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RAP, a specialized chaperone, prevents ligand-induced ER retention and degradation of LDL receptor-related endocytic receptors

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The multifunctional low density lipoprotein (LDL) receptor-related protein (LRP) forms a complex with a receptor-associated protein (RAP) within the secretory pathway. RAP inhibits ligand binding to LRP and is required for normal functional expression of LRP *in vivo*, suggesting a physiological function as a specialized chaperone. We have used RAP-deficient mice, generated by gene targeting, to investigate the role of RAP in the biosynthesis and biological activity of LRP and other members of the LDL receptor gene family in various organs and in embryonic fibroblasts. Our results demonstrate that RAP is required for the proper folding and export of the receptors from the endoplasmic reticulum (ER) by preventing the premature binding of co-expressed ligands. Overexpression of apolipoprotein E (apoE), a high affinity ligand for LRP, results in dramatically reduced cellular LRP expression, an effect that is prevented by co-expression of RAP. RAP thus defines a novel class of molecular chaperones that selectively protect endocytic receptors by binding to newly synthesized receptor polypeptides, thereby preventing ligand-induced aggregation and subsequent degradation in the ER.

Keywords: Alzheimer's disease/apoE/secretory pathway

Introduction

A growing number of endocytic cell surface receptors comprise the low density lipoprotein (LDL) receptor gene family. These proteins participate in diverse biological processes which include lipoprotein and vitellogenin metabolism and also the regulation of extracellular protease activity. Four genetically distinct receptors are known currently in mammalian species, the LDL receptor, a very low density lipoprotein (VLDL) receptor and two large multifunctional receptors, the LDL receptor-related protein (LRP) and its close homolog gp330/megalin, also known as the Heymann nephritis antigen. In contrast to the LDL receptor, which appears to be restricted to the cellular uptake of cholesterol-rich lipoproteins, an expanding number of functionally diverse ligands is being identified that interact with the other members of this gene family (reviewed in Krieger and Herz, 1994). The best characterized of these other receptors is the LRP, which participates in the uptake of dietary cholesterol into the liver, can modulate extracellular plasminogen activation and is involved in the neuronal metabolism of apolipoprotein E (apoE) and of protease-nexin II, also known as the amyloid precursor protein (APP) (Kounnas *et al.*, 1995). ApoE, APP and also tissue-type plasminogen activator (t-PA), another LRP ligand, are thought to mediate or modulate neurodegenerative processes that lead to Alzheimer disease (reviewed in Strittmatter *et al.*, 1993; Selkoe, 1994) or, in the case of t-PA, excitotoxic neuronal cell death (Tsirka *et al.*, 1995). Gene knockout experiments suggest that the VLDL receptor plays a role in the metabolism of adipose tissue and participates in fat accretion (Frykman *et al.*, 1995), while gp330/megalin is expressed primarily on resorptive epithelia and is thought to mediate the re-uptake of excreted proteins from the primary glomerular filtrate (Kerjaschki and Farquhar, 1983; Moestrup *et al.*, 1995).

The biosynthetic pathways and post-translational modifications of the LDL receptor and the other members of the family have been studied in detail. In the case of the LDL receptor and VLDL receptor, post-translational modifications include the addition of a substantial number of *O*-linked carbohydrate residues in a late Golgi compartment. Such *O*-linked sugar addition sites are missing in gp330/megalin and in LRP. However, LRP undergoes a unique proteolytic processing event in a *trans*-Golgi compartment. There, the resident endoproteinase furin cleaves the precursor polypeptide, LRP 600, into two subunits, LRP 515 and LRP 85 (Herz *et al.*, 1990; Willnow *et al.*, 1996). Both subunits remain non-covalently associated with each other. While the functional significance of these post-translational processing events is not known, they have proved useful for tracing newly synthesized receptor molecules as they travel through the secretory pathway en route to the cell surface.

Another functional difference between the LDL receptor and the three other receptors is the ability of the latter to bind tightly a receptor-associated protein (RAP). RAP was identified initially as a protein that co-purified with LRP (Kristensen *et al.*, 1990; Strickland *et al.*, 1990) and subsequently was shown to also bind strongly to gp330/megalin and to the VLDL receptor (Orlando *et al.*, 1992; Battey *et al.*, 1994), but only weakly to the LDL receptor (Mokuno *et al.*, 1994). Recombinant RAP potently inhibits ligand binding to the receptors *in vitro* and *in vivo* (Herz *et al.*, 1991; Willnow *et al.*, 1994a). This inhibitory property of RAP has been exploited in numerous studies (Herz *et al.*, 1991; Willnow *et al.*, 1994a; Kounnas *et al.*, 1995; Narita *et al.*, 1995); however, these experiments did not allow any conclusions about the functional role of endogenous RAP *in vivo*.

An indication of the physiological significance of RAP came from two different lines of experimental evidence.

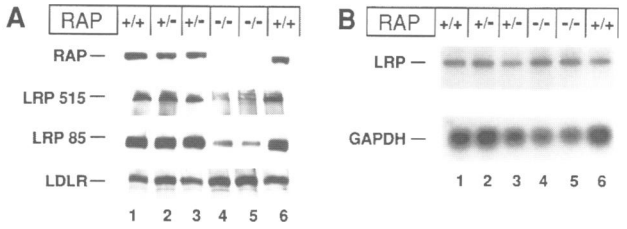

Fig. 1. Western and Northern blot analysis of LRP in mouse livers. (**A**) Membrane proteins were partially purified from wild-type (lanes 1 and 6), heterozygous (lanes 2 and 3) or homozygous (lanes 4 and 5) RAP-deficient mouse livers. Protein (20 μg/lane) was subjected to non-reducing 4–15% SDS–PAGE and immunoblot analysis using 5 μg/ml of rabbit polyclonal antibodies directed against RAP, LRP 515 and 85 or LDL receptor. Bound IgG was detected using the enhanced chemiluminescence system (ECL, Amersham). (**B**) Fifteen micrograms of total liver RNA from wild-type (lanes 1 and 6), heterozygous (lanes 2 and 3) or homozygous (lanes 4 and 5) RAP-deficient mice were separated on a 1% formaldehyde gel, transfered to nitrocellulose and hybridized with probes for LRP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The latter was used as a loading control.

