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Localization of apolipoprotein E receptor 2 to caveolae in the plasma membrane

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Abstract The LDL receptor (LDL-R) promotes the specific endocytosis and lysosomal delivery of extracellular lipoprotein ligands via clathrin-coated pits. It was widely assumed that other closely related members of the LDL-R gene family would have similar functions, but recent experimental evidence has revealed that one such protein, apolipoprotein E receptor 2 (apoER2), has a critical role as an "outside-in" signal transducer in the brain. ApoER2 signaling appears to require interaction between its cytoplasmic domain and adapter molecules such as Dab1, JIP 1 and JIP 2, and PSD-95. Many of the receptors for other signaling pathways affected by such adapter molecules are compartmentalized into specialized microdomains within the plasma membrane termed caveolae. Here, we show that apoER2, but not LDL-R, is localized to caveolae, supporting the concept that its physiological role is in cell signaling, rather than in endocytosing ligands.—Riddell, D. R., X-M. Sun, A. K. Stannard, A. K. Soutar, and J. S. Owen. **Localization of apolipoprotein E receptor 2 to caveolae in the plasma membrane.** ***J. Lipid Res.*** **2001.** 42: **998–1002.**

Supplementary key words intracellular signaling • lipid raft • LDL-R family • LRP8

The cDNA for the apolipoprotein E receptor 2 (apoER2) was cloned from a human placenta cDNA library on the basis of homology with the cytoplasmic-coated pit signaling domain of the LDL receptor (LDL-R) (1). ApoER2 shows strong homology with the LDL-R and the VLDL receptor, both in primary protein structure and in the position of intron:exon boundaries of the genes (2). In common with other LDL-R family members, apoER2 contains the cytoplasmic sequence NPxY required for internalization of the LDL-R via clathrin-coated pits (3). However, this motif is also a ligand for the phosphotyrosine binding and PDZ domains of signaling molecules (4), some of which can bind to apoER2 (5–7). We have shown that the cellular role of apoER2 is unlikely to involve lipoprotein uptake and degradation (8), a conclusion recently substantiated by direct analysis of the endocytic function of cytoplasmic apoER2 (9). This implies an alternative function for the NPxY motif in apoER2. Indeed, there is increasing evidence that apoER2 is a signal transducer molecule, regulating neuronal migration during brain development (5), and perhaps moderating platelet aggregation in the vasculature (10). Signaling appears to require interaction between the cytoplasmic domain of apoER2 and adapter molecules such as Dab1, JIP 1 and JIP 2, and PSD-95 (5–7).

Caveolae are specialized membrane microdomains that exist in many cells as vesicular invaginations of the plasma membrane. Enriched in cholesterol, sphingolipids, and structural proteins of the caveolin family, caveolae sediment with a low buoyant density (11, 12). Membranes of similar composition and properties, termed lipid rafts (12), have also been isolated from neurones, including neuroblastoma cell lines that contain little caveolin (13). Many of the intermediates of signaling cascades proposed to be initiated by apoER2 including src, fyn, abl, eNOS, and PSD-95 (14) are enriched in membranes of low buoyant density. The possibility, therefore, that caveolae or lipid rafts could serve as an important locus of apoER2-mediated signaling prompted us to examine the subcellular distribution of this protein. Here, we show by cell fractionation methods that apoER2, but not LDL-R, is enriched in low density caveolin-containing domains of the plasma membranes in recombinant Chinese hamster ovary (CHO) cells expressing human apoER2.

MATERIALS AND METHODS

Materials

Monoclonal antibodies against caveolin-1 (clone C060) and flotillin-1 (clone 18), and affinity-purified polyclonal rabbit anti-human caveolin (product C13630) were obtained from Trans-

Abbreviations: apoE, apolipoprotein E; apoER2, apoE receptor 2; CHO, Chinese hamster ovary; CLM, caveolin-rich light membranes; LDL-R, LDL receptor.

