Interaction of β2-glycoprotein I with members of the low density lipoprotein receptor family

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See also Pierangeli SS. In search for a receptor for antiphospholipid antibodies on target cells. This issue, pp 1678–9.

Summary. The antiphospholipid syndrome (APS) is a non-inflammatory autoimmune disease characterized by arterial and/or venous thrombosis and/or pregnancy morbidity in the presence of autoantibodies that recognize beta2-glycoprotein I (β2GPI) bound to phospholipids. We have previously demonstrated that dimerization of β2GPI by autoantibodies induces platelet activation, involving the platelet receptor apolipoprotein E receptor 2′ (apoER2′) a receptor belonging to the low-density lipoprotein receptor (LDL-R) family. Here, we show that dimeric β2GPI, but not monomeric β2GPI, interacts with four other LDL-R family members: the LDL-R related protein (LRP), megalin, the LDL-R and the very-low density lipoprotein receptor (VLDL-R). Interaction between dimeric β2GPI and LDL-R, apoER2′ and VLDL-R was best described with a one-site binding model (half-maximal binding; ∼20 nM for apoER2′ and VLDL-R and ∼300 nM for LDL-R), whereas the interaction between dimeric β2GPI and LRP or megalin was best described with a two-site binding model, representing a high- (∼3 nM) and a low-affinity site (∼0.2 μM). Binding to all receptors tested was unaffected by a tryptophane to serine (W316S) substitution in domain V of β2GPI, which is known to disrupt the phospholipid binding site of β2GPI. Also deletion of domain I or II left the interaction with the receptors unaffected. Deletion of domain V, however, significantly decreased the affinity for the receptors. In conclusion, our data show that dimeric β2GPI can interact with different LDL-R family

members. This interaction is dependent on a binding site within domain V of β2GPI, which does not overlap with the phospholipid-binding site within domain V.

Introduction

The antiphospholipid syndrome (APS) is a non-inflammatory autoimmune disease. APS has been identified as the association of arterial and/or venous thrombosis and/or pregnancy morbidity with the presence of antiphospholipid antibodies (aPL) in plasma of affected patients [1,2]. Assays that depend on the presence of antibodies against beta2-glycoprotein I (β2GPI) display the highest correlation with thrombosis [3]. The cause of hemostatic disbalance induced by β2GPI in the presence of β2GPI antibodies *in vivo* still remains to be unraveled. Several possibilities have been proposed, of which cell activation by β2GPI/anti-β2GPI antibodies has received most attention. The involvement of cellular receptors that can bind these immune complexes is essential in all these hypotheses [4–7].

β2-GPI is an abundant plasma protein (approximately 200 μg mL⁻¹). At present, the physiological function of β2GPI is unclear, but the protein has been described to have both pro- and anticoagulant properties *in vitro* [8–11]. In previous studies we have shown that β2GPI/antibody complexes can activate platelets. This effect could be mimicked by a recombinant form of dimeric β2GPI. A splice variant of the apolipoprotein E receptor 2 (apoER2), that was recently identified as apoER2′ on human platelets [12], has been demonstrated to be involved in the binding of dimeric β2GPI to human platelets [13]. ApoER2 (also known as low-density lipoprotein [LDL] receptor-related protein [LRP]-8) is among others expressed in brain, placenta and testis [14,15] and shares structural homology with other LDL receptor family

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members [16]. In general, these multiligand receptors consist of a number of discrete domains and their extracellular component contains ligand-binding domains that consist of complement-type repeats. Differential clustering of these repeats within a domain may expose specificity with respect to ligand recognition. These repeats are separated by one or more epidermal growth factor precursor homology domains that contain YWTD motifs, responsible for ligand dissociation. The receptors are anchored in the membrane via an intracellular tail that harbors motifs for endocytosis and signaling. At present, 12 members of the LDL-receptor family have been identified in mammals, including LRP, megalin, the low-density lipoprotein receptor (LDL-R), apoER2′, and the very LDL-R (VLDL-R). These receptors are expressed on an array of cell types. The concept that they are multiligand receptors does not necessarily mean that they recognize identical ligands. In contrast, ligand binding may be tightly regulated between different members of the LDL-receptor family.

In the present study we address a number of issues. Apart from apoER2′, other LDL-R homologs (LRP, megalin, LDL-R, VLDL-R) might recognize dimeric β₂GPI with different affinities. Furthermore, apoER2′-mediated platelet activation by β₂GPI only occurred in the presence of anti-β₂GPI antibodies. As these antibodies possess the property to dimerize antigens, we investigated whether anti-β₂GPI antibody-induced dimerization of β₂GPI also promotes the interaction with other members of the LDL-R family. It has been proposed that positive electrostatic surface potentials (mainly on domain I and V of β₂GPI), not the primary sequences, in different ligands constitute receptor-binding domains. Therefore, we used domain deletion mutants of dimeric β₂GPI to identify which domain of β₂GPI is involved in the interaction with the receptors. Finally, to investigate whether the molecular interactions between phospholipid binding and binding to the receptors is similar, we tested binding of a non-phospholipid binding β₂GPI mutant to LRP, megalin, apoER2′ and the VLDL-R.

Experimental procedures

Purification of plasma β₂GPI

Plasma β₂GPI was isolated from fresh citrated human plasma as described previously [17]. In short, dialyzed human plasma was applied subsequently to the following columns: DEAE-Sephadex A50, protein G-Sepharose, S-Sepharose, and finally heparin-Sepharose (all Sepharoses were obtained from Amersham Pharmacia Biotech; Piscataway, NJ, USA). Bound proteins were eluted with a linear salt gradient. Afterwards, β₂GPI was dialyzed against Tris-buffered saline (TBS). Purity of the protein was checked on a 4–15% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE). Plasma β₂GPI migrated with a molecular mass of 45 kDa under non-reducing conditions. Concentration of the protein was determined using the bicinchoninic acid (BCA) protein assay.

