The EMBO Journal (2007) 26, 3273–3282 | © 2007 European Molecular Biology Organization | All Rights Reserved 0261-4189/07
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THE EMBO JOURNAL

Identification of a VLDL-induced, FDNPVY-independent internalization mechanism for the LDLR

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The low-density lipoprotein (LDL) receptor (LDLR) binds to and internalizes lipoproteins that contain apolipoproteinB100 (apoB100) or apolipoproteinE (apoE). Internalization of the apoB100 lipoprotein ligand, LDL, requires the FDNPVY[807] sequence on the LDLR cytoplasmic domain, which binds to the endocytic machinery of coated pits. We show here that inactivation of the FDNPVY[807] sequence by mutation of Y807 to cysteine prevented the uptake of LDL; however, this mutation did not prevent LDLR-dependent uptake of the apoE lipoprotein ligand, β-VLDL. Comparison of the surface localization of the LDLR-Y807C using LDLR-immunogold, LDL-gold and β-VLDL-gold probes revealed enrichment of LDLR-Y807C-bound β-VLDL in coated pits, suggesting that β-VLDL binding promoted the internalization of the LDLR-Y807C. Consistent with this possibility, treatment with monensin, which traps internalized LDLR in endosomes, resulted in the loss of surface LDLR-Y807C only when β-VLDL was present. Reconstitution experiments in which LDLR variants were introduced into LDLR-deficient cells showed that the HIC[818] sequence is involved in β-VLDL uptake by the LDLR-Y807C. Together, these experiments demonstrate that the LDLR has a very low-density lipoprotein (VLDL)-induced, FDNPVY-independent internalization mechanism.

The EMBO Journal (2007) **26,** 3273–3282. doi:10.1038/sj.emboj.7601769; Published online 21 June 2007
Subject Categories: membranes & transport; cellular metabolism
Keywords: ARH; fibroblasts; LDL; LDLR; VLDL

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Received: 27 September 2006; accepted: 30 May 2007; published online: 21 June 2007

Introduction

The low-density lipoprotein (LDL) receptor (LDLR) plays a critical role in reducing the risk of atherosclerosis by binding to and internalizing atherogenic lipoproteins that contain apolipoproteinB100 (apoB100) or apolipoproteinE (apoE). Lipoprotein internalization involves first binding of the lipoprotein at the cell surface, followed by internalization of the LDLR–lipoprotein complex through clathrin-coated pits into clathrin-coated vesicles (Brown and Goldstein, 1986). These vesicles lose their clathrin coats and fuse with sorting endosomes, wherein the low pH of the endosomal lumen promotes the release of the lipoprotein from the LDLR (Maxfield and McGraw, 2004). The receptors recycle back to the cell surface, while the released lipoproteins remain in the endosomal lumen. Sorting endosomes mature into late endosomes through rab conversion and fuse with lysosomes, resulting in the degradation of the internalized lipoproteins (Goldstein *et al*, 2001; Bright *et al*, 2005; Rink *et al*, 2005).

The principal lipoproteins internalized by the LDLR are very low-density lipoprotein (VLDL), VLDL remnants and LDL (Goldstein and Brown, 1974; Kita *et al*, 1982). VLDL is synthesized in the liver and consists of a triglyceride-rich core surrounded by a shell of phospholipids, cholesterol and apolipoproteins. The apolipoprotein content includes a single copy of apoB100 and multiple copies of apoE (Shelness and Sellers, 2001). VLDL is converted to VLDL remnants in a process involving lipoprotein lipase, which releases fatty acids from the triglycerides carried by VLDL (Hahn, 1943). As compared to VLDL, VLDL remnants have a smaller size, an increased density and a decreased ratio of triglycerides to cholesterol esters (Berman *et al*, 1978; Innerarity *et al*, 1986). Internalization of VLDL and VLDL remnants by the LDLR requires apoE but not apoB100 (Weisgraber *et al*, 1983; Krul *et al*, 1985). In addition to uptake by the LDLR, VLDL remnants can also be eliminated by conversion to LDL, a pathway that accounts for most LDL production *in vivo* (Bilheimer *et al*, 1972; Phair *et al*, 1975). LDL consists of a cholesterol ester-rich core surrounded by a shell of phospholipids, cholesterol and a single copy of apoB100. The internalization of LDL by the LDLR requires the association of the LDLR with apoB100 (Shireman *et al*, 1977; Kita *et al*, 1981). LDL is the principal atherogenic lipoprotein in the circulation and the LDLR plays a central role in reducing the number of LDL both by increasing the rate of LDL clearance and by reducing the production of LDL via uptake of VLDL and VLDL remnants (Kita *et al*, 1982).

The importance of the LDLR in controlling LDL levels is revealed by loss-of-function mutations, which result in hypercholesterolemia and early onset of coronary artery disease. Among these loss-of-function mutations are variants that can support normal LDL binding, but fail to internalize LDL. The best studied of these internalization-defective alleles is the Y807C mutation, which has been extensively

studied using fibroblasts from individual JD, who has one Y807C allele and one null allele for the LDLR (Brown and Goldstein, 1976; Goldstein *et al*, 1977). JD fibroblasts bind a normal amount of LDL; however, these fibroblasts are largely unable to degrade LDL, because the Y807C LDLR fails to target LDL to clathrin-coated pits for internalization (Anderson *et al*, 1977). This observation led to the hypothesis that the LDLR has an internalization determinant in the cytoplasmic domain that allows the LDLR to associate with the endocytic machinery in clathrin-coated pits. Characterization of additional internalization-defective mutations led to the identification of the $FDNPVY^{807}$ sequence in the LDLR cytoplasmic domain as the internalization sequence that is required for LDL uptake by the LDLR (Davis *et al*, 1987; Chen *et al*, 1990; Hobbs *et al*, 1992).

The identification of the $FDNPVY^{807}$ sequence initiated a search for clathrin-coated pit-associated proteins that could bind the FDNPVY sequence. Many candidate proteins have been identified, including clathrin heavy chain, adaptor protein-2 (AP2), β-arrestin, disabled protein-1 (dab1), disabled protein-2 (dab2) and the autosomal recessive hypercholesterolemia protein (ARH) (Kibbey *et al*, 1998; Howell *et al*, 1999; Morris and Cooper, 2001; Boll *et al*, 2002; He *et al*, 2002; Mishra *et al*, 2002; Wu *et al*, 2003). Of these proteins, only dab2 and ARH appear to play a significant role in targeting the LDLR to coated pits (Michaely *et al*, 2004; Garuti *et al*, 2005; Keyel *et al*, 2006; Maurer and Cooper, 2006). As its name implies, ARH is associated with a recessive form of hypercholesterolemia (Garcia *et al*, 2001) and individuals lacking ARH ($ARH^{-/-}$) have LDL clearance rates that are as slow as those of individuals lacking the LDLR ($LDLR^{-/-}$) (Zuliani *et al*, 1999; Jones *et al*, 2003). This observation indicates that liver hepatocytes, which are responsible for LDL clearance *in vivo*, require ARH for LDLR-dependent uptake of LDL. By contrast, fibroblasts from $ARH^{-/-}$ individuals can internalize and degrade LDL (Harada-Shiba *et al*, 1991; Zuliani *et al*, 1995), because fibroblasts express both ARH and dab2, while hepatocytes express only ARH (Keyel *et al*, 2006).

