail domains are highly variable among different family members (78). Despite the lack of a hydrophobic signal peptide, the presence of annexin II on cell surfaces is well established (78), and approximately 4.3% of total endothelial annexin II is associated with the external plasma membrane (79). Annexin II mediates the binding of t-PA to endothelial cells through interactions with an LCKLSL sequence in the tail domain (53) and is also an endothelial cell receptor for Glu and Lys-plasminogen (54). Plasminogen binding may result from exposure of a C-terminal lysine (Lys^{307}) in the core domain following cleavage of the Lys^{307}-Arg^{308} bond (52, 55, 56), although others have presented compelling evidence for an important role of the N-terminal lysine residues of the p11 polypeptides in mediating the binding of plasminogen to the annexin II heterotetramer (70). Regardless, annexin II greatly enhances the catalytic efficiency of t-PA-mediated plasminogen activation on cell surfaces (54, 56), with even more potent enhancement mediated by the heterotetramer (55, 70).

Given the abundance of β_2GPI in plasma (plasma concentration, 2–4 μM) and the affinity with which it binds to annexin II, we would expect most endothelial cell surface annexin II molecules to be occupied by β_2GPI under normal conditions. This hypothesis is supported by recent immunohistochemical studies demonstrating an association of β_2GPI with endothelial cells *in vivo* (81). Preliminary studies performed in our laboratory suggest that this interaction may be of particular importance in the presence of circulating anti-β_2GPI antibodies. These antibodies, which are strongly associated with thrombo-

sis, activate endothelial cells *in vitro* only in the presence of β_2GPI (50, 51, 82, 83), and the elevated plasma levels of endothelial cell adhesion molecules (48) and von Willebrand factor (49, 84) in patients with antiphospholipid antibodies/anti-β_2GPI antibodies suggest that these antibodies may also disrupt endothelial function *in vivo*. We have observed that the same annexin II monoclonal antibodies that block β_2GPI binding to endothelial cells directly induce endothelial cell activation, as measured by the expression of endothelial adhesion molecules.[2] These results suggest that annexin II cross-linking induces signaling responses in endothelial cells that lead to cellular activation and the development of a proadhesive and procoagulant phenotype. Because annexin II does not span the cell membrane, this interaction may require an "adaptor" protein, the identity of which is under investigation. However, we hypothesize that "indirect" cross-linking of cell surface annexin II through ligation of bound β_2GPI by anti-β_2GPI antibodies might induce a similar response, and hence our studies may provide an explanation for β_2GPI-dependent endothelial cell activation by anti-β_2GPI antibodies. Alternatively, it has been suggested that annexin II might be capable of initiating signaling responses by mediating calcium channel activity (80). If so, the binding of β_2GPI and anti-β_2GPI antibodies might induce a conformational change in annexin II that stimulates this effect. At present, however, this hypothesis remains speculative.

Although the β_2GPI binding site on annexin II has not yet been determined, the anti-annexin II mAb Z014, as well as previously described anti-annexin II polyclonal antibodies (52), blocked binding of both t-PA and annexin II to endothelial cells. Although these studies suggested that the β_2GPI binding site may reside within the LCKLSL t-PA binding sequence (amino acids 8–13) in the tail domain of annexin II (53), β_2GPI itself did not inhibit the binding of ^{125}I-t-PA to endothelial cells (not shown). Hence, the t-PA and β_2GPI binding sites within annexin II are likely to be distinct, although spatially near one another in the tertiary structure of the molecule.

In summary, the demonstration that β_2GPI binds with high affinity to endothelial cell annexin II suggests that the paradigm in which the interaction of β_2GPI with endothelial cells is assumed to occur solely through binding to membrane phospholipid should be reconsidered. Further characterization of the β_2GPI-annexin II binding interaction, as well as definition of the role of annexin II in endothelial cell activation, may provide additional insight into the physiologic and pathophysiologic roles of these abundant proteins.

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