High Affinity Binding of $\beta_2$-Glycoprotein I to Human Endothelial Cells Is Mediated by Annexin II*

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$\beta_2$-Glycoprotein I ($\beta_2$GPI) is an abundant plasma phospholipid-binding protein and an autoantigen in the antiphospholipid antibody syndrome. Binding of $\beta_2$GPI to endothelial cells targets them for activation by anti-$\beta_2$GPI antibodies, which circulate and are associated with thrombosis in patients with the antiphospholipid antibody syndrome. However, the binding of $\beta_2$GPI to endothelial cells has not been characterized and is assumed to result from association of $\beta_2$GPI with membrane phospholipid. Here, we characterize the binding of $\beta_2$GPI to endothelial cells and identify the $\beta_2$GPI binding site. $^{125}$I-$\beta_2$GPI bound with high affinity ($K_d \sim 18$ nM) to human umbilical vein endothelial cells (HUVECs). Using affinity purification, we isolated $\beta_2$GPI-binding proteins of $\sim 78$ and $\sim 36$ 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 $\beta_2$GPI to cells was confirmed by the observations that annexin II-transfected HEK 293 cells bound $\sim 10$-fold more $^{125}$I-$\beta_2$GPI than control cells and that anti-annexin II antibodies inhibited the binding of $^{125}$I-$\beta_2$GPI to HUVECs by $\sim 90\%$. Finally, surface plasmon resonance studies revealed high affinity binding between annexin II and $\beta_2$GPI. These results demonstrate that annexin II mediates the binding of $\beta_2$GPI to endothelial cells.

$\beta_2$-Glycoprotein I ($\beta_2$GPI) 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$_{3-3}$, 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 $\beta_2$GPI, 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 $\beta_2$GPI is uncertain. A role in lipid metabolism is suggested by the observations that 30% of plasma $\beta_2$GPI circulates in complex with lipoproteins (2, 9) and that $\beta_2$GPI accelerates triglyceride clearance in mice (10). $\beta_2$GPI also binds with high affinity to the atherogenic lipoprotein, Lp(a) (11). A role for $\beta_2$GPI 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. $\beta_2$GPI may also promote the clearance of senescent cells (14, 15) and regulate the uptake of lipoproteins by macrophages (16).

Recently, $\beta_2$GPI 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 $\beta_2$GPI bound to the cardiolipin-coated microplates used in clinical “anticardiolipin” assays (19, 22–27). Binding of $\beta_2$GPI to cardiolipin or another appropriate surface results in either a conformational change in the protein, exposing antigenic neoepitopes (28–30), or concentration of $\beta_2$GPI to an antigenic density at which it is more avidly bound by low affinity anti-$\beta_2$GPI antibodies (31). Antiphospholipid antibodies may also recognize epitopes occurring as a consequence of the formation of adducts between $\beta_2$GPI and oxidized cardiolipin (32, 33).

It has been suggested that $\beta_2$GPI may contribute to the pathogenesis of APS-associated thrombosis by binding to platelets or endothelial cells and targeting them for anti-$\beta_2$GPI antibody-dependent activation. Binding of $\beta_2$GPI to these cells has been assumed to result from its interaction with membrane phospholipid. However, although $\beta_2$GPI 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, $\beta_2$GPI binds with only micromolar affinity (38, 39), if at all (40), to activated platelets. In addition, human anti-$\beta_2$GPI antibodies associated with the antiphospholipid antibody syndrome do not have high affinity for $\beta_2$GPI (31, 41). This is consistent with the view that $\beta_2$GPI may not play as significant a role in the pathogenesis of APS as was previously thought.
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-β₂GPI antibodies have been convincingly shown to activate endothelial cells in a β₂GPI-dependent manner (50, 51), although the mechanisms by which they do so remain undefined.