Construction of dimeric β₂GPI

Apple 4–C321S–β₂GPI (further referred to as dimeric β₂GPI) and the non-dimeric apple 2–β₂GPI were constructed as described previously [18]. Domain deletion mutants of dimeric β₂GPI were constructed using full-length apple 4–C321S–β₂GPI cDNA as a template [19]. In short, for deletion of domain I (delta I) one set of primers was used to construct domain II–V. For deletion of domain II (delta II) two sets of primers was used; one set to construct domain I and one set to construct domain III–V. The two created polymerase chain reaction (PCR) products served as a template to obtain the full-length domain II deletion. For the domain V deletion mutant (delta V) one set of primers was used to construct domain I–IV. To construct chimeric fusion proteins of the dimerization domain of factor XI (apple 4) and β₂GPI, the PCR product was cloned into the vector apple 4–C321S-tissue-type plasminogen activator, S478A. In the experiments we also used the non-phospholipid binding mutant, in which a tryptophane has been substituted to a serine (W316S). Construction of this mutant has been described previously [20]. As a control, we also used dimeric apple 4 [19]. Sequence analysis was performed to confirm correct amplification of the β₂GPI cDNA.

Construction of soluble VLDL-receptor

Soluble human (sh) apoER2′ was constructed as described previously [19]. Construction of sh-VLDL-R was performed as follows. Freshly isolated umbilical vein mRNA was prepared from endothelial cells to synthesize cDNA. This cDNA was the starting point for cloning sh-VLDL-R using Phusion DNA polymerase (Finnzymes; Espoo, Finland). The signal peptide was excluded and the stopcodon was deleted. The following primers were used. Forward primer sh-VLDL-R; TA ATA GGA TCC GGG AGA AAA GCC AAA TGT GAA CCC. Reverse primer sh-VLDL-R; TA ATA GCG GCC GCA GTA GCA AGA TCC ATT TGA TAG CC. The VLDL-R was subsequently cloned into the vector PTT3-SRα-GH-HISN-TEV. This expression vector is constructed from the pTT3 expression vector [21] and the pSGHV0 expression vector [22]. The sh-LDL-R was a generous gift of K. Mertens, Sanquin Bloodbank, Amsterdam, the Netherlands.

Transfection, expression, cell culture and purification of constructs

Transfection of BHK cells with the calcium phosphate method was performed as described previously [13]. Expression of all constructs was performed in conditioned serum-free medium (Dulbecco's modified Eagle's medium/F-12 medium supplemented with 0.5% UltroserG; ITK Diagnostics, the Netherlands). Protein expression was measured using a β₂GPI-ELISA [20]. Full-length and domain deletion mutants of dimeric β₂GPI were purified from cell culture medium using a monoclonal antibody (21B2; a generous gift of J. Arnout,

Leuven, Belgium) column. The purified proteins were further subjected to purification on a mono S column using fast performance liquid chromatography (Amersham Pharmacia Biotech). Apple 4 was purified using a monoclonal antibody (α-XI-1; a generous gift of J.C.M. Meijers, Academic Medical Center, Amsterdam, the Netherlands). Transfection of HEK293E cells with sh-apoER2′ and sh-VLDL-R was performed using the PEI transfection method according to Durocher et al. Production of sh-apoER2 and sh-VLDL-R was carried out in a 1-L suspension culture (in medium containing 90% freestyle, 10% calcium free DMEM, 0.1% fetal calf serum; Invitrogen, Carlsbad, CA, USA). Receptors were affinity purified using receptor associated protein (RAP)-sepharose or Ni-NTA sepharose and purity of the protein fractions was determined on a 4–15% SDS–PAGE, subsequently followed by dialyzation against TBS (50 mM Tris, 150 mM NaCl, pH 7.4). Protein concentrations were determined using a BCA protein assay (Pierce Biotechnology Inc, Rockford, IL, USA). Purified constructs were analyzed on a 7.5% SDS–PAGE. LRP fragments cluster II and cluster IV were purified as described previously [23]. LRP was obtained from BioMac (Instruchemie, Delfzijl, the Netherlands). Megalin was kindly provided by H. Pannekoek, Academic Medical Center, Amsterdam, the Netherlands.

Solid-phase immunosorbent assay for the interaction between β_2GPI and apoER2′ and the VLDL-R

Binding of β_2GPI and the domain deletion mutants to apoER2′ and the VLDL-R was measured using a solid-phase immunosorbent assay. Briefly, increasing concentrations (0–10 μg mL^{-1} in phosphate buffered saline; PBS) of receptors were immobilized on hydrophobic 96 wells plates (Costar, New York, NY, USA). After blocking with PBS/4%BSA (150 μL per well), proteins (3 μg mL^{-1} in PBS/1%BSA, 50 μL per well) were allowed to interact with the receptors. After incubation, bound β_2GPI was detected using subsequently a rabbit polyclonal anti-β_2GPI antibody and a peroxidase-labeled swine antirabbit polyclonal antibody (SWARPO, 1:500 in PBS/1%BSA, 50 μL per well), followed by a staining procedure using ortho-phenylenediamide.

Surface plasmon resonance analysis for the interaction between β_2GPI and LRP, megalin and the LDL-R

Surface plasmon resonance (SPR) binding assays were performed employing a Biacore 2000 system (Biacore AB, Uppsala, Sweden). LRP, cluster II or IV of LRP or megalin were immobilized on different CM5 sensor chips using the amine-coupling kit (Biacore). The LDL-R was immobilized on a C1 sensor chip using the amine-coupling kit (Biacore). Approximately 10 fmol mm^{-2} LRP, 26 fmol mm^{-2} cluster II, 16.5 fmol mm^{-2} cluster IV, 19.3 fmol mm^{-2} megalin and 29.4 fmol mm^{-2} LDL-R were immobilized. For each chip one channel was activated and blocked in the absence of protein and afterwards its signal (< 5% of binding to coated channels) was used to correct the signal from the coated channels for aspecific binding. SPR analysis was performed in buffer containing TBS/0.005% Tween 20, 1 μM CaCl$_2$ with a flow rate of 20 μL min^{-1} at 25 °C. Association and dissociation was followed for a period of 2 min. Regeneration of the surface was performed by application of 0.1 M sodium citrate containing 1 mM of EDTA and 1 M of NaCl, pH 5.0.

Binding of a β_2GPI/anti-β_2GPI antibody complex to LDL-receptor family members

Binding of plasma β_2GPI in the presence of a monoclonal anti-β_2GPI antibody (3B7) that recognizes domain I of β_2GPI and prolongs the clotting time (lupus anticoagulant), was investigated for LRP, megalin, the LDL-R, apoER2′ and the VLDL-R. For SPR analysis plasma β_2GPI (100 nM) was incubated with 3B7 (nM) for 30 min at 37 °C. Afterwards, interaction of the immune complex with immobilized LRP, megalin and the LDL-R was investigated. Association and dissociation was followed for a period of 2 min. Analysis of binding of the immune complex to the immobilized receptors was measured in TBS/0.005% Tween 20, 1 μM CaCl$_2$ with a flow rate of 20 μL min^{-1} at 25 °C. Because of the complexity of the interaction between antibody/β_2GPI complexes and immobilized receptor, the kinetic parameters of this interaction cannot be calculated using the available software programs. Interaction between β_2GPI/anti-β_2GPI antibody complex and coated apoER2– and the VLDL-R (5 μg mL^{-1}) was measured using the solid-phase immunosorbent assay. In this assay, plasma β_2GPI (3 μg mL^{-1}) was incubated with 3B7 (1 μg mL^{-1}) in PBS/1%BSA. Bound β_2GPI was visualized as described previously.