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duction Laboratories (Lexington, KY). Polyclonal antibodies against transferrin receptor and clathrin heavy chain were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Specific antisera against apoER2 and the LDL-R were produced by immunizing rabbits with peptides conjugated to keyhole limpet hemocyanin (residues TEEEDEDELHIGRTAC within the cytoplasmic tail of apoER2 or residues VGDRCERNEFQCQDGK in the first ligand-binding repeat of the LDL-R). Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes and the ECL Western-blotting detection system were purchased from Amersham Pharmacia Biotech (Little Chalfont, UK), as were some secondary antibodies; others were from DAKO Ltd (Ely, UK). Protease inhibitor cocktail tablets (Complete™ minus EDTA) were from Roche Diagnostics (Welwyn Garden City, UK). The different CHO cell lines expressing apoER2 and its splice variants (8) or the human LDL-R (15) have been previously described. All other reagents were obtained from Sigma (Poole, UK).

Isolation of caveolin-rich light membranes (CLM)

The CHO cell monolayers were grown to confluence in 175-cm^2 flasks. The CLM were prepared using a detergent-free method as described by Waugh et al. (16). All procedures were carried out at 4°C. Briefly, CHO cells from a 175-cm^2 flask were washed, scraped into 2 ml of MBS buffer (25 mM morpholinoethane sulfonic acid, 150 mM NaCl, pH 6.5) containing 500 mM sodium carbonate, protease inhibitors, and 1 mM N-ethylmaleimide (NEM), and disrupted by sonication (12 microns peak-to-peak, 6 × 10 s). Cell homogenates were mixed with 2 ml of 90% (w/v) sucrose in MBS and transferred to a 12-ml ultracentrifuge tube. A discontinuous sucrose gradient was formed above the homogenate by layering on 4 ml of 35% (w/v) sucrose in MBS, followed by 4 ml of 5% (w/v) sucrose in MBS. After centrifugation at 39,000 rpm for 18 h in a Beckman SW41 rotor at 4°C, 12 fractions (1 ml) were collected from the top of each tube and their protein content measured with the Bradford reagent (Bio-Rad, Hemel Hempstead, UK). Fractions 4–6 at the interface between the 5% and 35% (w/v) sucrose boundaries were designated the CLM fraction.

In some experiments, CLM were isolated from detergent-solubilized cells as described previously (17, 18). Briefly, cells from two 9-cm dishes were washed and scraped into 1 ml of 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 2 mM EGTA, 20 mM CHAPS, protease inhibitors, and 1.0 mM NEM, and solubilized by passage 10 times through a 25-g needle. Part of the extract (0.75 ml) was mixed with 90% (w/v) sucrose in MBS (0.75 ml) and, after sequentially overlaying with 1.5 ml each of the 35% and 5% sucrose solutions, was fractionated by centrifugation at 40,000 rpm in a Beckman 50.1 rotor for 18 h at 4°C. As before, 12 consecutive fractions (0.36 ml) were collected for analysis.

Immunoblotting

Samples were separated either by SDS-PAGE on 4–20% gradient gels (Invitrogen, The Netherlands) or on linear minigels containing 7.5–13% acrylamide. Proteins were transferred to Hybond ECL membranes and probed with antibodies against apoER2, LDL-R (both diluted 1:3,000), caveolin-1, clathrin heavy chain, transferrin receptor, or flotillin-1 (all at 1:1,000 dilution).

Immunoprecipitation

For the immunoisolation of caveolae, pooled CLM samples (fractions 4–6) were concentrated 10-fold by centrifugation (Vivascience concentrator with 10,000 MWCO; Sartorius Ltd, Epsom, UK) precleared with 20 μl of Dynabeads M-280 (Dynal, Bromborough, UK) coated with sheep anti-rabbit IgG. The cleared sample was incubated with anti-caveolin polyclonal Ig (final concentration, 1 μg/ml) for 90 min at 4°C, followed by the addition of 20 μl Dynabeads for 90 min at 4°C. Immunocomplexes were collected without centrifugation using magnetic separation, and washed four times in 20 mM Tris, 150 mM NaCl, pH 7.4. Immunoprecipitated caveolin was detected by immunoblotting using monoclonal caveolin antibody.