In contrast to LDL uptake, the role of the FDNPVY sequence on the uptake of apoE containing lipoproteins has not been examined. One reason to suspect that the FDNPVY sequence might be less important for LDLR-dependent uptake of apoE lipoprotein ligands is the observation that while the LDL clearance rates are similar in $LDLR^{-/-}$ and $ARH^{-/-}$ individuals, the circulating level of LDL is significantly lower in $ARH^{-/-}$ individuals as compared to $LDLR^{-/-}$ individuals (Arca *et al*, 2002; Pisciotta *et al*, 2006). This difference indicates that less LDL is produced in the absence of ARH than in the absence of the LDLR. A lower LDL production would be expected if ARH is not required for VLDL remnant uptake by the LDLR. Consistent with this possibility, $Arh^{-/-}$ mice have higher rates of clearance of β-VLDL and VLDL than $Ldlr^{-/-}$ mice (Jones *et al*, 2007). Here, we employed primary fibroblasts from normal, JD, $ARH^{-/-}$ and $LDLR^{-/-}$ individuals to test whether the FDNPVY sequence of the LDLR is required for uptake of β-VLDL, a VLDL remnant-like lipoprotein, that is internalized by the LDLR in an apoE-dependent manner (Mahley *et al*, 1980; Schneider *et al*, 1981). We show that inactivation of the $FDNPVY^{807}$ sequence by the Y807C mutation prevents LDL but not β-VLDL uptake, and provide evidence that the LDLR has a ligand-induced, FDNPVY-independent internalization mechanism that can support uptake of the apoE lipoprotein ligand, β-VLDL.

Results

Internalization of the apoB100 lipoprotein ligand, LDL, requires the $FDNPVY^{807}$ sequence of the LDLR (Davis *et al*, 1986; Chen *et al*, 1990). To determine if the $FDNPVY^{807}$ sequence is required for LDLR-mediated internalization of apoE lipoprotein ligands, we compared uptake of fluorescently labeled LDL and β-VLDL in primary skin fibroblasts from normal, JD, ARH-null ($ARH^{-/-}$) and LDLR-null ($LDLR^{-/-}$) individuals (Figure 1). Normal fibroblasts supported robust uptake of both LDL and β-VLDL. JD fibroblasts, which express only the Y807C variant of the LDLR (LDLR-Y807C), were unable to support LDL uptake, but showed substantial internalization of β-VLDL. $ARH^{-/-}$ fibroblasts internalized both LDL and β-VLDL. The LDLR was required for uptake of both LDL and β-VLDL because $LDLR^{-/-}$ fibroblasts failed to internalize either lipoprotein. Thus, the integrity of the canonical $FDNPVY^{807}$ internalization sequence of the LDLR is required for LDL but not β-VLDL internalization.

The $FDNPVY^{807}$ sequence binds to the clathrin adaptor proteins, ARH and dab2, which facilitate the association of the LDLR and its bound ligands with clathrin-coated pits (Morris and Cooper, 2001; He *et al*, 2002; Mishra *et al*, 2002; Michaely *et al*, 2004). To determine whether the LDLR-Y807C employed clathrin-coated pits for β-VLDL uptake, β-VLDL uptake was assayed in the presence of hypertonic media, which disrupts clathrin-coated pits (Heuser and Anderson, 1989). As shown in Supplementary Figure S1, hypertonic sucrose blocked uptake of β-VLDL uptake in JD cells, indicating that the LDLR-Y807C employs clathrin-coated pits for β-VLDL uptake.

The finding that the Y807C mutation eliminated LDL but not β-VLDL uptake suggested that β-VLDL might promote the clustering of LDLRs into clathrin-coated pits. To determine the effect of LDL and β-VLDL on the cell surface distribution of the LDLR in the four fibroblasts, we labeled LDLR on the cell surfaces with LDLR-immunogold, LDL-gold or β-VLDL-gold and visualized the LDLR distribution using electron microscopy (Figure 2). For LDLR-immunogold labeling, cells were fixed before immunogold labeling to determine the localization of LDLR in the absence of lipoproteins. For the LDL-gold and β-VLDL-gold labelings, cells were incubated with the labeled lipoprotein for 90 min before fixation, to determine the localization of the LDLR in the presence of lipoproteins. In normal cells, the LDLR-immunogold, LDL-gold and β-VLDL-gold labels were all enriched in coated pits, indicating that the LDLR is localized in clathrin-coated pits in the absence of lipoproteins and in the presence of either LDL or β-VLDL. By contrast, coated pits of JD cells displayed little enrichment of either the LDLR-immunogold label (1.59-fold enrichment) or LDL-gold label (1.65-fold enrichment); however, coated pits of JD cells displayed significant enrichment of the β-VLDL-gold label (14.77-fold). These observations indicate that the Y807C mutation inhibits the ability of the LDLR to accumulate in coated pits in the absence of lipoproteins or in the presence of LDL, but does not prevent the LDLR from accumulating in coated pits in the presence of β-VLDL. The LDL-gold results with normal and JD cells are

