The Modular Adaptor Protein ARH Is Required for Low Density Lipoprotein (LDL) Binding and Internalization but Not for LDL Receptor Clustering in Coated Pits

ARH is an adaptor protein required for efficient endocytosis of low density lipoprotein (LDL) receptors (LDLRs) in selected tissues. Individuals lacking ARH (ARH<sup>−/−</sup>) have severe hypercholesterolemia due to impaired hepatic clearance of LDL. Immortalized lymphocytes, but not fibroblasts, from ARH-deficient subjects fail to internalize LDL. To further define the role of ARH in LDLR function, we compared the subcellular distribution of the LDLR in lymphocytes from normal and ARH<sup>−/−</sup> subjects. In normal lymphocytes LDLRs were predominantly located in intracellular compartments, whereas in ARH<sup>−/−</sup> cells the receptors were almost exclusively on the plasma membrane. Biochemical assays and quantification of LDLR by electron microscopy indicated that ARH<sup>−/−</sup> lymphocytes had >20-fold more LDLR on the cell surface and a ~27-fold excess of LDLR outside of coated pits. The accumulation of LDLR on the cell surface was not due to failure of receptors to localize in coated pits since the number of LDLRs in coated pits was similar in ARH<sup>−/−</sup> and normal cells. Despite the dramatic increase in cell surface receptors, LDL binding was only 2-fold higher in the ARH<sup>−/−</sup> lymphocytes. These findings indicate that ARH is required not only for internalization of the LDL-LDLR complex but also for efficient binding of LDL to the receptor and suggest that ARH stabilizes the associations of the receptor with LDL and with the invaginating portion of the budding pit, thereby increasing the efficiency of LDL internalization.

The low density lipoprotein receptor (LDLR)<sup>1</sup> mediates the rapid endocytosis of apolipoprotein (apo) B-containing lipoproteins. Internalization of the LDLR occurs via clathrin-coated pits and is mediated by a tyrosine-containing motif (NPXY) in the cytoplasmic tail of the receptor (1). The molecular machinery that sorts the LDLR to coated pits and promotes its rapid internalization has not been fully defined. Recently a crucial component of the machinery that internalizes LDLR was identified by elucidation of the molecular basis of autosomal recessive hypercholesterolemia (ARH), a rare form of severe hypercholesterolemia. Patients with ARH have normal LDLR but markedly reduced clearance of circulating LDL by the liver (2, 3). The disorder is caused by mutations that inactivate a 308-amino acid adaptor protein named ARH (4).

ARH contains four highly conserved domains (5, 6). The first is a ~40-amino acid N-terminal domain of unknown function that is followed by a phosphotyrosine binding (PTB) domain (4). PTB domains are found in a variety of adaptor proteins involved in receptor trafficking and signaling (7). Typically PTB domains of adaptor proteins bind receptors via a consensus sequence, NPXY, in the cytoplasmic tail. In vitro studies with purified recombinant proteins indicate that the PTB domain of ARH binds to the unphosphorylated FDNPVY internalization sequence in LDLR in a sequence-specific manner (8, 9). PTB domains share structural similarity with phosphoinositide-binding pleckstrin homology domains (10), and some PTB domains, including the domain in ARH, bind phosphoinositides (8, 11, 12).

Downstream of the PTB domain in ARH is a canonical clathrin box sequence (LLDLE), a conserved motif that mediates binding of several adaptor proteins to the terminal domain of the clathrin heavy chain (13, 14). The clathrin box sequence in ARH can mediate high affinity binding to the heavy chain of clathrin (8, 9). Finally ARH contains a highly conserved sequence at its C terminus that binds the β2-subunit of AP2, a second structural protein in coated pits (9). The combination of these three functional regions in one protein coupled with the requirement of ARH for LDL endocytosis led to the proposition that ARH is required either to chaperone LDLR to the coated pit or to promote the internalization of the receptor (8, 9).