Inhibition studies in the presence of heparin or RAP

Inhibition experiments were performed using SPR (LRP and megalin) analysis and with the solid-phase immunosorbent assay (apoER2′ and VLDL-R). For heparin inhibition studies, dimeric β_2GPI was preincubated with increasing concentrations of heparin (0–2 mg mL^{-1}) in TBS/0.005% Tween 20 containing 1 μM CaCl$_2$ for 30 min at 37 °C prior to injection. For RAP inhibition studies, a preinjection with increasing concentrations of RAP (0–200 nM) was performed. Association- and dissociation was followed for a period of 2 min. This was subsequently followed by injection of 100 nM dimeric β_2GPI. For inhibition studies with apoER2′ and the VLDL-R, dimeric β_2GPI (3 μg mL^{-1}, 50 μL per well) in the presence of increasing concentrations heparin (0–2 mg mL^{-1}) or RAP (0–200 nM) in PBS/1%BSA was incubated on coated apoER2′ or the VLDL-R (5 μg mL^{-1}). Bound protein was detected as described previously.

Analysis of SPR data

Analysis of SPR data was performed using the GRAPHPAD PRISM program (GraphPad Prism version 4.0 for Windows,

GraphPad Software, San Diego, CA, USA). Data obtained from SPR analysis was used for the calculation of the steady state constants as follows. Responses at equilibrium (R_{eq}) derived from sensorgrams were plotted against protein concentration. The resulting binding isotherms were subsequently fitted to the following equations;

For a one-site binding model: $Y = \frac{B_{max} \times X}{K_d + X}$

For a two-site (heterologous) binding model:

$$Y = \frac{B_{max\,1} \times X}{K_{d1} + X} + \frac{B_{max\,2} \times X}{K_{d2} + X}$$

Scatchard plots were derived from the saturation curves to verify the use of a one-site- or a two-site (heterologous) ligand-binding model. Results are expressed as mean $\pm$ SD ($n = 3$).

Results

Binding of a β2GPI/anti-β2GPI antibody complex to LDL-receptor family members

To investigate the effect of dimerization of β2GPI by an antibody on its interaction with members of the LDL-R homologs, we assessed the binding of plasma β2GPI in the absence or presence of an anti-β2GPI antibody (3B7) to LRP, megalin, the LDL-R, apoER2′ and the VLDL-R. Interaction of β2GPI with LRP, megalin and the LDL-R was investigated by SPR analysis and binding to apoER2′ and the VLDL-R was assessed in a qualitative manner using an immunosorbent assay. As shown in Fig. 1, incubation of plasma β2GPI in the presence of 3B7 resulted in binding of the immune complex to all receptors tested, except for the LDL-R. Plasma β2GPI or 3B7 alone had less than 20% binding to the receptors. Thus, β2GPI binds to LRP, megalin, apoER2′ and the VLDL-R,
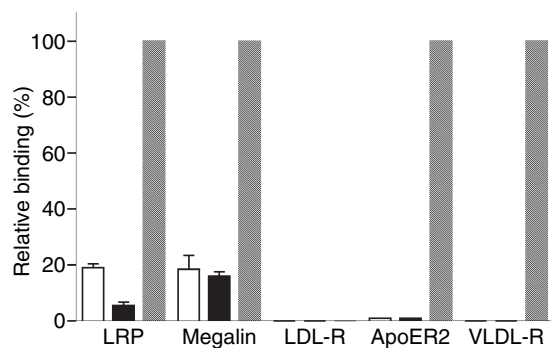
Fig. 1. Binding of a β2 glycoprotein I (GPI)/anti-β2GPI antibody complex to members of the low density lipoprotein (LDL)-receptor (R) family. Plasma β2GPI was incubated with an anti-β2GPI Moab (3B7) at 37 °C for 30 min. The formed immune complex could interact with LRP, megalin, apoER2′ and the very low density lipoprotein-receptor (VLDL-R). Binding of dimeric β2GPI (gray bars) in the presence of 3B7 to the receptors was set at 100%. Plasma β2GPI (white bar) and 3B7 (black bar) alone hardly bound to the receptors. Results represent mean $\pm$ SD ($n = 3$).

however dimerization of β2GPI is necessary for optimal binding.

Different members of the LDL-receptor family contain a binding site for recombinant dimeric β2GPI

Interaction between β2GPI/antibody complexes and the receptors is complicated to examine, as the analysis can be influenced by the affinity of the antibody for β2GPI. Therefore, we studied binding of a stable, recombinant form of dimeric β2GPI to LRP, megalin, the LDL-R, apoER2′ and the VLDL-R. In Fig. 2A, a representative sensorgram for LRP is shown, obtained from SPR analysis. Plasma β2GPI, apple 4 did, and the non-dimeric protein apple 2–β2GPI did not, bind to LRP. In contrast, dimeric β2GPI did bind to LRP. Upon replacement of dimeric β2GPI with buffer (arrow), the response signal declined gradually, indicating that dimeric β2GPI dissociates from LRP and that binding is reversible.