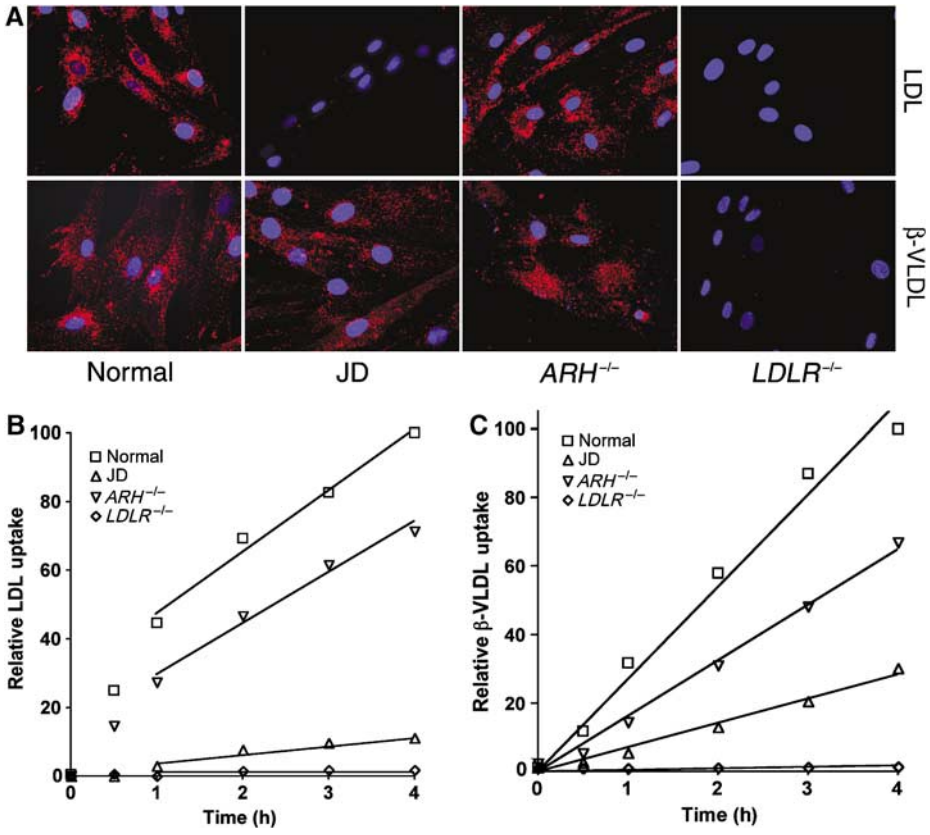
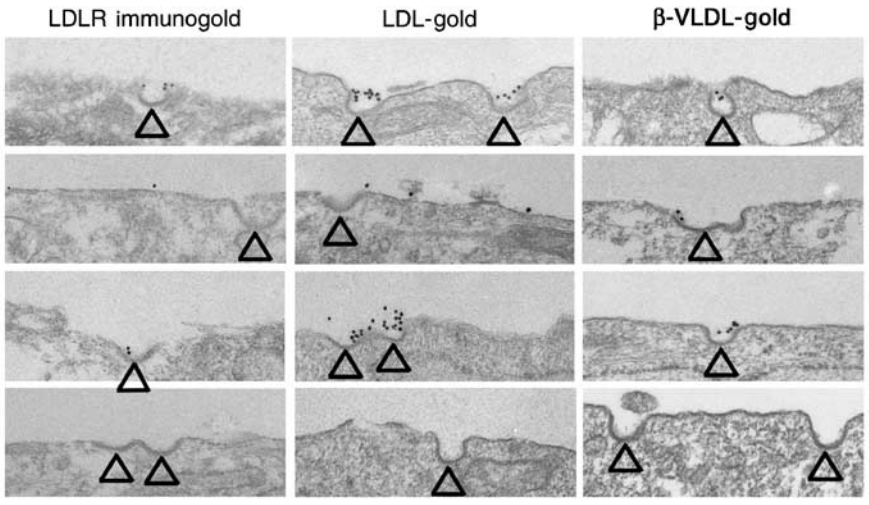


Figure 1 JD fibroblasts can internalize β-VLDL but not LDL. Normal, JD, $ARH^{-/-}$ and $LDLR^{-/-}$ fibroblasts were cultured in lipoprotein poor media for 48 h. (**A**) Cells were treated with 20 μg/ml Alexa546-labeled LDL or 10 μg/ml DiI-labeled β-VLDL for 2 h at 37°C and visualized by epifluorescent microscopy. (**B, C**), cells were treated with 10 μg/ml Alexa546-labeled LDL (B) or 5 μg/ml DiI-labeled β-VLDL (C) for 1 h at 4°C, and then shifted to 37°C for the times indicated. Cells were washed, suspended and fixed at 4°C. Mean fluorescent uptake of lipoproteins was determined by flow cytometry and is reported as a percentage of normal cell uptake at 4 h.

similar to previous results obtained with these fibroblasts using ferritin-labeled LDL (Anderson *et al*, 1977). In $ARH^{-/-}$ cells, all three gold labels were enriched in coated pits, indicating that the binding of ARH to the FDNPVY sequence of the LDLR is not required for the targeting of the LDLR to coated pits in fibroblasts, consistent with the ability of $ARH^{-/-}$ fibroblasts to internalize LDL (Harada-Shiba *et al*, 1991; Zuliani *et al*, 1995) and β-VLDL (Figure 1). The labeling of cells with LDLR-immunogold, LDL-gold and β-VLDL-gold was LDLR dependent because few gold particles were observed on $LDLR^{-/-}$ cells (Figure 2). Together, these observations indicate that the LDLR-Y807C supports targeting of β-VLDL but not LDL to clathrin-coated pits, and suggest that β-VLDL promotes the accumulation of the LDLR-Y807C in coated pits.

To directly test whether β-VLDL induced the internalization of the LDLR-Y807C, we inhibited LDLR recycling using the proton ionophore, monensin, and examined the loss of surface LDLR over time (Basu *et al*, 1981; Michaely *et al*, 2004). LDLRs on the cell surface were labeled with biotin using a non-cell-permeable biotinylation reagent. The biotinylated proteins were isolated using neutravidin–agarose and then immunoblotted to assess the relative amount of surface LDLR (Michaely *et al*, 2004). Treatment of normal cells with monensin alone, monensin plus LDL or monensin plus β-VLDL significantly reduced the number of LDLRs on the cell surface. By contrast, treatment of JD fibroblasts with monensin alone or monensin plus LDL did not change the number of surface LDLR-Y807C; however, treatment of JD fibroblasts with monensin plus β-VLDL sharply reduced the number of surface LDLR-Y807C (Figure 3A). Treatment with monensin and lipoproteins did not cause a global loss of surface proteins, because no change was observed in the cell surface level of CD44 (Figure 3A), a hyaluronic acid receptor that is not internalized through clathrin-coated pits (Tammi *et al*, 2001). The amount of LDLR in the cell lysates before separation of the biotinylated material was similar under all conditions, indicating that the loss of surface LDLR was not due to a loss of total cellular LDLR content (Figure 3B). These observations show that β-VLDL promotes internalization of LDLR-Y807C.