We hypothesized that endothelial cell activation mediated through this β₂GPI-dependent pathway would require a high affinity interaction between β₂GPI and a specific endothelial cell receptor, with receptor cross-linking subsequently induced indirectly through binding of anti-β₂GPI antibodies to receptor-bound β₂GPI. As an initial step in evaluating this hypothesis, we have characterized the binding of β₂GPI to endothelial cells. β₂GPI 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 (52–54). Preliminary studies also suggest a potential role for annexin II in mediating anti-β₂GPI 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 hypothalamus (57). Heparin-superflow was purchased from Sterogene (Chantilly, VA). Fetal bovine serum was from Hyclone (Logan, UT). Mediatech (Herndon, VA), and 96-well microplates were from Dynax (New Bedford, MA). Rabbit anti-annexin II polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Reflection autoradiographic film was from NEN Life Science Products, and polyvinylidene difluoride membranes were from Millipore (Billerica, MA). Tris-buffered saline containing 0.8 M NaCl, bound proteins were eluted by washing the column and eluting bound proteins, fractions were analyzed using 0.1 M glycine-HCl, pH 3.0. Fractions of 0.5 ml were collected, and super signal enhancement was used for detection. The binding of proteins within detergent extracts to a control column containing Affi-Gel-immobilized bovine serum albumin 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 β₂GPI and whether complexes of similar Mᵣ were detected when studies were performed using human MDA-MB-231 breast carcinoma cells as a control.

**Isolation of Endothelial Cell Surface β₂GPI-binding Proteins**—To determine whether β₂GPI might bind to an endothelial cell surface protein, we determined whether endothelial cell-bound 125I-β₂GPI was incorporated into an SDS-stable complex following exposure of cells to the membrane-impermeable, bifunctional cross-linker, BS⁵. HUVECs were incubated with 40 nM 125I-β₂GPI for 2 h and then exposed to 4 µM BS³ 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 β₂GPI and whether complexes of similar Mᵣ were detected when studies were performed using human MDA-MB-231 breast carcinoma cells as a control.

To further assess whether β₂GPI bound specifically to an endothelial cell protein, biotinylated endothelial cell surface proteins were affinity-purified using immobilized β₂GPI. Cells were biotinylated using sulfo-succinimidyl 6-(biotinamido) hexanoate, Aquasil, and Super signal chemiluminescence reagent were from Pierce. Affi-Gel-HZ was washed (to determine nonspecific binding), or with the specified concentration of antibody. After washing, cell-bound radioligand was determined.

**Binding of 125I-β₂GPI to HUVECs**—In initial studies, we observed that β₂GPI bound "specifically" to poly styrene 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 individually into complete medium until confluent. Prior to binding assays, cells were washed and incubated for 6 h with serum-free medium 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 125I-β₂GPI in the absence (to determine total binding) or presence (to determine nonspecific binding) of a 100-fold molar excess of unlabeled ligand. After washing, binding 125I-β₂GPI 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 (Kaleidagraph, Abelle Software, Reading PA), as well as by the method of Scatchard (62).

In selected experiments, the reversibility of 125I-β₂GPI binding was determined by incubating cells with 40 nM 125I-β₂GPI 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 β₂GPI. The radioactivity in cell supernatants at selected time points was then determined.

To assess the role of annexin II as an endothelial cell β₂GPI receptor, the ability of monoclonal and polyclonal anti-annexin II antibodies to inhibit the binding of 125I-β₂GPI to endothelial cells was measured. Cells were prepared for binding studies and then incubated for 2 h with 20 nM 125I-β₂GPI either alone, in the presence of 2 µM unlabeled β₂GPI (to determine nonspecific binding), or with the specified concentration of antibody. After washing, cell-bound radioligand was determined.
and BLAST) were performed to identify the tryptic peptides.

Effect of Transfection of HEK 293 Cells with Annexin II cDNA on β2GPI 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, non-specific, and specific binding of 125I-β2GPI to cells transfected with the empty and annexin II cDNA-containing vectors were then determined.

Measurement of the Binding of β2GPI to Annexin II by Surface Plasmon Resonance—To determine whether β2GPI 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). β2GPI was immobilized on a carboxymethylated dextran (CM-5) biosensor chip using either amine or aldehyde coupling (64). For amine coupling, the chip was exposed to β2GPI (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 β2GPI. For aldehyde coupling, carbohydrate residues on β2GPI were oxidized by adding 20 μl NaOCl to 1 ml of a 1 mg/ml solution of β2GPI 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 hydrazide bond was reduced by exposure to sodium cyanoborohydride. This resulted in the immobilization of 1062 RU of β2GPI.

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_d[annexin II]/[RU_max – RU_kd[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 125I-β2GPI to HUVECs—Preliminary attempts to measure the binding of 125I-β2GPI 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 125I-β2GPI 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 125I-β2GPI 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 125I-β2GPI 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 125I-β2GPI to endothelial cells.