The interaction of dimeric β2GPI with the LDL-R homologs was studied in more detail by calculating the steady state constants. The binding isotherms of the responses obtained at equilibrium are shown in Fig. 2. For the interaction between dimeric β2GPI and LRP (Fig. 2B) and megalin (Fig. 2C), Scatchard plot analysis (inserts) revealed two classes of binding sites. The correctness of fit (R^2) for the binding isotherms of both LRP and megalin was 0.999 for a two-site binding model and 0.962 and 0.934 for a one-site binding model. Therefore, the interaction between dimeric β2GPI and LRP and megalin could be adequately described employing a two-site (heterologous) binding model. Consequently, a high (3.1 $\pm$ 0.6 nM and 3.1 $\pm$ 0.7 nM, respectively) and a low affinity interaction (192.1 $\pm$ 13.2 nM and 241.2 $\pm$ 30.0, respectively) could be inferred from the data. After Scatchard plot analysis (Fig. 2 inserts) and careful interpretation of the correctness of fit from the binding isotherms, the interaction between dimeric β2GPI and the LDL-R (Fig. 2D), apoER2′ (Fig. 2E) and the VLDL-R (Fig. 2F) showed one class of binding sites (R^2: 0.993, 0.995 and 0.990, respectively). Therefore, the experimental data was best fitted according to a one-site binding model. Compared to the other receptors tested, the LDL-R showed one class of low affinity binding sites for dimeric β2GPI ($K_d = 341 \pm 54$ nM). Furthermore, the LDL-R has not been described in literature as being involved in signaling processes. Therefore, we decided to continue the experiments with only LRP, megalin, apoER2′ and the VLDL-R. The results are summarized in Table 1.

Effect of RAP on the dimeric β2GPI–LDL-receptor family interaction

The specificity of the interactions was examined by analyzing the interaction between dimeric β2GPI and individual receptors in the presence of RAP, a universal ligand-inhibitor for this receptor family. For SPR analysis, preinjections with five different concentrations of RAP (0–200 nM) were performed prior to injection of β2GPI. For immunosorbent analysis,
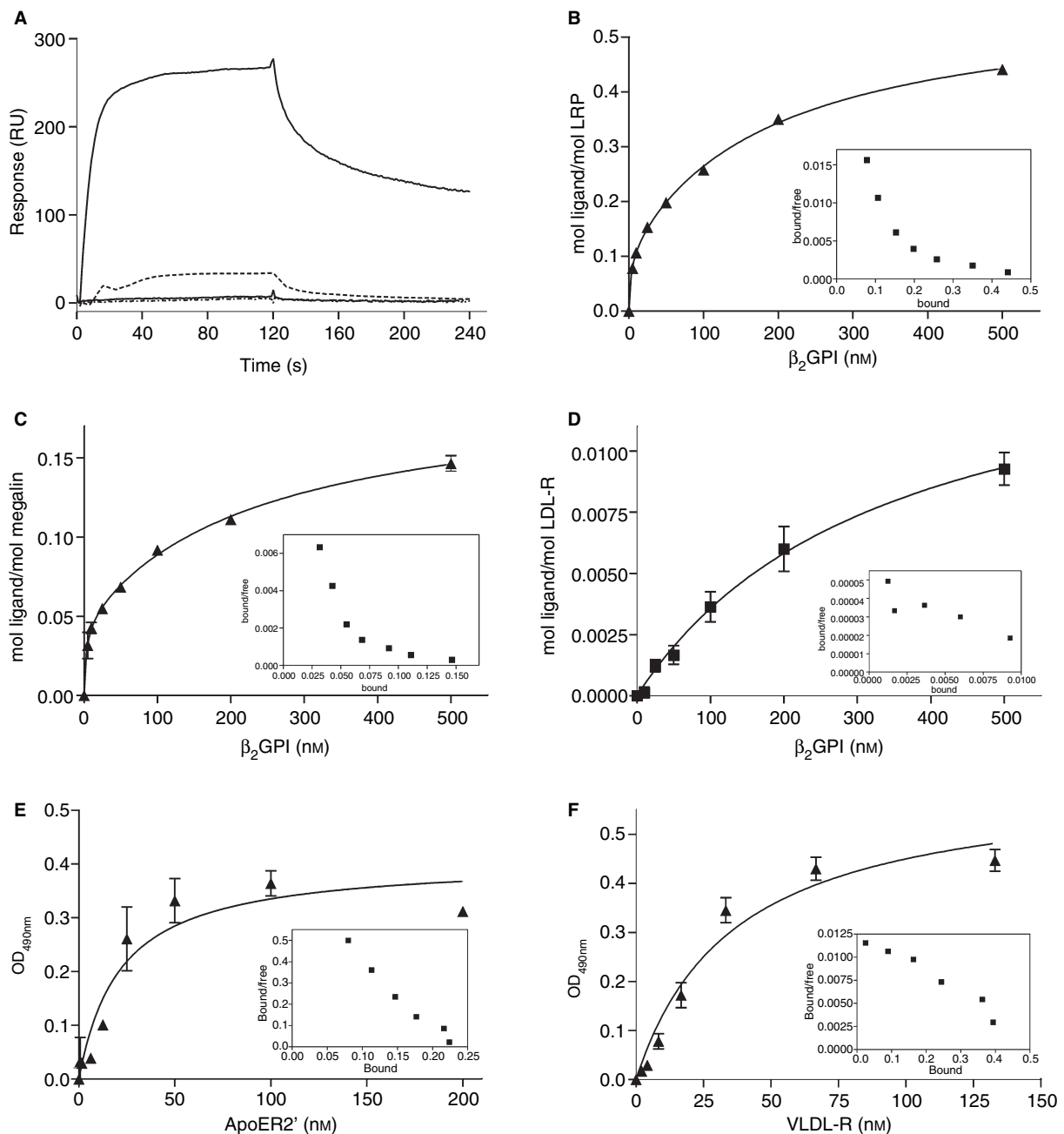
Fig. 2. Interaction of plasma and dimeric β_2 glycoprotein 1(GPI) with LDL-R homologs. (A) a reproducible sensorgram showing interaction of dimeric β_2GPI with low density lipoprotein-related protein (LRP) (solid line). Also the control proteins apple 2–β_2GPI (broken line), apple 4, and plasma β_2GPI are displayed. (B) LRP, (C) megalin or (D) LDL-R was immobilized on a CM5 or C1 sensor chip at a density of 10 fmol mm^{-2}, 26 fmol mm^{-2}, or 29.4 fmol mm^{-2} respectively. Seven different concentrations (5–500 nM) of dimeric β_2GPI were passed over the immobilized receptors. (E) apoER2′ or F, VLDL-R was coated with increasing concentrations (0–10 μg mL^{-1}) on a 96 wells plate and dimeric β_2GPI (3 μg mL^{-1}) was able to interact with the receptors. Lines represent the data curves and their fitted curves obtained with a one-site binding model or a two-site binding model. Scatchard plots (inserts) verify the use of the binding models. Data for LRP, megalin and the LDL-R is expressed as mol ligand bound to mol receptor. Steady state constants derived from the binding isotherms are listed in Table 1. Results represent mean ± SD ($n = 3$).

dimeric β_2GPI was incubated with RAP and binding to apoER2′ or the VLDL-R was analyzed. As shown in Fig. 3, binding of dimeric β_2GPI to LRP or megalin was inhibited up to 80% by RAP. In the presence of RAP, binding of dimeric β_2GPI to apoER2′ and the VLDL-R was inhibited up to 75%, and 100%, respectively.