We next compared the fate of the internalized lipoproteins by incubating the fibroblasts with LDL-gold or β-VLDL-gold at 37°C. LDL-gold accumulated in late endosomes and lysosomes of normal and $ARH^{-/-}$ cells, but not JD cells. In contrast, treatment of fibroblasts with β-VLDL-gold at 37°C resulted in accumulation of β-VLDL-gold in late endosomes and lysosomes of normal, $ARH^{-/-}$ and JD cells (Figure 4). The greater accumulation of LDL-gold in endosomes of normal and $ARH^{-/-}$ cells as compared to β-VLDL-gold is likely due to the more efficient trafficking of LDL to late endosomes (Lombardi *et al*, 1993; Jones *et al*, 2000). The relative ability of the four fibroblasts to transport LDL-gold and β-VLDL-gold to late endosomes and lysosomes correlated



Genotype	Membrane length (μm)	Gold particles	Gold per μm	Membrane length (μm)	Gold particles	Gold per μm	Coated pit enrichment*	
	----- Non-coated pit membrane -----			----- Coated pit membrane -----				
Normal	1573	341	0.22	25	84	3.36	15.50	LDLR immunogold
JD	1344	349	0.26	17	7	0.41	1.59	
ARH$^{-/-}$	1229	215	0.17	23	52	2.26	12.92	
LDLR$^{-/-}$	1298	16	0.01	18	0	0.00	0.00	
Normal	940	101	0.11	17	158	9.29	86.50	LDL-gold
JD	810	441	0.54	20	18	0.90	1.65	
ARH$^{-/-}$	906	157	0.17	20	92	4.60	26.55	
LDLR$^{-/-}$	967	11	0.01	27	0	0.00	0.00	
Normal	1346	96	0.07	37.2	113	3.04	42.58	β-VLDL-gold
JD	1425	135	0.09	14.3	20	1.40	14.77	
ARH$^{-/-}$	1601	163	0.10	40.5	164	4.05	39.76	
LDLR$^{-/-}$	1208	4	0.00	29.2	0	0.00	0.00	

*Coated pit enrichment=gold per μm in coated pits divided by gold per μm outside coated pits

Figure 2 β-VLDL-gold but not LDLR-immunogold or LDL-gold is present in the clathrin-coated pits of JD fibroblasts. Normal, JD, *ARH*$^{-/-}$ and *LDLR*$^{-/-}$ fibroblasts were cultured in lipoprotein-poor media for 48 h and then processed for LDLR-immunogold, LDL-gold or β-VLDL-gold surface labeling as described in Materials and methods. Arrowheads indicate coated pits. Quantification of the gold particle distributions is presented below the electron micrographs.

with their ability to degrade these lipoproteins (Supplementary Figure S2). Because *LDLR*$^{-/-}$ cells failed to internalize or degraded either LDL or β-VLDL, the uptake and degradation of both LDL and β-VLDL required the presence of the LDLR. Taken together, these results show that the LDLR-Y807C has a ligand-induced, FDNPVY-independent internalization mechanism that can support the coated pit targeting, internalization and degradation of β-VLDL but not LDL.

To identify the sequences in the LDLR-Y807C that are required for β-VLDL uptake, we generated a series of GFP-tagged LDLR constructs containing truncations or amino-acid substitutions and assayed their ability to support uptake of fluorescently labeled LDL and β-VLDL in transiently transfected *LDLR*$^{-/-}$ fibroblasts. Introduction of a stop codon at W792 (W792X) sharply reduced both LDL and β-VLDL uptake, indicating that the cytoplasmic domain is required for uptake of both LDL and β-VLDL (Figure 5 and Supplementary Figure S3). Consistent with this conclusion, neither LDL nor β-VLDL was internalized by fibroblasts from the hypercholesterolemic individual AH (Supplementary Figure S4), who is homozygous for the W792X allele of the LDLR (Lehrman *et al*, 1985). Introduction of a stop codon at residue 823 or 829 in the LDLR-Y807C had little effect on the ability of the LDLR to support β-VLDL uptake, whereas introduction of a stop codon at residue 812 or 817 of the LDLR-Y807C markedly reduced uptake of β-VLDL (Figure 5). These data suggest that the residues between 817 and 823 are involved in β-VLDL uptake by the LDLR-Y807C. Alanine substitution of amino acids 816–818 (but not 812–814, 814–816 or 818–820) markedly reduced internalization of β-VLDL by the LDLR-Y807C (Figure 5). β-VLDL uptake was not observed in cells not expressing the GFP-LDLR constructs, indicating that uptake required expression of the LDLR. Uptake was specific for β-VLDL because little uptake of LDL was observed in cells expressing GFP-LDLR constructs with the Y807C mutation (Supplementary Figure S3). These observations suggest that β-VLDL uptake by the LDLR-Y807C involves the HIC^{818} sequence.

To better assess the relative importance of the HIC^{818} sequence on β-VLDL uptake, we introduced the normal, HIC^{818}>AAA, Y807C, Y807C+HIC^{818}>AAA and W792X variants of the LDLR into LDLR$^{-/-}$ fibroblasts, using retroviruses. To avoid the possibility that fusion of GFP to the LDLR altered uptake, we generated retroviruses that allowed bicistronic expression of the LDLR and GFP through the presence of an internal ribosomal entry site, which separated the two coding sequences. Thus, because both the LDLR and GFP were produced from the same message, cells with GFP expression (green fluorescence) also expressed an LDLR and the amount of GFP expression is proportional to the amount of LDLR expression (Liu *et al*, 1997). Comparison of LDL uptake showed that cells expressing LDLR variants with the Y807C mutation were unable to internalize LDL, and that the HIC^{818}>AAA mutation had little impact on LDL uptake

(Supplementary Figure S5A). By contrast, comparison of β-VLDL uptake showed that the LDLR-Y807C was able to support approximately half the β-VLDL uptake of the normal LDLR, and that the HIC^{818}>AAA mutation reduced β-VLDL uptake of the LDLR-Y807C by half (Supplementary Figure S5B). These observations indicate that approximately half of the β-VLDL uptake activity of the LDLR-Y807C depends on the HIC^{818} sequence.