Elucidation of the specific role of ARH in LDLR endocytosis has been hampered by the fact that cultured skin fibroblasts, which provided critical insights into other aspects of LDLR function, do not recapitulate the defective LDLR internalization observed in ARH subjects (2). Whereas fibroblasts from LDLR<sup>−/−</sup> patients (homozygous familial hypercholesterolemia) take up radiolabeled LDL at less than 10% of the rate observed in fibroblasts from normal individuals, fibroblasts from most ARH subjects take up and degrade LDL at 50–100% of the normal rate (15). In contrast to fibroblasts, LDLR function is significantly impaired in immortalized lymphocytes from ARH patients (16, 17). To further define the role of ARH in LDLR...
function we examined the cellular distribution and ligand binding characteristics of the LDLR in ARH −/− lymphocytes.

EXPERIMENTAL PROCEDURES

Materials—All cell culture reagents were from Invitrogen. Rabbit anti-LDLR IgG used for immunofluorescence and immunoelectron microscopy was from Maine Biotechnology Services, Inc. (Portland, ME). Rabbit anti-LDLR (4548) used in biochemistry experiments was a gift from Joachim Herz. The mouse monoclonal antibody to LDL receptor (C7) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), mouse monoclonal anti-human transferrin receptor antibody was from Zymed Laboratories Inc. (San Francisco, CA), and mouse monoclonal anti-EEA-1 was from BD Transduction Laboratories. Alexa-Fluor 488-conjugated goat anti-rabbit IgG and Alexa-Fluor 568-conjugated goat anti-mouse IgG were from Molecular Probes (Eugene, OR), and 10-nm gold-labeled goat anti-rabbit IgG and PD-10 columns were from Amersham Biosciences. Formaldehyde was from Fluka (Buchs, Switzerland), and sulfo-NHS-LC-biotin (50 mM Tris-HCl, 150 mM NaCl, 2 mg/ml bovine serum albumin, pH 7.5) was from Pierce. Methylcellulose and uranyl acetate (20, 21) were from Electron Microscope Sciences. Formaldehyde was from Fluka (Buchs, Switzerland), and sulfo-NHS-LC-biotin (50 mM Tris-HCl, 150 mM NaCl, 2 mg/ml bovine serum albumin, pH 7.5) was from Pierce. Methylcellulose and uranyl acetate (20, 21) were from Electron Microscope Sciences. Immunofluorescence Microscopy—Immunolabeled normal, ARH −/−, and LDLR −/− lymphocytes were frozen in Medium B for 48 h prior to mounting on poly-l-lysine-coated glass coverslips. The coverslips were rinsed briefly with Hanks’ balanced salt solution and Buffer A (10 mM HEPES, pH 7.3) before fixation in freshly prepared 3% (v/v) formaldehyde in Buffer A for 30 min at room temperature. The coverslips were rinsed twice in PBS (0.15 M NaCl, 10 mM phosphate buffer, pH 7.3) and then incubated in 50 mM NH4Cl in PBS for 30 min. After rinsing twice in PBS, the fixed cells were permeabilized in PBS plus 0.1% (v/v) Triton X-100 for 7 min at 4 °C and then conditioned with 1% (w/v) bovine serum albumin in PBS for 30 min. For double immunofluorescence staining, rabbit anti-LDLR IgG (0.5 μg/ml) with either mouse monoclonal anti-human transferrin receptor (10 μg/ml) or mouse monoclonal anti-EEA-1 (25 μg/ml) antibodies were applied to the coverslips overnight at 4 °C. The coverslips were rinsed with PBS and then incubated for 2 h with Alexa-Fluor 488-conjugated goat anti-rabbit IgG (10 μg/ml) and Alexa-Fluor 568-conjugated goat anti-mouse IgG (10 μg/ml). The coverslips were rinsed in PBS prior to mounting and imaged using a Leica TCS SP confocal microscope.