Effect of heparin on the dimeric β_2GPI–LDL-receptor family interaction

β_2-GPI consists of two regions that are enriched in cationic residues. These residues are located on domain I and V, with the largest cationic patch on domain V ($\sim$2000 Å^2). As LDL-R

Table 1 Steady state constants of domain deletion mutants of β₂GPI. Binding isotherms were fitted accordingly to a one-site or a two-site binding model, dependent on the protein/receptor interaction. When a two-site binding model was used, two steady state constants are calculated (K_{d1} and K_{d2}); for a one-site binding model only one steady state constant (K_d) is calculated. Steady state constants are expressed in nmol L^{-1} (nM). Data represent mean $\pm$ SD ($n = 3$)

| Receptor	Dimeric β₂GPI	Delta I	Delta II	Delta V
LRP	3.1 $\pm$ 0.6	5.9 $\pm$ 1.6	2.0 $\pm$ 0.4	–
	192 $\pm$ 13	190 $\pm$ 17	244 $\pm$ 18	> 2·10³
Megalin	3.1 $\pm$ 0.7	4.1 $\pm$ 0.5	5.4 $\pm$ 1.2	–
	241 $\pm$ 30	283 $\pm$ 24	284 $\pm$ 81	212 $\pm$ 14
apoER2′	23 $\pm$ 4	16 $\pm$ 2	23 $\pm$ 6	–
VLDL-R	36 $\pm$ 8	26 $\pm$ 4	39 $\pm$ 6	–
LDL-R	341 $\pm$ 54	NT	NT	NT

GP1, glycoprotein 1; LDL-R, low density lipoprotein receptor; LRP, low density lipoprotein receptor-related protein; VLDL-R, very low density lipoprotein receptor; NT = not tested.
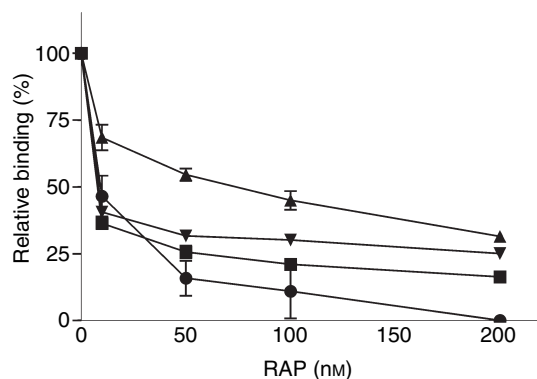
Fig. 3. Inhibition of dimeric β₂ glycoprotein 1(GPI)–LDL-receptor family interaction by RAP. For low density LRP and megalin, surface plasmon resonance (SPR) analysis was applied to study the effect of RAP. Pre-injections with five different concentrations of RAP were performed for 2 min, directly followed by injections of 100 nM dimeric β₂GPI on immobilized LRP (■) and immobilized megalin (▲) for 2 min. Interaction of dimeric β₂GPI with coated apoER2′ (▼) or VLDL-R (●) in the presence of RAP was measured using a solid phase immunosorbent assay. Binding of dimeric β₂GPI in the absence of RAP was set at 100%. Results represent mean $\pm$ SD ($n = 3$).

homologs are known to recognize such cationic residues, we investigated the ability of heparin (which also recognizes cationic residues) to inhibit the interaction between dimeric β₂GPI and LRP, megalin, apoER2′ and the VLDL-R. As shown in Fig. 4, heparin inhibited the interaction of dimeric β₂GPI with LRP or megalin according to a bi-phasic inhibition model. An initial rapid inhibition phase up to approximately 50–75% (residual binding of 46.2 $\pm$ 0.5% and 23.2 $\pm$ 1.9%, respectively) with 0.0625 mg mL^{-1} heparin and a slow inhibition phase up to > 95% with 2.0 mg mL^{-1} heparin. In contrast, binding of dimeric β₂GPI to apoER2′ or the VLDL-R was inhibited by heparin in accordance with a mono-phasic pattern. A gradual inhibition was observed up to > 95%. Heparin alone did not bind to the receptors (not shown). Half-
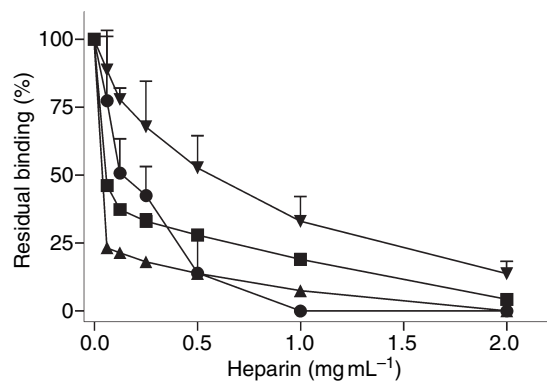

Fig. 4. Effect of heparin on the interaction between dimeric β₂ glycoprotein 1(GPI) and receptors. Dimeric β₂GPI (100 nM) was preincubated with increasing concentrations of heparin (0–2 mg mL^{-1}) at 37 °C for 30 min. Afterwards the effect of heparin on binding of dimeric β₂GPI to immobilized low density LRP (■) or megalin (▲) was investigated using SPR analysis. The effect of heparin on the interaction between dimeric β₂GPI and apoER2′ (▼) or the VLDL-R (●) was investigated using a solid phase immunosorbent assay. Percentage of inhibition in the presence of heparin was calculated as a result of binding of proteins to the receptors without heparin. Results represent mean $\pm$ SD ($n = 3$).

maximal inhibition was observed at 0.05 mg mL^{-1} for LRP and 0.03 mg mL^{-1} for megalin. In addition, for apoER2′ and the VLDL-R half-maximal inhibition was observed at 0.46 mg mL^{-1} and 0.17 mg mL^{-1}, respectively.