125I-β2GPI bound to HUVECs specifically and in a time-dependent manner (Fig. 1A). Binding was reversible in the presence of excess unlabeled ligand (Fig. 1B). Analysis of saturation isotherms revealed saturable, high affinity binding (K_d ~18

**TABLE I**

| Condition          | 125I-β2GPI specifically bound (fmol) |
|--------------------|-------------------------------------|
| No treatment       | 140.0 ± 24.2*                      |
| Aquasil            | 17.6 ± 7.5                         |
| Aquasil + fibronectin | 3.8 ± 1.1                         |
| Aquasil + HUVEC    | 7.2 ± 6.5                          |
| Aquasil + fibronectin + HUVEC | 87.2 ± 2.7                         |

* Data are expressed as mean of triplicate points ± S.D.

**FIG. 1.** Time course and reversibility of β2GPI 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 125I-β2GPI in the absence or presence of 4 μM unlabeled ligand. At various times thereafter, the amount of 125I-β2GPI specifically bound to cells was determined. B, reversibility of binding. HUVECs were incubated with 40 nM 125I-β2GPI for 2 h at 4°C. Supernatant containing the radiolabeled ligand was then replaced with cold PBS containing 4 μM unlabeled β2GPI (time = 0 min). At various times thereafter, supernatants were removed, and the amount of 125I-β2GPI that remained bound to the cells was measured. All points were determined in quadruplicate. These experiments are representative of three so performed.
Cells (right lane) were incubated with 60 nM 125I-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.

Specific binding of 125I-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 125I-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.

Identification of the Endothelial Cell 2GPI-binding Protein—We next wished to identify the endothelial cell 2GPI-binding proteins detected in the small-scale studies described above. However, due to the difficulty associated with culturing sufficient HUVECs to allow preparative scale isolation of such protein(s), we sought a transformed cell line that expressed similar proteins. No 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 2GPI-binding protein of higher Mr (Fig. 4, right lane). These studies suggested that EAHY.926 would be suitable for isolation of endothelial 2GPI-binding protein(s).

Definitive identification of endothelial cell 2GPI-binding proteins was pursued by affinity purification of extracts from 6 × 106 EAHY.926 cells, using a 5-ml 2GPI-Affi-Gel HZ affinity column. Coomassie Blue-stained gels of fractions eluted from the affinity column revealed bands of ~78 and ~60 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 2GPI.

Evidence That Annexin II Serves as an Endothelial Cell Receptor for 2GPI—To assess the role of annexin II as an endothelial cell 2GPI receptor, we determined whether anti-annexin II antibodies inhibited the binding of 125I-2GPI to endothelial cells. A 10-fold molar excess of a monoclonal anti-annexin II antibody (mAb Z014) inhibited the binding of 20 nM 125I-2GPI to endothelial cells to a similar extent (90%) as a 100-fold excess of unlabeled 2GPI. Binding was unaffected by a control, nonimmune murine IgG1 (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
binding of β₂GPI 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 β₂GPI binding, we measured the binding of ¹²⁵I-β₂GPI to HEK 293 cells transfected with either an empty vector (pCMV5) or the same vector containing 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 ¹²⁵I-β₂GPI than either untransfected cells or cells transfected with pCMV5 (Fig. 6). The effect of anti-annexin II antibodies on the binding of ¹²⁵I-β₂GPI to endothe- lial cells. HUVECs were prepared for binding studies and then incubated for 2 h at 4 °C with 20 nM ¹²⁵I-β₂GPI in the presence of 2 μM unlabeled β₂GPI (to determine nonspecific binding), 200 nM anti-annexin II mAb Z014, 200 nM MOPC-21 (mouse IgG1 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.

**Table II**

| Peptide sequence | Corresponding region in annexin II |
|------------------|-----------------------------------|
| 78-kDa band      |                                   |
| SLYYYIQQPDK     | 314–124                           |
| TNLQLQIEINR     | 126–135                           |
| 36-kDa band      |                                   |
| YDAGVK          | 199–204                           |
| DXYDAGVKR       | 197–205                           |
| WXXMTER         | 213–220                           |
| WXXXMoTER       | 213–220                           |
| QTVHEXXCK       | 2–10                              |
| DVFKWXXMTER     | 207–220                           |
| AXXEGHSTPSAAYGSK| 11–25                             |
| V3DYEYXQDQDAR   | 185–196                           |
| EDGSVX3DYEYXQDQAR | 181–196                         |

a X designates I or L.  
Mo designates oxidized methionine.
\( \beta_2 \)-Glycoprotein I Binds to Endothelial Cell Annexin II

**FIG. 8.** Binding of annexin II to immobilized \( \beta_2 \)-GPI—measurement by surface plasmon resonance. \( \beta_2 \)-GPI was immobilized on a carboxymethyl dextrans 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.

