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ORIGINAL ARTICLE

Platelets express three different splice variants of ApoER2 that are all involved in signaling

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Summary. *Background:* β2-Glycoprotein I is the most relevant antigen in antiphospholipid syndrome. We have shown that binding of dimerized β2-GPI to platelets via ApoER2′ sensitizes platelets for second activating stimuli. *Objective:* Determine the region of ApoER2 involved in the binding of dimeric β2-GPI. *Methods:* Cultured human megakaryocytes (MK) and three different human megakaryocytic cell lines were used for mRNA isolation to clone and express recombinant soluble platelet ApoER2. Domain deletion mutants of ApoER2 were constructed to identify the binding site for dimeric β2-GPI. The presence of ApoER2 splice variants in platelets was demonstrated by immuno-blotting. *Results:* Three different mRNA splice variants were isolated from all four types of megakaryocytic cells used. Sequence analysis identified the splice variants: (i) shApoER2Δ5 lacking low-density lipoprotein (LDL) binding domains 4, 5 and 6; (ii) shApoER2Δ4-5 lacking LDL binding domains 3, 4, 5, 6 and (iii) shApoER2Δ3-4-5 lacking LDL binding domains 3, 4, 5, 6 and 7. The presence of three splice variants of ApoER2 on platelets was confirmed by immuno-blotting, with ApoER2Δ4-5 being the most abundantly expressed splice variant. Upon stimulation with dimeric β2-GPI, all three splice variants were translocated to the cytosol; however, ApoER2Δ4-5 translocation was most prominent. Dimeric β2-GPI binds platelet ApoER2 variants via LDL-binding domain 1. *Conclusions:* Three different ApoER2 mRNA splice variants were isolated from MK and platelets express all three splice variants. All splice variants were shown to be functional by translocation upon stimulation with dimeric β2-GPI. All three splice variants express LDL-binding domain 1.

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Introduction

Patient studies have shown that in antiphospholipid syndrome the presence of anti-β2-GPI antibodies in plasma strongly correlates with the presence of thrombosis [1,2]. The mechanism by which anti-β2-GPI antibodies are involved in the patho-physiology of antiphospholipid syndrome is not fully understood. When β2-GPI interacts with anti β2-GPI antibodies, β2-GPI becomes dimerized and conformational changes are introduced into the structure of β2-GPI resulting in an enhanced affinity for anionic phospholipids [3,4]. Previous research has demonstrated that dimerization of β2-GPI by auto antibodies can also induce activation of several cell types such as monocytes [9,10], endothelial cells [11–13] and blood platelets [6,7,14] via interaction with cellular receptors such as TLR4 [5], GPIbα [6,7] and several members of the low-density lipoprotein (LDL)-receptor family [8]. These three cell types are considered to participate in the development of thrombotic complications.

One of the first cellular receptors identified for β2-GPI antibody complexes was ApoER2′, a receptor also present on the membrane of blood platelets [14]. ApoER2′ is a splice variant of ApoER2 in which exon 5 is omitted during mRNA splicing and therefore lacks LDL-binding domains 4, 5 and 6. Binding of auto-antibodies to β2-GPI results in dimerization of β2-GPI which can subsequently bind to ApoER2′ via a cationic patch in its domain V [15]. In neuronal cells, it has been shown that adaptor proteins attached to ApoER2 determine its cellular localization [16–18] and ligand binding can alter proteolytic cleavage of ApoER2 [19]. It is now clear that ApoER2 is not merely an endocytic receptor mediating uptake of ApoE but ApoER2 mainly has signaling properties as reviewed by Stolt *et al.* [20]. The intracellular portion of ApoER2 contains the NPxY motif localizing it to caveolae, cholesterol and sphingolipid-rich micro domains with known signaling properties [21]. The extracellular part of ApoER2 consists of three distinct functional and structural regions. These are: (i) the type A-binding repeats or LDL-binding domains of ± 40 residues displaying a negative-charged surface, responsible for receptor–ligand interactions; (ii) the

type B repeats which are homologous to regions in the epidermal growth factor precursor; and (iii) the protein stack comprised of modules of 50 residues of O-linked sugar domains spacing the LDL-binding domains from the cellular surface.