Function of the different domains of β₂GPI in LDL-receptor interaction

To further characterize the interaction between dimeric β₂GPI and LDL-receptor family members, we determined the binding characteristics of the domain deletion mutants for LRP, megalin, apoER2′ and the VLDL-R. SPR measurements and the immunosorbent assays were performed under the same conditions as for full-length dimeric β₂GPI. Deletion of domain V of β₂GPI appeared to be crucial for binding to LRP and megalin. A small increase in the response signal was observed with increasing concentrations of delta V, whereas a significant increase in the response signal was observed when delta I and delta II were passed over LRP or megalin (data not shown). After replacement with buffer, dissociation of delta V started rapidly and protein was completely gone before the dissociation time (120 s) was ended, whereas delta I and delta II dissociated gradually from LRP and megalin (similar to full-length dimeric β₂GPI). As shown in Fig. 5A, maximal binding to LRP was lower for delta I and higher for delta II compared with full-length dimeric β₂GPI. The results are summarized in Table 1. A model describing the interaction between delta I and delta II and two independent binding sites (two-site binding model) was found to provide the best fit of the experimental data for LRP (K_{d1} 5.9 $\pm$ 1.6 nM, K_{d2} 189.6 $\pm$ 16.4 nM and K_{d1} 2.0 $\pm$ 0.4 nM, K_{d2} 283.4 $\pm$ 80.7 nM, respectively). Delta V displayed a more than 600-fold decreased affinity (K_d > 2 × 10³ nM) for LRP. Binding characteristics for megalin
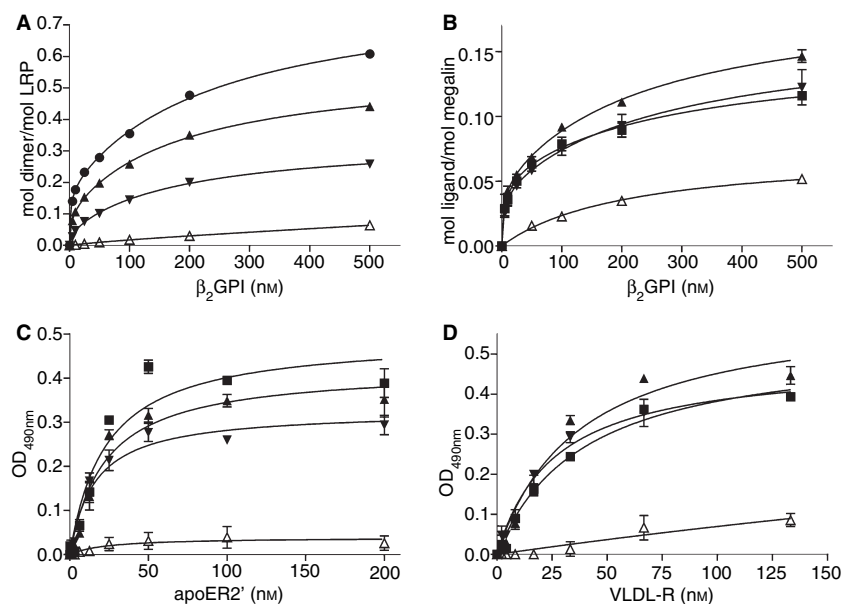
Fig. 5. Binding isotherms of the domain deletion mutants for members of the LDL receptor family. Binding characteristics for the domain deletion mutants for the LDL-R homologs was investigated using SPR analysis and with a solid phase immunosorbent assay. (A) increasing concentrations of full-length dimeric β_2GPI (▲), delta I (▼), delta II (■) or delta V (△) (0–500 nM) were able to interact with low density LRP. (B) interaction of the domain deletion mutants with megalin. Except for delta V, the experimental data was best fitted with a two-site binding model. Data for LRP and megalin is expressed as mol ligand bound to mol receptor. (C) and (D) increasing concentrations of apoER2′ and very low density lipoprotein-receptor (VLDL-R) were coated on a hydrophobic 96 wells plate. Full-length dimeric β_2 glycoprotein 1(GPI) (▲), delta I (▼), delta II (■) and delta V (△) (3 µg mL^{-1}) were able to interact with the receptors. The data obtained for the interactions was best fitted using a one-site binding model. Steady state constants derived from the binding isotherms are listed in Table 1. Results represent mean ± SD ($n = 3$).

Table 2 Maximal binding for all constructs of dimeric β_2GPI. Maximal binding to LRP and megalin is shown in response units (RU) and for apoER2′ and the VLDL-R the maximal response is expressed in OD280 nm. Data represent mean ± SD ($n = 3$)

Receptor	Dimeric β_2GPI	Delta I	Delta II	Delta V
LRP (RU)	507 ± 30	283 ± 40	608 ± 13	59 ± 22
Megalin (RU)	375 ± 14	343 ± 34	310 ± 20	120 ± 5
Cluster II (RU)	253 ± 9	194 ± 7	209 ± 3	28 ± 3
Cluster IV (RU)	201 ± 8	164 ± 6	180 ± 3	52 ± 2
apoER2′ (OD490 nm)	0.352 ± 0.06	0.294 ± 0.03	0.389 ± 0.05	0.026 ± 0.03
VLDL-R (OD490 nm)	0.437 ± 0.02	0.394 ± 0.01	0.404 ± 0.01	0.086 ± 0.02

GP1, glycoprotein 1; LRP, low density lipoprotein receptor- related protein; VLDL-R, very low density lipoprotein receptor.

were similar to LRP; interaction of delta I and delta II with megalin (Fig. 5B) displayed no major differences in maximal response compared with full-length dimeric β_2GPI (Table 2). Except for delta V (K_d 211.4 ± 13.7 nM), a two-site binding model could be applied for both delta I and delta II (K_{d1} 4.1 ± 0.5 nM, K_{d2} 283.1 ± 24.1 nM and K_{d1} 5.4 ± 1.2 nM, K_{d2} 283.4 ± 80.7 nM, respectively). For apoER2′ (Fig. 5C) and the VLDL-R (Fig. 5D) similar binding characteristics were observed; delta V displayed reduced binding to both receptors, whereas delta I and delta II displayed a dose-dependent interaction that was comparable with full-length dimeric β_2GPI.