Electron Microscopy—Cultured lymphocytes were immersion-fixed in 3% (w/v) paraformaldehyde in Buffer A for 60 min. The lymphocytes were suspended in 2% (w/v) agar and then infused in 2 × sucrose containing 15% (v/v) polyvinylpyrrolidone (10 kDa). Frozen ultrathin sections were prepared using a Leica Ultracut UCT ultramicrotome equipped with a Leica EMFC5 cryochamber. The sections were lifted onto nickel grids in 1.8% (v/v) methylcellulose and 2.3 μm sucrose (1:1) and stored overnight on gelatin at 4 °C (20–22). Before immunolabeling, the gelatin was liquefied at 37 °C, and the nickel grids were removed. The sections were washed by floating the sections on droplets of PBS.

For immunogold localization, the grids with the attached thin sections were conditioned on droplets containing 1% (w/v) bovine serum albumin, 0.01% (v/v) Triton X-100, and 0.01% (v/v) Tween 20 in PBS (Buffer B) for 10 min at room temperature. The grids were incubated for 2 h in the presence of rabbit anti-LDLR IgG antibody and diluted in Buffer B to a final concentration of 1 μg/ml. The sections were rinsed on droplets of PBS and then incubated with 10-nm gold-labeled goat anti-rabbit IgG (diluted 1:40) in Buffer B containing 10% normal goat serum. Finally the grids with the attached thin sections were rinsed in PBS, fixed with 2% (v/v) glutaraldehyde in PBS for 10 min, embedded, and stained with methylcellulose and uranyl acetate (20, 21).

Immunoblotting, the lymphocytes were formaldehyde-fixed as described above, rinsed in Buffer B, and incubated with rabbit anti-LDLR IgG antibody (1 μg/ml) in Buffer B for 17 h. The primary antibodies were localized by incubating the cells for 2 h with 10-nm gold-labeled goat anti-rabbit IgG (diluted 1:40) in Buffer B. After extensive washings, the cells were fixed with 2% glutaraldehyde and postfixed in 1% (w/v) osmium tetroxide. Subsequently the specimens were rinsed in distilled water, dehydrated with graded ethanol, and then Epon-embedded according to the manufacturer’s protocol (Electron Microscope Science). Ultrathin sections (−80 nm) were cut with a diamond knife using a Leica Ultracut B ultramicrotome and placed on Formvar/carbon-coated nickel grids. Ultrathin sections were stained with 3% aqueous uranyl acetate (15 min) and lead acetate (5 min).

Electron micrographs were taken using a JEOL 1200 electron microscope operating at 80 kV.

Colloidal gold-conjugated LDL was produced as described previously (23). Briefly 100 ml of 10% (w/v) gold chloride was added to 100 ml of boiling H2O. After 10 s, 2 ml of 1% trisodium citrate was added, and the mixture was maintained at 100 °C for 5 min. The reaction was cooled to room temperature, adjusted to pH 6 with dilute HCl, and then centrifuged at 800 × g for 30 min at 4 °C to remove aggregates. The colloidal gold was collected by centrifugation at 17,500 × g for 40 min at 4 °C. The centrifuged material was aspirated to a volume of 1 ml. The colloidal gold pellet was resuspended and added to an equal volume of human LDL (1 mg/ml), which had been dialyzed overnight in 50 mM EDTA, pH 6.0. After allowing the mixture to equilibrate to room temperature (60 min), the mixture was overlaid on a 55% sucrose cushion and centrifuged at 17,500 × g for 60 min at 12 °C. The colloidal gold-LDL in the pellet was resuspended in PBS (2.5 ml), solvated-exchanged over a PD-10 column equilibrated in PBS, and dialyzed twice against 3 liters of PBS at 4 °C. The colloidal gold-labeled LDL was used within 5 days of synthesis.

Immortalized lymphocytes were seeded at 5 × 106 cells/ml in Medium B and cultured for 2 days. Cells were then counted and resuspended at 5 × 106 cells in 1 ml of Medium B at either 4 or 37 °C prior to addition of 10 μg/ml colloidal gold-labeled LDL. The cells were maintained at either 4 or 37 °C for 90 min prior to centrifugation at 300 × g for 5 min at 4 °C and then washed three times using Buffer C (50 mM Tris-HCl, 150 mM NaCl, 2 mg/ml bovine serum albumin, pH 7.4). Cells were fixed, embedded, sectioned, and placed on nickel grids as described above.

