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## Binding of Low Density Lipoprotein to Platelet Apolipoprotein E Receptor 2' Results in Phosphorylation of p38<sup>MAPK</sup> \*

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**Binding of low density lipoprotein (LDL) to platelets enhances platelet responsiveness to various aggregation-inducing agents. However, the identity of the platelet surface receptor for LDL is unknown. We have previously reported that binding of the LDL component apolipoprotein B100 to platelets induces rapid phosphorylation of p38 mitogen-activated protein kinase (p38<sup>MAPK</sup>). Here, we show that LDL-dependent activation of this kinase is inhibited by receptor-associated protein (RAP), an inhibitor of members of the LDL receptor family. Confocal microscopy revealed a high degree of co-localization of LDL and a splice variant of the LDL receptor family member apolipoprotein E receptor-2 (apoER2') at the platelet surface, suggesting that apoER2' may contribute to LDL-induced platelet signaling. Indeed, LDL was unable to induce p38<sup>MAPK</sup> activation in platelets of apoER2-deficient mice. Furthermore, LDL bound efficiently to soluble apoER2', and the transient LDL-induced activation of p38<sup>MAPK</sup> was mimicked by an anti-apoER2 antibody. Association of LDL to platelets resulted in tyrosine phosphorylation of apoER2', a process that was inhibited in the presence of PPI, an inhibitor of Src-like tyrosine kinases. Moreover, phosphorylated but not native apoER2' co-precipitated with the Src family member Fgr. This suggests that exposure of platelets to LDL induces association of apoER2' to Fgr, a kinase that is able to activate p38<sup>MAPK</sup>. In conclusion, our data indicate that apoER2' contributes to LDL-dependent sensitization of platelets.**

Platelets and low density lipoproteins (LDL)<sup>1</sup> are key elements in the development of atherothrombotic complications.

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<sup>1</sup> The abbreviations used are: LDL, low density lipoprotein; apoB100, apolipoprotein B100; apoER2, apolipoprotein E receptor-2; BSA, bovine serum albumin; FAK, p125 focal adhesion kinase; GFP, gel-filtered platelets; GST, glutathione S-transferase; LDL-R, LDL receptor; LRP1,

The interplay between both elements is apparent from the notion that LDL particles markedly enhance the responsiveness of platelets to various aggregation-inducing agents (1–4). These agonists mediate the release of growth factors, vasoactive substances, and chemotactic agents that are known to stimulate atherosclerotic plaque formation. Sensitization of platelets by LDL involves the major LDL constituent apolipoprotein B100 (apoB100) (5), a 4563 amino acid protein that is wrapped around the LDL particle (6). LDL particles are recognized by the classical hepatic LDL receptor (LDL-R) through the apoB100 moiety, and in particular through a region within the apoB100 protein that is enriched in positively charged amino acids, the so-called B-site (7). Like LDL, a synthetic peptide mimicking this B-site associates to the platelet surface (5). Moreover, this peptide interferes with binding of LDL to platelets (5), suggesting that both elements share similar binding sites. This possibility is supported by the observation that binding of either LDL or the B-site peptide to the platelet results in a near immediate activation of the intracellular enzyme p38 mitogen-activated protein kinase (p38<sup>MAPK</sup>) (5, 8). Activation of this Ser/Thr kinase is associated with downstream phosphorylation and activation of cytosolic phospholipase A<sub>2</sub>, which leads to the formation of thromboxane A<sub>2</sub> (9, 10). Finally, enhanced platelet function occurs via exposure of the integrin  $\alpha_{IIb}\beta_3$  and fibrinogen binding (11).

The mechanism by which LDL particles signal to p38<sup>MAPK</sup> is yet unclear, but it seems conceivable that it involves a receptor-dependent pathway. The finding that the signaling pathway is initiated by the apoB100 component of LDL may point to the involvement of an LDL-binding receptor. However, platelets are known to lack the classical LDL-R as well as LDL receptor-related protein-1 (LRP1) (12, 13). Recently, a splice variant of apolipoprotein E receptor-2 (apoER2) has been identified in platelets and megakaryocytic cell lines (14). ApoER2, which is also known as LDL receptor-related protein-8, is a member of the LDL receptor family, and is mainly expressed in brain, testes, and vascular cells but not in the liver (15, 16). Its structure is most closely related to the LDL-R and VLDL-R (17). However, transcriptional analysis has revealed that multiple alternative splicing variants of apoER2 exist (18, 19), one

LDL receptor-related protein-1; p38<sup>MAPK</sup>, p38 mitogen-activated protein kinase; PBS, phosphate-buffered saline; PECAM-1, platelet endothelial cell adhesion molecule-1; PGI<sub>2</sub>, prostacyclin; PRP, platelet-rich plasma; RAP, receptor-associated protein; SPR, surface plasmon resonance; SR-A, scavenger receptor A; SR-BI, scavenger receptor BI; TBS, Tris-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate.

of which, apoER2', is present in platelets (14). Platelet apoER2' mRNA encodes a 130-kDa protein, which consists of a single ligand-binding domain that comprises four complement type A repeats (compared with seven repeats in full-length apoER2), an epidermal growth factor-like homology region, an O-linked sugar domain, and a single transmembrane domain that connects the extracellular region to the cytoplasmic tail.

Several studies have shown the ability of apoER2 to bind and internalize apoE-containing lipid vesicles (14, 15). However, the contribution of apoER2 to the general lipid metabolism is probably limited, since mice genetically deficient for apoER2 do not suffer from increased plasma lipoprotein levels (20). Because also the endocytosis rate of apoER2 is almost 20-fold lower compared with LRP1 (21), it seems conceivable that apoER2 serves an alternative physiological function as well. Indeed, there is firm support for a role of apoER2 in cellular signaling processes. For example, apoER2 has been reported to contribute to the neuronal signaling pathway that governs the layering of the developing cortex (20). In this process, apoER2 functions as a receptor for the signaling molecule reelin, and transmits the reelin signal to the intracellular adaptor protein disabled-1, which associates to the cytoplasmic tail of apoER2 (20, 22, 23, 24). This tail contains an  $\psi$ XNPXY sequence (where  $\psi$  is a hydrophobic amino acid residue), which may function as a potential binding site for phosphotyrosine binding domains of signaling molecules, including disabled-1. In addition, the cytoplasmic region of apoER2 contains three proline-rich areas that correspond to the consensus sequence (PXXP) for Src homology-3 recognition (14). All of these motifs that potentially link apoER2 to various signaling pathways are also present in the platelet variant of this receptor (14).

In the present study, we investigated whether apoER2' has the potential to transmit the LDL signal to intracellular signaling components, with particular reference to p38<sup>MAPK</sup>. We found that LDL bound efficiently to soluble apoER2' and that an anti-apoER2 antibody was similar to LDL particles in activating p38<sup>MAPK</sup>. Furthermore, the absence of apoER2 in platelets was associated with a lack of LDL-dependent p38<sup>MAPK</sup> activation, while inhibition of ligand binding to apoER2' by the 39 kDa receptor-associated protein (RAP) interfered with LDL-dependent phosphorylation of p38<sup>MAPK</sup>. Finally, incubation of platelets with LDL particles resulted in a rapid tyrosine phosphorylation of apoER2'. This process was mediated by Src-like kinases and allowed the association with the signaling molecule Fgr. In view of our data, we propose that apoER2' serves a critical role in the LDL-initiated signaling pathway that governs platelets with increased sensitivity toward its aggregation-inducing agents.

#### EXPERIMENTAL PROCEDURES

**Mice**—Wild-type C57Bl/6 mice and mice genetically deficient for the LDL receptor (LDL-R<sup>-/-</sup>), CD36 (CD36<sup>-/-</sup>), scavenger receptor BI (SR-BI<sup>-/-</sup>), and scavenger receptor A (SR-A<sup>-/-</sup>) were used in this study. C57Bl/6 mice were obtained from Charles River (Maastricht, The Netherlands). Mice genetically deficient for apoER2 have been described previously (20). Homozygous LDL-R<sup>-/-</sup> mice, originally generated by Ishibashi *et al.* (25), were obtained from the Jackson Laboratory (Bar Harbor, ME) as mating pairs, and bred at the Gorlaeus Laboratories (Leiden, The Netherlands). CD36<sup>-/-</sup> mice were kindly provided by Dr. M. Febbraio (Department of Medicine, Weill Medical College of Cornell University, New York) (26), SR-BI<sup>-/-</sup> mice by Dr. M. Krieger (Department of Biology, Massachusetts Institute of Technology, Cambridge, MA) (27), and Mex-4 SR-A<sup>-/-</sup> mice by Dr. T. Kodama (Department of Molecular Biology and Medicine, University of Tokyo, Tokyo, Japan) (28). All mice were backcrossed at least 4 generations to the C57Bl/6 background. Mice had unlimited access to water and regular chow diet, containing 4.3% (w/w) fat with no added cholesterol (RM3, Special Diet Services, Witham, UK). All experimental protocols were approved by the local ethics committees for animal experiments.

**Materials**—Nonfat dry milk was obtained from Nutricia (Zoetermeer,

the Netherlands). Bovine serum albumin (BSA), a monoclonal antibody against  $\alpha$ -actinin, protease inhibitor mixture, peroxidase-conjugated anti-goat IgG, and sodium vanadate (NaVO<sub>3</sub>) were obtained from Sigma. Prostacyclin (PGI<sub>2</sub>) was from Cayman Chemical (Ann Arbor, MI). Protein G-Sepharose was obtained from Amersham Biosciences. PP1 was from Alexis Biochemicals (San Diego, CA). Renaissance chemiluminescence Western blot reagent was from PerkinElmer Life Sciences. Polyclonal antibodies against p38<sup>MAPK</sup> and dual phosphorylated p38<sup>MAPK</sup> (phosphoplu p38<sup>MAPK</sup>), and horseradish peroxidase-labeled anti-rabbit IgG were from New England Biolabs (Beverly, MA). An antibody against the ligand binding domain of apoER2 (anti-apoER2 antibody 186) has been described previously (24). A polyclonal antibody directed against c-Fgr, a polyclonal antibody directed against Src kinases, and a goat polyclonal antibody directed against the ectodomain of apoER2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-LDL-R monoclonal antibody was obtained from Oncogene Research Products (Boston, MA). Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology (Bucks, UK). The anti-human apoB100 monoclonal antibody 2D8 used for confocal microscopy was obtained from the University of Ottawa Heart Institute (Ontario, Canada), and has been described previously (29). The anti-apoB100 antibody used in the solid phase assay was from BiosPacific (Emeryville, CA). Fibrinogen  $\gamma$ -chain derived dodecapeptide HHLG-GAKQAGDV ( $\gamma$ 400–411) was kindly provided by the Department of Biochemistry at the University of Utrecht (Utrecht, The Netherlands). SDZ-GPI-562 (GPI-562) was a kind gift of Dr. H. G. Zerwes (Novartis Pharmaceuticals, Basel, Switzerland).

**Proteins**—RAP fused to glutathione S-transferase (GST-RAP) (30) was prepared as described previously (31). The peptide RL-TRKRLGLKLA ( $M_r = 1311$ ) designated B-site peptide, represents the receptor binding domain within apoB100. The peptide was synthesized by standard solid phase peptide synthesis and purified by C18 reverse-phase chromatography (HPLC, Genosphere biotechnologies, Paris, France). The purity of the peptides was >99% as determined by HPLC, and the molecular weights were verified by matrix-assisted laser desorption mass spectrometry by the manufacturer. Preparation of recombinant murine soluble apoER2' (s-apoER2') fused to mannose-binding protein was performed as described (32).

**Lipoprotein Isolation**—Lipoproteins were isolated as described before (11). In short, fresh, non-frozen plasma from 3 healthy subjects each containing less than 100 mg of lipoprotein(a)/liter was pooled, and LDL (density range 1.019–1.063 kg/liter) was isolated by sequential flotation in a Beckman L-80 ultracentrifuge (33). Centrifugations (175,000  $\times g$ , 20 h, 10 °C) were carried out in the presence of Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub> and EDTA. The quality of these preparations has been described (11). Lipoproteins were stored at 4 °C under nitrogen for not longer than 14 days and before each experiment dialyzed overnight against 10<sup>4</sup> volumes 150 mmol/liter NaCl. ApoB100 and lipoprotein(a) concentrations were measured using the Behring Nephelometer 100. The concentration of LDL was expressed as grams of apoB100 protein/liter.

**Platelet Isolation**—Freshly drawn venous blood from healthy volunteers was collected with informed consent into 0.1 volume 130 mmol/liter trisodium citrate. The donors claimed not to have taken any medication during 2 weeks prior to blood collection. Platelet-rich plasma (PRP) was prepared by centrifugation (200  $\times g$ , 15 min, 22 °C). Gel-filtered platelets (GFPs) were isolated by gel filtration through Sepharose 2B equilibrated in Ca<sup>2+</sup>-free Tyrode's solution (137 mmol/liter NaCl, 2.68 mmol/liter KCl, 0.42 mmol/liter NaH<sub>2</sub>PO<sub>4</sub>, 1.7 mmol/liter MgCl<sub>2</sub>, and 11.9 mmol/liter NaHCO<sub>3</sub>, pH 7.25) containing 0.2% BSA and 5 mmol/liter glucose. GFPs were adjusted to a final count of  $2 \times 10^{11}$  platelets/liter.

For the isolation of murine platelets, mice were anesthetized by subcutaneous injection of a mixture of xylazine (5 mg/ml), ketamine (40 mg/liter), and atropine (0.05 mg/ml), and blood was subsequently collected into 0.1 volume of 130 mmol/liter trisodium citrate and 0.1 volume of ACD buffer (2.5 g of trisodium citrate, 1.5 g of citric acid, and 2 g of D-glucose in 100 ml of distilled water) by heart puncture. PRP was obtained by centrifugation (87  $\times g$ , 15 min, 20 °C). The remainder of the blood was diluted with Hepes-Tyrode buffer (145 mmol/liter NaCl, 5 mmol/liter KCl, 0.5 mmol/liter Na<sub>2</sub>HPO<sub>4</sub>, 1 mmol/liter MgSO<sub>4</sub>, 10 mmol/liter Hepes, 5 mmol/liter D-glucose, pH 6.5), and 0.1 volume of ACD buffer and centrifuged again (87  $\times g$ , 15 min, 20 °C). PRP samples were pooled, and platelets were isolated from PRP by centrifugation (350  $\times g$ , 15 min, 20 °C) in the presence of 0.1 volume of ACD buffer and resuspended in Hepes-Tyrode buffer (pH 6.5). PGI<sub>2</sub> was added to a final concentration of 10 ng/ml, and the washing procedure was repeated once. The platelet pellet was resuspended in Hepes-Tyrode buffer (pH 7.2). Platelet count was adjusted to  $1 \times 10^{11}$  platelets/liter.



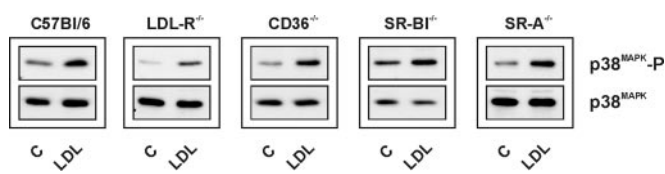
**p38<sup>MAPK</sup> Assay**—Human GFPs or washed murine platelets were incubated at 37 °C with LDL, anti-apoER2 antibody 186 or anti-LDL-R antibody as indicated. After incubation, 100- $\mu$ l aliquots were mixed (1:10 v/v) with cold lysis buffer consisting of 10% (v/v) Nonidet P-40, 5% (w/v) octylglucoside, 50 mmol/liter EDTA, 1% (w/v) SDS supplemented with 5 mmol/liter NaVO<sub>3</sub> and 10% (v/v) protease inhibitor mixture and subsequently taken up in Laemmli sample buffer. Samples were heated prior to SDS-polyacrylamide gel electrophoresis (12%). Proteins were electrophoretically transferred (1 h, 100 volts) to a nitrocellulose membrane using a mini-protein system (Bio-Rad). The blots were blocked in 5% (w/v) nonfat dry milk, 0.1% (v/v) Tween 20 in phosphate-buffered saline (PBS) (1 h, 4 °C) and incubated with the phosphoporus p38<sup>MAPK</sup> (recognizing p38<sup>MAPK</sup> phosphorylated at Thr<sup>180</sup> and Tyr<sup>182</sup>) or p38<sup>MAPK</sup> antibody, which recognizes both phosphorylated and non-phosphorylated isoforms (1:2000 (v/v) in 1% (w/v) nonfat dry milk, 0.1% (v/v) Tween in PBS, 16 h, 4 °C). Both antibodies are raised against residues 171–186 of human p38<sup>MAPK</sup>. After washing, the membranes were incubated with horseradish peroxidase-labeled anti-rabbit (1:2000 (v/v), 1 h, 4 °C) and p38<sup>MAPK</sup> was visualized using the enhanced chemiluminescence reaction. For semi-quantitative determination of the amount of dual phosphorylated or total p38<sup>MAPK</sup>, the density of the bands was analyzed using ImageQuant software (Molecular Dynamics).

**Solid Phase Binding Assay**—Microtiter plates were incubated with Tris-buffered saline (TBS), 2 mmol/liter CaCl<sub>2</sub> containing 10  $\mu$ g/ml of s-apoER2' (16 h, 4 °C). After blocking (1 h, 22 °C) in blocking solution (2% BSA in TBS, 2 mmol/liter CaCl<sub>2</sub>, 0.05% (v/v) Tween), wells were incubated with different concentrations of human LDL diluted in blocking solution in the presence or absence of 10 mmol/liter EDTA. Bound LDL was detected by anti-apoB antibody (diluted 1:500 in blocking solution) followed by peroxidase-conjugated secondary antibody (diluted 1:40,000 in blocking solution). All incubations were carried out at room temperature for 1 h. For the color reaction, a solution of 0.1 mol/liter sodium acetate, pH 6.0 containing 0.1 mg/ml of 3,3',5,5'-tetramethylbenzidine and 10 mmol/liter H<sub>2</sub>O<sub>2</sub> was used. The reaction was stopped after 5 min by the addition of 0.3 mol/liter H<sub>2</sub>SO<sub>4</sub>, and bound secondary antibody was photometrically quantified at 450 nm.

**Surface Plasmon Resonance Analysis**—Surface plasmon resonance (SPR) binding experiments were performed using a Biacore2000 biosensor system. A biotinylated peptide corresponding to the apoB100 B-site was immobilized at a density of 239 fmol/mm<sup>2</sup>. A control channel was prepared by the immobilization of a biotinylated irrelevant monoclonal antibody. Binding of s-apoER2' was corrected for binding to this control channel (less than 2%). SPR analysis was performed in 150 mmol/liter NaCl, 2.5 mmol/liter CaCl<sub>2</sub>, 0.005% (v/v) Tween-20, 25 mmol/liter Hepes (pH 7.4) at 25 °C with a flow rate of 5  $\mu$ l/min. Regeneration of the sensor chip surface was performed by incubating with 10 mmol/liter taurodeoxycholic acid, 100 mmol/liter Tris (pH 9.0) for 2 min. Data were analyzed as described previously (34).

**ApoER2' Tyrosine Phosphorylation**—Human GFPs were incubated at 37 °C with LDL or B-site peptide as indicated, mixed with ice-cold lysis buffer (1:10 v/v) as described above and subsequently taken up in Laemmli sample buffer. ApoER2' was precipitated using a goat polyclonal antibody directed against apoER2 (1  $\mu$ g/ml) and protein G-Sepharose for 3 h at 4 °C. Precipitates were washed three times with lysis buffer (containing 1 mmol/liter phenylmethylsulfonyl fluoride, 1 mmol/liter NaVO<sub>3</sub>, and 1  $\mu$ g/ml leupeptin) and taken up in non-reducing Laemmli sample buffer. Samples were analyzed by SDS-PAGE and Western blotting. Tyrosine phosphorylation of apoER2' was visualized by incubation with 4G10, an antibody directed against phosphorylated tyrosine residues (0.5  $\mu$ g/ml, 16 h, 4 °C), followed by incubation with peroxidase-linked anti-mouse IgG (1:5000 (v/v), 1 h, 4 °C), and the enhanced chemiluminescence reaction. As a control for equal lane loading, the blots were stripped and incubated with a monoclonal anti- $\alpha$ -actinin antibody (1:5000 (v/v), 16 h, 4 °C), followed by incubation with peroxidase-linked anti-mouse IgG (1:5000 (v/v), 1 h, 4 °C). For semi-quantitative determination of apoER2' phosphorylation, the density of the bands was analyzed using ImageQuant software. Complex formation between apoER2' and Src family tyrosine kinases was monitored by immunoprecipitation of apoER2' from lysates of LDL-stimulated platelets, followed by Western blotting with a polyclonal antibody against c-Fgr (0.4  $\mu$ g/ml, 16 h, 4 °C), and a subsequent incubation with horseradish peroxidase-labeled anti-rabbit IgG (1:10,000 (v/v), 1 h, 4 °C).

**Immunofluorescence Studies**—Human GFPs were incubated at 37 °C with LDL (1.0 g/liter) in the presence or absence of GST-RAP (0.3  $\mu$ mol/liter, 10 min). Then, the platelets were applied to coverslips after fixation with 1% (v/v) paraformaldehyde, washed with PBS, blocked for 10 min with PBS containing 1% (w/v) BSA and 0.1% (v/v) glycine (pH



**FIG. 1. LDL-induced p38<sup>MAPK</sup> phosphorylation may proceed independently of LDL-R, CD36, SRBI, and SR-A.** Platelets from wild-type C57Bl/6 mice and mice genetically deficient for the LDL-R (LDL-R<sup>-/-</sup>), CD36 (CD36<sup>-/-</sup>), SR-BI (SR-BI<sup>-/-</sup>), and SR-A (SR-A<sup>-/-</sup>) were stimulated with LDL (1.0 g/liter, 1 min, 37 °C). Samples were drawn and centrifuged (30 s, 9000  $\times$  g, 22 °C) and resuspended in sample buffer. Samples were split, and dual phosphorylated p38<sup>MAPK</sup> was identified in one part by SDS-PAGE and Western blotting using a phosphospecific anti-p38<sup>MAPK</sup> polyclonal antibody (upper panel). Another part was analyzed for equal loading by detecting total p38<sup>MAPK</sup> using an antibody against p38<sup>MAPK</sup> (lower panel).

7.4), and incubated with the monoclonal anti-apoB100 2D8 (5) and the goat polyclonal anti-apoER2 antibody. Afterward, the coverslips were incubated with a tetramethylrhodamine B isothiocyanate (TRITC)-labeled anti-mouse antibody (BD Biosciences, San Jose, CA) and a fluorescein isothiocyanate (FITC)-labeled anti-goat antibody, diluted 1:20 (v/v) in PBS for 45 min at 37 °C, followed by washing with PBS. Coverslips were embedded in Mowiol and analyzed by confocal laser microscopy on a Leica confocal laser microscope.

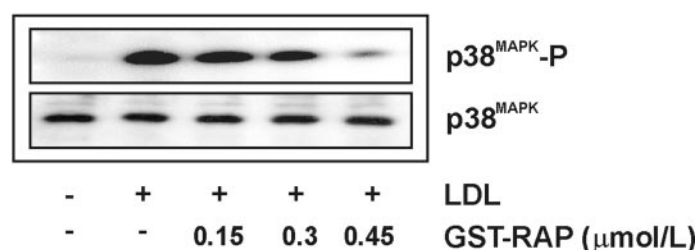
**Statistics**—Data are expressed as means  $\pm$  S.E. with number of observations  $n$  and were analyzed with the Student's  $t$  test for unpaired observations. Differences were considered significant at  $p < 0.05$ .

## RESULTS

**LDL-dependent Activation of p38<sup>MAPK</sup> in the Absence of LDL-R, CD36, SR-BI, or SR-A**—LDL-particles are recognized by the classical hepatic LDL-R through the apoB100 moiety (7). The scavenger receptors SR-BI and CD36 also bind LDL, in contrast to SR-A that only binds LDL after modification (35). To investigate the contribution of these receptors to LDL-dependent platelet sensitization, platelets were obtained from wild-type C57Bl/6 mice and mice that were genetically deficient for one of the following receptors: LDL-R, CD36, SR-BI, or SR-A. These platelets were incubated with freshly purified LDL (1 g/liter) for 5 min, and activation of p38<sup>MAPK</sup> was subsequently assessed by comparing the binding of antibodies directed against dual-phosphorylated p38<sup>MAPK</sup> to the binding of antibodies recognizing total p38<sup>MAPK</sup>. In the absence of LDL, low levels of phosphorylated p38<sup>MAPK</sup> relative to the total amount of the kinase were present in platelets from wild-type as well as the various knockout mice (Fig. 1). As expected, a substantial increase in phosphorylation was observed upon incubation of wild-type platelets with LDL. Moreover, an increase in phosphorylation of p38<sup>MAPK</sup> was also observed when platelets obtained from the various knockout mice were incubated with LDL (Fig. 1). Thus, at least in mice, LDL-mediated p38<sup>MAPK</sup> activation is a process that may occur independently of LDL-R, CD36, SR-BI, and SR-A, suggesting that other LDL-binding receptors are involved.

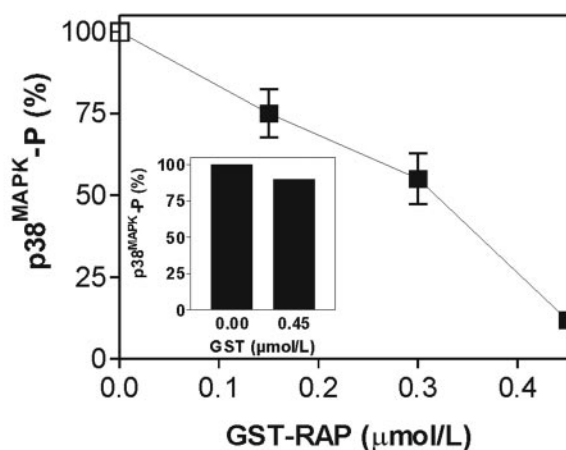
**LDL-induced Platelet p38<sup>MAPK</sup> Activation Requires the Presence of ApoER2'**—Ligand binding to members of the LDL receptor family is blocked in the presence of RAP. To study whether LDL-induced platelet signaling involves a RAP-sensitive receptor, LDL-dependent phosphorylation of p38<sup>MAPK</sup> in human platelets was determined in the absence and presence of GST-RAP (0.15–0.45  $\mu$ mol/liter). As shown in Fig. 2A, similar amounts of p38<sup>MAPK</sup> were present in all samples, and the kinase was efficiently phosphorylated upon incubation of the human platelets with LDL (1 g/liter for 1 min). The presence of GST-RAP, however, was associated with a dose-dependent decrease in p38<sup>MAPK</sup> phosphorylation, with over 70% inhibition at 0.45  $\mu$ M GST-RAP (Fig. 2B). GST alone (0.45  $\mu$ M) failed to affect LDL-induced p38<sup>MAPK</sup> phosphorylation (Fig. 2B, inset). This

A

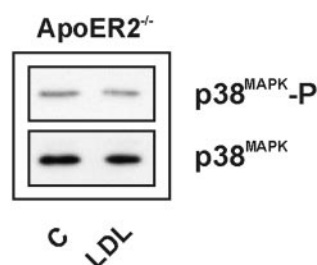


**FIG. 2. LDL-induced p38<sup>MAPK</sup> phosphorylation in the presence of GST-RAP and in platelets lacking apoER2'.** A, platelets were incubated with GST-RAP (0.15, 0.30, and 0.45 μM, 10 min, 37 °C) prior to incubation with LDL (1 g/liter, 1 min, 37 °C). p38<sup>MAPK</sup> phosphorylation was determined as described in the legend to Fig. 1. B, blots were semi-quantified, and the data were expressed as percentage of the p38<sup>MAPK</sup> phosphorylation in the absence of GST-RAP (open symbol). Inset, platelets were incubated with GST alone (0.45 μmol/liter, 10 min, 37 °C) prior to stimulation with LDL. Data are expressed as means ± S.E., *n* = 4. C, platelets from apoER2-deficient mice were stimulated with LDL (1 g/liter, 1 min, 37 °C), and analyzed for p38<sup>MAPK</sup> phosphorylation as described in the legend to Fig. 1.

B



C

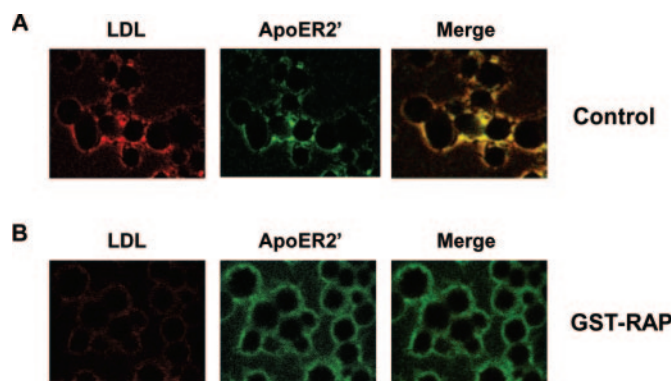


indicates that LDL-dependent activation of p38<sup>MAPK</sup> is mediated by a RAP-sensitive receptor, only one of which has been reported to be present in platelets: apoER2' (14). As such, apoER2' is a candidate receptor for LDL particles at the platelet surface. To test the contribution of this receptor, p38<sup>MAPK</sup> activation was monitored using platelets obtained from apoER2-deficient mice. As shown in Fig. 2C, LDL was unable to induce the phosphorylation of p38<sup>MAPK</sup> in the absence of apoER2'. Thus, these data show that apoER2' is an essential link in the process of LDL-dependent p38<sup>MAPK</sup> activation in platelets.

**ApoER2' and LDL Co-localization on the Platelet Surface**—As apoER2' is indispensable for LDL-dependent phosphorylation of p38<sup>MAPK</sup>, we further examined whether LDL particles indeed are able to interact with this receptor. Therefore, we first analyzed the location of apoER2' and of platelet-bound LDL employing confocal immunofluorescence microscopy analysis. Platelets were incubated with LDL (1 g/liter) in the presence or absence of GST-RAP (0.3 μmol/liter). By using antibodies directed against apoER2 and the LDL constituent apoB100, it was found that both proteins were selectively present at the platelet surface (Fig. 3). Merging of both images suggested a

high degree of co-localization of apoB100 and apoER2', indicating that LDL may bind to or in close proximity of apoER2'. Furthermore, in the presence of GST-RAP only minor amounts of apoB100 were detectable at the platelet surface, demonstrating that GST-RAP interferes with LDL binding to the platelet surface.

**Interaction between s-ApoER2' and LDL**—Since LDL and ApoER2' appear to co-localize at the platelet surface, direct binding studies were performed to address the interaction between both components. First, various concentrations of LDL (0–10 μg/ml) were incubated with immobilized murine ApoER2' (1 μg/well), and bound LDL was detected employing an anti-apoB100 directed antibody. A dose-dependent and saturable binding isotherm was observed, with half-maximal binding at 0.28 μg/ml LDL (Fig. 4A). Little, if any, binding was observed in the presence of EDTA. In a complementary approach, the interaction between this receptor and the LDL constituent apoB100 was examined by SPR analysis. To this end, various concentrations of murine s-apoER2' (50–300 nmol/liter) were perfused over a biotinylated peptide corresponding to the B-site of apoB100 immobilized on a streptavidin sensorchip (239 fmol/mm<sup>2</sup>). Association of soluble apoER2'

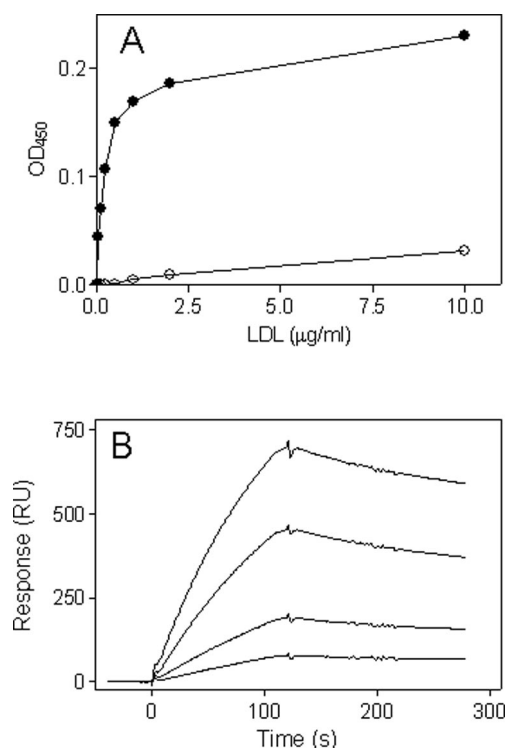


**FIG. 3. Co-localization of LDL with apoER2' on the platelet surface.** LDL-stimulated platelets were incubated with TRITC-labeled monoclonal anti-apoB100 2D8 (red, left panel) and apoER2-FITC (green, middle panel) as described under "Experimental Procedures" with and without preincubation with GST-RAP (0.30  $\mu\text{mol/liter}$ , 10 min) as indicated. The right panel shows a merged picture in which co-localization of apoER2' and LDL can be visualized (yellow).

to the peptide was found to be dose-dependent as the highest response was observed for the highest concentration (Fig. 4B), whereas binding to the control channel was less than 2% compared with the peptide-coated channel (not shown). Analysis of the sensorgrams revealed that the association and dissociation curves were best fitted to a model describing the interaction with a single class of binding sites. The apparent dissociation and association rate constants were calculated to be  $1.21 \pm 0.12 \times 10^{-3} \text{ s}^{-1}$  and  $2.96 \pm 0.33 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ , resulting in an apparent affinity constant of 41 nmol/liter. Hence, the soluble form of apoER2' constitutes a binding site for LDL, and more specifically, the ligand-binding region of the LDL constituent apoB100.

**An Anti-ApoER2 Antibody and LDL Are Similar in Activation of p38<sup>MAPK</sup>**—To further assess the contribution of apoER2' to the activation of p38<sup>MAPK</sup>, human platelets were incubated with anti-apoER2 antibody 186 (1:1000 (v/v) (24)). This antibody has previously been reported to induce dimerization of ApoER2, and does not cross-react with other members of the LDL receptor family (24). As a control, p38<sup>MAPK</sup> phosphorylation was determined similarly after incubation of platelets with LDL (1 g/liter) or in the presence of an anti-LDL-R antibody at the indicated concentrations. As expected, the anti-LDL-R antibody was unable to induce p38<sup>MAPK</sup> activation (Fig. 5C), whereas the presence of LDL was associated with a rapid increase in the amount of phosphorylated p38<sup>MAPK</sup> with a maximum at 1 min (Fig. 5A). This rapid increase was followed by a gradual decline to baseline levels of phosphorylated p38<sup>MAPK</sup> in the following 10 min. A similar time-dependent pattern was detected when platelets were incubated with anti-apoER2 antibody 186, with maximal phosphorylation of p38<sup>MAPK</sup> observed after 1 min (Fig. 5B). This suggests that ligand binding to and/or dimerization of apoER2' is associated with a rapid activation of p38<sup>MAPK</sup>.

**LDL-dependent Phosphorylation of Platelet ApoER2'**—LDL receptor-related proteins have been implicated in various signaling events, and participation in signaling processes may be associated with intracellular phosphorylation of these receptors. We therefore addressed the possibility that association of LDL particles to the platelet surface results in phosphorylation of apoER2'. Human platelets were incubated with LDL (1 g/liter), and at various time intervals apoER2' was withdrawn by immunoprecipitation employing a polyclonal anti-apoER2 antibody. Samples were analyzed for the presence of phosphorylated tyrosine residues within apoER2' using 4G10, an antibody that recognizes tyrosine-phosphorylated proteins. Gel-



**FIG. 4. Interaction between recombinant murine s-apoER2 and LDL.** A, microtiter plates were coated with 10  $\mu\text{g/ml}$  s-apoER2'. After incubation with the indicated amounts of LDL in the presence or absence of 10 mmol/liter EDTA, bound LDL was detected with anti-apoB100 and peroxidase-conjugated anti-goat antibodies as described under "Experimental Procedures."  $A_{450}$  represents optical density at 450 nm. B, various concentrations of soluble murine apoER2' (50–300 nmol/liter) were perfused over a biotinylated peptide corresponding to the B-site of apoB100, which was immobilized onto a streptavidin-sensor chip at a density of 239 fmol/ $\text{mm}^2$ . Perfusion was allowed for 2 min in 150 mmol/liter NaCl, 2.5 mmol/liter  $\text{CaCl}_2$ , 0.005% (v/v) Tween-20, 25 mmol/liter HEPES (pH 7.4) at 25  $^\circ\text{C}$ , after which dissociation was initiated by perfusion with buffer alone. Binding was corrected for binding to a control channel (biotinylated irrelevant monoclonal antibody), which was less than 2% of binding to the peptide-coated channel.

loading was monitored employing an antibody directed against  $\alpha$ -actinin, a protein that co-precipitated in a nonspecific manner. In non-LDL exposed platelets minor amounts of tyrosine-phosphorylated apoER2' could be detected (Fig. 6A). However, incubation of platelets with LDL particles resulted in a marked increase in tyrosine phosphorylation of apoER2', a process that appeared to be maximal after 30 s. Prolonged incubations resulted in a gradual decay of phosphorylated apoER2', although after 20 min its levels were still above basal levels (Fig. 6A). Phosphorylation of apoER2' also proved to be dependent on the concentration of LDL (Fig. 6B). Since the synthetic peptide corresponding to the B-site of the LDL component apoB100 induces p38<sup>MAPK</sup> activation (5), we further tested whether this peptide induced tyrosine phosphorylation of platelet apoER2'. Indeed, incubation of platelets with this peptide was associated with phosphorylation of apoER2' to a similar extent as compared with LDL (Fig. 6C). Previously, it has been suggested that the integrin  $\alpha_{\text{IIB}}\beta_3$  serves as a receptor for LDL on platelets (36, 37). However, tyrosine phosphorylation of apoER2' by LDL remained unaffected in the presence of the integrin  $\alpha_{\text{IIB}}\beta_3$  inhibitors, fibrinogen  $\gamma$ -chain-derived dodecapeptide ( $\gamma 400$ –411) and GPI-562 (Fig. 6D). Thus, both the synthetic apoB100 B-site peptide and native LDL particles induce reversible phosphorylation of the platelet surface receptor apoER2' in a manner that is independent of integrin  $\alpha_{\text{IIB}}\beta_3$ .

**Recruitment of Src Kinases upon LDL Binding to ApoER2'**—Several tyrosine kinases may mediate LDL-dependent tyrosine



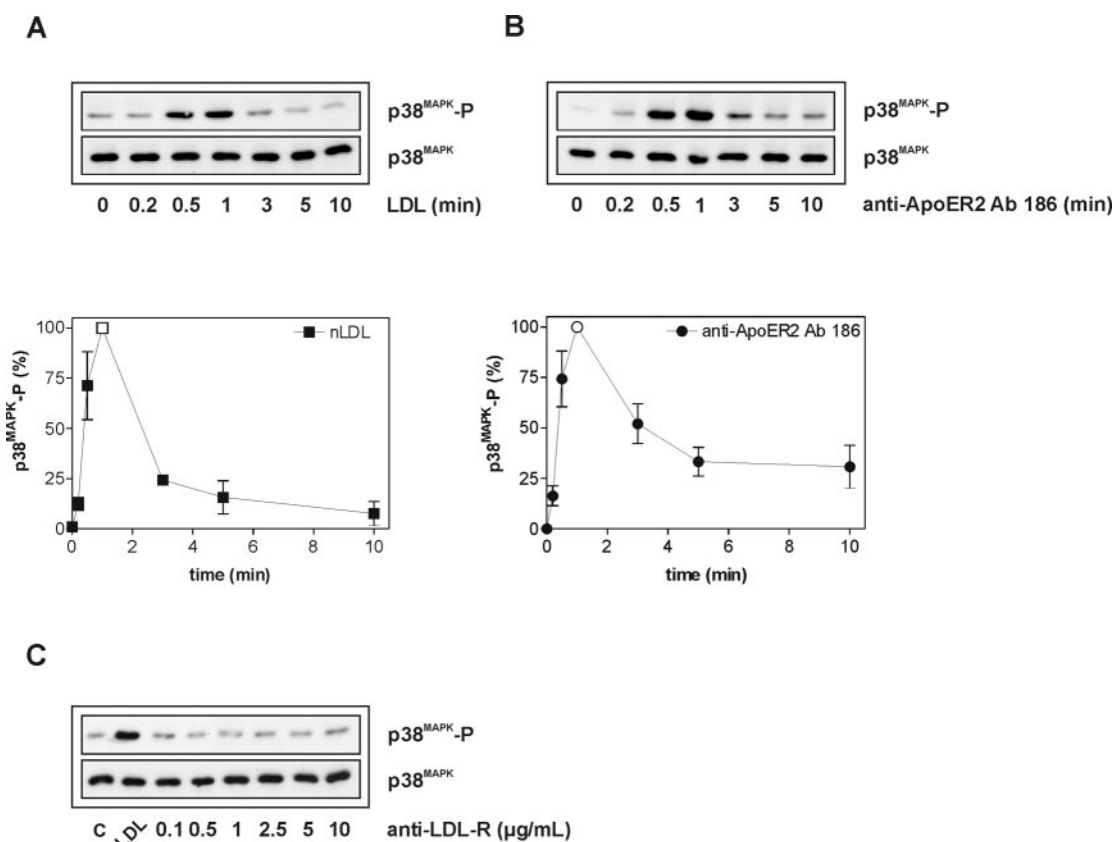


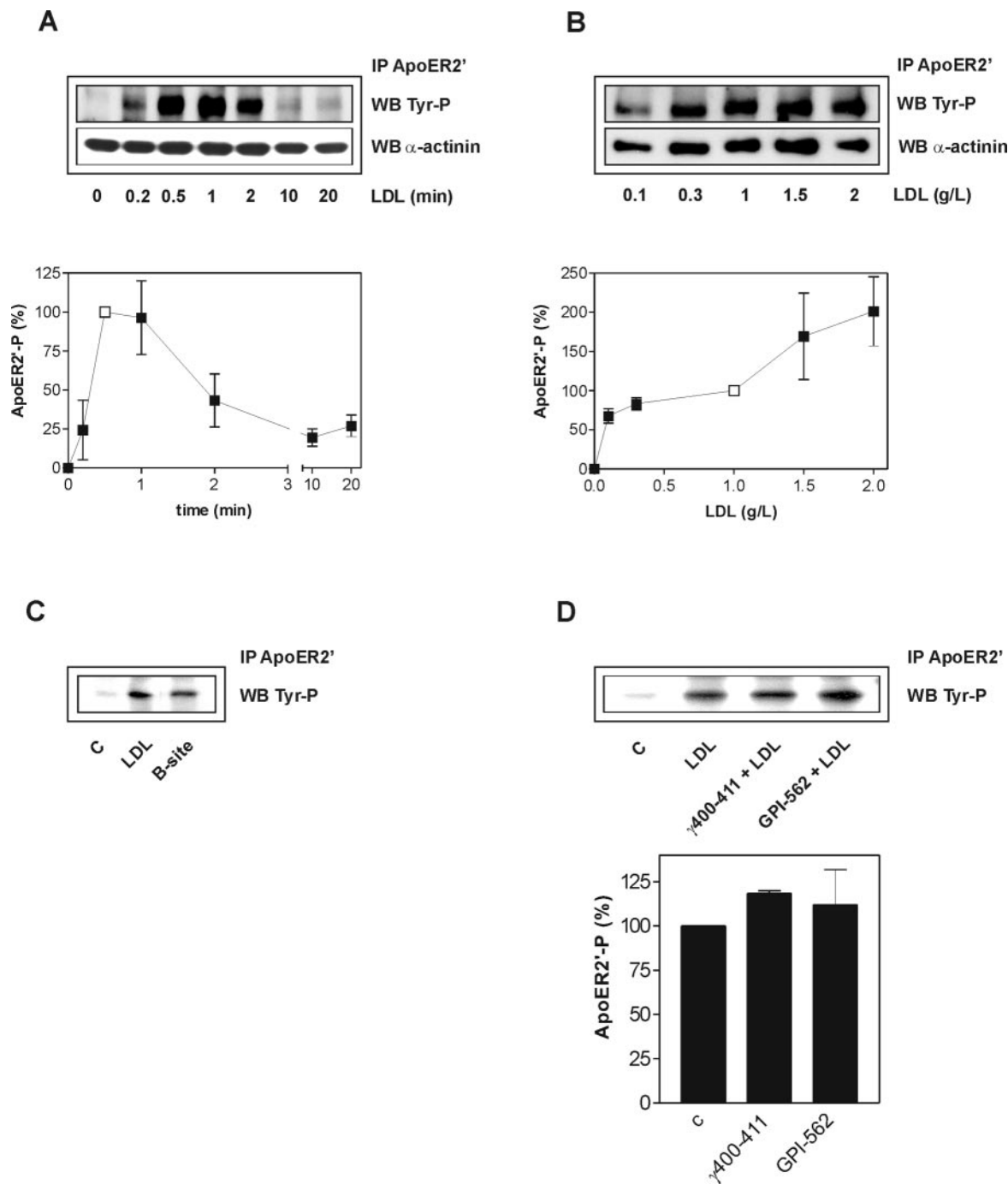
FIG. 5. **Anti-apoER2 antibody-induced phosphorylation of p38<sup>MAPK</sup>.** Human platelets were incubated with LDL (1.0 g/liter, A), anti-apoER2 antibody 186 (1:1000; B) or an anti-LDL-R antibody (0.1–10 μg/ml, C) at 37 °C for the indicated time periods. p38<sup>MAPK</sup> phosphorylation was determined as described in the legend to Fig. 1. The graphs show the semi-quantification of dual-phosphorylated p38<sup>MAPK</sup> from the blots. Data were expressed as percentage of the p38<sup>MAPK</sup> phosphorylation after 1 min of incubation (open symbol). Means ± S.E., *n* = 3.

phosphorylation of the cytoplasmic tail of platelet ApoER2'. It has been reported by others that phosphorylation of LRP1 in fibroblastic cells involves the Src family of tyrosine kinases (38). In addition, we have previously shown that the Src kinase inhibitor PP1 interferes with LDL-dependent phosphorylation of p38<sup>MAPK</sup> (5). Therefore, PP1 was used to examine the role of the Src kinase family in the phosphorylation of apoER2' by LDL. In the absence of LDL and PP1, low levels of phosphorylated apoER2' were present, whereas a marked increase was observed upon incubation with LDL (Fig. 7A). However, preincubation of platelets with PP1 (1, 5 and 10 μmol/liter) reduced LDL-induced apoER2' tyrosine phosphorylation up to 80% (Fig. 7A). This strongly suggests that Src-like tyrosine kinases play a dominant role in the phosphorylation of the apoER2' cytoplasmic tail. To identify the Src-like kinase that contributes to this process, LDL-treated platelets were used for co-immunoprecipitation experiments employing a polyclonal anti-apoER2 antibody. In preliminary experiments, analysis of the precipitates with polyclonal antibodies that recognize multiple members of the Src family, including Fyn, c-Src, Yes, and Fgr, revealed a band migrating with an apparent molecular mass of 55 kDa (data not shown). As the molecular mass of Fgr corresponds to 55 kDa, the precipitates were re-examined employing an antibody specifically directed against Fgr. Minor association of Fgr to apoER2' could be detected in platelets that were not exposed to LDL (Fig. 7B). In contrast, incubation with LDL resulted in a transient association of Fgr to apoER2' with maximal co-precipitation of Fgr at 1–2 min. Thus, association of LDL with the platelet surface leads to association of apoER2' with the Src kinase Fgr.

## DISCUSSION

Association between platelets and LDL particles is believed to result in the formation of hyperreactive platelets, enhancing the risk for thrombotic complications and atherosclerotic plaque formation. Several studies have been directed to the identification of LDL-induced signaling pathways that are responsible for the increased responsiveness of platelets to its various agonists (8, 39, 40). Although much insight has been gained in these signaling pathways, the identification of the platelet surface receptor(s) mediating LDL binding has remained inconclusive. Previously, the platelet integrin  $\alpha_{IIb}\beta_3$  has been suggested to serve as a receptor for LDL, as inferred from ligand blotting and immunofluorescence studies (36, 37). However, specific antibodies directed against integrin  $\alpha_{IIb}\beta_3$  proved unable to inhibit the binding of LDL to platelets. Furthermore, similar binding characteristics were found with platelets obtained from controls and Glanzmann's thrombasthenia patients, who lack integrin  $\alpha_{IIb}\beta_3$  (11). Thus, the involvement of  $\alpha_{IIb}\beta_3$  in LDL binding at the platelet surface is inconclusive (11, 41), leaving the possibility that other receptors are involved in LDL-induced platelet signaling.

In order to identify the platelet receptor for LDL-particles, we focused on LDL-dependent activation of the Ser/Thr kinase p38<sup>MAPK</sup> (8). Phosphorylation of this kinase was not affected by the genetic deletion of various receptors (LDL-R, CD36, SR-BI, and SR-A) (Fig. 1). This could be compatible with a mechanism of redundancy that compensates for the absence of one of these receptors. Alternatively, LDL-dependent p38<sup>MAPK</sup> activation may be mediated independently of these receptors. Indeed, phosphorylation of p38<sup>MAPK</sup> was almost completely inhibited in the presence of GST-RAP, pointing to the involvement of a



**FIG. 6. Tyrosine phosphorylation of apoER2' by LDL.** A, platelets were incubated with LDL (1 g/liter, 37 °C). At the indicated time points, apoER2' was immunoprecipitated from platelet lysates and tyrosine phosphorylation was detected by SDS-PAGE and Western blotting with 4G10, an antibody directed against tyrosine-phosphorylated proteins (upper lane). After stripping, the membranes were reprobed with a monoclonal antibody directed against  $\alpha$ -actinin as a control for equal lane loading (lower panel). The graph shows the semi-quantification of tyrosine phosphorylation of apoER2' relative to the density of the bands representing  $\alpha$ -actinin. Data were expressed as percentage of the density after 1 min of incubation with LDL (open symbol; means  $\pm$  S.E.,  $n = 3$ ). B, platelets were incubated with the indicated concentrations of LDL (1 min, 37 °C), and tyrosine phosphorylation of apoER2' was monitored as described above. Data were expressed as percentage of the density after incubation with 1 g/liter LDL (open symbol; means  $\pm$  S.E.,  $n = 3$ ). C, platelets were incubated with LDL (1 g/liter, 1 min, 37 °C) or B-site peptide (100  $\mu$ mol/liter, 1 min, 37 °C). Tyrosine phosphorylation of apoER2' was determined as described above. D, platelets were incubated with the fibrinogen  $\gamma$ 400–411 peptide (100  $\mu$ mol/liter, 2 min) or the  $\alpha_{IIb}\beta_3$  blocker GPI-562 (10 nmol/liter, 1 min) prior to incubation with LDL (1 g/liter, 1 min, 37 °C). ApoER2' tyrosine phosphorylation was detected as described. Data were expressed as percentage of apoER2' tyrosine phosphorylation in the absence of the blockers of ligand binding to integrin  $\alpha_{IIb}\beta_3$ . (Means  $\pm$  S.E.,  $n = 3$ ).

member of the LDL receptor family in LDL-dependent activation of p38<sup>MAPK</sup> (Fig. 2). This possibility is supported by our observations that (i) the absence of apoER2 in platelets of mice genetically deficient for this receptor resulted in a lack of LDL-dependent p38<sup>MAPK</sup> activation (Fig. 2C); (ii) the LDL-component apoB100 co-localized at the platelet surface with

the platelet receptor apoER2' (Fig. 3A); (iii) GST-RAP interfered with binding of LDL to the platelet surface (Fig. 3B); (iv) soluble apoER2' interacted efficiently with LDL and the apoB100-derived B-site peptide (Fig. 4); (v) LDL particles and an anti-apoER2 antibody were similar in mediating p38<sup>MAPK</sup> activation (Fig. 5); and (vi) apoER2' was rapidly phosphorylated



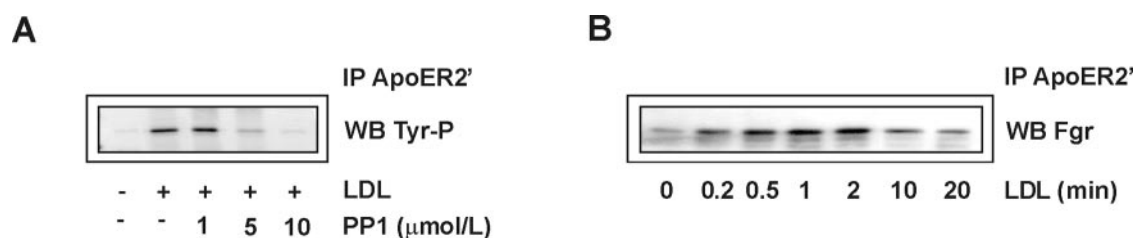


FIG. 7. **LDL-induced phosphorylation of apoER2' is dependent on Src family tyrosine kinases.** A, platelets were incubated with the Src family tyrosine kinases inhibitor PP1 (1, 5, and 10  $\mu\text{mol/L}$ , 15 min) prior to incubation with LDL (1 g/liter, 1 min, 37 °C). Tyrosine phosphorylation of apoER2' was detected as described in the legend to Fig. 5. B, complex formation between apoER2' and Src family tyrosine kinases was monitored by incubating platelets with LDL (1 g/liter, 1 min, 37 °C), followed by the immunoprecipitation of apoER2' from platelet lysates and Western blotting with a polyclonal antibody against c-Fgr.

ated upon the incubation of platelets with LDL particles or with a peptide corresponding to the B-site of the LDL constituent apoB100 (Fig. 6).

The finding that apoER2' serves as a receptor for apoB100-containing LDL particles may seem unexpected in view of the report by Kim *et al.* (16), who observed only minimal binding of  $^{125}\text{I}$ -labeled LDL and VLDL to CHO cells expressing full-length apoER2. In contrast, efficient binding of apoE-rich  $\beta$ -VLDL particles was observed. It should be mentioned, however, that in those experiments the lipoprotein concentrations did not exceed 5 mg/liter, which is far below the physiological LDL plasma concentration (normal range: 0.6 to 1 g/liter) that is used in our experiments. Of note, the binding isotherm of LDL particles to immobilized purified s-apoER2' indicates saturation of the receptor at LDL concentrations below 0.01 g/liter (Fig. 4A), while LDL concentrations of 0.1–0.3 g/liter are sufficient to induce p38<sup>MAPK</sup> activation (8). This suggests that platelet apoER2' would be saturated in normal humans, considering the physiological levels of 0.6–1 g/liter LDL.

Whereas binding of LDL to apoER2' appears to be mediated by the apoB100 moiety, binding of  $\beta$ -VLDL to apoER2 involves the apoE component of these lipoprotein particles. Interestingly, incubation of platelets with full-length apoE or synthetic peptides corresponding to the Arg/Lys-rich sequence of apoE resulted in a decrease in ADP-induced platelet aggregation, a process that could be inhibited by RAP (14). Apparently, apoB100 and apoE seem to have different upstream effects upon binding to apoER2' in that apoB100 governs platelets an increased response toward agonists, whereas apoE has the opposite effect. In this regard, the LDL component apoB100 acts in a similar fashion as dimeric  $\beta$ 2-glycoprotein I, which was recently shown to enhance platelet deposition to collagen and thrombus size via interaction with apoER2' (42). Thus, different ligands may initiate different effects through the same receptor. This conclusion becomes even more intriguing given the observation that the effects of apoE, apoB100 and dimeric  $\beta$ 2-glycoprotein I can be neutralized by GST-RAP (this study and Refs. 14 and 42). In principle, GST-RAP should be considered as a ligand for apoER2', as it shares its interactive region within the receptor with the other ligands. One possibility that may explain these different responses may be related to ligand-dependent association of apoER2' with other cell surface receptors. For instance, LDL-dependent activation of p38<sup>MAPK</sup> in platelets is followed by a de-activation step of this kinase that is mediated by platelet endothelial cell adhesion molecule-1 (PECAM-1) (43). The mechanism underlying the molecular cross talk between apoER2' and PECAM-1 remains to be elucidated, but the possibility exists that both receptors meet at the platelet surface in an LDL-dependent manner. It should be noted in this respect that in preliminary co-immunoprecipitation experiments no association between apoER2' and PECAM-1 could be observed upon incubation with LDL (data not shown).

Incubation of platelets with LDL resulted in the tyrosine phosphorylation of apoER2', a process that was inhibited in the presence of the Src kinase inhibitor PP1. This suggests that apoER2' phosphorylation is mainly dependent on kinases of the Src family (Fig. 7). ApoER2' phosphorylation coincided with association to the Src kinase Fgr (Fig. 7). Thus, ligand binding may facilitate the interaction with Fgr, which in turn might phosphorylate the receptor. Alternatively, the receptor may become phosphorylated upon ligand binding by a so far unidentified Src kinase, which subsequently results in association to the signaling molecule Fgr. This kinase has the potential to act as an activator of p38<sup>MAPK</sup> in neutrophils (44). It seems conceivable that Fgr may serve a similar role in platelets. Fgr also mediates the activation of p125 focal adhesion kinase (FAK) (39). Indeed, incubation of platelets with LDL particles results in a prompt activation of FAK (39). A similar pattern of FAK phosphorylation was observed upon incubation of platelets with anti-apoER2 antibody 186 (data not shown). Apparently, distinct pathways can be initiated upon ligation of apoER2'.

In conclusion, our study strongly suggests that ApoER2' may act as a receptor for LDL at the platelet surface. Furthermore, we demonstrate that LDL binding to platelets results in phosphorylation of apoER2', and this step appears to be critical to activate further signaling cascades. Thus, apoER2' serves a so far unrecognized role in the pre-activation of platelets, and may therefore be used as a target for the development of therapeutic strategies aiming to reduce platelet hyperreactivity. Interestingly, a polymorphism within the *apoER2* gene has recently been reported to be associated with the development of Alzheimer disease (45). It would be of interest to investigate whether this polymorphism or others predispose to thrombotic complications or display a protective effect.

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**Binding of Low Density Lipoprotein to Platelet Apolipoprotein E Receptor 2' Results  
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