Interaction of dimeric β_2GPI with recombinant LRP clusters

LRP consists of four clusters enriched in complement-type repeats, of which clusters II and IV encompass the main ligand-

binding domains of LRP. The observation that interaction between dimeric β_2GPI and LRP was best characterized with a two-site binding model may suggest heterogeneity of LRP. To identify LRP regions involved in binding dimeric β_2GPI, purified recombinant cluster II and IV were used. When plasma β_2GPI was passed over both clusters II and cluster IV, no significant increase in the resonance signal was observed (data not shown). Binding of dimeric β_2GPI displayed a reversible and dose-dependent binding to both ligand-binding clusters. The experimental data for each cluster fitted best using a two-site binding model. Therefore, as shown in Table 3, a high and a low affinity binding site could be calculated; for cluster II 1.6 ± 0.6 nM (K_{d1}) and 158.5 ± 30.5 nM (K_{d2}) and for cluster IV 1.7 ± 0.4 nM (K_{d1}) and 171.9 ± 14.6 nM (K_{d2}). To further characterize the interaction, binding characteristics of the domain deletion mutants were investigated. Delta V showed reduced binding to both cluster II and IV (K_d 365.4 ± 31.7 nM

Table 3 Steady state constants of the domain deletion mutants for cluster II and IV of LRP. Binding isotherms were fitted accordingly to a one-site or a two-site binding model, dependent on the type of interaction. When a two-site binding model was used, two binding constants (K_{d1} and K_{d2}) are calculated; for a one-site binding model only one K_d is calculated. Steady state constants are expressed in nmol L^{-1} (nM). Data represent mean $\pm$ SD ($n = 3$)

Receptor fragment	Dimeric β₂GPI	Delta I	Delta II	Delta V
Cluster II of LRP	1.6 ± 0.6	2.4 ± 0.8	2.0 ± 0.3	–
	159 ± 30	205 ± 38	157 ± 19	365 ± 32
Cluster IV of LRP	1.7 ± 0.4	1.4 ± 1.2	4.2 ± 0.7	–
	172 ± 15	249 ± 53	186 ± 22	143 ± 7

GP1, glycoprotein 1; LRP, low density lipoprotein receptor-related protein.

and 143.1 ± 6.9 nM, respectively). Delta I and delta II showed similar binding characteristics compared with full-length apple 4–β₂GPI.

Effect of a mutation in the phospholipid-binding site in domain V of dimeric β₂GPI

To address the possibility that binding of dimeric β₂GPI to members of the LDL-receptor family has similar characteristics as binding to anionic phospholipids, we performed binding experiments with the non-phospholipid-binding mutant of dimeric β₂GPI. As shown in Fig. 6, the mutant displayed dose-dependent association with LRP (Fig. 6A), megalin (Fig. 6B), apoER2′ (Fig. 6C) and the VLDL-R (Fig. 6D). In

Table 4 Steady state constants of the Trp316Ser mutant vs. full-length dimeric β₂GPI. Binding isotherms were fitted accordingly to a one-site or a two-site binding model, dependent on the type of interaction. When a two-site binding model was used, two binding constants (K_{d1} and K_{d2}) are calculated; for a one-site binding model only one K_d is calculated. Steady state constants are expressed in nmol L^{-1} (nM). Data represent mean $\pm$ SD ($n = 3$)

Receptor	Dimeric β₂GPI	Trp316Ser
LRP	3.1 ± 0.6	3.7 ± 0.4
	192 ± 13	162 ± 47
Megalin	3.1 ± 0.7	2.6 ± 0.5
	241 ± 30	198 ± 17
Cluster II of LRP	1.6 ± 0.6	1.7 ± 0.19
	158 ± 30	197 ± 10
Cluster IV of LRP	1.7 ± 0.4	1.2 ± 0.3
	172 ± 15	175 ± 13
apoER2′	22 ± 4	34 ± 9
VLDL-R	38 ± 8	60 ± 17

GP1, glycoprotein 1; LRP, low density lipoprotein receptor-related protein; VLDL-R, very low density lipoprotein receptor.

contrast to full-length dimeric β₂GPI, the maximal response of the mutant was lower for LRP and megalin. As shown in Table 4, there are no significant differences in the affinity constants (K_d) of the mutant for either the LDL-R homologs or both clusters of LRP.

Discussion

In the present study we have investigated interaction between β₂GPI and five members of the LDL-R family. Among these are:
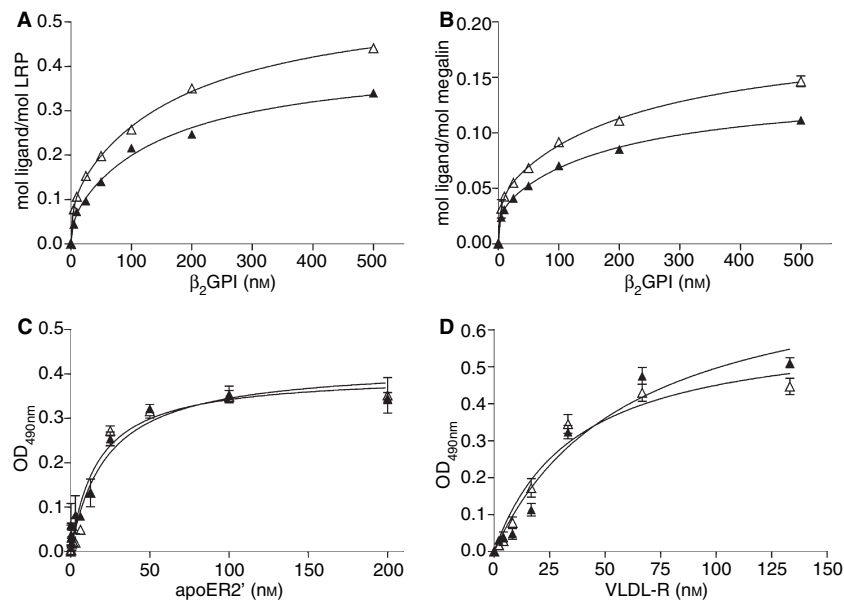
Fig. 6. Effect of a mutation in the phospholipid-binding region in domain V of β₂ glycoprotein 1(GPI). For SPR analysis, increasing concentrations (0–500 nM) of dimeric β₂GPI (△) and Trp316Ser mutant (▲) were past over (A), immobilized low density LRP, or (B) megalin. Association and dissociation was allowed for two minutes with a flow of 20 μL min^{-1} at 25 °C. Afterwards, binding isotherms corresponding to seven different concentrations were fitted accordingly to a one-site- or a two-site binding model. Data is expressed as mol ligand bound to mol receptor. (C) and (D) increasing concentrations of apoER2′ and very VLDL-R (0–10 μg mL^{-1}) were coated on a hydrophobic 96 wells plate; dimeric β₂GPI (△) and Trp316Ser mutant (▲) were able to interact with the receptors. Experimental data were best fitted using a one-site binding model. Results represent mean $\pm$ SD ($n = 3$). Steady state constants derived from the binding isotherms are listed in Table 4.