Quantification of Gold Labeling—Electron microscopy images were obtained by randomly taking 100 photographs of each cell type (normal, ARH −/−, and LDLR −/−) at the same magnification and an identical exposure. All of the photographs were labeled with the LDL-specific antibody and 25 photographs of each cell type labeled with the colloidal gold-LDL. 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. 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 in the experiments using LDL were counted as one since these likely represented aggregates of LDL. Gold particles separated by a gap larger than twice their diameter were counted as two separate particles.

Quantification of Cell Surface LDLR—A total of 5 × 106 lymphocytes were washed with ice-cold Medium C (1 × Eagle’s modified minimal essential medium supplemented with 20 mM HEPES, pH 7.4) and incubated on a rotator with 125I-labeled monoclonal antibody to human LDLR (C7) or a rabbit polyclonal antibody to the human LDLR (4548) (7 μg/ml) for 90 min at 4 °C. Cells incubated with 4548 were washed five times in Buffer C and then resuspended in 1 ml of Buffer C with 1 μCi of 125I-Protein A prior to rotating for 60 min at 4 °C. After incubation with either 125I-C7 or 125I-Protein A, cells were washed three times with Buffer C and twice with Buffer D (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and then resuspended in 1 ml of Buffer D. Cellular protein was determined by the method of Bradford (24), and the 125I counts were determined using half of the sample. The counts were normalized to the cold protein. In the case of C7, counts were converted to fmin of C7. All experiments were performed in triplicate.

An additional method used to quantify cell surface LDLR was by biotinylating the cell surface proteins (25). Briefly 2.5 × 106 cells were washed twice with cold PBS and resuspended in 1 ml of PBS plus sulfo-NHS-LC-biotin (1 mg/ml) for 30 min at 4 °C. The cells were then washed in PBS and incubated with Buffer D for 30 min on ice to quench any residual biotin reagent. Cells were washed twice with cold PBS and lysed with 60 μl of lysis buffer (1% Triton X-100, 4 mM EDTA, 10 mM Tris-HCl, pH 8) at room temperature for 30 min with end-over-end mixing. Cells were subjected to centrifugation at 15,000 rpm for 5 min in a microcentrifuge tube coated with insoluble debris. The pellets were resuspended in a lysis buffer (Lysate). A total of 40 μl of lysis was added to 100 μl of a 50% slurry of neutravidin-agarose and 660 μl of lysis buffer. The mixture was rotated for 60 min at 4 °C and then pelleted. The supernatants consisted of unbiotinylated material, which contained internal proteins.
The beads were washed three times with 15 mM Tris-HCl, 4 mM EGTA, 500 mM NaCl, and 0.5% Triton X-100, pH 8.0 and then in the same buffer without NaCl. Proteins that remained associated with the beads were biotinylated and represented proteins exposed on the cell surface. This material was eluted by incubating the beads in SDS sample buffer at 90°C for 5 min (Surface). Samples were then size-fractionated by 5–17% gradient SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and processed for immunoblot analysis using a polyclonal anti-LDLR antibody (4548).

**Fig. 1.** ARH−/− cells fail to endocytose LDL receptors. The rate of endocytosis of the receptor was estimated using 25 μM monensin to prevent recycling of receptor back to the plasma membrane. Shown in A is an immunoblot of biotinylated LDLR showing the relative amount of LDLR on the surface of either normal (upper blot) or ARH−/− (lower blot) cells. The quantification of the immunoblots is shown in B.