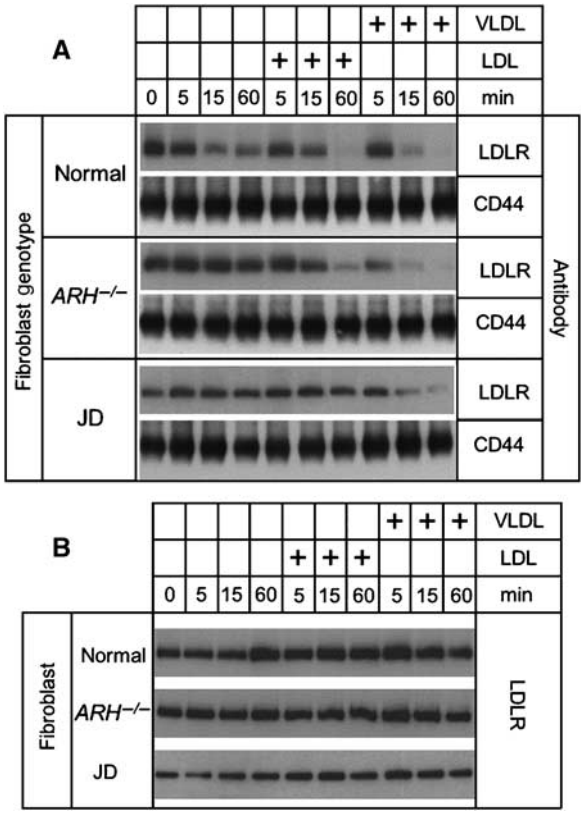


Figure 3 β-VLDL can drive uptake of the LDLR-Y807C of JD fibroblasts. Normal, JD and ARH$^{-/-}$ fibroblasts were cultured in lipoprotein-poor media for 48 h and then treated with monensin alone for 0, 5, 15 or 60 min, or in combination with 20 μg/ml LDL for 5, 15 or 60 min, or in combination with 10 μg/ml βVLDL for 5, 15 or 60 min. Cells were then surface biotinylated and lysed. The biotinylated proteins were isolated from the whole-cell lysates by neutravidin agarose precipitation. (**A**) Biotinylated proteins were run on 5–17% SDS–PAGE gels, transferred to nylon membranes and immunoblotted for the presence of LDLR or for CD44. (**B**) The whole-cell lysates were run on 5–17% SDS–PAGE gels, transferred to nylon membranes and immunoblotted for the presence of LDLR.

Uptake at steady state involves endocytosis, release of bound lipoprotein in the endosome and recycling of unbound receptors back to the cell surface. To determine whether the reduced β-VLDL uptake observed with the LDLR-Y807C + HIC^{818}>AAA was caused by a reduced rate of β-VLDL endocytosis, we measured initial rates of β-VLDL endocytosis. For these experiments, cells infected with viruses that direct expression of normal, HIC^{818}>AAA, Y807C, Y807C + HIC^{818}>AAA LDLR variants were sorted by fluorescence-activated cell sorting (FACS) to produce cell populations in which >95% of the cells expressed GFP and had LDLR on their cell surfaces (data not shown). The total LDLR expression by the infected fibroblasts was similar to the endogenous level expressed by normal human fibroblasts (Figure 6A). Comparison of the initial rates of uptake of ^{125}I-β-VLDL showed that the HIC^{818}>AAA mutation reduced by half the rate of ^{125}I-β-VLDL endocytosis supported by the LDLR-Y807C (Figure 6B and C). These observations indicate that the reduced uptake of β-VLDL by the LDLR-Y807C + HIC^{818}>AAA results from a reduced ability to internalize β-VLDL.

Discussion

The central finding of this study is that the Y807C mutation in the LDLR (LDLR-Y807C), which disrupts the canonical internalization motif (FDNPVY^{807}), prevented internalization of LDL but not β-VLDL. JD fibroblasts, which express only the LDLR-Y807C, were able to internalize β-VLDL in a clathrin-coated pit-dependent manner, but were unable to internalize LDL (Figure 1 and Supplementary Figure S1). When β-VLDL-gold was added to JD fibroblasts, the label accumulated in coated pits, which is consistent with uptake occurring through clathrin-coated pits (Figure 2). Uptake was specific for β-VLDL because no coated pit enrichment was seen when the LDLR-Y807C was labeled with LDLR-immunogold or

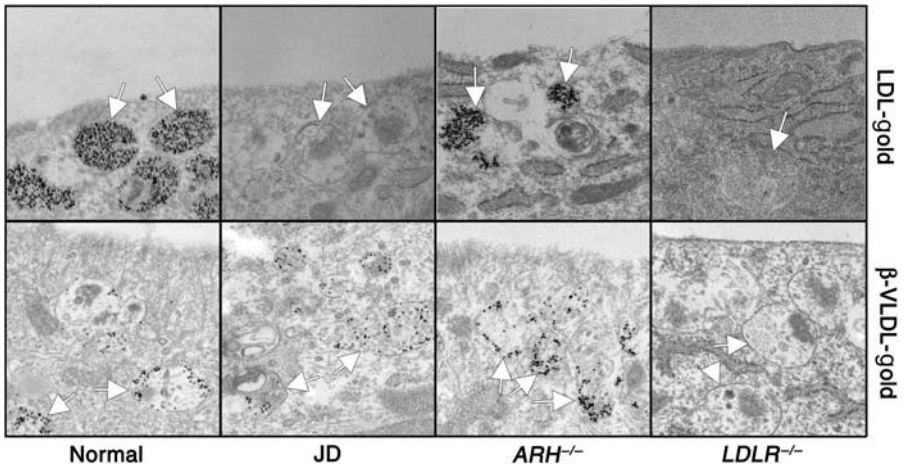
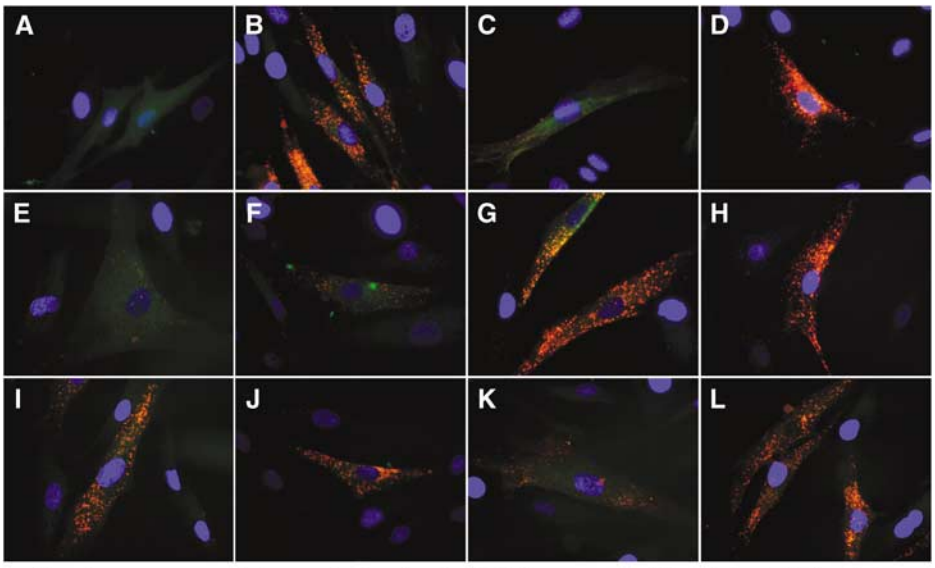
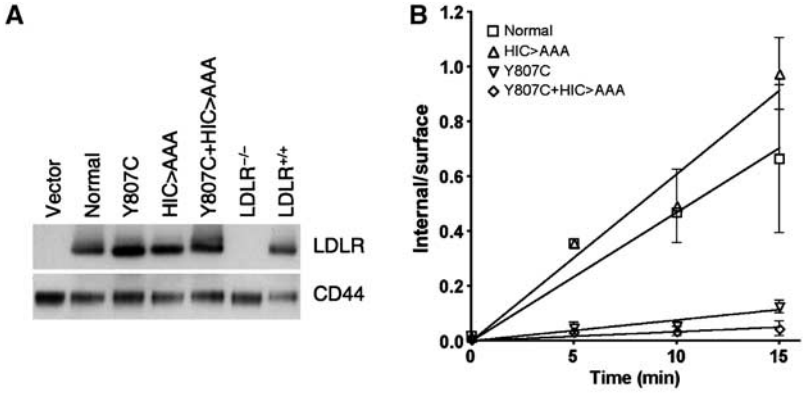


Figure 4 JD fibroblasts can target β-VLDL but not LDL to late endosomes. Normal, JD, ARH$^{-/-}$ and LDLR$^{-/-}$ fibroblasts were cultured in lipoprotein-poor media for 48 h and then treated with LDL-gold or β-VLDL-gold for 90 min at 37°C. Arrows indicate multivesicular bodies, a morphologically distinctive late endosome.



Panel	Cytoplasmic domain (K790–A833)	LDL uptake	β-VLDL uptake
A		–	–
B	-KNWRLKNINSINFDNPVYQKTTEDEVHICHNQDGYSYPSRQMVSLEDDVA	+	+
C	X	–	–
D	C	–	+
E	C X	–	–
F	C X	–	–
G	C X	–	+
H	C X	–	+
I	C AAA	–	+
J	C AAA	–	+
K	C AAA	–	–
L	C AAA	–	+

Figure 5 The HIC^{818} sequence is required for β-VLDL uptake by the LDLR-Y807C. LDLR$^{-/-}$ fibroblasts were transfected with plasmids that direct the expression of GFP (**A**), GFP-LDLR (**B**), GFP-LDLR-W792X (**C**), GFP-LDLR-Y807C (**D**), GFP-LDLR-Y807C + E812X (**E**), GFP-LDLR-Y807C + I817X (**F**), GFP-LDLR-Y807C + G823X (**G**), GFP-LDLR-Y807C + R829X (**H**), GFP-LDLR-Y807C + EDE^{814} > AAA (**I**), GFP-LDLR-Y807C + EVH^{816} > AAA (**J**), GFP-LDLR-Y807C + HIC^{818} > AAA (**K**) or GFP-LDLR-Y807C + CHN^{820} > AAA (**L**). Cells were then plated on coverslips, treated with lipoprotein poor media, incubated at 37°C with DiI-β-VLDL for 2 h and visualized for the presence of β-VLDL (red), GFP- (green) and DAPI-stained nuclei (blue). Shown below the fluorescent images is a diagram detailing the mutations and their effect on lipoprotein uptake.



C

	Vector			Normal			Y807C			HIC>AAA			Y807C+HIC>AAA		
Time	Internal	Surface	Ratio	Internal	Surface	Ratio	Internal	Surface	Ratio	Internal	Surface	Ratio	Internal	Surface	Ratio
0	5	122	0.045	57	4809	0.012	95	18 511	0.005	73	5201	0.014	111	17 387	0.006
5	6	111	0.055	1124	3606	0.29	491	11 426	0.046	719	3387	0.208	542	15 071	0.035
10	6	118	0.046	1363	3889	0.351	803	12 001	0.068	1396	3466	0.403	589	10 813	0.056
15	23	119	0.209	1107	2086	0.531	1353	11 797	0.117	3625	4551	0.81	662	11 235	0.059

Figure 6 Mutation of the HIC^{818} sequence reduces the rate of β-VLDL uptake by the Y807C LDLR by half—LDLR$^{-/-}$ fibroblasts were infected with bicistronic retroviruses encoding just GFP (vector) or the normal, Y807C, HIC^{818} > AAA or Y807C + HIC^{818} > AAA variants of the LDLR, together with GFP. Cells were sorted based upon green fluorescence to > 95% homogeneity. (**A**) A 5 μg weight of total cell lysate from each fibroblast was run on SDS–PAGE gels, transferred to nylon membranes and immunoblotted for the LDLR or for CD44. (**B, C**) Cells were incubated with 10 μg/ml ^{125}I-β-VLDL for 1 h at 4°C, and then shifted to 37°C for 0, 5, 10 or 15 min. Internalized and surface-bound ^{125}I-β-VLDL were separated as described in Materials and methods, and the ratio is presented in panel B Individual values for internalized and surface-bound β-VLDL are presented in panel C Each point is the mean of four determinations. Error bars show the standard deviation. The experiment shown is representative of three independent experiments.

LDL-gold (Figure 2), and because β-VLDL but not LDL promoted the internalization of the LDLR-Y807C (Figure 3). The internalized β-VLDL trafficked to late endosomes and lysosomes (Figure 4) and was degraded (Supplementary Figure S2) by the JD fibroblasts. The inability of $LDLR^{-/-}$ fibroblasts to bind (Figure 2), internalize (Figures 1 and 4) or degrade (Supplementary Figure S2) β-VLDL indicated that β-VLDL uptake by JD fibroblasts required the LDLR-Y807C. These findings suggest that the LDLR has an ancillary mechanism, independent of the FDNPVY sequence, which allows ligand-dependent uptake of β-VLDL in JD cells. This novel mechanism appears to involve the HIC^{818} sequence in the LDLR cytoplasmic domain, because mutation of these residues to alanine reduced the ability of the LDLR-Y807C to support β-VLDL uptake (Figures 5 and 6 and Supplementary Figure S5).

Together, these observations show that the LDLR is more than a simple, constitutive, endocytic receptor. Endocytic receptors can be classified into two categories: constitutive and ligand dependent (Hopkins *et al*, 1985). Ligand-dependent endocytic receptors, such as the EGF receptor, are efficiently targeted to coated pits and internalized only in the presence of their ligands (Gorden *et al*, 1978; Wiley *et al*, 1991). By contrast, constitutive endocytic receptors, such as the transferrin receptor, are targeted to coated pits and internalized in both the presence and absence of ligand (Willingham *et al*, 1984; Ajioka and Kaplan, 1986). The LDLR qualifies as a constitutive receptor because the it is present in coated pits in the absence of LDL (Anderson *et al*, 1982; Figure 2), and because the LDLR can be internalized in the absence of LDL (Basu *et al*, 1981; Figure 3); however, the results of this study indicate that the LDLR can also function as a ligand-dependent endocytic receptor for the apoE-dependent uptake of the VLDL remnant-like lipoprotein, β-VLDL. Thus, the LDLR functions as a hybrid endocytic receptor with both constitutive and ligand-induced properties.