1 LRP, a promiscuous and ubiquitously expressed receptor involved in several physiological processes.

2 ApoER2′, which participates in neuronal development but is also expressed on platelets.

3 LDL-R.

4 VLDL-R, which is expressed for instance on endothelial cells.

5 Megalin, a multifunctional receptor expressed in various resorptive epithelia (including the proximal renal tubule) pointing to a predominant role in endocytosis and transport.

The current report addresses a number of specific questions. In previous experiments we have shown that platelets could be activated by a complex of β_2GPI and an anti-β_2GPI antibody, involving the receptor apoER2′ [13]. The interaction between β_2GPI and apoER2′ results in phosphorylation of apoER2′ followed by p38 MAPK phosphorylation and thromboxane synthesis [4].

This raised the question if other LDL-R homologs also recognize a β_2GPI/anti-β_2GPI antibody complex. Indeed, we found that LRP, megalin and the VLDL-R recognize only a complex of β_2GPI/anti-β_2GPI but not β_2GPI alone (Fig. 1) and this interaction can be mimicked by our recombinant form of dimeric β_2GPI (Fig. 2). Two binding models were identified for the interactions; interaction between dimeric β_2GPI, apoER2′, the LDL-R and the VLDL-R could be described with a one-site binding model, whereas with LRP or megalin a two-site (heterologous) binding model was more suitable. In contrast to the other receptors tested, the LDL-R showed one class of low affinity binding sites (340 nM) for dimeric β_2GPI. The LDL-R is a classical endocytosis receptor, whereas other LDL-R family members have been shown to regulate intracellular signaling processes [24], suggesting the minor role of the LDL-R in β_2GPI-mediated signaling.

We could not observe a two-site binding model in the solid-phase binding assay, presumably because the multiple washing steps in this system may amplify association or dissociation defects. SPR analysis measures binding under equilibrium conditions and is therefore not affected by washing steps. In order to confirm whether data obtained from SPR analysis or from solid-phase binding assays are comparable, we analyzed interaction between dimeric β_2GPI and LRP using the solid-phase binding assays (as described for apoER2′ or the VLDL-R). The resulting saturation curve could be adequately described employing a one-site binding model, representing a high affinity-binding site with half-maximal binding of 2.7 ± 1.0 nM (data not shown). This was in concordance with the high-affinity site (3.1 ± 0.6 nM) derived from the SPR data.

In this study we have further investigated the receptor/protein interaction by using several recombinant constructs of dimeric β_2GPI. We demonstrated that domain V of β_2GPI is the crucial domain for recognition by the LDL-R homologous tested (Fig. 5) and that this recognition site is not identical to the phospholipid-binding site within domain V of β_2GPI (Fig. 6). It has been proposed that positive electrostatic surface potentials, not a primary sequence, in different ligands constitute receptor-recognition domains [25,26]. Our observations from this study suggest that indeed positively charged residues are important (Fig. 4). We cannot exclude that this observed inhibition is due to steric interference by heparin. However, in a previous study we have shown that interaction between dimeric β_2GPI and apoER2′ on platelets can be inhibited by a peptide spanning amino acids 282–287 in domain V of β_2GPI [19]. Furthermore, it has been reported that Lys (284), Lys (286) and Lys (287) in domain V are essential for the interaction of β_2GPI with heparin [27]. This implies that both electrostatic interactions and amino acid sequences are important for the interaction between dimeric β_2GPI and LDL-R homologs. Interaction of dimeric β_2GPI with LRP or megalin was best described with a two-site binding model, suggesting the presence of two interaction sites on the surface of the receptors or on β_2GPI. For this purpose, we analyzed the interaction between β_2GPI and cluster II and IV of LRP. We could still observe a two-site binding model with both clusters, suggesting that LRP recognizes two surface potentials on β_2GPI. Two domains that expose cationic charges were of interest; domain I and domain V of β_2GPI. However, delta I interacted similar with the receptors or both clusters of LRP compared to full-length dimeric β_2GPI. Therefore, it is likely that domain V contains both regions involved in the receptor-interaction. Site-directed mutagenesis is required to establish the relative importance of cationic residues in domain V for the assembly of the β_2GPI/LDL-R complex.

Besides members of the LDL-R family, a number of other cellular receptors have been described for β_2GPI. The contribution of LDL-R family members in β_2GPI-mediated cell-activation in comparison with these other receptors is unknown. One of the candidate receptors, annexin A2 [28,29], does not have a transmembrane and intracellular domain, making it unlikely that annexin A2 is directly involved in signal transduction. It has been reported that anti-β_2GPI antibodies display a signaling cascade in endothelial cells comparable to that induced by members of the toll-like receptor (TLR) family. TLR2 [30] and TLR4 [31] have been implicated in this event. Interesting, also GPIb, another possible member of the TLR family, has been shown to bind β_2GPI/anti-β_2GPI antibody complexes [32]. Our next challenge is to understand how cell signaling induced by anti-β_2GPI antibodies is coordinated by all these different receptors.

Besides β_2GPI, other proteins have been implicated as possible target for aPL, in particular prothrombin. However, the majority of the performed studies indicate that anti-β_2GPI antibodies correlate best with the observed clinical manifestations [33]. In addition, addition of purified antiprothrombin antibodies to whole blood did not result in increased platelet deposition (P. G. de Groot, unpubl. obs.). Therefore, we do not believe that interaction of prothrombin–antiprothrombin antibody complexes with cells result in cellular activation.

An important step in resolving the pathways that explain clinical symptoms in APS is the identification of cellular

receptors that can interact with the protein of interest. The classical clinical manifestations observed in APS are thrombosis and pregnancy morbidity [33–35]. However, patients often suffer from other aPL-associated clinical manifestations, such as heart valve abnormalities, thrombocytopenia, proteinuria, chorea, neuropathy and livedo reticularis [36–40], which are undoubtedly frequently observed in patients with aPL antibodies in their plasma. Here we have shown that β_2GPI/antiβ_2GPI-antibodies complexes can interact with different LDL-R homologs. It is clear now that these receptors have the potential to induce signaling processes in different cell types [41–43]. Different members of the LDL-R family are expressed on almost all cell types. It is interesting to speculate that β_2GPI/anti-β_2GPI antibody complexes can activate a large number of different cells via LDL-R homologs, resulting in responses specific for that cell type. We speculate that the heterogeneous clinical manifestations observed in APS are due to interaction between β_2GPI/anti-β_2GPI antibody complexes and LDL-R family members on different cell types.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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