**Fig. 2.** Indirect immunofluorescence using antibodies against LDLR and EEA1 shows that loss of ARH shifts the LDLR distribution from the endosomal to the plasma membrane compartment. Staining is shown for normal (A, D, and G), ARH−/− (B, E, and H), and LDLR−/− (C, F, and I) lymphocytes. A, B, and C show the fluorescence staining for the Alexa-Fluor 488-visualized LDLR. D, E, and F show the Alexa-Fluor 555-visualized EEA1. G, H, and I are merged images with white signal signifying co-staining. Immunofluorescence was performed as described under "Experimental Procedures."
PBS to inhibit further endocytosis, washed with cold PBS, resuspended in 1 ml of PBS plus sulforafinimidyl-6-biotinamido hexanoate (1 mg/ml), and incubated for 30 min at 4 °C with end-over-end mixing. Samples were then processed for surface expression of LDLR using neutravidin-agarose as described above.

Lipoprotein Binding Assays—Radiolaabeled human 125I-LDL and rabbit 125I-labeled β-migrating very low density lipoprotein (125I-VLDL) binding assays were performed in triplicate as described previously (18). Acid and EDTA washes were conducted at 4 °C using Buffer E containing 50 mM Tris maleate, 150 mM NaCl, and 2 mM CaCl₂, pH 7.6 (control), Buffer E at pH 5.0 (acid wash), or Buffer E with 10 mM EDTA (EDTA wash) for 1 h with end-over-end mixing prior to addition of 125I-LDL.

The experiments used to compile the data for the Scatchard plots included the following modifications. In each experiment, 2.5 × 10⁶ lymphocytes were suspended in 100 μl of ice-cold Medium C. The cells were then incubated with 5 μg/ml 125I-LDL together with increasing concentrations of cold LDL (up to 5 mg/ml). The specific activity of the labeled LDL in each assay was recalculated based upon the total LDL concentration. Bound and free counts were separated by centrifugation of the cells through a cushion of Medium C plus 10% sucrose at 10,000 × g for 5 min. Tubes were then frozen in liquid nitrogen and cut to separate the bound and free LDL. The amount of free and bound LDL in each assay was calculated using the specific activity of LDL in each sample. In A, LDLR⁻/⁻, normal, and ARH⁻/⁻ cells were treated with non-cell-permeable biotinylation reagent, washed, and lysed with Triton X-100. After removal of insoluble debris, one-fourth of the lysate was set aside, while two-thirds of the lysate was further processed for biotinylated proteins. Shown in the upper portion of A is an immunoblot of lysates (lanes 1–3); biotin-free material (lanes 4–6), and biotinylated material (lanes 7–9). Lanes 1, 4, and 7 were from LDLR⁻/⁻ lymphocytes; lanes 2, 5, and 8 were from normal cells; and lanes 3, 6, and 9 were from ARH⁻/⁻ cells. The lower portion of A shows a quantification of the immunoblot normalized to sample size. In B, the relative number of LDLRs on the cell surface was determined by incubating LDLR⁻/⁻, ARH⁻/⁻, and normal lymphocytes with 125I-labeled mouse monoclonal antibody against the LDLR (C7) at 4 °C for 90 min. Cells were then washed, lysed, and counted on a γ counter. The cellular protein content was determined and used to normalize each sample. In C, the relative number of LDLRs on the cell surface was determined by incubating LDLR⁻/⁻, normal, and ARH⁻/⁻ lymphocytes and a no-cell control with a rabbit polyclonal antibody against the LDLR at 4 °C. Cells were then washed, incubated with 125I-Protein A, washed again, lysed, and counted on a γ counter. The cellular protein content was used to normalize each sample.

RESULTS

In normal cells, LDL binds to the LDLR on the cell surface, and the LDL-LDLR complex is internalized and delivered to endosomes. In the low pH environment of the endosome, the receptor dissociates from the lipoprotein and recycles to the cell surface. LDL that is released from the receptor in the endosome is degraded, delivering cholesterol to the cell. The degradation of 125I-LDL is severely compromised in ARH⁻/⁻ lymphocytes despite an increased amount of LDL binding to the surfaces of these cells (16, 17). To determine whether the decreased LDL degradation in ARH⁻/⁻ cells is due to a failure of LDL internalization, ARH⁻/⁻ and normal lymphocytes were treated with monensin to block LDLR recycling, and the rates of disappearance of LDLR from the cell surface were compared in the two cell lines (Fig. 1). The half-life of LDLR at the plasma membrane was ~8 min in normal lymphocytes, a value similar to that seen in fibroblasts (26). In contrast, no loss of surface LDLR was detected in ARH⁻/⁻ cells after 60 min. These results are consistent with a failure of LDLR to be internalized in ARH⁻/⁻ lymphocytes.