Uptake by endocytic receptors involves internalization determinants that function by associating with components of clathrin-coated pits. The NPXY and YXXΦ motifs are examples of constitutive determinants that bind to coated pit components in the presence or absence of bound ligands (Bonifacino and Traub, 2003). Ligand-induced determinants frequently involve post-translational modifications that are triggered by ligand binding. Common examples include phosphorylation and ubiquitination events (Bonifacino and Traub, 2003; Lefkowitz and Whalen, 2004). The LDLR can be phosphorylated (Kishimoto *et al*, 1987), however, the phosphorylation site at S833 does not appear to be involved in β-VLDL uptake by the LDLR-Y807C, because truncation of the LDLR-Y807C at R829 did not appear to reduce β-VLDL uptake (Figure 5). Ubiquitination of the LDLR is also unlikely because ubiquitination of receptors frequently results in their degradation (Hurley and Emr, 2006), yet JD cells showed no loss in their ability to internalize β-VLDL over time (Figure 1). Ubiquitination may play a role through clathrin adaptor proteins that can be ubiquitinated (Lefkowitz and Whalen, 2004), and future experiments will address this possibility. Ligand-induced endocytosis can also involve receptor dimerization (Bonifacino and Traub, 2003). The LDLR can form dimmers, and interestingly, C818 is likely in or near the dimerization surface because dimerization requires residues 812–839 and is sensitive to the sulfhydryl alkylating agent, *N*-ethylmaleimide (van Driel *et al*, 1987). β-VLDL binding may promote LDLR dimerization because β-VLDL, like other VLDL species, has multiple copies of apoE and can engage multiple LDLR simultaneously (Windler *et al*, 1980). VLDL-induced dimerization may generate a novel binding site for a coated pit component. Future work with cells stably expressing LDLR variants will address this possibility.

A second question is why the LDLR has a separate, ligand-induced mechanism for the internalization of VLDL. One possibility suggested by our data is that the VLDL-induced mechanism augments VLDL uptake by the normal receptor. β-VLDL-gold labeling of the normal LDLR showed higher coated pit enrichment (44-fold) than the immunogold labeling (15-fold) in the absence of lipoproteins (Figure 2), suggesting that the binding of β-VLDL-gold promoted the accumulation of the LDLR in coated pits. In support of this possibility, co-treatment of normal fibroblasts with monensin and β-VLDL resulted in a more complete loss of LDLR from the cell surface than was observed with monensin alone (Figure 3).

The presence of a VLDL-specific mechanism for the uptake of the LDLR suggests that the LDLR can function solely as an endocytic receptor for VLDL. The degree to which the LDLR functions in LDL or VLDL uptake may be modulated by the relative expression or activity of adaptor proteins, which bind to the FDNPVY sequence. Perhaps by modulating the expression of these adaptors, the function of the LDLR can be altered from a dual specificity apoB100/apoE receptor to a ligand-induced, apoE-specific receptor. This change may play a role in maintaining lipoprotein homeostasis under different nutritional states, or allow uptake of VLDL but not LDL in specific tissues. Lung, for example, expresses moderate levels of the LDLR, but does not express ARH or dab2 (Fazili *et al*, 1999; Garcia *et al*, 2001), and organ perfusion studies have shown that lung will absorb VLDL and VLDL remnants but not LDL (Pietra *et al*, 1976). Liver may also employ the FDNPVY-independent mechanism because $Arh^{-/-}$ mice are able to clear VLDL and β-VLDL faster than $Ldlr^{-/-}$ mice (Jones *et al*, 2007). Interestingly, ARH deficiency in humans is characterized by the formation of large xanthomas, which are cholesterol ester-rich deposits formed by macrophages (Khachadurian and Uthman, 1973; Harada-Shiba *et al*, 2003). VLDL remnants such as β-VLDL are potent activators of cholesterol ester production in macrophages (Goldstein *et al*, 1980; Mahley *et al*, 1980), and an FDNPVY-independent mechanism of VLDL remnant uptake may contribute to xanthoma formation in ARH deficiency. Consistent with this possibility, previous studies suggest that macrophages internalize β-VLDL via a mechanism that is distinct from that of LDL uptake (Tabas *et al*, 1990; Myers *et al*, 1993).

In summary, we have shown that the LDLR has a ligand-induced, FDNPVY-independent internalization mechanism that can support uptake of β-VLDL but not LDL when the FDNPVY sequence is inactivated by the Y807C mutation. This novel internalization mechanism appears to involve the HIC^{818} sequence in the LDLR cytoplasmic domain.

Materials and methods

Materials

All cell culture reagents were purchased from Gibco (Carlsbad, CA). The $LDLR^{-/-}$ and JD fibroblasts were a gift from Michael Brown

and Joseph Goldstein (Department of Molecular Genetics, UT Southwestern Medical Center, Dallas, TX). The Normal and *ARH*$^{-/-}$ fibroblasts were a gift from Sebastiano Calandra and Roberta Tiozzo (Department of Biomedical Sciences, University of Modena, Italy). Rabbit anti-LDLR IgG used for electron microscopy was from Maine Biotechnology Services (Portland, ME). Rabbit anti-LDLR IgG used for immunoblotting was a gift from Joachim Herz (Department of Molecular Genetics, UT Southwestern Medical Center, Dallas, TX). Goat anti-rabbit IgG (10 nm gold labeled) and PD-10 columns were from Amersham (Piscataway, NJ). Formaldehyde was from Fluka (Buchs, Switzerland). Alexa546 succinimidyl ester was from Molecular Probes (Carlsbad, CA). All other chemicals were from Sigma (St Louis, MO). LDL was prepared from freshly drawn human plasma, as previously described (Goldstein *et al*, 1983). Beta migrating VLDL (β-VLDL) was prepared from the serum of cholesterol fed rabbits, as previously described (Kovanen *et al*, 1981).

Cell culture

Fibroblasts, isolated from normal, ARH-deficient and LDLR-deficient subjects, and from JD, were cultured in media A (DMEM media supplemented with 10% (v/v) fetal bovine serum, 20 mM HEPES pH 7.5, 100 U/ml penicillin G and 100 μg/ml streptomycin). LDLR expression was upregulated by replacing media A with media B (DMEM media supplemented with 10% (v/v) lipoprotein-poor serum, 20 mM HEPES pH 7.5, penicillin G (100 U/ml) and streptomycin (100 μg/ml)) for 48 h.

Electron microscopy

Colloidal gold-conjugated LDL (LDL-gold) and colloidal gold-conjugated β-VLDL (β-VLDL-gold) were produced using methods established for the production of LDL-gold (Handley *et al*, 1981; Michaely *et al*, 2004). Cell surface immunolabeling was performed as previously described (Michaely *et al*, 2004). Surface labeling and uptake with LDL-gold and β-VLDL-gold were performed by incubation at either 4 or 37°C, in the presence of 10 μg/ml of LDL-gold or β-VLDL-gold for 90 min. The cells were washed three times using buffer A (PBS plus 2 mg/ml BSA, pH 7.4) and fixed in buffer B (3% (w/v) paraformaldehyde in PBS). The cells were then embedded, sectioned and visualized as previously described, using a JEOL 1200 electron microscope operated at 80 kV (Michaely *et al*, 2004).

Quantification of gold labeling

Electron microscope images were obtained by taking 50 micrographs of each cell type (normal, *ARH*$^{-/-}$, *LDLR*$^{-/-}$ and JD) that had been labeled with LDLR-immunogold, LDL-gold or β-VLDL-gold. Micrographs were coded and the length of the non-coated pit membranes, the diameter of the coated pits and the number of gold particles associated with each of these two regions were determined by a naive observer. The labeling intensity was expressed as the number of gold particles per micrometer length of the different regions of the plasma membrane. Clusters of tightly associated gold particles were assumed to be aggregates and were counted as one particle. Gold particles separated by a gap greater than twice their diameter were counted as single particles.

Lipoprotein degradation assays

Human ^{125}I-LDL and rabbit ^{125}I-β-VLDL degradation assays were performed in triplicate at 37°C for 5 h using 15 μg/ml ^{125}I-LDL or 15 μg/ml ^{125}I-β-VLDL (Goldstein *et al*, 1983).

Monensin treatment and analysis of surface biotinylated proteins

Fibroblasts were treated with 25 μM monensin alone or in combination with 20 μg/ml LDL or 10 μg/ml β-VLDL for the times indicated. Cells were washed with ice-cold PBS and incubated with buffer C (1 mg/ml Sulfo-NHS-(LC)-biotin (Pierce) in PBS, pH 8.0) for 30 min at 4°C. Cells were then washed with ice-cold PBS and incubated with buffer D (100 mM NaCl and 20 mM Tris pH 8.0) for 30 min at 4°C. Cells were lysed in 1 ml buffer E (1% Triton X-100, 4 mM EGTA, 10 mM Tris pH 8.0) and lysates clarified by centrifugation for 15 min at 20 000 *g* at 4°C. A 100 μl volume of the lysate was set aside to compare total LDLR content. Biotinylated proteins from 800 μl of the lysate were precipitated by addition of 100 μl of a 50% slurry of neutravidin agarose (Pierce), followed by end over end mixing for 1 h at 4°C. Biotinylated proteins bound to the agarose were washed three times with buffer F (15 mM Tris pH 8.0, 500 mM NaCl, 4 mM EGTA and 0.5% Triton X-100) and once with buffer G (15 mM Tris pH 8.0, 4 mM EGTA and 0.5% Triton X-100). Biotinylated proteins were eluted from neutravidin agarose by the addition of an equal volume (50 μl) of 5 × SDS–PAGE sample buffer and heating to 100°C for 10 min. Samples were loaded on 5–17% SDS–PAGE gels, transferred to PVDF membranes and immunoblotted for LDLR or for CD44 (Chemicon).

Introduction of LDLR variants into LDLR$^{-/-}$ fibroblasts

For the GFP-LDLR fusions, the signal sequence of the human LDLR was inserted into the *Age*I site at the 5′ end of GFP in the pEGFP-C2 vector. The remainder of the human LDLR was cloned into the *Hin*dIII/*Kpn*I sites at the 3′ end of GFP. Mutations were introduced into the LDLR by site-directed mutagenesis (Quikchange, Stratagene). GFP-LDLR expression plasmids were introduced into *LDLR*$^{-/-}$ fibroblasts by electroporation (AMAXA). For the LDLR retroviruses, LDLR variants were cloned into the pMX-IRES-GFP bicistronic retroviral vector (Liu *et al*, 1997). Retroviral vectors were cotransfected with the pAmpho vector (Clontech) into 293T cells to produce infectious, replication-defective retroviruses. Retroviruses were added to *LDLR*$^{-/-}$ fibroblasts in the presence of hexadimethrine bromide to promote viral entry.

Flow cytometry

Cells were incubated with either 10 μg/ml Alexa546-labeled LDL or 5 μg/ml DiI-labeled β-VLDL in media C (bicarbonate-free DMEM supplemented with 20 mM HEPES pH 7.5, and 10% HLPPS) for 1 h at 4°C. The media was then replaced with warm media B containing 10 μg/ml 546-LDL or 5 μg/ml DiI-β-VLDL for the times indicated. Cells were washed with ice-cold PBS, suspended by scraping in PBS and fixed in the presence of 3% paraformaldehyde. Cells were washed with PBS and analyzed by flow cytometry on a FACS Calibur (BD Biosciences). For the determination of LDL and β-VLDL uptake by endogenous LDLRs, the mean fluorescence intensities were recorded for 10 000 events. For the determination of LDL and β-VLDL uptake by LDLRs introduced by retroviruses, intact GFP-positive cells were gated and the mean green (FL1) and red (FL2) fluorescence values were recorded. The FL2 fluorescence values were divided by the FL1 fluorescence values and are reported as a percentage of the uptake supported by the normal LDLR.

FACS

Cells infected with retroviruses were sorted twice based on green (GFP) fluorescence, using a MoFlo High Performance Cell Sorter (Dako). Purity was 90% after the first sorting and >95% after the second sorting.

Initial endocytic rates

Initial endocytic rates were determined as previously described (Lombardi *et al*, 1993). Briefly, cells were incubated with 10 μg/ml ^{125}I-β-VLDL for 1 h at 4°C in media C. Media was replaced for the times indicated with warm media B, also containing 10 μg/ml ^{125}I-β-VLDL. Cells were extensively washed with ice-cold PBS and incubated with 1 mg/ml Protease K in buffer H (PBS + 1 mM EDTA) for 2 h at 4°C. The cell suspension was then centrifugated at 5000 *g* for 10 min over a cushion of 10% sucrose in PBS. The tubes were frozen in liquid nitrogen, cut to separate the cells (internal) from the solution (surface-bound material released by protease K) and counted on a gamma counter. Nonspecific activity was assessed in parallel experiments in the presence of 250 μg/ml unlabeled β-VLDL. Nonspecific activities were subtracted from mean values for each data point.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank Richard Anderson for many helpful discussions, Joachim Herz for the anti-LDLR antibody, Michael Brown and Joseph Goldstein for the *LDLR*$^{-/-}$ and JD fibroblasts, and Sebastiano Calandra and Roberta Tiozzo for the normal and *ARH*$^{-/-}$ fibroblasts. This work was supported by NIH grants HL085218-01 (PM) and HL20948 (HH).

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