\( \beta_2 \)-GPI 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 \( \beta_2 \)-GPI to intact endothelial cells. Hence, although these results do not exclude the possibility that other cell surface components may stabilize or promote the \( \beta_2 \)-GPI-annexin II interaction, they demonstrate that such components are not essential for binding.

**DISCUSSION**

These studies demonstrate that annexin II mediates the binding of \( \beta_2 \)-GPI to endothelial cells. This conclusion is supported by 1) the affinity purification of annexin II from endothelial cells using \( \beta_2 \)-GPI-Affi-Gel, 2) the near-complete inhibition of \( \beta_2 \)-GPI binding to endothelial cells by anti-annexin II antibodies, 3) enhanced binding of \( \beta_2 \)-GPI to annexin II-transfected HEK 293 cells, and 4) the direct demonstration of high affinity binding of annexin II to \( \beta_2 \)-GPI using surface plasmon resonance.

Identification of annexin II (\( M_r \sim 36 \)) as the primary \( \beta_2 \)-GPI binding site on unactivated endothelial cells is consistent with the pattern of protein bands affinity-purified using \( \beta_2 \)-GPI-Affi-Gel. Mass spectrometric sequencing of tryptic peptides from the \( \sim 56 \)- and \( \sim 78 \)-kDa bands isolated in both the small and large scale affinity purification procedures confirmed that each of these contained annexin II. The \( \sim 78 \)-kDa band proved likely identical to an spontaneously forming homodimer present in preparations of recombinant annexin II. Mass spectrometric sequencing of tryptic peptides from the \( \sim 60 \)-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 \( \sim 98 \)-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 \( \beta_2 \)-GPI-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 \( \beta_2 \)-GPI (74), or perhaps another low density lipoprotein receptor family member. Taken together with the endothelial cell studies, however, these results suggest that \( \beta_2 \)-GPI 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 \( \beta_2 \)-GPI 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 LCKL5L 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\(^{397}\)) in the core domain following cleavage of the Lys\(^{397}\)-Arg\(^{399}\) 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 \( \beta_2 \)-GPI in plasma (plasma concentration, 2–4 nm) and the affinity with which it binds to annexin II, we would expect most endothelial cell surface annexin II molecules to be occupied by \( \beta_2 \)-GPI under normal conditions. This hypothesis is supported by recent immunohistochemical studies demonstrating an association of \( \beta_2 \)-GPI with endothelial cells in \emph{vivo} (81). Preliminary studies performed in our laboratory suggest that this interaction may be of particular importance in the presence of circulating anti-\( \beta_2 \)-GPI antibodies. These antibodies, which are strongly associated with thrombo-

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**TABLE III**

| \( K_d \) values for the binding of annexin II to immobilized \( \beta_2 \)-GPI using surface plasmon resonance | \( \beta_2 \)-GPI coupling method |
|---|---|
| Amine | Aldehyde |
| \( K_d \) (Global Fit) | 89.9 | 33.4 |
| \( K_d \) (fast)\(^a\) | 12.75 | 5 |
| \( K_d \) (slow)\(^a\) | 127.5 | 71 |

\( \text{ru} \)

\(^a\) Designates the use of the fast or slow association rate constant for calculation of the \( K_d \).

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sis, activate endothelial cells in vitro only in the presence of \(\beta_2\)GPI (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-\(\beta_2\)GPI antibodies suggest that these antibodies may also disrupt endothelial function in vivo. We have observed that the same annexin II monoclonal antibodies that block \(\beta_2\)GPI 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 \(\beta_2\)GPI by anti-\(\beta_2\)GPI antibodies might induce a similar response, and hence our studies may provide an explanation for \(\beta_2\)GPI-dependent endothelial cell activation by anti-\(\beta_2\)GPI 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 \(\beta_2\)GPI and anti-\(\beta_2\)GPI antibodies might induce a conformational change in annexin II that stimulates this effect. At present, however, this hypothesis remains speculative.

Although the \(\beta_2\)GPI binding site on annexin II has not yet been determined, the anti-annexin II mAb Z014, as well as monoclonal antibodies that block in vivo 2GPI antibodies suggest that these antibodies may also dis-...
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