RESULTS AND DISCUSSION

Subcellular localization of apoER2 in CLM fractions

The CLM were isolated from recombinant CHO-A7 cells expressing either human apoER2 cDNA (CHO^{ER2}) or the human LDL-R cDNA (CHO$^{LDL\text{-}R}$). Initially, the cells were solubilized by sonication in buffer containing 500 mM sodium carbonate and extracts fractionated by discontinuous density-gradient ultracentrifugation. Immunoblotting of gradient fractions for caveolin-1 and flotillin-1 showed that these two integral membrane protein markers for caveolae were enriched in fractions 4–6 at the interface between the 5% and 35% sucrose layers (**Fig. 1**), corresponding to the CLM fraction (16). In keeping with the findings of others (16), the CLM fractions were found to

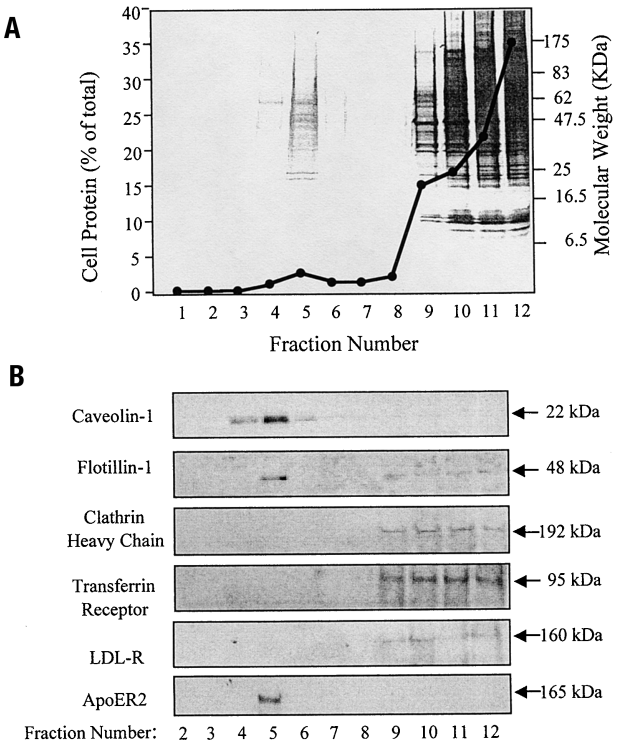

Fig. 1. ApoER, but not the LDL-R, localizes in CLM isolated by density-gradient untracentrifugation on non-detergent-solubilized cells. Cells from one 175-cm^2 flask of CHO cells expressing either the LDL-R or apoER2 were sonicated in 2 ml of buffer containing 500 mM sodiom carbonate, and the homogenates fractioned on a 5–35% (w/v) discontinuous sucrose density gradient. A: Fractions (12 × 1 ml) were collected and assayed for protein concentration and content by reduced SDS-PAGE, followed by silver staining (4–20% acrylamide gels; 15 μl of each fraction per lane); the position of molecular weight marker proteins is shown on the right. B: Replicate gels were immunoblotted with antibodies against caveolin-1, flotillin-1, clathrin heavy chain, transferrin receptor, apoER2, and LDL-R as indicated; the approximate molecular mass weight of each protein is shown on the right.

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contain less than 5% of the total cellular protein, the bulk of which remained in the 45% sucrose layer at the bottom of the ultracentrifuge tube. The clathrin-coated pit-associated proteins, clathrin heavy chain and transferrin receptor, also remained at the bottom of the tube (Fig. 1).

When we probed the gradient fractions of $CHO^{LDL\text{-}R}$ cells with a polyclonal antibody to a peptide at the amino terminus of the LDL-R, we found that the receptor remained, as expected (19, 20), in the 45% sucrose layer together with clathrin heavy chain and transferrin receptor. Interestingly, in extracts of CHO^{ER2} cells, apoER2 was not detected in these fractions but, instead, was concentrated in the CLM fraction with caveolin-1 and flotillin-1 (Fig. 1). Similar results were obtained when the cells were solubilized in buffer containing the zwitterionic detegent, CHAPS, which has also been used to isolate caveolae-enriched membrane fractions (17, 18). As shown in **Fig. 2**, the LDL-R and transferrin receptor both remained at the bottom of the gradient with clathrin, whereas apoER2 floated at the 5–35% interface with caveolin-1. Thus, using two different methods of isolating CLM, apoER2 has been found to co-localize with caveolin.

Co-immunoprecipitation of apoER2 with caveolin

Intact caveolae were isolated from CLM with a polyclonal anti-caveolin-1 serum, and the immunocomplexes were collected using secondary antibody-covered magnetic Dynabeads (16). By this means, most of the caveolin-1-containing vesicles in the CLM fraction were immunoprecipitated (**Fig. 3**). Immunoblotting of the immune complexes with the antipeptide antiserum to apoER2 showed that the majority of apoER2 present within the CLM fraction was co-immunoprecipitated with caveolin-1. Thus, a significant portion of apoER2 is closely associated with caveolae in low density caveolin-1-containing domains of the plasma membrane.

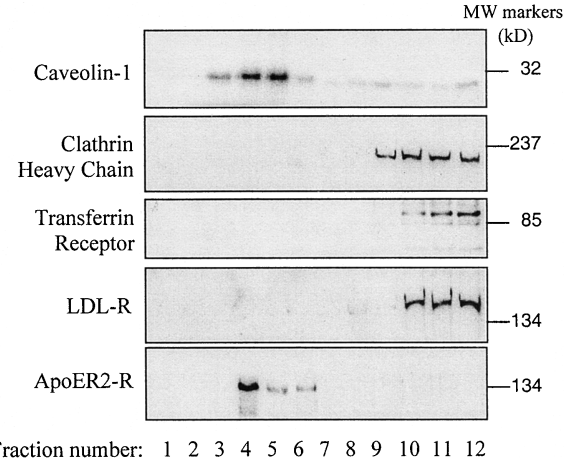

Fig. 2. ApoER2 also localizes in CLM isolated from detergent-solubilized cells. Cells from two 9-cm diameter dishes were solubilized by sonication in 1 ml of buffer containing 20 mM CHAPS, and fractionated by discontinuous sucrose density-gradient centrifugation as described. Fractions were analyzed by SDS-PAGE (15 μl of each fraction per lane), and replicate gels immunoblotted with antibodies to caveolin-1, clathrin heavy chain, transferrin receptor, apoER2, and LDL-R as indicated. The position of an appropriate molecular weight marker protein is shown on each blot.

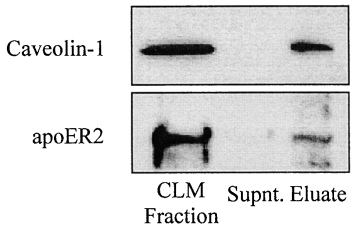

Fig. 3. Co-immunoprecipitation of caveolin and apoER2 from CLM fractions. The CLM fraction from one 175-cm^2 flask of CHO^{ER2} cells was isolated as described in Fig. 1, concentrated 10-fold, and incubated sequentially with polyclonal anti-caveolin Ig and magnetic beads coated with secondary antibody. Equivalent volumes of the whole concentrate (CLM Fraction), the post-adsorption supernatant (Supnt.), and the adsorbed proteins eluted from the beads (Eluate) were analyzed by SDS-PAGE and immunoblotting on nitrocellulose membranes. Appropriate sections of the same blot were probed with apoER2 antiserum or anti-caveolin-1 monoclonal antibody.

Localization of splice variants of apoER2

ApoER2 differs from other members of the LDL-R gene family in that several splice variants of the receptor have been detected in significant amounts in mRNA from human tissues (8, 21). Variant forms of apoER2 mRNA are predicted to encode proteins that lack the novel 59-amino acid insert in the cytoplasmic tail (apoER2Δins), the serine and threonine-rich O-linked sugar domain (apoER2Δsugar), the ligand-binding repeats 4–6 (apoER2Δ4–6), or combinations of these. The functional significance, if any, of these different splice variants is unknown. Conceivably, one of these domains may influence the ability of apoER2 to localize to caveolae, and we analyzed, therefore, the distribution of apoER2 variants in the gradient fractions of CHO cells expressing the cDNA for these variants. In each case, localization to the CLM fraction was observed (**Fig. 4**).

In summary, we have shown, using two different methods of solubilizing cell membranes, that apoER2 is localized to the caveolin-containing light membrane fraction of CHO cells expressing the cDNA for human apoER2. We have also shown that apoER2 can be immunoadsorbed from the CLM fraction with an antibody to caveolin-1. By contrast, the LDL-R localizes to and is internalized via clathrin-coated pits in human skin fibroblasts (20). This is consistent with our findings that the LDL-R remains at the bottom of the gradient with clathrin in fractionated extracts of CHO cells. Although we have studied cells overexpressing recombinant receptor proteins, because we wished to investigate the different apoER2 splice variants and because endogenous expression of apoER2 is relatively low, it is likely that our data reflect the physiological situation. Thus, when the LDL-R was expressed at higher than endogenous levels, there was no artifactual shift into the CLM fraction, implying that the distribution of recombinant apoER2 also reflects that of the endogenous receptor. Importantly, the localization of apoER2 in low density caveolin-enriched membrane domains on the cell surface

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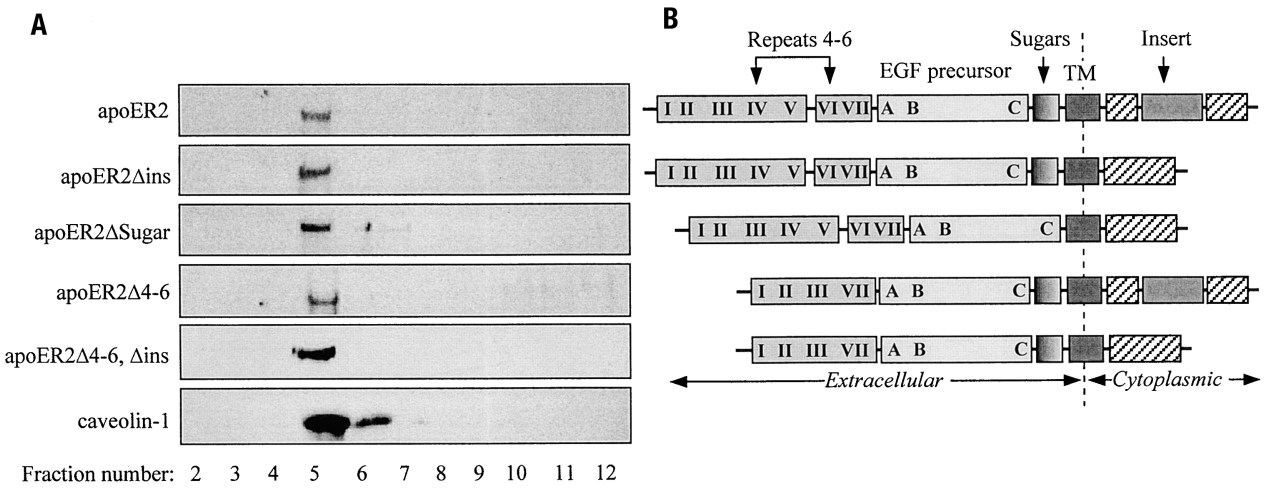

Fig. 4. Localization of different splice variants of apoER2. CHO cells expressing apoER2, apoER2ΔIns, apoER2ΔSugar, apoER2Δ4-6, or apoER2Δ4-6ΔIns were solubilized in 500 mM sodium carbonate and fractionated as outlined in Fig. 1. A: Aliquots of each fraction (15 μl) were separated by SDS-PAGE, and the gels immunoblotted with antibodies against apoER2 and caveolin-1. B: Diagram of the deleted domain structure of apoER2, showing the regions omitted by the variant splicing. Repeats 4–6, the ligand-binding domain excised from the disulfide-rich repeats I-VII; EGF precursor, epidermal growth factor precursor-like domain with disulfide-rich repeats A-C; Sugars, the domain rich in glycosylated serine and threonine residues; TM, transmembrane domain; Insert, 59-amino acid sequence inserted in the cytoplasmic tail of apoER2.

provides support for the view that its physiological role is in cell signaling, rather than in mediating endocytosis of ligands. Such co-localization of receptors and caveolin has previously been indicative of caveolar localization of receptor activation (12, 13). At present, we cannot define what structural or functional differences between apoER2 and the LDL-R determine their very different partitioning in the cell membrane. Initially, we suspected that the 59-amino acid insert in the cytoplasmic domain of apoER2 that is absent in the LDL-R might be responsible for its compartmentalization into caveolae. This proved not to be the case, as all of the splicing variants of apoER2 that we tested also localize to the caveolin-containing membrane fraction. Clearly, more subtle differences between the structure of the LDL-R and apoER2 are responsible for their different localization in cell surface membranes.

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