A potential consequence of the failure to internalize LDLR is an accumulation of receptors on the cell surface. We examined the localization of the LDLR in normal and ARH⁻/⁻ cells using immunofluorescence. In normal lymphocytes LDLR staining was observed predominantly in internal compartments (Fig. 2). The internal LDLR staining co-localized with the early endosome marker EEA1 (Fig. 2) and partially co-localized with the lysosomal marker lysosome-associated membrane protein-1 (data not shown). In contrast, LDLR was predominantly on the cell surface of ARH⁻/⁻ lymphocytes. The distribution of LDLR in the ARH⁻/⁻ lymphocytes mirrors the distribution observed previously in hepatocytes from ARH⁻/⁻ mice (28). The relative abundance of surface LDLR on normal and ARH⁻/⁻ cells was quantified by biotinylating cell surface proteins, precipitating with neutravidin-agarose, and immunoblotting for the LDLR. ARH⁻/⁻ cells had ~20-fold more LDLR on the cell surface than normal cells (Fig. 3A). A similar increase in LDLR number was
FIG. 4. Immunogold labeling of LDLRs in ARH−/− and normal lymphocytes shows that LDLRs are concentrated in endosomes in normal cells and on the plasma membrane in ARH−/− cells. Lymphocytes were cryosectioned and processed for immunogold labeling using a rabbit polyclonal antibody against the LDLR as described under “Experimental Procedures.” A shows the distribution of LDLR in ARH−/− cells, while B shows the distribution of LDLR in normal cells. The solid arrows point to immunogold particles on the cell surfaces. The large open arrows point to endosomes.

| Cell line | Temperature | Cell number | Non-coated pit membrane | Coated pit membrane | Coated pit enrichment |
|-----------|-------------|-------------|-------------------------|---------------------|----------------------|
|           | °C          |             | Membrane length | Gold particles | Membrane length | Gold particles | Membrane length | Gold particles |                  |
| Normal    | 37          | 102         | μm              | 53              | 0.03             | 3.5          | 17             | 4.85          | 162               |
| ARH−/−    | 37          | 96          | μm              | 1529             | 0.83             | 4.04         | 22             | 5.45          | 6.6               |
| LDLR−/−   | 37          | 100         | μm              | 15               | 0.01             | 2.65         | 0              | 0             | 0                 |

* Coated pit enrichment = coated pit particles per μm/non-coated pit particles per μm. Gold particles on the cell surface of normal, ARH−/−, and LDLR−/− cells were quantified from 100 random photographic images and binned into either coated pit or non-coated pit categories based upon the presence of an electron-dense coat under the membrane and an invaginated morphology typical of coated pits.

seen when cell surface LDLRs were detected with 125I-labeled monoclonal anti-LDLR antibody or with polyclonal anti-LDLR followed by 125I-Protein A (Fig. 3, B and C).

To determine whether the accumulation of LDLR on the plasma membranes of ARH−/− lymphocytes reflected a failure to cluster the receptors in coated pits, the surface distribution of LDLR was examined by immunoelectron microscopy. In agreement with the immunofluorescence data (Fig. 2), LDLR was located predominantly on the cell surfaces of ARH−/− lymphocytes, while in normal cells the LDLR was associated with internal vesicular structures resembling endosomes and lysosomes (Fig. 4). Quantification of immunogold particles on the cell surface revealed a ≈23-fold increase in the number of LDLRs/μm of membrane in the ARH−/− cells (Table I). Surprisingly there were at least as many LDLRs/μm of membrane in the coated pits of ARH−/− cells as in normal cells (Table I). The large increase (≈27-fold) in the number of LDLRs in the non-coated pit membrane of the ARH−/− cells resulted in a significant reduction in the proportion of surface LDLRs in coated pits of the ARH−/− cells as compared with normal cells (1 versus 31%). Thus, the loss of ARH resulted in a redistribution of LDLR from endosomes to the non-coated pit portion of the plasma membrane without significantly affecting the level of LDLR in coated pits.