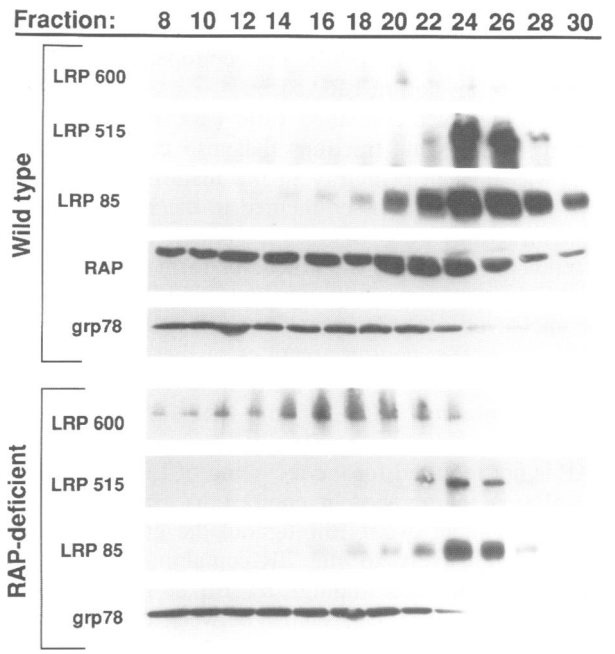

Fig. 2. Subcellular fractionation of mouse liver membranes. Microsomal liver membranes from wild-type (upper panel) and RAP-deficient mice (lower panel) were separated on a linear sucrose gradient by ultracentrifugation as described in Materials and methods. Thirty fractions of 1.3 ml each were collected from the bottom of each gradient and 0.1 ml of each fraction subjected to non-reducing 3–10% SDS–PAGE and subsequent immunoblot analysis using 5 μg/ml of rabbit polyclonal antibodies directed against LRP 600, LRP 515 and 85, RAP and grp78. Bound IgG was detected by ECL.

First, studies in cultured cells have demonstrated that RAP is an endoplasmic reticulum (ER) protein. Bu *et al.* (1995) showed that the carboxy-terminal tetrapeptide HNEL of RAP mediates this ER retention. They also suggested that RAP associates with LRP in the ER and that complexes of LRP and RAP are transported to the *cis*-Golgi compartment where RAP is released from the complex and recycled to the ER. Another study by Biemesderfer *et al.* (1993) previously had reported the association of newly synthesized and partially unfolded gp330/megalin molecules with RAP in the ER.

Further information regarding the physiological role of RAP has been obtained from gene knockout experiments in mice (Willnow *et al.*, 1995). RAP was found to be required for the functional expression of LRP *in vivo*. In RAP-deficient mice, the amount of immunodetectable LRP in liver and brain was reduced by 75%. However, the underlying mechanism by which RAP controls LRP expression in mice has remained unclear.

Here we have elucidated this mechanism by analyzing the biosynthesis of LRP, gp330/megalin and the VLDL receptor in cells and tissues expressing or lacking RAP. Our data show that RAP is necessary to protect newly synthesized receptor molecules in the ER from aggregation and subsequent degradation. This aggregation may be caused by the premature binding of co-expressed ligands to newly synthesized receptor molecules which then interferes with their export through the secretory pathway. These results define RAP as a novel, specialized escort protein that protects a family of endocytic receptors during biosynthesis from ligand-induced degradation.

Results

We have reported previously that RAP is required for the efficient functional expression of LRP in mice (Willnow *et al.*, 1995). Hepatic LRP expression (LRP 515 and LRP 85) was reduced by ~75% in animals in which both alleles of the RAP gene have been disrupted by homologous recombination (Figure 1A, lanes 4 and 5, –/–) as compared with wild-type littermates (lanes 1 and 6, +/+). LRP and RAP expression was not altered significantly in heterozygous mice carrying one functional RAP allele (lanes 2 and 3, +/–).

To distinguish whether RAP exerts its effect on LRP expression at the transcriptional level or through a post-transcriptional mechanism, we compared hepatic LRP mRNA levels in wild-type (Figure 1B, lanes 1 and 6), heterozygous (lanes 2 and 3) and homozygous RAP-deficient animals (lanes 4 and 5). In contrast to LRP protein, LRP mRNA levels were unchanged in the –/– mice, suggesting that RAP acts during LRP biosynthesis and does not affect LRP gene transcription.

Taken together with the findings of Biemesderfer *et al.* (1993) and Bu *et al.* (1995), these results suggested a possible physiological function for RAP in which RAP is required for stabilizing newly synthesized LRP during biosynthesis in the ER or for its export through the secretory pathway. To investigate the fate of LRP in the absence of RAP, we fractionated subcellular organelles from wild-type and $RAP^{-/-}$ animals and determined the distribution of LRP, RAP and grp78, a resident ER protein, by immunoblot analysis (Figure 2). Hepatic microsomal membranes were separated by sucrose density centrifugation as described by Bartles and Hubbard (1990). This technique allowed us to separate intracellular compartments of the secretory pathway (i.e. ER) from endosomes and plasma membrane-containing fractions. To distinguish between mature LRP that has traversed the secretory pathway to the cell surface and the fraction of LRP that has not yet completed its journey to the plasma membrane, we took advantage of the proteolytic cleavage of LRP in the late Golgi. The precursor form of LRP, LRP 600, can be distinguished from LRP 515 by an epitope-specific

antibody that recognizes the carboxy-terminal tail of the receptor (Herz *et al.*, 1990). This epitope is also present on LRP 85 but not on LRP 515.

Wild-type liver contained little LRP 600 which was present in the same fractions that also contained grp78. LRP was present primarily in the mature LRP 515 and LRP 85 forms, which sedimented in the higher (i.e. less dense) fractions with a distribution pattern that was clearly different from that of the precursor form. In contrast, in RAP-deficient livers, large amounts of LRP remained in the uncleaved 600 kDa precursor form that was present in the lower (i.e. denser) fractions of the gradient. These fractions also contained grp78 and were therefore enriched in ER membranes. LRP 515 and LRP 85 were found in the same fractions as in wild-type livers, suggesting that RAP is not required for the recycling of LRP to and from the plasma membrane. In contrast to grp78, RAP was distributed more evenly throughout the gradient and not limited exclusively to the ER-containing fractions, in agreement with the findings by Bu *et al.* (1995) that suggested that RAP can shuttle between the ER and the *cis*-Golgi.

The results of these subcellular fractionation experiments are consistent with the hypothesis that RAP is required for the transport of LRP from the early to the late compartments of the secretory pathway where proteolytic processing occurs. To confirm that LRP 600 accumulated in the ER of RAP$^{-/-}$ mice, we analyzed the structure of the carbohydrates attached to LRP. This was done by separating pooled gradient fractions containing mainly LRP 600 by SDS–PAGE. Proteins were transfered to nitrocellulose and filters were blotted with digoxigenin-coupled lectins (Figure 3A) specific for the high mannose form of *N*-linked carbohydrates (*Galanthus nivalis* agglutinin, GNA; lanes 1 and 2) or with *Sambucus nigra* agglutinin (SNA; lanes 3 and 4) which recognizes terminal sialic acids acquired in the *trans*-Golgi. As seen in lane 2, in RAP$^{-/-}$ livers a single large glycoprotein of the size of LRP 600 reacted preferentially with GNA as compared with the wild-type sample. The relative intensity with which other proteins (indicated by *) reacted with the lectin was not affected by the genotype. Conversely, the intensity of a band of similar mobility was reduced when a parallel filter was probed with SNA which would be expected to react with LRP 515. The same membrane used for incubation with GNA was reprobed using the epitope-specific antibody that recognizes LRP 600 (lanes 5 and 6). Overlay of the ECL immunoblot with the filter confirmed that the same band that reacted with GNA also bound anti-LRP antibody. Thus, LRP 600 accumulates in RAP-deficient hepatocytes containing carbohydrates in the high mannose form, indicating that it has not left the ER.

The abnormal conformation of newly synthesized proteins is known to prevent their export from the ER. To determine whether proper folding of LRP is impaired in the absence of RAP, we solubilized liver membrane proteins under mild conditions and subjected them to immunoblot analysis using the anti-carboxy-terminal antibody (Figure 3B). As has been shown in Figures 1A and 2, the absolute amount of mature LRP, as determined by the amount of LRP 85 present in the samples, was reduced substantially in the membrane extracts from RAP$^{-/-}$ mice (lanes 3 and 4) as compared with wild-type controls (lanes

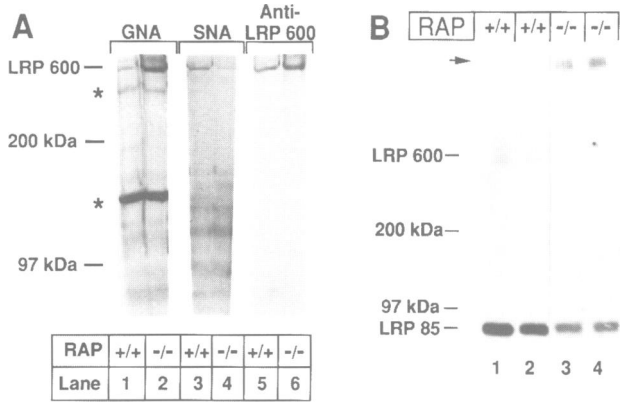

Fig. 3. Aggregation of LRP 600 in the ER of RAP$^{-/-}$ hepatocytes. (**A**) Wild-type (lanes 1, 3 and 5) and RAP-deficient (lanes 2, 4 and 6) microsomal liver membranes from sucrose gradient fractions 10–12 (Figure 2) were pooled, subjected to non-reducing 3–10% SDS–PAGE (0.1 ml/ lane) and transferred to nitrocellulose membranes. Parallel nitrocellulose strips were incubated with either GNA (lanes 1 and 2) or SNA (lanes 3 and 4) and bound agglutinin was detected as described in Materials and methods. Nitrocellulose strips in lanes 1 and 2 were reprobed subsequently with anti-LRP tail IgG. The position of LRP 600 as well as myosin (200 kDa) and phosphorylase B (97 kDa) in the gel is indicated. Asterisks denote unknown glycoproteins which react with GNA. (**B**) Partially purified membrane proteins from either wild-type (lanes 1 and 2) or RAP-deficient (lanes 3 and 4) mouse livers were solubilized in 2.3% SDS under mild conditions at 22°C without boiling, separated on a 3–10% SDS–polyacrylamide gel and transferred to nitrocellulose. Membranes were incubated with 5 μg/ml of polyclonal antibody directed against the carboxy-terminus of LRP present in both LRP 600 and LRP 85. The position of migration of LRP 600, LRP 85, myosin (200 kDa) and phosphorylase B (97 kDa) in the gel is indicated. The arrow denotes high molecular weight LRP aggregates in RAP-deficient liver membrane preparations (lanes 3 and 4).

1 and 2). A striking result was that, under these conditions, LRP 600 was virtually undetectable in RAP$^{-/-}$ liver extracts (lanes 3 and 4). Instead, the antibody detected a high molecular weight complex that barely entered the gel (indicated by the arrow), suggesting that, in the absence of RAP, LRP 600 forms insoluble aggregates in the ER.

This biochemical finding was supported further by the intracellular immunohistochemical detection of LRP in RAP-deficient cortical neurons (Figure 4). We chose neurons instead of liver parenchymal cells, because our previous characterization of the RAP$^{-/-}$ mice had shown that LRP biosynthesis in the brain was impaired similarly to that in the liver. Furthermore, neurons abundantly express LRP (Wolf *et al.*, 1992), thus facilitating its immunohistochemical subcellular localization. Wild-type neurons (Figure 4A and C) showed a punctate staining pattern over the surface of the cell bodies (open arrows) and apical dendrites (solid arrows), consistent with a distribution of the protein in vesicles and coated pits on the plasma membrane. The diffuse and punctate staining of LRP in the tissue sections is caused by its presence in peripheral cell processes. In contrast, in RAP-deficient neurons, LRP immunoreactivity was restricted almost exclusively to the cytoplasm of the cell bodies with a smooth appearance of the plasma membrane (Figure 4B and D), a staining pattern that is consistent with an ER localization.

In addition to LRP, RAP also binds with high affinity to two other known members of the LDL receptor gene

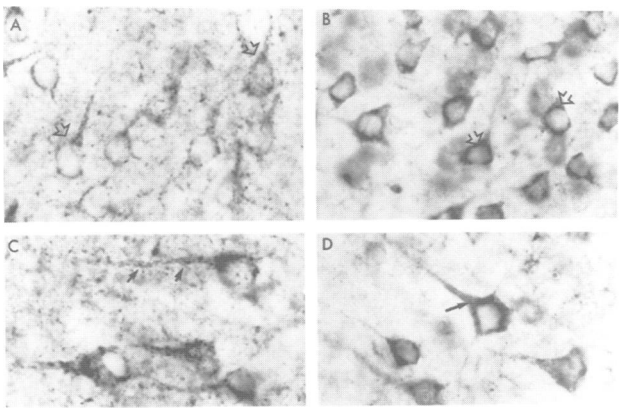

Fig. 4. Intracellular accumulation of LRP in RAP-deficient neurons. Brains from wild-type (**A** and **C**) or RAP-deficient mice (**B** and **D**) were perfused and fixed for 2 h in 4% paraformaldehyde, 0.1 M phosphate buffer (PB) and subsequently incubated in 20% sucrose, 0.1 M PB for 24 h at 22°C. Frontal sections (25 μm) were cut from the cortex, permeabilized in PBS containing 1% Triton X-100, and blocked with 30% normal calf serum and 20% normal goat serum in PBS overnight. Sections were stained overnight at 4°C with rabbit anti-LRP carboxy-terminal antibody (1:1000) and subsequently with goat anti-rabbit IgG (Nordic, 1:80) for 40 min at 37°C followed by peroxidase–anti-peroxidase complex (Nordic, 1:200) for 1 h at 37°C. Sections were developed with 0.05% 3,3′-diaminobenzidine, 0.15% $NiCl_2$ and 0.00525% H_2O_2. No staining was seen in sections incubated with rabbit non-immune antiserum (not shown). Open arrows denote cell bodies of cortical neurons (A and B), filled arrows show apical dendrites of large pyramidal cells (C and D). Magnification 500×.

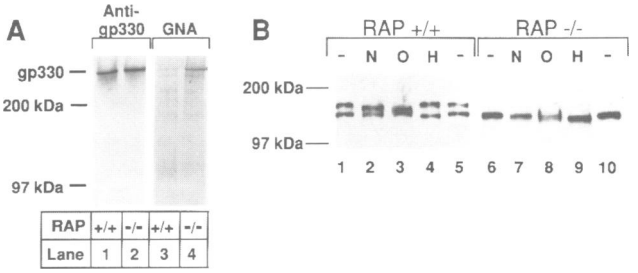

Fig. 5. Expression of gp330/megalin and VLDL receptor in wild-type and RAP-deficient mouse kidneys and hearts. (**A**) Twenty micrograms of partially purified membrane proteins from wild-type (lanes 1 and 3) and RAP-deficient (lanes 2 and 4) mouse kidneys were subjected to non-reducing 4–15% SDS–PAGE and transfer to nitrocellulose. Replicate filters were either incubated with 5 μg/ml of polyclonal antibody directed against gp330 (lanes 1 and 2) or with GNA (lanes 3 and 4). Bound IgG and lectins were detected as described in Materials and methods. The position of migration of gp330/megalin (gp330), myosin (200 kDa) and phosphorylase B (97 kDa) in the gel is indicated. (**B**) Twenty micrograms of partially purified membrane proteins from wild-type (lanes 1–5) and RAP-deficient mouse hearts (lanes 6–10) were either untreated (lanes 1, 5, 6 and 10), incubated with neuraminidase alone (N, lanes 2 and 7), with neuraminidase and *O*-glycanase (O, lanes 3 and 8) or with endoglycosidase H (H, lanes 4 and 9) as described in Materials and methods. Proteins were separated on non-reducing 4–15% SDS–PAGE and analyzed by immunoblotting using 5 μg/ml polyclonal antibody directed against the mouse VLDL receptor. Bound IgG was detected by ECL. The position of migration of myosin (200 kDa) and phosphorylase B (97 kDa) in the gel is indicated.

family, gp330/megalin and the VLDL receptor. To investigate whether RAP is required for the normal expression of gp330/megalin, we examined the expression level of this protein in kidney membrane extracts of $RAP^{-/-}$ (Figure 5A, lane 2) and wild-type (lane 1) mice by immunoblotting using a specific polyclonal antibody. In contrast to LRP in liver and brain, gp330 expression was not reduced significantly by the disruption of the RAP gene. Nevertheless, a substantially higher fraction of total gp330 contained high mannose carbohydrates in $gp330^{-/-}$ kidneys (lane 4) than in wild-type kidneys (lane 3), indicating that gp330 export from the ER is also affected in the knockout animals. Similarly, expression of total VLDL receptor protein was not affected by the absence of RAP in numerous tissues examined (data not shown). However, VLDL receptor transport to the cell surface was blocked completely in the heart of $RAP^{-/-}$ mice (Figure 5B), but not in other tissues including skeletal muscle, adipose tissue and brain (not shown) as revealed by immunoblot analysis. Total heart membrane extracts from wild-type (lanes 1–5) and $RAP^{-/-}$ animals (lanes 6–10) were solubilized in Triton X-100-containing buffer and subjected to digestion with neuraminidase (lanes 2 and 7), *O*-glycanase and neuraminidase (lanes 3 and 8) or endoglycosidase H (lanes 4 and 9). Control samples were incubated in buffer in the absence of enzyme (lanes 1, 5, 6 and 10). In wild-type mouse heart VLDL receptor was present in two forms. The lower molecular weight form was sensitive to treatment with endoglycosidase H (lane 4), indicating that it has not undergone Golgi-specific modification of its carbohydrate side chains and therefore still resides in the ER. The higher molecular weight form, in contrast, was not sensitive to treatment with endoglycosidase H, but was digested readily by neuraminidase (lane2) and *O*-glycanase (lane 3), indicating that the protein has completely traversed the Golgi complex. In the hearts of RAP-deficient animals, VLDL receptor was present only in the lower molecular weight, precursor form that was sensitive to endoglycosidase H digestion (lane 9), but did not contain terminal sialic acid residues (lane 7) or *O*-linked carbohydrates (lane 8).

These results suggested that RAP is required for efficient export of LRP, gp330 and VLDL receptor from the ER and through the secretory pathway. To determine, whether, under normal conditions, RAP might have regulatory properties by limiting LRP or VLDL receptor expression and whether the transient expression of RAP in $RAP^{-/-}$ mice would correct the phenotype, we explored two different approaches to overexpress RAP in mice. First, we used intravenously injected recombinant adenovirus (AdCMV-RAP) for gene transfer of RAP into the livers of wild-type (Figure 6A, lanes 3 and 4) or $RAP^{-/-}$ mice (lanes 6 and 7). An adenovirus containing the *Escherichia coli* β-galactosidase gene served as a control (lanes 1, 2 and 5). Overexpression of RAP in wild-type mice (lanes 3 and 4) did not increase endogeneous LRP 515 expression levels over those in the control animals (lane 1 and 2). In contrast, LRP expression was restored to normal levels by the transfer of the RAP cDNA in knockout livers (lanes 6 and 7). This effect was limited to the liver which can be quantitatively infected with adenovirus. No increase in LRP expression levels was seen in the brains of the same mice in response to RAP gene transfer; the brain is an organ that is not infected efficiently by adenovirus via the intravenous route. Similarly, transgenic animals (Figure 6B, lanes 3 and 4) that overexpressed RAP several fold over wild-type levels (lanes 1 and 2) in their livers and hearts showed unaltered levels of expression and normal biosynthetic processing of LRP and VLDL receptor in

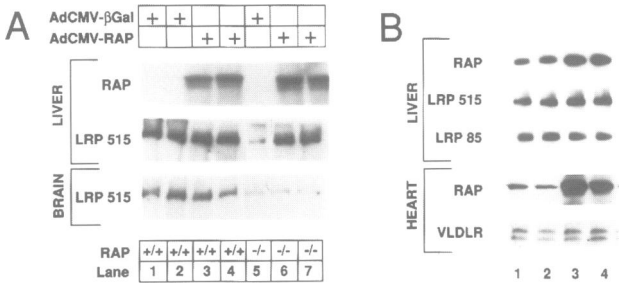

Fig. 6. Expression of LRP and VLDL receptor in RAP-overproducing mice. (**A**) Wild-type (lanes 1–4) and RAP-deficient mice (lanes 5–7) were injected via the external jugular vein with 2×10^{11} particles of recombinant adenovirus carrying the β-galactosidase gene (AdCMV-βGal, lanes 1, 2 and 5) or the rat RAP cDNA (AdCMV-RAP, lanes 3, 4, 6 and 7) in 200 μl of Tris-buffered saline. Five days after virus administration, animals were sacrificed and membrane proteins purified from liver and brain. Twenty micrograms of protein/lane were analyzed by non-reducing 4–15% SDS–PAGE and immunoblotting with antibodies directed against RAP and LRP 515. Bound IgG was detected with ^{125}I-labeled goat anti-rabbit IgG and autoradiography. (**B**) Partially purified membrane proteins were obtained from livers and hearts of either wild-type (lanes 1 and 2) or transgenic mice (lanes 3 and 4) overexpressing RAP. Twenty micrograms of protein/lane were subjected to non-reducing 4–15% SDS–PAGE and subsequent immunoblot analysis with polyclonal antibodies directed against RAP, LRP 515 and 85 and VLDL receptor. Bound IgG was detected by ECL.

their livers and hearts, respectively, demonstrating that RAP is not limiting or regulating LRP or VLDL receptor expression under normal conditions.

The universal ability of RAP to prevent ligand binding to LRP and its restricted ER expression pattern suggest that RAP may function to prevent the premature binding of simultaneously synthesized ligands to newly synthesized LRP, rather than directly assisting in the folding of the receptor. Such premature ligand binding might prevent proper folding and thus might result in aggregation and subsequent degradation of newly synthesized receptors. To test this hypothesis, we first prepared primary murine embryonic fibroblasts from wild-type (EF-1) and RAP-deficient (EF-7) embryos. In contrast to liver and brain cells, RAP-deficient fibroblasts expressed only marginally less LRP than wild-type cells (not shown), indicating a differential requirement for RAP in different tissues. We then used increasing amounts of a recombinant adenovirus to overexpress apoE (AdCMV-apoE), a high affinity ligand for LRP, in these fibroblasts that normally do not produce this ligand (Figure 7A, lanes 1–4 and 11–14). In parallel dishes, cells were infected with AdCMV-RAP (lanes 5–7 and 15–17) or with AdCMV-apoAI (lanes 8–10 and 18–20), a recombinant adenovirus expressing apolipoprotein AI which does not bind to LRP. Expression of LRP in the fibroblasts was analyzed 60 h post-infection by immunoblotting. In both cell lines, LRP expression was reduced with increasing apoE expression, while overexpression of RAP or apoAI had no effect. The LDL receptor, which also tightly binds apoE but interacts with RAP only poorly, was not affected by the apoE expression. Overexpression of apoE in the fibroblasts resulted in LRP aggregation and the formation of SDS-stable complexes (not shown) similar to those seen in RAP-deficient livers (Figure 3B).

This overexpression experiment did not allow us to conclude whether apoE reduced LRP expression by binding to the receptor inside the cell or whether cellular

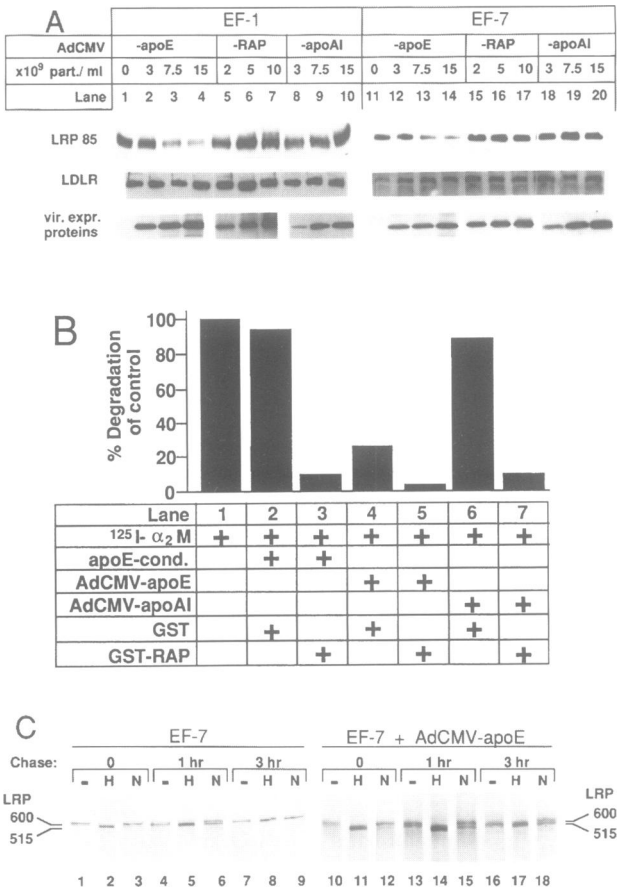

Fig. 7. ApoE expression induces LRP accumulation in the ER of murine embryonic fibroblasts. (**A**) 5×10^5 cells/well of either wild-type (EF-1, lanes 1–10) or RAP-deficient murine embryonic fibroblasts (EF-7, lanes 11–20) were grown in 6-well dishes as described in Materials and methods. Replicate monolayers of cells were either not infected (lanes 1 and 11) or infected with the indicated amount of recombinant adenoviruses containing the human apoE3 cDNA (AdCMV-apoE, lanes 2–4 and 12–14), rat RAP cDNA (AdCMV-RAP, lanes 5–7 and 15–17) or human apoAI cDNA (AdCMV-apoAI, lanes 8–10 and 18–20). At 60 h after infection, the cells were lysed and 20 μg of protein were analyzed by immunoblotting with antibodies directed against LRP 85, LDL receptor, RAP, human apoE and human apoAI. Bound IgG was detected by ECL. (**B**) Replicate monolayers of RAP-deficient EF-7 cells were either not infected (lanes 1–3) or infected with 3×10^9 particles/ml of AdCMV-apoE (lanes 4 and 5) or AdCMV-apoAI (lanes 6 and 7). After 48 h, cells received 1 ml of DMEM, 0.2% BSA (lanes 1 and 4–7) or DMEM, 0.2% BSA conditioned with human apoE as described in Materials and methods (lanes 2 and 3). LRP-mediated endocytosis was assayed by determining the rate of [^{125}I]α_2-macroglobulin degradation in the presence of either 50 μg/ml GST (lanes 2, 4 and 6) or GST–RAP (lanes 3, 5 and 7). After incubation at 37°C for 8 h, the amount of ^{125}I-labeled degradation products secreted into the medium was determined as described in Materials and methods. Each value represents the mean of duplicate incubations of multiple experiments. The 100% value represents degradation rates in the absence of added reagents (lane 1) and range from 420 to 870 ng/mg protein/8 h. (**C**) 5×10^5 EF-7 cells/well, either not infected (lanes 1–9) or infected with 7.5×10^9 particles/ml of AdCMV-apoE (lanes 10–18) were pulse-labeled with Trans[^{35}S]-label and subsequently chased with complete medium for the indicated periods of time, followed by immunoprecipitation with polyclonal anti-LRP antibody. Immunoprecipitates were split in equal parts that were either not treated (–) or treated with endoglycosidase H (H) or neuraminidase (N). Samples were then separated on 5% SDS–PAGE under reducing conditions and labeled proteins were visualized by fluorography. The position of LRP 600 and LRP 515 in the gel is indicated.

secretion of apoE and re-uptake by LRP led to receptor aggregation and degradation in the endocytic pathway. To distinguish between these two possibilities, we directly measured functional LRP activity in RAP-deficient EF-7 cells that had either been infected with AdCMV-apoE or had been grown in conditioned medium harvested from apoE-overexpressing cells (Figure 7B). LRP activity was assayed by measuring the ability of the cells to degrade ^{125}I-labeled α_2-macroglobulin. Untreated EF-7 cells efficiently degraded the ^{125}I-labeled ligand (Figure 7B, lane 1). ApoE-conditioned medium had no effect (lane 2), but LRP activity was reduced by 70% in cells that had been infected with AdCMV-apoE (lane 4), consistent with the significant reduction of LRP protein expression (Figure 7A). Cells infected with AdCMV-apoAI (lane 6) degraded ^{125}I-labeled α_2-macroglobulin as efficiently as uninfected cells (lane 1). LRP-mediated ligand degradation was blocked under all conditions by a fusion protein of glutathione-*S*-transferase and RAP (GST–RAP, lanes 3, 5 and 7) but not by GST alone (lanes 2, 4 and 6).

To demonstrate directly that apoE overexpression interfered with LRP transport to the cell surface, we performed a pulse–chase experiment in the RAP-deficient EF-7 fibroblasts (Figure 7C). Cells were either not infected (lanes 1–9) or infected (lanes 10–18) with AdCMV-apoE, pulse-labeled with [^{35}S]methionine 24 h later and chased for the indicated times. After immunoprecipitation with anti-LRP antibody, samples were either not treated (lanes 1, 4, 7, 10, 13 and 16) or treated with endoglycosidase H (lanes 2, 5, 8, 11, 14 and 17) or with neuraminidase (lanes 3, 6, 9, 12, 15 and 18) before SDS–gel electrophoresis. In both uninfected and infected cells, ~50% of the labeled LRP was sensitive to neuraminidase after 1 h chase (lanes 6 and 15). Consistent with previous results (Herz *et al.*, 1990), virtually all labeled LRP was sensitive to neuraminidase (lane 9) and resistant to endoglycosidase H (lane 8) treatment after 3 h chase in uninfected cells. In contrast, in AdCMV-apoE-infected cells, a substantial fraction of the labeled LRP remained sensitive to endoglycosidase H (lane 17) and was resistant to neuraminidase (lane 18) treatment after 3 h chase, indicating that this fraction of LRP has not left the ER.

Adenoviral gene transfer of RAP restored normal LRP expression in RAP$^{-/-}$ livers (Figure 6A). To test whether RAP would be able to prevent the ligand (apoE)-induced aggregation and degradation of LRP, we compared the levels of LRP 515 and LRP 85 in uninfected EF-7 fibroblasts (Figure 8, lane 1), and cells that had been infected with apoE virus (lanes 2–4), RAP virus (lane 3) and apoAI virus (lane 4). Infection with AdCMV-apoE substantially reduced LRP expression. Superinfection of the cells with AdCMV-RAP (lane 3) but not with AdCMV-apoAI (lane 4) restored LRP expression to the level of uninfected cells. LDL receptor expression was unaffected under any condition.

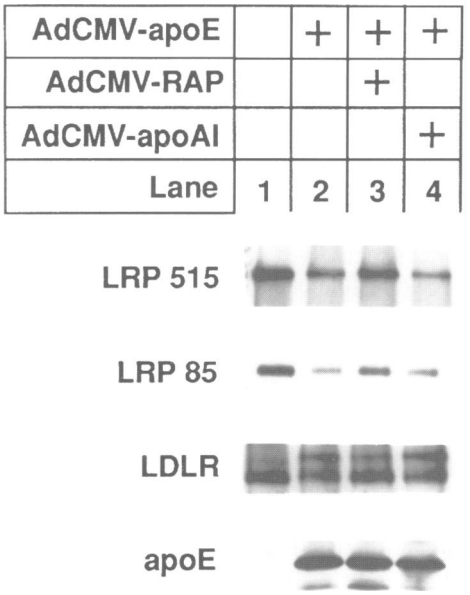

Fig. 8. Co-expression of RAP prevents apoE-induced decrease of LRP expression. 5×10^5 EF-7 cells per well were seeded into 6-well dishes and grown for 24 h in DMEM containing 10% fetal calf serum. Replicate monolayers of cells were either not infected (lane 1) or infected with 3×10^9 particles/ml of AdCMV-apoE (lanes 2, 3 and 4). Two hours after infection, the cells were superinfected with 2×10^{10} particles/ml of AdCMV-RAP (lane 3) or 3×10^{10} particles/ml of AdCMV-apoAI (lane 4) and cultured for an additional 60 h. Twenty micrograms of cell lysate of each sample were subjected to non-reducing 4–15% SDS–PAGE and immunoblot analysis with antibodies directed against LRP 515 and 85, LDL receptor and apoE. Bound IgG was detected using ECL.

Discussion

Here we have used RAP-deficient knockout mice to examine the mechanism by which RAP selectively promotes the biosynthesis and intracellular transport of the endocytic receptors of the LDL receptor gene family. Our findings demonstrate that RAP functions physiologically as a specialized chaperone for this gene family. (i) In the absence of RAP, LRP has a propensity to aggregate in the ER in livers and neurons of knockout mice, and the export of gp330/megalin from the ER is also impaired. (ii) In the heart, VLDL receptor export from the ER is prevented in the absence of RAP. (iii) LRP expression levels are greatly reduced in liver and brain of RAP-deficient mice, but not in embryonic fibroblasts derived from homozygous knockout animals, suggesting tissue-specific differences in the requirement for RAP in receptor biosynthesis and transport. (iv) Co-expression of apoE, a high affinity ligand for LRP, greatly reduces LRP expression in cultured fibroblasts and impedes transport of LRP through the secretory pathway. (v) When RAP is expressed together with apoE, LRP expression levels are restored to normal. Taken together, these findings suggest that RAP is necessary to protect newly synthesized receptors in the ER from premature ligand binding and subsequent aggregation and degradation.

Chaperone functions similar to the one proposed here for RAP have been described for example for the invariant chain which facilitates the export of MHC class II molecules from the ER (Roche and Cresswell, 1990; Teyton *et al.*, 1990; Bijlmakers *et al.*, 1994) or for the chaperone 7B2 which blocks the activity of the processing protease PC2 before it has reached its proper compartment in the Golgi (Braks and Martens, 1994). Disruption of the gene for invariant chain in mice results in intracellular accumulation and poor expression of dysfunctional MHC class II antigen on the cell surface (Viville *et al.*, 1993). In contrast to the case of the invariant chain and MHC

class II molecules, RAP mainly seems to protect LRP from aggregation and subsequent degradation, whereas it is not required for the functional maturation of LRP molecules that escape to later compartments. The few LRP molecules that are transported to the cell surface in RAP-deficient livers appear to be fully functional in ligand uptake (Willnow *et al.*, 1995).

In contrast to the findings in liver and brain, LRP expression and activity was virtually unaffected in $RAP^{-/-}$ fibroblasts, which indicates that RAP is not always required for normal LRP folding or transport. The differences between the effects of RAP deficiency in liver and in fibroblasts may relate to the larger amounts of LRP ligands produced in liver. This organ synthesizes several ligands which are not expressed at high levels in fibroblasts, including apoE and α_2-macroglobulin. Under tissue culture conditions, fibroblasts may therefore not need RAP to prevent premature ligand binding to LRP. Such a mechanism of ligand-induced aggregation and degradation of LRP in the absence of RAP is supported by the apoE overexpression experiments (Figures 7 and 8). Infection of RAP-deficient fibroblasts with AdCMV-apoE caused markedly reduced LRP expression by preventing the exit of LRP from the ER. This effect was prevented when the cells were co-infected with AdCMV-RAP. Overexpression of apoE also reduced LRP in wild-type fibroblasts, probably because the large amounts of apoE that were produced effectively competed for RAP binding sites on LRP.