Moreover, blood platelets play a major role in the development of arterial thrombosis. Blood platelets can be used as a model cell system to study cell activation by dimeric β2-GPI because platelets are easily isolated from humans and they respond strongly to many stimuli.

To better understand the interaction of dimeric β2-GPI with blood platelets via ApoER2′, we started to investigate which LDL-binding domain of ApoER2′ is involved in binding of dimeric β2-GPI. Here we found that platelets express three splice variants of ApoER2 and all three variants are able to signal via β2-GPI-antibody complexes.

Methods

Reagents

Polyclonal rabbit anti β2-GPI antibody was produced by immunization of rabbits with human β2-GPI. Plasma was obtained after sequential boosting by plasmaphereses. Monoclonal mouse anti-human β2-GPI antibody 21B2 was a generous gift from Professor J. Arnout (Leuven, Belgium). For purification of dimeric β2-GPI, 21B2 was coupled to CNBr-Sepharose according to the manufacturer's protocol (Amersham Pharmacia Biotech, Uppsala, Sweden). We produced a monoclonal mouse anti-human ApoER2 antibody (MP4-3) by immunizing mice with a peptide derived from the LDL binding domain 1 (WRCDEDDDCLDHSDED). Hybridoma cell lines were produced using a standard PEG fusion protocol with SP2.0 cells and specificity was tested using solid phase binding assays and immuno-blotting procedures.

Purification of plasma β2-GPI

Plasma β2-GPI was isolated from fresh citrated human plasma as described previously [22]. In short, dialyzed human plasma was applied to the following columns in consecutive order: DEAE-Sephadex A50, protein G-Sepharose, S-Sepharose, and finally heparin-Sepharose (all Sepharoses were obtained from Amersham Pharmacia Biotech). Bound proteins were eluted with linear salt gradients. Afterwards, β2-GPI was dialyzed against tris buffered saline (TBS). Purity of the protein was checked by 4–15% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) showing a single band of 47 kD under reduced conditions. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay.

Cell culture and blood platelet isolation

Human megakaryocytes (MK) were isolated as described previously [23]. Megakaryocytic cell lines Meg01, Dami and CHRF were cultured as described by Den Dekker *et al*. [24]. Human blood platelets were isolated as previously described [25].

Cloning and expression of dimeric β2-GPI

Recombinant dimeric β2-GPI was constructed and purified as described previously [26]. In short, the sequence encoding the mature β2-GPI protein was amplified with the primers β2-GPI-*Xho*I (C CCT CGA GGA CGG ACC TGT CCC AAG CC) and β2-GPI-*Xba*I (GC TCT AGA AAA CAA GTG TGA CAT TTT ATG TGG A) by polymerase chain reaction (PCR). To construct chimerical fusion proteins of the dimerization domain of factor XI (apple4) and β2-GPI, the PCR product was cloned with *Xho*I and *Xba*I into the vector zPL7-apple4-tissue-type plasminogen activator (tPA)-S478A [27] replacing tPA. The dimeric β2-GPI construct was then transfected into BHK cells and stable transfectants were selected in MTX-containing medium. Dimeric β2-GPI was affinity purified using monoclonal anti β2-GPI antibody 21B2 and further purified using ion exchange (MonoQ; Amersham).

Cloning of soluble human ApoER2′

Mature MK were cultured from citrated umbilical cord blood as described previously [23]. Messenger RNA from freshly isolated MK and three megakaryocytic cell lines (Meg-01, Dami and CHRF) were isolated using Nucleospin® RNA II (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and cDNA was synthesized using superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. CopyDNA was amplified using Phusion DNA polymerase (Finnzymes, Espoo, Finland). Primer design was such that, to obtain a soluble receptor, the forward primer started at the ATG sequence of the signal peptide adding a kozak sequence and a *Hin*DIII site for cloning and expression. Reverse primer started amplifying at the 2nd EGF domains thereby omitting the protein stack, transmembrane portion and intra-cellular tail adding an *Eco*RI site. Forward primer: Sol. H. ApoER2 *Hin*DIII forward: TATAA AAGCTT GCCACC ATGGGCCTCCCCGAGCCGG. Reverse primer: Sol. H. ApoER2 *Eco*RI reverse: TAATA GAATTC CTTGCAGTTCTTGGTCAGTAGG . Amplified cDNA products were separated using agarose gel electrophoreses and cloned into cloning vector Topo®-blunt (Invitrogen) according to the manufacturer's protocol for sequence analysis. Sequence analysis was performed using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol. When secretion of expressed recombinant proteins using pcDNA6-V5-HIS failed, constructs from the cloning strategy were used as template for growth hormone fusion to recombinant protein thereby omitting the signal peptide starting directly at the beginning of LDL-binding domain 1: forward primer: shApoER2′ *Bam*HI GGA TCC GGG CCG GCC AAG GAT TGC GAA AAG G; reverse primer: shApoER2′

*Not*I GC GGC CGC CTT GCA GTT CTT GGT CAG TAG GTC C.

Cloning LDL-binding domain deletion mutants of soluble human ApoER2′

Constructs containing either LDL-binding domain 1 or LDL-binding domain 2 were constructed from cDNA coding for shApoER2-650. For the deletion of LDL-binding domain 2 (shApoER2-BD1), two PCRs were performed which were combined by a third PCR afterwards. PCR 1: LDL-binding domain 1 was amplified using primer 1: shApoER2′-BD1 *Bam*HI Forward TAATAGGATCC GGGCCGGCCAAG-GATTGC G and primer2: shApoER2′ ex2 overlap ex7 reverse primer GTTGTGCAGACACTC GTTCAGCCTGGGG-CAGTCGTCCTCGTCGCGCTGTG. The EGF domains were amplified with PCR 2 using primer3: shApoER2′ ex7 overlap ex2 forward CACAGCGACGAGGACGACTG-CCCCAGGCTGAACGAGTGTCTGCACAAC and primer4: shApoER2′ + EGF *Not*I reverse TAATA GCGGCCGCCTTGCAGTTCT TGGTCAGTAGGTCC. Both PCR products were then combined with PCR 3 using primers 1 and primer 4 to obtain a construct containing the LDL-binding domain 1 fused to the EGF domains of ApoER2′.

For deletion of domain 1 (shApoER2-BD2), one PCR was performed using primer 5: shApoER2′-BD2 + EGF *Bam*HI TAATA GGATCCCCCAAGAAGACCTGTGCAGA C and primer 4: shApoER2′ *Not*I reverse TAATA GCGGCCGC CTTGCAGTTCTT GGTCAGTAGGTCC. PCR products were analyzed by agarose gel electrophoreses to determine product size, excised from the agarose and purified using a DNA extraction kit (Macherey-Nagel) and cloned into cloning vector Topo®-blunt (Invitrogen) according to manufacturer's protocol for sequence analysis. Sequence analysis was performed using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to manufacturer's protocol.

Expression of soluble human ApoER2′ splice variants and LDL-binding domain deletion mutants

Soluble human ApoER2 splice variants and domain deletion mutants were cloned into the expression vector PTT3-SRα-GH-HISN-TEV. The expression vector is constructed from the pTT3 [28] expression vector and the pSGHV0 expression vector [29]. HEK293-EBNA cells were transfected with the constructs using the DNA-PEI method according to Durocher *et al*. [28]. Soluble human ApoER2 splice variants and domain deletion mutants were grown to produce in a 1-liter suspension culture [medium containing 90% freestyle, 10% calcium free Dulbecco's modified Eagle's minimal essential medium (DMEM), 0.1% fetal calf serum, (Invitrogen)] for 4 days. Soluble human ApoER2 splice variants and domain deletion mutants were affinity purified using NiNTA Sepharose and subsequent RAP-Sepharose. Concentration was measured with a BCA protein assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as a standard. Protein purity and molecular weight were assessed by SDS–PAGE followed by PageBlue™ Protein Staining Solution (Fermentas) according to manufacturer's protocol.

Immuno-blotting of platelet lysate

Washed platelets of three different donors were prepared as described by Weeterings *et al*. [25], set at 200 000 μL^{-1} and 20 μL was then lysed by adding 5 μL 5× reducing Laemmli sample buffer (62.5 mM Tris–HCl pH6.8, 10% glycerol, 2% SDS and 5% β-mercaptoethanol). Samples were separated on 8% SDS-polyacrylamide gel and transferred onto an Immobilon-P polyvinylidene difluoride membrane. Blots were blocked with Tris-buffered saline (25 mM Tris, 150 mM NaCl) with 0.1% (v/v) Tween-20 (TBST) containing 2% (w/v) BSA (Sigma-Aldrich, St Louis, MO, USA) for 1 h at room temperature. The blots were then incubated with mouse anti ApoER2 antibody (MP4-3, 1 μg mL^{-1}) overnight in TBST with 1% BSA and washed three times with TBST. After incubation with peroxidase labeled rabbit anti-mouse antibodies for 1 h at RT (1:2500; Dako, Glostrup, Denmark), blots were washed again with TBST and developed with enhanced chemiluminescence reagent plus (PerkinElmer Life Sciences, Waltham, MA, USA)

Solid phase binding assay

Binding of dimeric β2-GPI to different splice variants and domain deletion mutants was measured in a solid phase binding assay. Recombinant soluble human ApoER2 splice variants and domain deletion mutants were coated at 10 and 5 μg mL^{-1} in TBS (50 mM Tris, 150 mM NaCL pH = 7.4) in soft-well ELISA plates (Vinyl Costar 96-well protein assay plates). TBS-treated wells were included as a negative control. Wells were then blocked using 4% Protifar (Nutricia, Zoetermeer, the Netherlands) for 1 h at 37°C. Plates were washed three times with TBS + 0.1% Tween-20 (Riedel-de Haën, Seelze, Germany) and wells were incubated with rabbit anti β2-GPI (1:500) antibody in TBS + 1% Protifar for 1 h at 37°C. After washing the plates five times with TBS + 0.1% Tween-20, wells were incubated with goat anti-rabbit HRP 1:1000 (Dako A/S, Glostrup, Denmark) in TBS + 1% Protifar for 1 h at 37°C. Plates were washed again with TBS + 0.1% Tween-20 and wells were incubated with rabbit anti-goat HRP 1:1000 (Dako) in TBS + 1% Protifar for 1 h at 37°C. Finally plates were washed for the last time and wells were incubated with Ortho-PhenyleneDiamide for staining.

Translocation of ApoER2′

Next, 500-μL aliquots of washed platelets (220.000/μL) resuspended in HEPES/Tyrode buffer pH 7.3 were incubated with TBS or dimeric β2-GPI at 50 μg mL^{-1} for 0.5, 1, 3, 5 or 10 min at 37°C in the presence or absence of inhibitors. Platelets were incubated with either TBS or dimeric β2-GPI for 5 min while

stirring and lyzed by adding 1/10th volume of 10× Triton lysis buffer [10% Triton-100, 200 mM Tris, 50 mM EGTA and EDTA-free proteinase inhibitor cocktail tablets according to manufacture's protocol (Sigma-Aldrich)]. Lysates were spun for 30′ at 16 110 *g* and supernatant was aspirated (cytosolic fraction). The precipitate (cytoskeleton fractions) was washed twice with 1× Triton lysis buffer. The cytoskeleton fraction was dissolved in 40 μL of 1× reducing Laemmli sample buffer [0.001% (w/v) bromophenol blue, 2% (w/v) SDS, 10% (v/v) glycerol in 62.5 mM Tris, pH 6.8 and 5% β2-mercaptoethanol], and boiled for 5 min. Five microlitres of 5× reducing Laemmli sample buffer was added to 20 μL of the cytosolic fraction. Samples were separated on an 8% SDS–polyacrylamide gel and transferred onto an Immobilon-P polyvinylidene difluoride membrane. Blots were blocked with Tris-buffered saline (25 mM Tris, 150 mM NaCl) with 0.1% (v/v) Tween-20 (TBST) containing 2% (w/v) BSA (Sigma) for 1 h at room temperature. The blot was then incubated with mouse anti ApoER2 antibody (MP4-3, 1 μg mL^{-1}) overnight in TBST with 1% BSA and washed three times with TBST. After incubation with peroxidase labeled rabbit anti-mouse antibodies for 1 h at RT (1:2500; Dako) blots were washed again with TBST and developed with enhanced chemiluminescence reagent plus (PerkinElmer Life Sciences).

Results

To identify the binding site for dimeric β2-GPI on ApoER2′ expressed on blood platelets, we isolated mRNA from freshly isolated human MK and three different megakaryocytic cell lines. This mRNA served as a template to amplify the extra cellular LDL-binding and EGF region of ApoER2′. Three different mRNA forms were isolated and evaluated using sequencing analysis to confirm alternative splicing.

Apart from the soluble form of ApoER2Δ5 or ApoER2′ lacking LDL-binding domains 4, 5 and 6, the presence of which on blood platelets is well described [30], we also isolated two shorter forms of ApoER2, shApoER2Δ4-5 lacking LDL-binding domains 3-4-5 and 6 and shApoER2Δ4-5-6 lacking LDL-binding domains 3, 4, 5, 6 and 7. We also cloned and expressed two additional domain deletion mutants shAPoER2-BD-1 and shAPoER2-BD2. Fig. 1B is a schematic representation of the mRNA splice variants. The mRNA splice variants isolated from megakaryocytic cells of all used origin (freshly isolated MK, CHRF, Meg01 and DAMI) were identical (Fig. 1A, data only shown for CHRF and freshly isolated MK).

To confirm the presence of different splice variants of ApoER2 on the membrane surface of blood platelets, platelet lysates from three different donors were analyzed by immunoblotting using monoclonal antibody (mAb) MP4-3 directed against the LDL-binding domain 1 of ApoER2. Three protein bands were detected with a molecular weight of approximately 80, 100 and 120 kDa (respectively ApoER2Δ3-4-5, ApoER2Δ4-5 and ApoER2Δ5, Fig. 1C). ApoER2Δ4-5 seems to be the dominant splice variant expressed in all four donors.

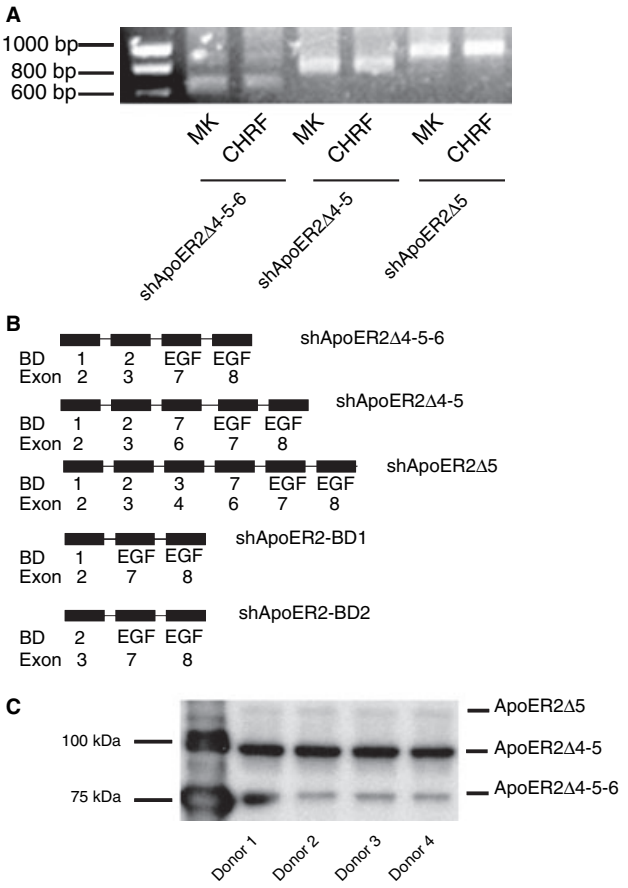

Fig. 1. (A) Freshly isolated megakaryocytes (MK) and three different megakaryocytic cell lines contain mRNA coding for three different splice variants of ApoER2. Freshly isolated MK and the megakaryocytic cell lines CHRF, Meg01 and CHRF were used to isolate mRNA and subsequent cDNA synthesis. Polymerase chain reaction was performed to amplify soluble ApoER2′ and cDNA was cloned into TOPO blunt for further analysis. Three different forms of soluble ApoER2′were isolated from all three cell lines (data shown only for MK and CHRF). (B) Schematic representation of the three isolated splice variants and two constructed domain deletion mutants of ApoER2. (C) Platelets express three different splice variants of ApoER2. Platelet lysate of three different donors was analyzed by immuno-blotting using a monoclonal antibody directed against a peptide stretch present in the low-density lipoprotein-binding domain 1 of ApoER2 (MP4-3). Platelets express three different forms of ApoER2.

The three mRNA splice variants isolated from MK and the two LDL-binding domain deletion mutants that only express either binding domain 1 or binding domain 2, were cloned into an expression vector for eukaryotic expression in 293EBNA cells and recombinant proteins were affinity purified. The soluble form of ApoER2′ (shApoER2Δ5) was detected at a molecular weight of 47 kD. The shorter splice variant shApoER2Δ4-5 had a molecular weight of 42 kD and shApoER2Δ3-4-5 was 38 kD (Fig. 2). Both domain deletion mutants we constructed (shApoER2-BD1 and shApoER2-BD2) had similar molecular weights of approximately 34 kD.

To characterize the binding site for dimeric β2-GPI on apoER2, we used the purified splice variants and domain

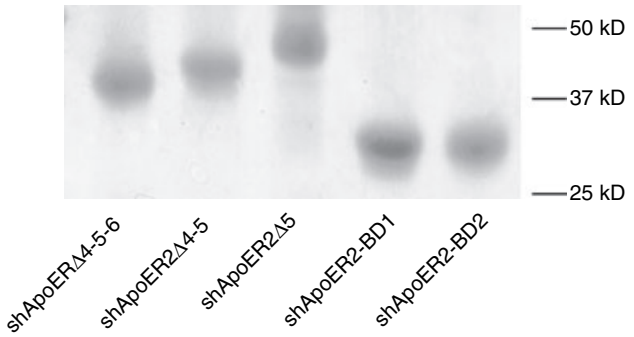

Fig. 2. Expression and purification of the three isolated mRNA splice variants and domain deletion mutants of ApoER2. Recombinant proteins were expressed by 293EBNA cells. Proteins were purified by NiNTA and subsequent RAP sepharose affinity purification. Purified proteins were run on a 10% sodium dodecylsulfate–polyacrylamide gel to check protein purity. Each recombinant protein showed only one band.

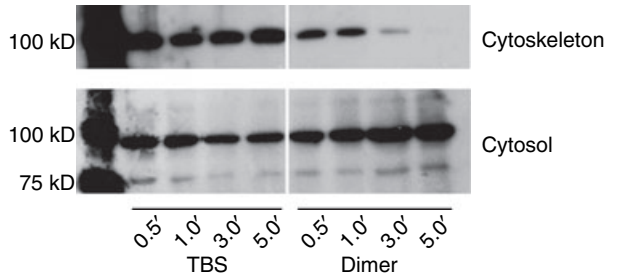

Fig. 4. Incubation of platelets with dimeric β2-GPI shows a time-dependent translocation of ApoER2 splice variants away from the platelet cytoskeleton. Platelets were stimulated with either TBS or dimeric β2-GPI. When cytosolic and cytoskeletal fractions were separated and blotted using MP4-3, a time-dependent dissociation from ApoER2-Δ5, ApoER2Δ4-5 and ApoER2-Δ3-4-5 away from the cytoskeleton was observed with ApoER2-Δ4-5 translocation being the most prominent.

deletion mutants in a solid phase binding assay. All three cloned splice variants of ApoER2 were able to bind dimeric β2-GPI in a similar fashion. Upon deletion of LDL-binding domain 1, (shApoER2-BD2), binding of dimeric β2-GPI was lost (Fig. 3).

To study the cellular re-distribution of ApoER2 splice variants after stimulation with dimeric β2-GPI, platelets were incubated with either TBS or dimeric β2-GPI. Platelets were lyzed and the RIPA soluble cytoskeleton fraction was separated from the Tx100 soluble cytosolic fraction. ApoER2Δ5, ApoER2Δ4-5 and ApoER2Δ3-4-5 time-dependently dissociated from the cytoskeleton and ended up in the cytosolic fraction; however, of the three splice variants expressed, ApoER2Δ4-5 seems to be the dominant signaling receptor. (Fig. 4, blots representative for three separate experiments).

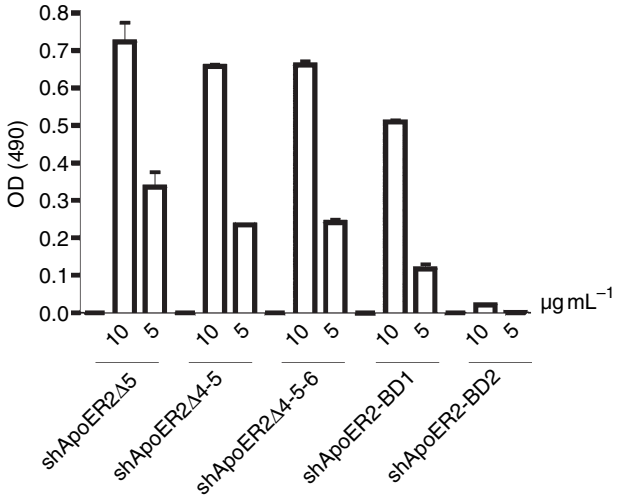

Fig. 3. Dimeric β2-GPI binds to ApoER2′ via the low-density lipoprotein (LDL)-binding domain 1 on ApoER2′. Purified splice variants and domain deletion mutants were coated at 10 or 5 μg mL^{-1} and wells were blocked. Coated wells were incubated with dimeric β2-GPI and binding detected using in-house rabbit polyclonal antiserum raised against β2-GPI. Upon deletion of LDL-binding domain1, binding of dimeric β2-GPI was completely abolished.

Discussion

There is now ample evidence that antibodies directed against β2-GPI correlate best with the clinical manifestations in the antiphospholipid syndrome. No physiological function has been described for β2-GPI so far. However, after complex formation with auto-antibodies, dimerized β2-GPI is able to activate a whole variety of cells, including blood platelets [5,31–34]. The interaction of β2-GPI-antibody complexes with platelets has been described to be mediated in part by ApoER2′ [14], so far the only member of the LDL-receptor super family known on platelets [30]. The binding site for dimeric β2-GPI on ApoER2′ has not been identified.