The relative increase in the number of cell surface LDLRs in ARH−/− lymphocytes observed in this study (≈15–20-fold) is substantially higher than the relative increase in LDL binding (2-fold) observed previously (16, 17, 29). Therefore, we tested the capacity of ARH−/− cells to bind two well characterized LDLR ligands: β-VLDL particles, which bind to LDLR through apoE, and LDL, which binds to LDLR through apoB-100. The surface binding capacity of 125I-β-VLDL to ARH−/− cells was markedly greater (14-fold) than the binding capacity of the normal cells (Fig. 5A), which is consistent with the large increase in cell surface LDLR in the ARH−/− cells. By contrast 125I-LDL binding was only 2-fold higher in ARH−/− cells than in normal cells (Fig. 5B) in agreement with prior estimates of the relative LDL binding capacity of ARH−/− lymphocytes (16, 17, 29).

To determine whether the decrease in LDL binding was due to a reduction in the affinity of the LDLR for LDL in the ARH−/− cells, we performed more detailed binding studies. Scatchard plot analysis indicated that the 2-fold increase in LDL binding to the ARH−/− cells was due to a ≈2-fold increase in the number of high affinity LDL binding sites rather than to a large increase in low affinity binding sites (Fig. 5C). Together these binding experiments indicated that the majority (≈90%) of LDLR on the cell surface of ARH−/− cells was unable to bind lipoproteins via apoB-100.

To determine which LDLRs on the cell surface bind LDL, we...
incubated the ARH−/− and normal cells with colloidal gold-labeled LDL (LDL-gold) for 1 h at either 4 or 37 °C and performed electron microscopy (Fig. 6). At 4 °C the ARH−/− cells bound ∼2-fold more LDL-gold than did normal cells, which is consistent with the 125I-LDL binding assays (Table II and Fig. 5). At 37 °C, ARH−/− cells also had more surface-associated LDL-gold than did normal cells, although the increase was somewhat less than 2-fold. Within coated pits, the number of LDL-gold particles/μm of membrane was proportional to the number of LDLR-immunogold particles/μm of membrane both in normal and in ARH−/− cells (Tables I and II). In contrast, LDL-gold binding to the surface of non-coated pit membranes was much less efficient in ARH−/− cells than in normal cells. Thus, in ARH−/− lymphocytes the LDLR binds LDL in coated pits but largely fails to bind LDL outside coated pits (>95% failure).

How does ARH promote LDL binding to the LDLR outside the coated pit? One possibility is that the LDLR in ARH−/− cells undergoes a post-translational modification that interferes with LDL binding. We explored this possibility by comparing the electrophoretic mobility on denaturing gels of the LDLR from the ARH−/− and normal cells; no differences in mobility were detected (data not shown). Moreover 125I-LDL ligand blots performed using Triton X-100 extracts from the two cell lines showed no difference in LDL binding activity (Fig. 7). Thus, the defect in LDL binding in ARH−/− cells appears not to be intrinsic to the LDLR but rather is specific to the cellular context of the receptor.

Next we tested whether the inability of most LDLRs on ARH−/− cells to bind LDL was due to non-covalent interactions between the ectodomain of LDLR and another protein, such as receptor-associated protein or Boca (30, 31). Cells were washed with acidic or EDTA-containing buffers to release any attached ligands prior to assaying for LDL binding. No increases in the LDL binding capacity were seen in the ARH−/− cells after these treatments (Fig. 8). We also did not see any difference in the electrophoretic mobility of LDLR from ARH−/− cells on non-denaturing blue native gels nor did we detect any difference in chemical cross-linking behavior of LDLRs (32) from the two cell types (data not shown). Thus, the failure of LDLR to bind LDL did not appear to be due to association of alternative ligands to the receptor ectodomain.