In other experiments not shown here, we also used recombinant adenoviruses to overexpress other LRP ligands (t-PA, urokinase, plasminogen activator inhibitor-1) besides apoE. In contrast to the results obtained with apoE, however, none of these ligands induced LRP aggregation and degradation in fibroblasts. A possible reason for this difference might be that several apoE molecules, after association with lipids in the ER, can form small multivalent lipoprotein particles that could conceivably bind simultaneously to several nascent and only partially folded receptor molecules. Moreover, a total of four potential apoE binding domains are present in a single LRP molecule (Herz *et al.*, 1988), which would further favor the formation of large multivalent complexes. As a consequence, completion of the normal folding process might be prevented. All the other ligands mentioned above presumably interact only with a single site on the receptor (Willnow *et al.*, 1994b) and would thus be unable to induce such a multivalent interaction.

Nevertheless, apoE cannot be the only ligand that induces LRP aggregation. First, LRP expression was reduced in neurons to an extent similar to that in liver parenchymal cells, although neurons do not normally produce apoE (Boyles *et al.*, 1985). We would thus predict that the affected brain cells produce one or more multivalent ligands for LRP. Furthermore, we have tested directly whether apoE might be solely responsible for ligand-induced LRP aggregation and degradation in RAP knockout livers by cross-breeding apoE-deficient mice (Piedrahita *et al.*, 1992) with the RAP knockout animals. If apoE were the only ligand that can induce LRP aggregation, LRP expression should have been restored to wild-type levels in the double knockouts. However, hepatic LRP levels were still reduced to the same extent in $RAP^{-/-}$; $apoE^{-/-}$ doubly deficient animals as in $RAP^{-/-}$ mice (data not shown), indicating that other non-apoE-mediated mechanisms are also at work.

An alternative explanation for the differential cellular effect of the RAP knockout would be the existence of a related protein that is capable of compensating for RAP in some tissues. The variable requirement for RAP in different tissues is also exemplified by its relatively minor effect on gp330 expression levels in the kidney (Figure 5A), the absence of any effect on expression of the homologous LDL receptor in RAP-deficient livers (Figure 1A) and by the absolute requirement for RAP in VLDL receptor biosynthesis in the heart (Figure 5B), but not in other tissues.

How does RAP work? Our study, taken together with the findings by Biemesderfer *et al.* (1993) and Bu *et al.* (1995), favors a model in which RAP interacts selectively with nascent receptors of the LDL receptor gene family in the ER, blocking the binding sites for its numerous ligands on the emerging polypeptide chain. Whether this occurs through an allosteric or a direct competitive mechanism is not certain, although reverse competition experiments by Bu *et al.* (1992) favor the latter. The precise nature of the sites on the receptors to which RAP binds is also unknown. To resolve these questions, future studies that aim at elucidating the structural basis of the interaction of RAP with the endocytic receptors of the LDL receptor gene family will be needed.

Materials and methods

Reagents

Antibodies directed against rat grp78 were purchased from StressGene (Victoria, Canada); antibodies against human apoE and human apoAI were from Calbiochem (La Jolla, CA). GST or a GST–RAP fusion protein were produced as described (Herz *et al.*, 1991). Methylamine-activated human α_2-macroglobulin was a gift from Dudley Strickland (American Red Cross, Rockville, MD). Protein iodinations were performed using the IODO-GEN method (Fraker and Speck, 1978). Mice used in these studies were age- and sex-matched C57Bl/6J×129SvJ hybrid mice bred in-house and fed *ad libitum* throughout the course of the experiments (Teklad 6% mouse/rat diet 7001; Teklad, Madison, WI). Animal care and experimental procedures involving animals were conducted in accordance with institutional guidelines. The generation of recombinant adenoviruses carrying the cDNA of rat RAP, the cDNA of human apoAI or the β-galactosidase gene under the control of the cytomegalovirus (CMV) promoter were described previously (Kopfler *et al.*, 1994; Willnow *et al.*, 1994a). A derivative of adenovirus AdRR5 containing the human apoE3 cDNA under the control of the CMV promoter (AdCMV-apoE) was generated essentially as described for other recombinant adenoviruses (Willnow *et al.*, 1994a). All viruses were purified and titrated in 293 cells. Plaque-forming units (p.f.u.) were related to viral particle numbers and were similar for independent virus preparations (2×10^{11} particles ~ 5×10^{9} p.f.u.).

Isolation and analysis of membrane proteins

Membrane proteins were prepared from various mouse tissues or cell lines as published previously (Willnow *et al.*, 1995). Protein concentrations were determined using the Coomassie plus protein assay reagent (Pierce) and 20 μg protein/lane were subjected to non-reducing 3–10% or 4–15% SDS–PAGE and transfer to nitrocellulose filters at 4°C. Filters were incubated with 5 μg/ml polyclonal rabbit IgG and bound IgG was detected with either ^{125}I-labeled goat anti-rabbit IgG (1×10^{6} c.p.m./ml) or by enhanced chemiluminescence (ECL) using horseradish peroxidase-coupled donkey anti-rabbit IgG and the ECL system (Amersham) according to the manufacturer's recommendations. Alternatively, nitrocellulose filters were probed with digoxigenin-labeled *Galanthus nivalis* agglutinin or *Sambucus nigra* agglutinin. Bound lectins were detected with an anti-digoxigenin antibody coupled to alkaline phosphatase and subsequent incubation in 4-nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (X-phosphate)

substrate solution (DIG Glycan differentiation kit, Boehringer Mannheim). For analysis of the protein glycosylation pattern, 20 μg of membrane proteins were treated with either 70 mU neuraminidase (Sigma) in 20 mM sodium citrate, 2 mM $CaCl_2$, 150 mM NaCl, pH 6.5; with 20 mU endoglycosidase H (Genzyme, Cambridge, MA) in 50 mM sodium citrate, pH 6.0; or with 5 mU *O*-glycanase (Genzyme, Cambridge, MA) in 10 mM sodium phosphate, pH 6.0. Incubations were carried out for 16 h at 22°C, after which the proteins were analyzed by non-reducing SDS–PAGE.

Isolation of microsomal liver membranes

Microsomal liver membranes were isolated and fractionated essentially as described (Bartles and Hubbard, 1990) and resuspended in 1.9 ml of 0.25 M sucrose buffer per gram of wet liver weight. Five ml of the microsomal membranes were layered on top of a 32 ml linear sucrose gradient (density 1.10–1.25 g/ml) buffered with 3 mM imidazole, pH 7.4, and centrifuged for 18 h at 83 000 *g* in a swingout bucket rotor (Sorvall AH 628). Thirty fractions of 1.3 ml each were collected from the bottom of the gradient.

Generation of transgenic mice and murine embryonic fibroblast cell lines

Transgenic mice overexpressing RAP were generated by egg injection of a 14 kb genomic DNA fragment carrying the murine RAP gene region. This DNA fragment was isolated from a commercial genomic library (Stratagene) by hybridization screening with the rat RAP cDNA probe. Murine embryonic fibroblasts were isolated from wild-type and RAP-deficient mouse embryos at day 15.5 of gestation as described (Willnow and Herz, 1994).

Cellular degradation of ^{125}I-labeled α_2-macroglobulin

Murine embryonic fibroblasts (2×10^5/well) were seeded into 12-well plates and grown for 16 h. Subsequently, parallel wells were either not infected or infected with 3×10^9 particles/ml of AdCMV-apoE or AdCMV-apoAI in Dulbecco's modified Eagle's medium (DMEM; without glutamine) containing 0.2% (w/v) bovine serum albumin (BSA). After 48 h incubation at 37°C, the conditioned medium of AdCMV-apoE-infected cells was collected. For measurement of cellular degradation of ^{125}I-labeled α_2-macroglobulin, cells were incubated in apoE-conditioned or unconditioned DMEM (without glutamine) containing 0.2% (w/v) BSA and 2.5 μg/ml of ^{125}I-labeled α_2-macroglobulin (sp. act. 860–2000 c.p.m./ng) for the indicated periods of time. Cellular degradation of ^{125}I-labeled α_2-macroglobulin was measured as previously described (Goldstein *et al.*, 1983) and is expressed as nanograms of ^{125}I-labeled trichloroacetic acid-soluble (non-iodide) material released into the culture medium per mg of total cell protein.

Metabolic labeling and immunoprecipitation of LRP

Murine embryonic fibroblasts (5×10^5/well) were grown in 6-well dishes for 16 h prior to infection with 7.5×10^9 particles/ml of AdCMV-apoE. After 24 h, cells were pulse labeled, chased and analyzed by immunoprecipitation using polyclonal rabbit anti-LRP IgG followed by neuraminidase and endoglycosidase H treatment as described (Willnow *et al.*, 1996).

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References

Bartles,J.R. and Hubbard,A.L. (1990) *Methods Enzymol.*, **191**, 825–841.
Battey,F.D., Gafvels,M.E., FitzGerald,D.J., Argraves,W.S., Chappel, D.A., Strauss,J.F. and Strickland,D.K. (1994) *J. Biol. Chem.*, **269**, 23268–23273.
Biemesderfer,D., Dekan,G., Aronson,P.S. and Farquhar,M.G. (1993) *Am. J. Physiol.*, **264**, F1011–F1020.
Bijlmakers,M.J., Benaroch,P. and Ploegh,H.L. (1994) *EMBO J.*, **13**, 2699–2707.
Boyles,J.K., Pitas,R.E., Wilson,E., Mahley,R.W. and Taylor,J.M. (1985) *J. Clin. Invest.*, **76**, 1501–1513.
Braks,J.A. and Martens,G.J. (1994) *Cell*, **78**, 263–273.
Bu,G., Williams,S., Strickland,D.K. and Schwartz,A.L. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 7427–7431.
Bu,G., Geuze,H.J., Strous,G.J. and Schwartz,A.L. (1995) *EMBO J.*, **14**, 2269–2280.
Fraker,P.J. and Speck,J.C. (1978) *Biochem. Biophys. Res. Commun.*, **80**, 849–857.
Frykman,P.K., Brown,M.S., Yamamoto,T., Goldstein,J.L. and Herz,J. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 8453–8457.
Goldstein,J.L., Basu,S.K. and Brown,M.S. (1983) *Methods Enzymol.*, **98**, 241–260.
Herz,J., Hamann,U., Rogne,S., Myklebost,O., Gausepohl,H. and Stanley,K.K. (1988) *EMBO J.*, **7**, 4119–4127.
Herz,J., Kowal,R.C., Goldstein,J.L. and Brown,M.S. (1990) *EMBO J.*, **9**, 1769–1776.
Herz,J., Goldstein,J.L., Strickland,D.K., Ho,Y.K. and Brown,M.S. (1991) *J. Biol. Chem.*, **266**, 21232–21238.
Kerjaschki,D. and Farquhar,M.G. (1983) *J. Exp. Med.*, **157**, 667–686.
Kopfler,W.P., Willard,M., Betz,T., Willard,J.E., Gerard,R.D. and Meidell,R.S. (1994) *Circulation*, **90**, 1319–1327.
Kounnas,M.Z., Moir,R.D., Rebeck,G.W., Bush,A.I., Argraves,W.S., Tanzi,R.E., Hyman,B.T. and Strickland,D.K. (1995) *Cell*, **82**, 331–340.
Krieger,M. and Herz,J. (1994) *Annu. Rev. Biochem.*, **63**, 601–637.
Kristensen,T., Moestrup,S.K., Gliemann,J., Bendtsen,L., Sand,O. and Sottrup-Jensen,L. (1990) *FEBS Lett.*, **276**, 151–155.
Moestrup,S.K., Cui,S., Vorum,H., Bregengard,C., Bjorn,S.E., Norris,K., Gliemann,J. and Christensen,E.I. (1995) *J. Clin. Invest.*, **96**, 1404–1413.
Mokuno,H., Brady,S., Kotite,L., Herz,J. and Havel,R.J. (1994) *J. Biol. Chem.*, **269**, 13238–13243.
Narita,M., Bu,G., Herz,J. and Schwartz,A.L. (1995) *J. Clin. Invest.*, **96**, 1164–1168.
Orlando,R.A., Kerjaschki,D., Kurihura,H., Biemesderfer,D. and Farquhar,M.G. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 6698–6702.
Piedrahita,J.A., Zhang,S.H., Hagaman,J.R., Oliver,P.M. and Maeda,N. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 4471–4475.
Roche,P.A. and Cresswell,P. (1990) *Nature*, **345**, 615–618.
Selkoe,D.J. (1994) *Annu. Rev. Cell Biol.*, **10**, 373–403.
Strickland,D.K., Ashcom,J.D., Williams,S., Burgess,W.H., Migliorini,M. and Argraves,W.S. (1990) *J. Biol. Chem.*, **265**, 17401–17404.
Strittmatter,W.J., Saunders,A.M., Schmechel,D., Pericak-Vance,M., Enghild,J., Salvesen,G.S. and Roses,A.D. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 1977–1981.
Teyton,L., O'Sullivan,D., Dickson,P.W., Lotteau,V., Sette,A., Fink,P. and Peterson,P.A. (1990) *Nature*, **348**, 39–44.
Tsirka,S.E., Gualandris,A., Amaral,D.G. and Strickland,S. (1995) *Nature*, **377**, 340–344.
Viville,S., Neefjes,J., Lotteau,V., Dierich,A., Lemeur,M., Ploegh,H., Benoist,C. and Mathis,D. (1993) *Cell*, **72**, 635–648.
Willnow,T.E. and Herz,J. (1994) *J. Cell Sci.*, **107**, 719–726.
Willnow,T.E., Sheng,Z., Ishibashi,S. and Herz,J. (1994a) *Science*, **264**, 1471–1474.
Willnow,T.E., Orth,K. and Herz,J. (1994b) *J. Biol. Chem.*, **269**, 15827–15832.
Willnow,T.E., Armstrong,S.A., Hammer,R.E. and Herz,J. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 4537–4541.
Willnow,T.E., Moehring,J.M., Inocencio,N.M., Moehring,T.J. and Herz,J. (1996) *Biochem. J.*, **313**, 71–76
Wolf,B.B., Lopes,M.B.S., VandenBerg,S.R. and Gonias,S.L. (1992) *Am. J. Pathol.*, **141**, 37–42.

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