rapid communication

Identification and characterization of LRP8 (apoER2) in human blood platelets

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Abstract **Recently, we reported that apoE inhibits platelet reactivity by stimulating NO release and postulated apoE-receptor activation of intracellular NO synthase (eNOS). Here, we implicate a low density lipoprotein receptor (LDL-R) family member by studying ligand requirements using purified apoE isoforms, synthetic peptides, and the receptor antagonist, receptor-associated protein (RAP). Then, using a homology cloning approach and degenerate PCR primers to amplify the conserved Cys-rich binding domain of the LDL-R family, this receptor was identified as LRP8 (formerly termed, apoER2), a newly described brain protein with several splice variants. Immunoprecipitation of platelet membranes with anti-peptide antisera confirmed protein expression, while analysis of RNA from platelets and two megakaryocytic cell lines (Meg-01 and HEL) disclosed that the major LRP8 transcript lacked binding repeats 4–6 (LRP8Δ4–6) but contained the full-length cytoplasmic tail. Sequence analysis of cytoplasmic LRP8 revealed several peptide motifs with potential for cellular signaling and we propose this as a rational mechanism through which apoE inhibits platelet aggregation.**—Riddell, D. R., D. V. Vinogradov, A. K. Stannard, N. Chadwick, and J. S. Owen. **Identification and characterization of LRP8 (apoER2) in human blood platelets.** *J. Lipid Res.* **1999.** 40: **1925–1930.**

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Apolipoprotein E (apoE) is a 34-kDa polymorphic protein in the surface of circulating lipoproteins (1). Of the three major human alleles (ε2, ε3, ε4) the rarest variant, apoE2, is associated with recessive forms of type III hyperlipidemia and differs from apoE3, the common or wild-type form, by an Arg158Cys substitution. The apoE4 allele (Cys112Arg) produces a dominant hyperlipidemia and is a risk factor for restenosis (2) and neurodegenerative diseases, including Alzheimer's disease. ApoE mediates hepatic clearance of lipoproteins through two receptors, the low density lipoprotein receptor (LDL-R) and LRP1 (LDL receptor-related protein 1), although there is a significant allelic influence (E4>E3>>E2) (1). Plasma apoE is largely liver-derived, but apoE released by macrophages has important local effects (3). Low apoE is a risk factor for coronary heart disease and severe hyperlipidemia and atherosclerosis ensue in humans or animals if apoE is dysfunctional or absent (reviewed in ref. 2).

Recently, we discovered that apoE potently inhibits platelet reactivity by stimulating intracellular nitric oxide synthase (eNOS or NOSIII), the NO released acting on soluble guanylate cyclase to elevate levels of anti-aggregatory cGMP (4). A receptor-mediated process was postulated as platelets bound both HDL-E (5) and apoE–phospholipid vesicles (6). ApoE binds to members of the LDL-R family (7), but none have yet been detected in platelets. Here, we identify a splice variant of LRP8 (the HUGO-NC approved gene symbol for apoE receptor 2 (apoER2), also termed LR7/8B) in platelets, which contains several cytoplasmic peptide motifs with potential for cellular signaling, and speculate that binding of apoE by this receptor inhibits platelet aggregation.

MATERIALS AND METHODS

Materials

Recombinant human apoE4, apoE3, and RAP were generous gifts from Dr. Weisgraber (Gladstone Institute) and Dr. Gliemann (University of Aarhus). ApoE2 was purified from VLDL obtained from type III hyperlipidemic patients (8). Synthetic peptides corresponding to sequences within apoE ($E_{263-286}$, $E_{141-155}$, and

Abbreviations: apoE, apolipoprotein E; apoER2, apoE receptor 2 (LRP8); DMPC, dimyristoylphosphatidylcholine; DEPC, diethyl pyrocarbonate; HDL, high density lipoprotein; LDL-R, low density lipoprotein receptor; LRP, LDL receptor-related protein; eNOS, endothelial nitric oxide synthase; PRP, platelet-rich plasma; PTB, phosphotyrosine-binding; RAP, receptor-associated protein; RT-PCR, reverse transcriptase-polymerase chain reaction; SH3, Src-homology 3; VLDL-R, very low density lipoprotein receptor.

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$E_{(141-155)2}$) and LDL-R-deficient CHO cells expressing full-length LRP8 (CHO^{LRP8}) were from Dr. Harmony (University of Cincinnati) and Drs. Soutar and Sun (Hammersmith Hospital), respectively. All chemicals, unless otherwise stated, were supplied by Sigma.

Platelet aggregation

ApoE isoforms and peptides were incorporated into small, unilamellar vesicles of dimyristoylphosphatidylcholine (DMPC) and, as described previously (4), used in platelet aggregation studies with ADP as an agonist. All experiments were repeated several times and were qualitatively reproducible. Results are expressed as means ± SEM, and statistical differences were determined by Student's two-tailed unpaired *t* test.

mRNA extraction and cDNA production

Polyadenylated RNA was extracted from washed platelets (200 ml of fresh blood) and from HEL, Meg-01 and CHO^{LRP8} cells (10^7 cells) using a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech). One μg mRNA was converted to cDNA using the GeneAmp RNA PCR Kit (PE Biosystems).

Homology cloning

Oligonucleotide primers used for PCR amplification were based on alignment of published cDNA sequences corresponding to the highly conserved Cys-rich LDL-R class A binding domains from human LDL-R family members. The primer sequences incorporated restriction sites to facilitate cloning of PCR products. The sense primers (A1: 5′-GATCGGATCCTG(C/T)(C/G)(A/C)(G/T)GATGG(C/T)TC(T/C/A)GATGA-3′ and A2: 5′-GATCGGATCCTCTGGGGA(C/T)(G/A)(G/A)CAGTGA(C/T)-3′) contained a BamHI site (underlined), and the antisense primer (A3: 5′-GATCGAATTC(C/T)C(G/A/C)CC(A/G)TC(A/G)CA(G/T)(C/A)(G/T)CCA-3′) an EcoRI site (double underlined). Five μl of the reverse transcription reaction mixtures was subjected to "hot-start" PCR using the GeneAmp AmpliTaq Gold PCR DNA polymerase system (PE Biosystems) with 10 μm A1/A2 and 10 μm A3 in a total volume of 50 μl. Amplification proceeded for 40 cycles, with primer annealing at 53°C. One-tenth (5 μl) of the reaction mixture was then subjected to a second round of PCR amplification to increase detection of rare transcripts. Products of interest were digested with EcoRI and BamHI, gel purified, and cloned into pUC18 for automated fluorescent sequencing.

Specific amplifications of platelet, HEL, and Meg-01 cDNA

To confirm the presence of LRP8, a specific sequence from the O-linked sugar domain to the cytoplasmic insert was amplified, carrying out "hot-start" PCR on platelet, HEL and Meg-01 cDNA as outlined above (sense primer: 1 μm of oligonucleotide 2092 5′-GGAGG(C/A)TGTGAATACCT(G/A)TGC-3′; antisense primer: 1 μm of oligonucleotide 2696 5′-CGATCAAAGCTGCTG ATTGC-3′). To detect LRP8 splice variants, the region corresponding to the ligand-binding domain was amplified using the conditions and primers (oligonucleotide 24: 5′-TCTCCGGCT TCTGGCGCT-3′ and oligonucleotide 1114: 5′-TCTGGTCCAGG AGCTGGAA-3′) described elsewhere (9). Because multiple products of unexpected length were obtained due to mispriming, the ligand-binding domain PCR products were subjected to Southern blotting using 10 fmol/ml of an internal oligo 5′-^{32}P-labeled probe (oligonucleotide 71: 5′-TGCGGCTCCAGCATCTTG-3′) for hybridization. Finally, to amplify the full-length open reading frame of LRP8, the Advantage-GC PCR kit (Clontech) was used (sense primer: oligonucleotide 24; antisense primer: oligonucleotide 2918 5′-GAGGCACGAAGGGGGTGAT-3′, both at 300 nm). Reaction products were cloned into pCRII (Invitrogen) and restriction mapped using BlpI, EcoRI, BstYI, HindIII, SmaI, AvaII, SacI, HincII, PstI, BbsI and XhoI; appropriate fragments were sub-cloned into pUC18 and sequenced.

Antibody production and immunoprecipitation

A 17-amino acid sequence (residues 865–881: clgetrepedpapalke) within the cytoplasmic insert of human LRP8 was conjugated to KLH and used to immunize rabbits for production of antipeptide antisera. Platelets (10^9 cells) and CHO^{LRP8} (10^6 cells) were surface labeled using the ECL protein biotinylation module (Amersham Pharmacia Biotech), lysed with 1% Triton X-100 and subjected to immunoprecipitation. Precipitated proteins were analyzed by Western blotting and any biotin-labeled, cell-surface proteins detected with streptavidin-horseradish peroxidase and chemiluminescence.

RESULTS

Inhibition of platelet aggregation by RAP, synthetic apoE peptides, and apoE isoforms

Several approaches have shown that an Arg/Lys-rich sequence within apoE (residues 140–150) is bound by the "class A repeats" at the N-terminus of LDL-R family members (reviewed in ref. 1). To assess whether the anti-platelet action of apoE requires a similar interaction, we studied ligand requirements. RAP, the 39-kDa intracellular chaperone glycoprotein that facilitates folding and processing of LDL-R family members, competes with apoE for receptor binding (7, 10). When apoE–DMPC vesicles and RAP were coincubated, the anti-platelet action of apoE was significantly reduced (30.6 ± 6.6 vs. 62.5 ± 2.9% inhibition; $P < 0.001$, n = 3) (**Fig. 1A**,**B**). By contrast, RAP alone had no effect on aggregation compared to untreated controls (9.8 ± 6.6 vs. 0.0 ± 2.8% inhibition; $P > 0.05$, n = 3) (Fig. 1A), implying that the receptor–ligand interaction stimulating release of NO (4) was specific to apoE.

The monomeric peptide ($E_{141-155}$; lrklrkrllrdaddl) and its tandem ($E_{(141-155)2}$; lrklrkrllrdaddl-lrklrkrllrdaddl) both inhibited ADP-induced aggregation in a dose-dependent manner (26.5 ± 2.9% and 26.4 ± 5.8% inhibition at 3.0 μm peptide, respectively; both $P < 0.05$, n = 3; Fig. 1C), although less potent than full-length apoE (70.1 ± 7.3% inhibition; $P < 0.001$, n = 3). By contrast, a control peptide within the carboxyl-terminal 10-kDa of apoE ($E_{263-286}$; swfeplvedmqrqwaglvekvqaa) lacked anti-platelet activity (4.2 ± 3.1% inhibition at 3.0 μm peptide, $P > 0.05$, n = 3).

The three major apoE isoforms, E3, E2, and E4, suppressed ADP-induced aggregation of washed platelets to a similar degree (70.0 ± 8.3%, 81.8 ± 8.4%, and 74.9 ± 7.7% inhibition, respectively, at 50 μg (1.47 μm) protein/ml of apoE–DMPC; $P > 0.05$, n = 3; Fig. 1D). This suggests that the LDL-R or LRP1 are not involved as these bind apoE2 inefficiently (11, 12). By contrast, the VLDL-R, which has similar affinities for each apoE isoform (13), or one of the newly recognized apoE receptors, LRP2-LRP8 or LR11 (7, 14), remain candidates.

Identification of the platelet receptor

The above data implicate an LDL-R family member as the mediator through which apoE suppresses platelet ag-

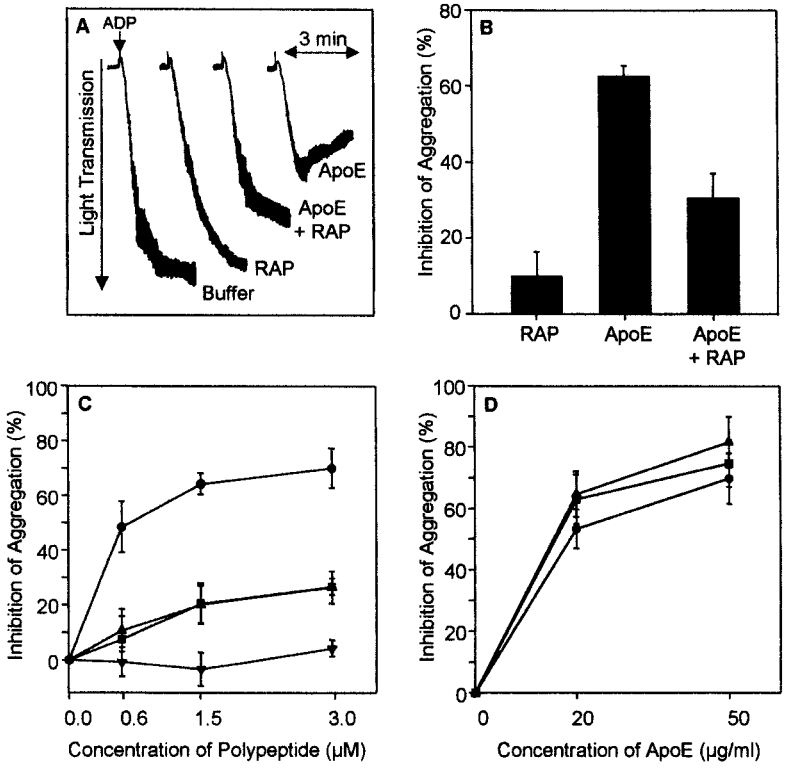

Fig. 1. Effects of RAP, synthetic apoE peptides and apoE isoforms on ADP-induced platelet aggregation. A, B: aliquots of PRP ($1–2 \times 10^8$ cells/ml) were pre-incubated with either 1.5 μm apoE-DMPC or 1.5 μm RAP, or both apoE-DMPC and RAP, for 10 min at 20°C. A pre-determined threshold concentration of ADP (4) was then added and the extent of aggregation was measured after 3 min at 37°C and expressed as a percentage of controls with buffer alone. Typical aggregation traces are shown in (A), while the mean percentage inhibition of aggregation (±SEM) for three different platelet preparations is given in (B). C, D: apoE or apoE peptide sequences were complexed with DMPC vesicles and increasing amounts were pre-incubated with washed platelets (3×10^8 cells/ml) for 30 s at 37°C before initiation of aggregation by addition of ADP. The inhibitory effects of $E_{141-155}$ (■) and $E_{(141-155)2}$ (▲) are compared to those of $E_{263-286}$ (▼) and apoE3-DMPC (●) in (C), while the anti-platelet actions of apoE2-DMPC (●), apoE3-DMPC (▲), or apoE4-DMPC (■) are shown in (D). All points are the mean percentage inhibition of aggregation (±SEM) for three individual platelet suspensions.

gregation. A homology cloning approach was used to detect a known or unknown platelet receptor of this gene family, targeting the conserved Cys-rich binding domain. Two different degenerate sense primers (A1 and A2) and one antisense degenerate primer (A3) were designed with the potential to recognize all known members of the LDL-R family but, because human peripheral blood platelets are anucleate fragments of precursor megakaryocytes and contain only small amounts of residual mRNA, two well-characterized human leukemia megakaryoblastic cell lines, HEL and Meg-01 (15), were also studied.

Although use of the A1/A3 and A2/A3 primer pairs and HEL mRNA did not detect LDL-R, VLDL-R, LRP1, LRP2 (GP330), LRP3–LRP7 or LR11, it did generate a

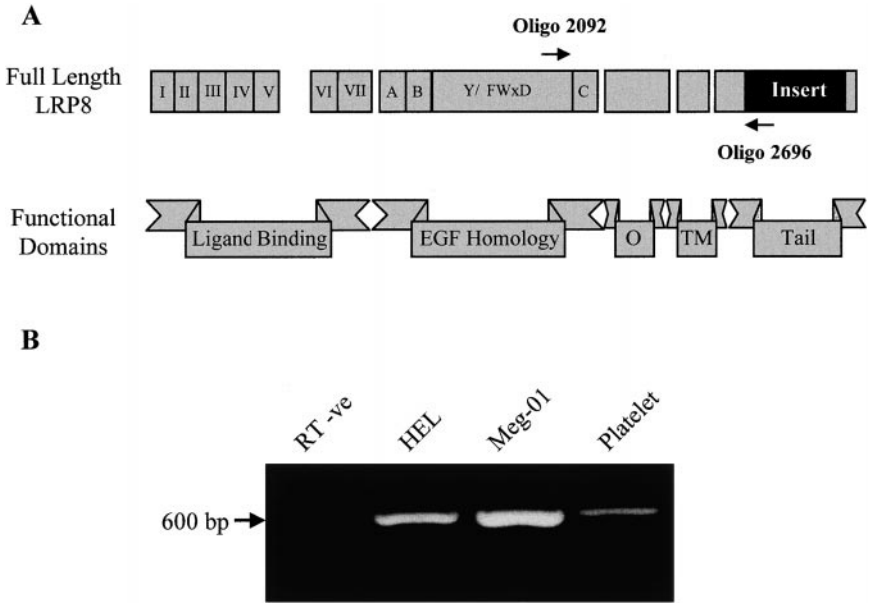

Fig. 2. Identification of LRP8 in platelets by specific RT-PCR amplification. A: the main structural features of LRP8 are indicated, including the LDL-R class A repeats (I–VII), epidermal growth factor (EGF) homology repeats, Y/FWxD repeats, O-linked sugar domain (O), transmembrane region (TM), and cytoplasmic tail containing the 59 amino acid insert (insert). B: HEL, Meg-01, and platelet cDNA, synthesized from mRNA using reverse transcriptase, were subjected to PCR amplification with specific primers (indicated in A) and the products were separated on 2% agarose gels.

120 bp product with 100% identity to human LRP8, a receptor expressed predominantly in brain (16, 17). To confirm the presence of LRP8 in cells of the megakaryocytic lineage, RT-PCR was performed on mRNA from platelets, HEL, and Meg-01 cells using specific primers, the antisense primer being directed against the insertion sequence of 177 bp (59 amino acids) found only in cytoplasmic LRP8 (**Fig. 2A**). As predicted, a ~600 bp product was amplified from each cell type (Fig. 2B) and sequencing confirmed this was from human LRP8 cDNA.

Alternative splicing of platelet LRP8

Genomic and cDNA studies of brain LRP8 show several splice variants, including those lacking binding repeats 4–6 or 4–7 (LRP8Δ4–6/7) and/or the cytoplasmic insert (9). To determine which variant megakaryocytic cells express, long-range RT-PCR was carried out on HEL and Meg-01 mRNA. A ~2500 bp product was obtained from both cells, substantially shorter than the 2894 bp of full-length LRP8 present in CHO^{LRP8} (**Fig. 3A**). Cloning, restriction mapping and sequence analysis identified this product as LRP8Δ4–6, i.e., without repeats 4–6 in the binding domain but containing the 59-amino acid cytoplasmic insert. To confirm the absence of binding repeats 4–6, RT-PCR was carried out on HEL, Meg-01, and platelet mRNA using published primers that flank the ligand-binding domain of LRP8. As previously reported (9), multiple products of unexpected length were obtained, most probably due to mispriming. However, Southern hybridization using an internal probe radiolabeled with ^{32}P gave the predicted major band of 704 bp (Fig. 3B).

Immunoprecipitation of platelet LRP8

Identification of platelet LRP8 mRNA by the sensitive technique of RT-PCR does not prove expression of protein, while the instability of residual platelet mRNA impedes Northern blot analysis. Therefore, immunoprecipitation of platelet lysates was used to verify that receptor protein was present. A band with reduced molecular mass of ~130 kDa was readily detected using polyclonal antisera raised against a 17-mer synthetic peptide within the cytoplasmic insert of LRP8 (Fig. 3C), confirming that mature LRP8 was expressed. The difference in molecular mass between CHO cell and platelet LRP8 (~180 kDa vs. ~130 kDa), presumably reflects the deleted 4–6 ligand binding domains in the platelet receptor.

DISCUSSION

Earlier, we reported that apoE regulates platelet homeostasis by augmenting intracellular NO synthesis in re-

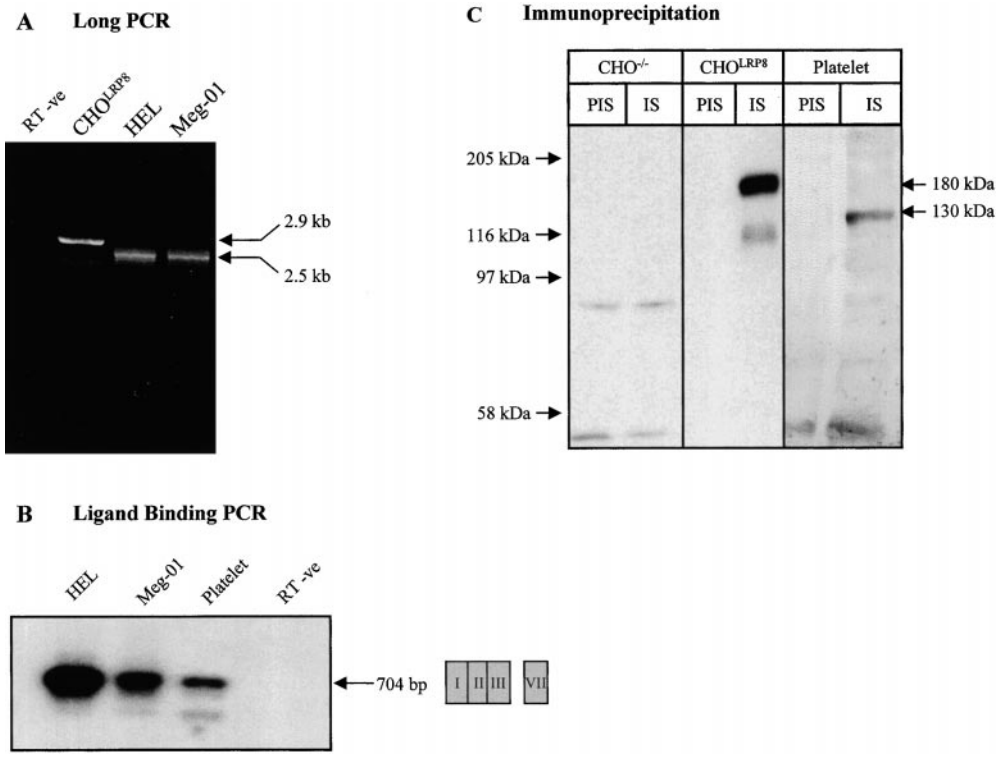

Fig. 3. Expression of LRP8Δ4–6 in platelets. A: the full-length open reading frame of LRP8 was amplified by long-range PCR of CHO^{LRP8}, HEL, and Meg-01 cell cDNA. B: the ligand-binding domain of platelet, HEL, and Meg-01 cDNA was amplified in PCR reactions, the products separated in 1% agarose gels and then Southern blotted with a 5′-^{32}P-labeled internal oligonucleotide probe. C: intact platelets, CHO^{LRP8} and non-transfected CHO cells (CHO$^{-/-}$) were biotinylated, solubilized, and immunoprecipitated with either pre-immune sera (PIS) or immune sera (IS) raised against a 17-mer synthetic peptide within cytoplasmic LRP8. Precipitated cell-surface proteins were reduced, separated by 8% SDS-PAGE, and electrotransferred to nitrocellulose for streptavidin-horseradish peroxidase detection.

sponse to an agonist-induced flux of Ca^{2+}. We speculated that occupation of specific receptors by circulating apoE "primes" platelets to help attenuate activation when challenged by agonists or other stimuli (4). The data presented herein indicate that LRP8 is the likely means through which apoE inhibits platelet aggregation.

Platelet apoE receptors were first implicated by binding studies with radiolabeled ligands (5, 6). Our current aggregation studies indicated an LDL-R family member, consistent with the observation that apoE in free solution or with neutralized arginine residues, both of which prevent interaction with the LDL-R (1), was not inhibitory (4). Thus, the receptor antagonist RAP (10), effectively blocked the apoE anti-platelet effect, whereas the synthetic peptide, $E_{141-155}$, encompassing the apoE recognition domain inhibited aggregation. However, the LDL-R or LRP1 are unlikely to be involved: the dimeric peptide $E_{(141-155)2}$, which has a higher α-helical content and greater affinity for these receptors (18), was no more effective than the monomeric peptide; and apoE2, which interacts weakly with the LDL-R or LRP1 (1, 12), was as anti-aggregatory as apoE3.

We opted for an homology cloning approach to identify the platelet apoE receptor, rather than sets of specific primer pairs, as this would potentially amplify an unidentified LDL-R family member. This possibility was plausible: there are recent additions to the LDL-R family (7, 14), while the multiple class A domains which bind apoE and other ligands also occur in several related proteins. Hence, we designed primers directed against the Cys-rich class A binding domains with sufficient degeneracy to recognize all known members of the LDL-R family. In addition, as the domains are highly conserved within each receptor (7), as well as amongst known LDL-R family members, our primers would potentially recognize several annealing sites and thus increase detection opportunities. Nevertheless, the only receptor sequence amplified was that of LRP8, an unexpected finding as this receptor is abundant in human brain and placenta but reported absent from other tissues (16, 17).

LRP8 has five functional domains (Fig. 2A), but while the VLDL-R has eight class A repeats, LRP8 and LDL-R contain seven. However, the ligand-binding domain structure of LRP8 more closely resembles that of the VLDL-R; their class A repeats have 45–63% amino acid homology and both bind RAP and apoE-containing lipoproteins with high affinity (16, 19). Indeed, our finding that apoE2 is an effective inhibitor of platelet aggregation is consistent with a VLDL-R-like mediator (13). Expression of LRP8 protein in platelets and megakaryocytic cells was confirmed by anti-peptide LRP8 antibodies and, although the splice variant lacked binding repeats 4–6, the significance of this is uncertain as LRP8Δ4–7 and full-length LRP8 bind apoE-rich β-VLDL with equal affinity (9, 19).

Does identification of the platelet apoE receptor as LRP8 allow a rational explanation for inhibition of platelet aggregation? LRP8, like other LDL-R family members, contains the cytoplasmic sequence ΨxNPxY (where Ψ is hydrophobic) required for internalization via clathrin-coated pits (7). However, this motif can act as a ligand for the phosphotyrosine binding (PTB) domains of signaling molecules, including Fe65 neuronal protein and mDab (20, 21) and may also couple LRP1 to intracellular GTPase activity (22). Significantly, the cellular role of LRP8 is unlikely to involve lipoprotein uptake and degradation (19), implying an alternative function for this motif in LRP8. We have also identified three proline-rich motifs within the cytoplasmic insert which fulfill minimal consensus sequences (PxxP) for Src homology 3 (SH3) recognition; the first, **R**e**P**ed**P**a**P**, is a strong candidate for high-affinity binding as it is flanked by arginine and an additional proline (23). Finally, Thr^{815} (RKNT) and Thr^{829} (RKTT) are potential targets for cGMP- or cAMP-dependent protein kinases (24). It is tempting, therefore, to speculate that the anti-aggregatory effect of apoE is mediated through LRP8 and PTB- and/or SH3-dependent protein–protein interactions; though poorly understood, eNOS activity is regulated by protein–protein interactions and phosphorylation at multiple sites (25).

In conclusion, we have established that the apoE–NO link in platelets is mediated by an LDL-R family member and, after identifying and characterizing a LRP8 variant in human blood platelets, have proposed cellular signaling as a rational mechanism by which apoE inhibits aggregation. Unfortunately, the anucleate nature of platelets makes it difficult to manipulate experimentally. Hence, probing functional coupling of LRP8 and eNOS and unravelling mechanistic details must await further studies, including co-expression of these proteins in cultured cells.

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