The results of this study indicate that the modular adaptor protein ARH plays a crucial role in both ligand binding and internalization of the LDLR. Loss of ARH resulted in failure of LDLR endocytosis and a dramatic redistribution of LDLR such that the vast majority of receptors were on the cell surface rather than in internal compartments. Electron microscopy revealed that most of the LDLRs on the surfaces of ARH−/− cells were outside coated pits and were unable to bind LDL. Biochemical studies confirmed that despite a >20-fold increase in immunoreactive LDLR on the cell surface, LDL cell surface binding was only modestly (2-fold) increased in ARH−/− cells. Within coated pits, ARH−/− cells had at least as many LDLR and LDL binding sites as did normal cells. Remarkably, however, the LDLR and LDL were not efficiently endocytosed from these pits. Thus, ARH was not required for localization of the LDLR to coated pits but rather appeared to be required for endocytosis of the LDLR from pits. In addition the failure of >90% of LDLR in ARH−/− cells to bind LDL suggested that ARH potentiated the ability of LDLR to bind LDL in vivo.

We have shown previously that ARH is required for normal uptake and degradation of LDL in human lymphocytes and in mouse liver (17, 28), but the specific role of ARH in LDLR-mediated endocytosis has not been defined. The observation that ARH can bind to LDLR, clathrin, and AP2 suggested that ARH couples the LDLR to the endocytic machinery and may direct LDLR to coated pits or anchor the receptor in the pit during endocytosis. Our present results indicated that the number of LDLRs in coated pits was similar in normal and ARH−/− lymphocytes and that the number of LDL particles bound in coated pits was normal in ARH−/− cells. Unlike normal cells, ARH−/− cells did not accumulate LDL-gold in multivesicular bodies characteristic of late endosomes and lysosomes when maintained at 37 °C (Fig. 6). We were also unable...
to detect LDLR internalization after monensin treatment in ARH−/− cells. Thus, despite the presence of both LDLR and LDL in coated pits of ARH−/− cells, neither was internalized. The failure to internalize LDLR is consistent with the reduced uptake and degradation of 125I-LDL observed previously in ARH−/− lymphocytes (16, 17, 29). These observations suggested that the loss of ARH arrested the LDLR trafficking cycle at the point of endocytosis of LDLR from coated pits. How might ARH be involved in the endocytosis of LDLR from coated pits?

ARH may facilitate the endocytosis of LDLR and LDLR-LDL complexes from coated pits by stabilizing the interaction between the receptor and the structural components of the pit. Efficient trapping of receptors in coated pits can be mediated by a so called “rugged energy landscape” in which multiple interactions of relatively low but varying dissociation constants act in concert to reduce the dissociation rate of receptors from coated pits (33). In accordance with this mechanism, coated pits contain many potential binding partners for LDLR including AP2 (34), PTB domain-containing adaptor proteins such as Dab2 (12, 28) and the clathrin heavy chain (35). The associations of these molecules individually or collectively may enable ARH−/− cells to accumulate LDLR in coated pits but may be insufficient to hold the receptor in the invaginating portion of the budding pit. In fibroblasts, many LDLRs appear to be

**Fig. 6.** Localization of LDL-gold in normal and ARH−/− lymphocytes at 4 and 37 °C. Lymphocytes were incubated with LDL-gold for 90 min at either 4 or 37 °C. Cells were then washed, fixed, sectioned, and processed for electron microscopy (see “Experimental Procedures”). Electron micrographs are shown of normal (A, C, E, and G) and ARH−/− (B, D, F, and H) lymphocytes from experiments at 4 °C (A, B, E, and F) or 37 °C (C, D, G, and H). A–D show typical examples of coated pits containing LDL-gold, while E–H show examples of multivesicular bodies. Open arrowheads indicate coated pits. