The low density lipoprotein (LDL) receptor family comprises several proteins with similar structures including the LDL receptor and apoE receptor 2 (apoER2). The human brain expresses two major splice variants of apoER2 mRNA, one of which includes an additional exon that encodes 59 residues in the cytoplasmic domain. This exon is absent from the LDL receptor and contains three proline-rich (PXXP) motifs that may allow apoER2 to function as a signal transducer. To investigate the role of this insert, we took advantage of the well-characterized low density lipoprotein receptor pathway. Chimeras comprising the ectodomain and transmembrane domain of the LDL receptor fused to the cytoplasmic domain of apoER2 lacking the PXXP-containing residues are able to mediate clathrin-dependent endocytosis of LDL as effectively as cells expressing the LDL receptor but not if the PXXP insert is present in the protein. Although expressed on the cell surface, the PXXP-containing chimeric receptor is excluded from clathrin vesicles as judged by its failure to co-localize with adaptor protein-2 possibly due to interaction with intracellular adaptors or scaffolding proteins. Chimeras with the transmembrane domain of apoER2, predicted to be longer than that of the LDL receptor by several residues, fail to mediate endocytosis of LDL or to co-localize with adaptor protein-2 regardless of the presence or absence of the PXXP insert. Thus features of apoER2 that distinguish it as a signaling receptor, rather than an endocytotic receptor like the LDL receptor, reside in or near the transmembrane domain and in the proline-rich motifs.

The low density lipoprotein (LDL) receptor family comprises a number of proteins with a similar domain organization including the LDL receptor itself, the very low density lipoprotein receptor (VLDLR), and apoER2. All three proteins have an amino-terminal region composed of multiple cysteine-rich repeats that mediates ligand binding in the LDL receptor. Adjacent to this is a region with homology to the epidermal growth factor precursor followed by a serine- and threonine-rich region that is heavily glycosylated in the LDL receptor. This is followed by a single transmembrane segment and finally followed by a carboxyl-terminal cytoplasmic domain containing an NPVY motif that comprises the internalization signal of the LDL receptor (1).

The LDL receptor is responsible for specific and regulated binding and uptake of cholesterol-carrying lipoproteins by cells, and defects in its gene cause familial hypercholesterolemia, one of the most common single gene disorders (2). The VLDLR and apoER2 can bind lipoproteins containing apoE, but their primary role in vivo does not appear to involve lipoprotein catabolism, and their pattern of expression differs from that of the LDL receptor. ApoER2 is expressed mainly in the brain, and the first evidence for its physiological role came from genetically modified mice. Mice lacking either the VLDLR or apoER2 have no apparent defects in lipoprotein metabolism, but double knockout mice lacking both manifest a phenotype resembling that of reeler and scrambler mice with mutations in the genes for Reelin or Disabled-1 (Dab-1). In these mice, normal patterning of neurons in the brain is disrupted, giving rise to the characteristic defect in motor function (3).

Thus the data suggest that Reelin, apoER2, and Dab-1 lie on a single pathway involved in signaling in the brain (4), although it is still not clear how the VLDLR is involved. Mice with single knockout of either apoER2 or VLDLR show minor alterations of dentate organization; migration defects were more prominent in apoER2 knockout mice (5). Although they may not be directly involved in lipoprotein catabolism, the ability of apoER2 and VLDLR to bind apoE suggests that they may play a role in the pathology of Alzheimer’s disease or brain injury (6). ApoE has proved to be an important factor in the repair of neural injury, and it also appears to interact with β-amyloid proteins (6). However, the structural differences among the LDL receptor, VLDLR, and apoER2 that underlie these apparently very different functions are not fully understood.

ApoER2 contains one distinct structural feature in its cytoplasmic domain, encoded by a single exon, namely an additional 59 amino acids containing three PXXP motifs (7, 8). PXXP motifs bind to Src homology domains (9, most of which are found in proteins involved in signal transduction pathways (10, 11). The PXXP insert in apoER2 has also been shown to bind to scaffolding proteins, for example the c-Jun amino-terminal kinase-interacting proteins JIP-1 and JIP-2 (12), also known to be involved in cell signaling (13). We have shown previously that apoER2 cannot bind LDL and is unable to internalize and degrade even apoE-containing lipoproteins (14), which is consistent with the view that the main function.
of apoER2 is not endocytosis of lipoproteins. We also found that while the LDL receptor localizes with clathrin-containing membrane fragments apoER2 tends to associate with tighter, caveolin-containing membranes (15).

We have also demonstrated by RNase protection assays, rather than by PCR, which can produce artifactualy short fragments, that several differently spliced isoforms of apoER2 are present in brain tissue at significant levels. All these isoforms can be expressed as stable proteins in transfected cells, demonstrating their potential for expression in vitro (14). Of the differently spliced forms, there are two variants of the cytoplasmic domain that either contain or lack the PXXP insert involved in signaling. It is intriguing that both forms are equally expressed in the human brain as judged both by RNase protection assay (14) and by an in situ hybridization assay (16). More interestingly, in brain stem cells only mRNA for the form of apoER2 lacking the insert is expressed, and no mRNA for the “plus insert” form, JIP-1, or JIP-2 can be detected. However, when these cells differentiate in vitro, mRNA for all three of these proteins is expressed in addition to the “minus insert” form of apoER2 (12). These data suggest that different isoforms of the receptor protein may have different functions required at different developmental stages. The purpose of this study was to investigate how the presence of the cytoplasmic insert influences the function of apoER2.

The only known physiological ligand for apoER2 is Reelin, an extracellular matrix signaling molecule with a molecular mass of more than 400 kDa (17, 18). To overcome difficulties associated with working with Reelin in vitro, we took advantage of the well studied ligand-receptor interaction between LDL and the LDL receptor by studying chimera of the LDL receptor and apoER2. The results show that both the transmembrane domain and the cytoplasmic domain of apoER2 influence the properties of the protein probably because they determine its ability to localize in different regions of the cell membrane.

MATERIALS AND METHODS

Plasmid Constructs—The human LDL receptor/apoER2 chimeras were constructed by PCR, as described in detail in the supplementary information, and cloned into the mammalian expression vector pcDNA3 (Invitrogen). Site-directed mutagenesis of three PXXP motifs in the apoER2 cytoplasmic tail (motif 1, REP’’EDP’’AP’’P’’P’’); motif 2, LPGQNHAP’’P’’; and motif 3, LPQINEQ’’P’’) was carried out using the QuikChange mutagenesis kit (Stratagene, Amsterdam, Netherlands) according to the manufacturer’s instructions. Details are shown as supplementary information. All constructs were verified by nucleotide sequencing.

Cell Culture and Transfections—CHO ldl A7 cells, a gift from Dr. M. Krieger, were maintained in culture as described previously (19). For transient transfection, cells were transfected with FuGENE 6 according to the manufacturer’s instructions (Roche Applied Science). In brief, cells were plated on glass coverslips and allowed to adhere overnight before transfection with plasmid using FuGENE 6 (Roche Applied Science). For staining of cell surface receptors 24 h after transfection, the cells were blocked in phosphate-buffered saline/0.1% bovine serum albumin for 15 min at room temperature and then incubated on ice for 5 min before incubating with mouse monoclonal anti-human LDLR IgGc7 (diluted 1:50, Progen Biotec GmbH, Heidelberg, Germany) for 1 h at 4 °C. Cells were washed three times at 4 °C in phosphate-buffered saline, 0.1% bovine serum albumin and fixe in 4% paraformaldehyde for 5 min on ice and for an additional 15 min at room temperature. The fixed cells were then washed three times with phosphate-buffered saline/bovine serum albumin and then incubated with ALEXA 568-conjugated goat anti-mouse IgG (Molecular Probes Europe BV, Leiden, Netherlands) for 45 min. For analysis of receptor internalization, cells were incubated with rabbit anti-LDLR (Progen Biotec GmbH, diluted 1:200) as described above and then washed and incubated at 37 °C for the times indicated. Cells were then fixed as described above and permeabilized by incubation in 0.1% Triton X-100, 10 mM glycine in phosphate-buffered saline for 10 min at ambient temperature. After washing, the cells were incubated with either mouse anti-AP2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, diluted 1:100) or mouse anti-EEA1 (BD Biosciences, diluted 1:100) for 60 min. After three washes, the cells were incubated with ALEXA 488-conjugated goat anti-rabbit IgA and ALEXA 568-conjugated goat anti-mouse Ig (Molecular Probes) for 45 min. The coverslips were mounted on glass slides with Vectashield antifade mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories Inc., Peterborough, UK). Fluorescent images were obtained on a Leica laser-scanning confocal microscope using the 100× oil objective and analyzed using Leica confocal software (Leica Microsystems Ltd., Milton Keynes, UK).

RESULTS

Construction and Expression of LDLR/ApoER2 Chimeras—Plasmid constructs expressing chimeras of apoER2 and the LDL receptor were constructed as shown in Fig. 1. The chimeras (Fig. 1A) contained the extracellular domains of the LDL receptor fused to the transmembrane and cytoplasmic domains of apoER2 (LDLR/ER2-TM) or the extracellular and transmembrane domains of the LDLR fused to the cytoplasmic domain of apoER2 (LDLR-TM/ER2). Variants of both LDLR/ER2-TM and LDLR-TM/ER2 were constructed that either contained (+) or lacked (−) the 59 amino acids containing the PXXP motifs in

Endocytosis of Chimeras of the LDLR and ApoER2

The human LDL receptor/apoER2 chimeras were constructed by PCR, as described in detail in the supplementary information, and cloned into the mammalian expression vector pcDNA3 (Invitrogen). Site-directed mutagenesis of three PXXP motifs in the apoER2 cytoplasmic tail (motif 1, REP’’EDP’’AP’’P’’P’’); motif 2, LPGQNHAP’’P’’; and motif 3, LPQINEQ’’P’’) was carried out using the QuikChange mutagenesis kit (Stratagene, Amsterdam, Netherlands) according to the manufacturer’s instructions. Details are shown as supplementary information. All constructs were verified by nucleotide sequencing.

Cell Culture and Transfections—CHO ldl A7 cells, a gift from Dr. M. Krieger, were maintained in culture as described previously (19). For transient transfection, cells were transfected with FuGENE 6 according to the manufacturer’s instructions (Roche Applied Science). In brief, cells were plated on glass coverslips and allowed to adhere overnight before transfection with plasmid using FuGENE 6 (Roche Applied Science). For staining of cell surface receptors 24 h after transfection, the cells were blocked in phosphate-buffered saline/0.1% bovine serum albumin for 15 min at room temperature. After washing, the cells were incubated with either mouse anti-AP2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, diluted 1:100) or mouse anti-EEA1 (BD Biosciences, diluted 1:100) for 60 min. After three washes, the cells were incubated with ALEXA 568-conjugated goat anti-rabbit IgA and ALEXA 488-conjugated goat anti-mouse Ig (Molecular Probes) for 45 min. The coverslips were mounted on glass slides with Vectashield antifade mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories Inc., Peterborough, UK). Fluorescent images were obtained on a Leica laser-scanning confocal microscope using the 100× oil objective and analyzed using Leica confocal software (Leica Microsystems Ltd., Milton Keynes, UK).

RESULTS

Construction and Expression of LDLR/ApoER2 Chimeras—Plasmid constructs expressing chimeras of apoER2 and the LDL receptor were constructed as shown in Fig. 1. The chimeras (Fig. 1A) contained the extracellular domains of the LDL receptor fused to the transmembrane and cytoplasmic domains of apoER2 (LDLR/ER2-TM) or the extracellular and transmembrane domains of the LDLR fused to the cytoplasmic domain of apoER2 (LDLR-TM/ER2). Variants of both LDLR/ER2-TM and LDLR-TM/ER2 were constructed that either contained (+) or lacked (−) the 59 amino acids containing the PXXP motifs in
the cytoplasmic domain (residues 852–910 of native apoER2). In addition, mutants of LDLR-TM/ER2+, designated LDLR-TM/ER2-P1, -P2, and -P3, were constructed in which key proline residues in each of the three PXXP motifs were replaced with alanine (Fig. 1B). Stable G418-resistant cell lines expressing each variant were produced in CHO ldl A7 cells that lack endogenous LDL receptor activity.

Relative protein expression was confirmed by blotting of whole cell extracts with an anti-LDL receptor antibody that detects the extracellular region of the LDL receptor (Fig. 2A). Confocal microscopy of non-permeabilized cells stained at 4 °C with anti-LDL receptor antibody showed that all the expressed chimeras were present on the cell surface (Fig. 2B).

Binding, Uptake, and Degradation of [125I]-LDL by Cells Expressing ApoER2/LDLR Chimeras—Stable cell lines that expressed similar levels of the proteins were assayed for their ability to mediate specific, saturable uptake and degradation of [125I]-labeled LDL. As shown in Fig. 3A, cells expressing LDLR-TM/ER2− were able to mediate uptake and degradation of LDL as well as cells expressing the normal LDL receptor, but cells expressing LDLR-TM/ER2− were totally unable to take up or degrade LDL. The amount of cell-associated LDL in cells expressing LDLR-TM/ER2− was as low as that in mock-transfected control cells, showing that this chimeric receptor was unable even to bind LDL on the cell surface. Cells expressing LDLR-TM/ER2−+ gave identical results to those expressing LDLR-TM/ER2−. Thus the transmembrane domain of apoER2 cannot substitute for that of the LDL receptor regardless of the presence or absence of the 59-amino acid residue insert in the cytoplasmic domain of apoER2. One explanation for this may be that the predicted transmembrane helical domain of apoER2 appears to be longer by 2–3 residues than that of the LDL receptor as shown in Fig. 4A (22); the same difference is predicted between the murine LDL receptor and apoER2. Although the amino acid sequences of the intracellular domains of apoER2 and the LDL receptor are very similar, there are considerable sequence differences in the extracellular region adjacent to the membrane (Fig. 4B). Whether these residues merely exclude apoER2 from associating with clathrin-coated areas of the membrane or actively direct it to caveolae or rafts (15) remains to be determined.

As shown in Fig. 3, inclusion of the 59 amino acids containing the PXXP motifs rendered LDLR-TM/ER2− completely unable to bind or mediate LDL uptake. However, mutation of the proline residues to alanine in any one of the three PXXP motifs restored the ability to mediate uptake and degradation of LDL (P1, P2, and P3 in Fig. 3B). Thus the chimeric receptor protein comprising the LDL receptor substituted with the cytoplasmic
endothelial cell-monolayer expressing chimeric proteins were preincubated for 18 h in medium containing lipoprotein-deficient serum and then for 37 h at 37 °C with 125I-labeled LDL. Saturable binding plus uptake or degradation of LDL was determined as the difference in the amount of trichloroacetic acid-soluble, non-iodide radioactivity in the medium or cell-associated radioactivity of cells incubated in the presence or absence of an excess of unlabeled LDL (1 mg/ml). The data shown are means of triplicate dishes and are representative of three independent experiments with similar results. A, LDLR-TM/ER2 (LDLR-TM); B, LDLR-TM/ER2; C, empty vector; D, LDLR-TM/ER2-P1 (LDLR-TM/ER2-P1); E, LDLR-TM/ER2-P2 (LDLR-TM/ER2-P2); F, LDLR-TM/ER2-P3 (LDLR-TM/ER2-P3); G, LDLR-TM/ER2-P1, -P2, and -P3 restores the ability to mediate degradation of LDL receptor, LDLR-TM/ER2 (LDLR-TM/ER2-P1, -P2, and -P3). Thus substitution of the proline residues with alanine in the PXXP motifs essentially abolished their ability to bind to the PID of JIP-2. The observation that disruption of the proline-rich motifs in LDLR-TM/ER2+ and LDLR-TM/ER2− with a cross-linking reagent that is known to cross-link LDL receptor dimers efficiently (19). However, only the LDL receptor can be cross-linked as dimers in intact cells as shown in Fig. 6 where the cross-linked receptor protein is visible as an immunoreactive band with increased molecular weight (top band in lanes 6 and 8). Neither LDLR-TM/ER2+ (lane 10) nor LDLR-TM/ER2− (lane 4) can be cross-linked by this reagent. This suggests that the difference in their ability to bind LDL is not due to differences in their ability to dimerize at least as detected by cross-linking with a reagent that detects LDL receptor dimers. While we cannot exclude that the high molecular weight bands do not contain adducts of the LDL receptor with other unknown proteins, clearly the inability of the chimeras to form dimers is not related to the ability to bind LDL.

Localization of Chimeras by Confocal Microscopy—We considered that differences in the localization of the chimeras in microdomains on the cell surface might determine their ability to bind LDL. To examine this, CHO ldl A7 cells transiently expressing the chimeras were stained with specific antisera to the LDL receptor and to the a-subunit of the clathrin-coated vesicle component AP2 (26) and examined by laser-scanning confocal microscopy (Fig. 7). Single cells are shown in Fig. 7 for...
Endocytosis of Chimeras of the LDLR and ApoER2

Fig. 5. Interaction of the cytoplasmic domain of apoER2 with the PID of JIP-2. A. Coomassie Blue-stained gel of purified GST fusion proteins comprising the cytoplasmic tail of normal apoER2 with the 59 amino acids containing the PXXP motifs (+ PXXP motif) or mutants P1, P2, and P3 (see Fig. 1). B, each fusion protein (~3 μg of protein) was incubated with Myc-tagged, [35S]methionine-labeled PID of JIP-2 (translated in vitro) bound to anti-Myc complexed with Protein A + G-agarose beads. Bound proteins were eluted from the beads in 50 mM Tris (pH 8.0), 0.5 M NaCl, 1 mM EDTA, 1% Nonidet P-40, 5% glycerol, 1 mM PMSF, and 10 μg/ml of rabbit anti-apoER2 (15) to detect bound fusion protein.

| transfected construct | vector | LDLR-TM/ER2- (A and D) co-localized | LDLR-ER2 (H) | LDLR (L) | LDLR-TM/ER2+ |
|-----------------------|--------|-----------------------------------|--------------|----------|-------------|
| vector                | - +    | -                                 | -            | - +      | - +         |
| LDLR-TM/ER2- (A and D) co-localized | + - | -                                 | -            | + -      | + -         |

Fig. 6. Cross-linking of surface-expressed receptor proteins in CHO ldl A7 cells. Intact stably transfected cells expressing chimeric proteins as indicated were incubated with (+) or without (−) 1 μg/ml dithiothreitol (dithiol/disulfide reductant) for 30 min at 4 °C; total cell extracts (25 μg of protein) were fractionated by 9% SDS-PAGE, immunobotted with rabbit anti-LDLR antibody, and detected by chemiluminescence. Cells expressing the LDLR at high (H) or low (L) levels were included to match approximately the level of expression of the chimeras. The low molecular weight band visible in the LDLR (H) lanes is either the immature precursor form or a partial breakdown product of the LDLR (19).

cloarity, but the patterns observed were typical of several cells scanned in each field. LDLR-TM/ apoER2- (A and D) co-localized on the cell surface extensively with AP2 (B and E) in a manner that was identical to that found with the normal LDL receptor (data not shown). This can be seen from the punctate yellow pattern on the edge of the transfected cells (overlays C) and the linear pattern on the cell surface (overlay F). The linear pattern of AP2 α-subunits on the cell surface is consistent with previous observations (27). In contrast, the LDLR-TM/ER2+ (G and J) did not co-localize with AP2 (H and K) as very few yellow dots can be seen on the edge of the cells (I) and hardly any are seen on the surface (L), and the linear pattern of AP2 appears disrupted. LDLR-TM/ER2-P1, -P2, and -P3, in which proline residues were substituted with alanine in the PXXP motifs, localized with AP2 to the same extent as LDLR-TM/ER2-; LDLR/ER2-TM did not co-localize with AP2 (data not shown).

The LDL receptor has been reported to recycle constitutively regardless of ligand binding and to enter the early endosomal compartment rapidly (28), and therefore we determined whether the chimeras that were unable to bind LDL and mediate its uptake could nonetheless be internalized by the cells. Cells were incubated with anti-LDL receptor antibody at 4 °C, then washed, and incubated at 37 °C for either 5 or 10 min (Fig. 7, lower panels). After incubation of the cells at 37 °C, LDLR-TM/ER2- was co-localized with EEA1 after 5 min of incubation at 37 °C but not after 10 min of incubation. This is consistent with a previous study of internalization of the LDL and transferrin receptors via clathrin vesicles in which the t½ for entering early endosomes was found to be 5 min and the t½ for recycling back to the plasma membrane was about 10 min (28). Surprisingly, although LDLR-TM/ER2+ could not mediate uptake of LDL, the receptor chimera was internalized by the cells. However, it partially co-localized with the early endosome marker EEA1 only after incubation at 37 °C for 10 min and not after incubation for 5 min, implying that its route of internalization differed from that of the LDL receptor.

DISCUSSION

The LDL receptor and apoER2 both have an identical FD-NPYY motif in their cytoplasmic domain that is believed to be the signal for clustering into clathrin-coated pits on the cell surface (29). Therefore both should, in theory, be able to mediate endocytosis of bound ligands via the clathrin pathway. The major finding of this study is that two features of apoER2 prevent it from functioning in this way. First the transmembrane domain of apoER2 appears to be important for determining its location on the cell surface and, therefore, its function.
Although we have not mapped the transmembrane domains biochemically, the helical transmembrane domain of apoER2 is predicted to be several residues longer than that of the LDL receptor, which may influence the type of lipid bilayer with which it preferentially associates (30). We replaced the whole of the region encoded by exon 16 together with part of that encoded by exon 17 of the LDL receptor with the equivalent region from apoER2; thus it is possible that important residues are adjacent to the membrane on the intracellular side rather than within it. Residues at the intracellular junction of the transmembrane domain of influenza virus hemagglutinin (31) or neuraminidase (32) were found to be important for localization with cholesterol-rich rafts and apical sorting.

The apoER2 receptor construct with the LDL receptor transmembrane domain, but lacking the proline-rich insert, does indeed contain a functional signal for clustering into clathrin-coated pits, but this signal is somehow masked when the proline-rich insert in its cytoplasmic tail is present. This conclusion is based on the observation that LDLR-TM/ER2\(^{\text{–H11001}}\) co-localized with AP2, a component of clathrin-coated pits (26), but LDLR-TM/ER2\(^{\text{+H11001}}\) did not. Furthermore cells expressing LDLR-TM/ER2\(^{\text{+H11001}}\), but not those expressing LDLR-TM/ER2\(^{\text{–H11001}}\), can bind, internalize, and degrade LDL as efficiently as cells expressing the LDL receptor, which is typically endocytosed via the clathrin-coated vesicle pathway.

The FDNPVY motif has been predicted to form a reverse turn conformation, and it is believed that this conformation is important for directing the receptor to clathrin-coated pits (33, 34). The 59 amino acids in the cytoplasmic domain of LDLR-TM/ER2\(^{\text{+H11001}}\), which are located only 26 amino acids downstream from FDNPVY, appear to disrupt the function of this motif. LDLR-TM/ER2\(^{\text{+H11001}}\) is unable to co-localize with AP2 in clathrin vesicles or to bind and mediate internalization of LDL. The underlying cause for this may be that the PXXP motifs can bind to proteins such as JIP-1 and JIP-2 (12) and thereby disrupt the conformation of the reverse turn. JIPs are scaffolding proteins that can interact with themselves and recruit many other signaling proteins to form large complexes (35). In support of this we have shown that replacement of the proline residues with alanine in any one of the three PXXP motifs prevents binding of the PID of JIP-2 to the cytoplasmic domain of apoER2 and thereby allows the LDLR-TM/ER2\(^{\text{+H11001}}\) mutants to mediate cellular uptake of LDL. However, the exact nature of the proteins in CHO cells that might interact with the cytoplasmic PXXP motif of apoER2 is not known. Available antisera were not sufficiently specific to allow unambiguous detection of JIP-1 and JIP-2, but it is unlikely that they are expressed in these cells. Presumably other JIP-like proteins must be expressed in CHO cells that fulfill a similar scaffolding function.

Alternatively the disruption of internalization could be caused by other signaling events involving the PXXP insert as RhoGEF (RhoA activation factor) can also bind to the PID of JIPs (13). It is possible that apoER2 may compete with RhoGEF for binding to a JIP-like protein in turn releasing RhoGEF to sites for activating RhoA. Activated RhoA can promote actin stress fiber formation and thereby inhibit internalization, endocytosis, and cell migration. For example, activated RhoA has been shown to inhibit clathrin-mediated receptor endocytosis (36–38). It has also been shown that activated RhoA prevents neuronal cell differentiation and induces cell rounding and that inactivated RhoA has the opposite effect (39). Thus, the apoER2\(^{\text{+}}\) isoform may be involved in a RhoA signaling pathway to control neuronal differentiation under physiological conditions.

Even if it cannot be internalized via clathrin-coated pits, we do not know why the LDLR-TM/ER2\(^{+}\) cannot bind LDL on the cell surface since the ectodomains and transmembrane domains are the same in LDL receptor, LDLR-TM/ER2\(^{\text{+H11001}}\), and LDLR-TM/ER2\(^{\text{–H11001}}\). However, the cytoplasmic tail of some receptors, for example in the integrin receptor, has been shown to be able to send a signal out (inside-out signal) and thereby change the conformation of the ectodomain (40). We sought a monoclonal antibody that might be sensitive to changes in conformation, but none of the three different monoclonal antibodies against the human LDL receptor detected any differences in the ectodomains of the chimeras. Thus we only can postulate that RhoA activation leading to actin fiber changes triggers a change in the microenvironment of the cell membrane that in turn changes the conformation of the ectodomain of LDLR-TM/ER2\(^{\text{+H11001}}\). Indirect evidence for the involvement of the cytoskeleton in the apoER2 signaling pathway is that the molecular motor of kinesin has been found to interact with JIPs and to co-immunoprecipitate with apoER2 (13).

We have tried to determine whether LDLR-TM/ER2\(^{\text{+H11001}}\) is localized in caveolae first by preparation of detergent-insoluble membranes (15). However, since carrying out these early experiments we have obtained less consistent results with the receptor proteins expressed in transfected cells, suggesting that they may move between different membrane fractions under different conditions. We have also examined whether the chimeras are localized in caveolae in transfected cells by confocal microscopy and frequently observed partial co-localization of caveolin-1, a component protein of caveolae, with LDLR-TM/ER2\(^{\text{+H11001}}\) but not with the LDL receptor or LDLR-TM/ER2\(^{\text{–H11001}}\) (data not shown). However, co-localization was variable and incomplete, and we cannot conclude from our studies that LDLR-TM/ER2\(^{\text{+H11001}}\) localizes in caveolae. We speculate that its localization may be very dynamic and could be ligand-dependent. However, we have shown that cell surface LDLR-TM/ER2\(^{\text{+H11001}}\) co-localizes with EEA1, an early endosome marker, after 10 min at 37 °C but not if only incubated for 5 min. This suggests that LDLR-TM/ER2\(^{\text{+H11001}}\) may take a different endocytosis pathway from that of the LDL receptor because one round trip for the LDL receptor from the cell surface into the cell and back to the surface again takes 9–12 min. Cell surface LDL receptor appears in the early endosomes after about 2–5 min (28), and we have shown that LDLR-TM/ER2\(^{\text{–}}\) also appears in the early endosomes after only 5 min of incubation at 37 °C.

We chose to carry out these experiments in CHO cells lacking endogenous LDL receptor activity to allow unambiguous assessment of the ability of the chimeras to mediate specific receptor-dependent uptake of LDL. However, apoER2 is normally expressed in neuronal cells in vivo, and we cannot exclude the possibility that apoER2 chimeras might function differently in neuronal cells. No cell lines are known that express detectable levels of apoER2, including neuronal cell lines such as SH-SY-5Y or SK-N-SH, so it is not possible to determine whether endogenous apoER2 can mediate endocytosis. We also investigated the possibility of expressing the chimeras in these neuronal cell lines, but surprisingly the level of expression of the endogenous LDL receptor was too high to allow analysis of the ability of the chimeras to mediate LDL uptake cells (data not shown).

We conclude that apoER2 is a signaling receptor and that it has been diverted, like other signaling receptors, away from the recycling pathway. Presumably apoER2-mediated signaling involves interaction of the PXXP motifs with intracellular signaling adaptors, and thus it is unclear what function the splice variant without the PXXP-containing motif fulfills. Although our data show that it is unlikely to mediate endocytosis via the clathrin-dependent pathway, various non-clathrin-dependent
Endocytosis of Chimeras of the LDLR and ApoER2

pathways are known including uptake via caveolae (41). We found that although some of the chimeric receptors were unable to mediate endocytosis of LDL, they were nonetheless internalized by cells albeit at a slower rate than those that were internalized by cells that entered by the clathrin-dependent pathway. Overall it is clear that apoER2 receptors that include the FXXP motif are likely to have a different function from those without, but the factors that regulate which splice variant of apoER2 is expressed, or when, remain to be determined.

Acknowledgments—We thank Dr. M. Krieger for providing CHO ldl A7 cells and Dr. C. Bonny for a cDNA clone encoding the PID of JIP-2.

REFERENCES
1. Willnow, T. E., Nykjaer, A., and Herz, J. (1999) Nat. Cell Biol. 1, E157–E162
2. Goldstein, J. L., Hobbs, H., and Brown, M. S. (1995) in The Metabolic and Molecular Bases of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) Vol. II, 7th Ed., pp. 1981–2030, McGraw-Hill, New York
3. D’Arcangelo, G., and Curran, T. (1998) Bioessays 20, 235–244
4. Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimpf, J., Hammer, R. E., Richardson, J. A., and Herz, J. (1999) Cell 97, 689–701
5. Drakew, A., Deller, T., Heimrich, B., Gebhardt, C., Del Turco, D., Tielsch, A., Forster, E., Herz, J., and Frotscher, M. (2002) Exp. Neurol. 176, 12–24
6. LaDu, M. J., Shah, J. A., Reardon, C. A., Getz, G. S., Bu, G., Hu, J., Guo, L., and Van Eerd, L. J. (2001) Brain Res. 899, 15–24
7. Kim, D. H., Iijima, H., Goto, K., Sakai, J., Ishii, H., Kim, H. J., Suzuki, H., Kondo, H., Saeki, S., and Yamamoto, T. (1996) J. Biol. Chem. 271, 8373–8380
8. Kim, D. H., Magori, K., Inoue, T. R., Mao, C. C., Kim, H. J., Suzuki, H., Fujita, T., Endo, Y., Saeki, S., and Yamamoto, T. R. A. (1997) J. Biol. Chem. 272, 4936–4940
9. Alexandropoulos, K., Cheng, G., and Baltimore, D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3110–3114
10. Buday, L. (1999) Biochim. Biophys. Acta 1422, 187–204
11. Bork, P., Schultz, J., and Ponting, C. P. (1997) Trends Biochem. Sci. 22, 296–298
12. Stockinger, W., Brandes, C., Fasching, D., Hermann, M., Gotthardt, M., Herz, J., Schneider, W. J., and Nimpf, J. (2000) J. Biol. Chem. 275, 25625–25632
13. Verhey, R. J., Meyer, D., Dechan, R., Blesis, J., Schnapp, B. J., Rapoport, T. A., and Margolis, B. (2001) J. Cell Biol. 152, 959–970
14. Sun, X. M., and Soutar, A. K. (1999) Eur. J. Biochem. 262, 230–239
15. Riddell, D. R., Sun, X. M., Stannard, A. K., Soutar, A. K., and Owen, J. S. (2001) J. Lipid Res. 42, 998–1002
16. Clatworthy, A. E., Stockinger, W., Christie, R. H., Schneider, W. J., Nimpf, J., Hyman, B. T., and Rebeck, G. W. (1999) Neurosciences 90, 903–911
17. Curran, T., and D’Arcangelo, G. (1996) Brain Res. Rev. 25, 285–294
18. D’Arcangelo, G., Homayouni, R., Keshhvara, L., Rice, D. S., Sheldon, M., and Curran, T. (1999) Neuron 24, 471–479
19. Patel, D. D., Soutar, A. K., and Knight, B. L. (1999) Biochem. J. 345, 569–573
20. Sun, X. M., Patel, D. D., Bhatnagar, D., Knight, B. L., and Soutar, A. K. (1995) Arterioscler. Thromb. Vasc. Biol. 15, 219–227
21. Negri, S., Oberson, A., Steinmann, M., Sausser, C., Nicod, P., Waeher, G., Schorderet, D. F., and Bonny, C. (2000) Genomics 64, 324–330
22. Müller, S., Croning, M. D., and Apte, R. (2001) Bioinformatics 17, 646–653
23. van Driel, I., Davis, C. G., Goldstein, J. L., and Brown, M. S. (1987) J. Biol. Chem. 262, 16127–16134
24. Bessiegel, U., Schneider, W. J., Goldstein, J. L., Andersen, R. G., and Brown, M. S. (1981) J. Biol. Chem. 256, 11923–11931
25. Stockinger, W., Brandes, C., Fasching, D., Hermann, M., Gotthardt, M., Herz, J., Schneider, W. J., and Nimpf, J. (2000) J. Biol. Chem. 275, 25625–25632
26. Schimid, S. L. (1997) Annu. Rev. Biochem. 66, 511–548
27. Bennett, E. M., Chen, C. Y., Engqvist-Goldstein, A. E., Drubin, D. G., and Brodsky, F. M. (2001) Traffic 2, 801–858
28. Mukherjee, S., Ghosh, R. N., and Maxfield, F. R. (1997) Physiol. Rev. 77, 759–803
29. Chen, W. J., Goldstein, J. L., and Brown, M. S. (1990) J. Biol. Chem. 265, 3116–3123
30. Simons, K., and Ikonen, E. (1997) Nature 387, 569–572
31. Scheiffele, P., Roth, M. G., and Simons, K. (1997) EMBO J. 16, 5501–5508
32. Barman, S., and Nayak, D. P. (2000) J. Virol. 74, 6538–6545
33. Collawn, J. F., Kuhn, L. A., Liu, L. F., Tainer, J. A., and Trowbridge, I. S. (1991) EMBO J. 10, 3247–3253
34. Kibbey, R. G., Rizo, J., Gierasch, L. M., and Anderson, R. G. (1998) J. Cell Biol. 142, 59–67
35. Yasuda, J., Whitmarsh, A. J., Carvagh, J., Sharma, M., and Davis, R. J. (1999) Mol. Cell. Biol. 19, 7245–7254
36. Lamaze, C., Chuang, T. H., Terlecky, L. J., Bokoch, G. M., and Schmid, S. L. (1996) Nature 382, 177–179
37. Lamaze, C., Fujimoto, I. M., Yin, H. L., and Schmid, S. L. (1997) J. Biol. Chem. 272, 20332–20335
38. Ridley, A. J. (2001) Traffic 2, 303–310
39. Gehbink, M. F., Kronenburg, O., Poland, M., van Heuck, F. F., Houssa, B., and Moenlaen, W. H. (1997) J. Cell Biol. 137, 1603–1613
40. Schaeffer-Reckinger, E., Hons, N. H., and Kieffer, N. (2001) Thromb. Haemostasis 85, 716–723
41. Nichols, B. J., and Lippincott-Schwartz, J. (2001) Trends Cell Biol. 11, 406–412
The Transmembrane Domain and PXXP Motifs of ApoE Receptor 2 Exclude It from Carrying out Clathrin-mediated Endocytosis
Xi-Ming Sun and Anne K. Soutar

J. Biol. Chem. 2003, 278:19926-19932. doi: 10.1074/jbc.M302047200 originally published online March 5, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302047200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2003/03/24/M302047200.DC1

This article cites 41 references, 18 of which can be accessed free at
http://www.jbc.org/content/278/22/19926.full.html#ref-list-1