In our search for the binding site for dimeric β2-GPI on ApoER2′ we have isolated three different mRNA splice variants of ApoER2 expressed by freshly isolated MK and three different megakaryocytic cell lines. The largest variant lacks exon 5 resulting in a splice variant depleted of LDL-binding domains 4, 5 and 6 (shApoER2Δ5). Expression of this splice variant by platelets has been described previously [30]. The second variant lacks exon 4 and 5 resulting in the absence of LDL-binding domains 3, 4, 5 and 6 (shApoER2Δ4-5). The shortest splice variant of ApoER2 lacks exons 4, 5 and 6 and therefore misses LDL-binding domains 3, 4, 5, 6 and 7 (shApoER2Δ4-5-6). Both splice variants ApoER2Δ4-5 and ApoER2Δ4-5-6 have not been described before and might be platelet and megakaryocyte specific.

Possible expression of ApoER2 splice variants was studied on circulating platelets. We found platelets express three splice variant of ApoER2 but based on the intensity on the bands in Western blotting, ApoER2-Δ4-5 is expressed most prominent. All variants contain LDL-binding domain 1 as mAb MP4-3 used for detection, was raised against a peptide present in LDL-binding domain 1.

All three cloned mRNA splice variants of ApoER2 were able to bind dimeric β2-GPI but when LDL-binding domain 1 was omitted, binding of dimeric β2-GPI was lost. We

Table 1 Localization and signaling properties of ApoER2 splice variants expressed on platelets

Name	Cytosol	Cytoskeleton	Signaling
ApoER2-120 kDa	+/–	–	+
ApoER2-100 kDa	+ + + +	+ + + +	+ + + +
ApoER2-80 kDa	+	+	+/–

furthermore show translocation of ApoER2Δ5, ApoER2Δ4-5 and ApoER2Δ4-5-6 away from the cytoskeleton to the cytosol upon stimulation with dimeric β2-GPI. Trafficking and re-location of ApoER2 in neuronal cells has been described during neuronal development and appears to be essential for correct development [16,17,35]. This is the first report of ApoER2 translocation in blood platelets and we assume that translocation of ApoER2 is part of the signal pathway in platelets after stimulation with β2-GPI-antibody complexes. As described in neurons, translocation of ApoER2 could be part of a physiological function of ApoER2 splice variants in platelets (Table 1). Dimerization of the VLDL-R and ApoER2 has been implicated in the initiation of signaling via VLDL-R and ApoER2 by the group of Strasser *et al*. [36]. It is possible that dimerization o f β2-GPI leads to dimerization or even clustering of ApoER splice variants thereby, transmitting a signal across the platelet membrane inside the platelet.

Why blood platelets express three different ApoER2 variants is still unclear as we demonstrate binding of dimeric β2-GPI to all three splice variants isolated followed by translocation of all three spice variants. Besides differences in expression levels, no differences between ApoER2 splice variants were observed. In general, the presence of different splice variants on platelets could play a role in binding of other receptor ligand like LDL. Differences for ligand binding by ApoER2 splice variants varying in the LDL-binding region has been described before [37]. In neuronal cells, splicing of the intracellular domain has also been described [38].

As there is no evidence for the occurrence of circulating immuno-complexes of β2-GP and antibodies directed against β2-GPI, we assume that these complexes are formed on the platelet surface upon exposure of anionic phospholipids. Further investigations are required to elucidate the exact sequence of events leading to the formation of β2-GPI-anti-β2-GPI complexes and binding to GPIbα and ApoER2 splice variants.

In conclusion, we have cloned and expressed soluble forms of three different splice ApoER2 splice variants from cells of megakaryocytic origin varying in the extra-cellular LDL-binding region. We identified LDL-binding domain 1 to be the binding moiety for dimeric β2-GPI. We furthermore show the presence of three different splice variants of ApoER2 on human blood platelets which all translocate to the cytosol upon stimulation with dimeric β2-GPI. The most abundant splice variant of ApoER2 present on platelets is ApoER2Δ4-5 lacking LDL-binding domains 3, 4, 5, 6.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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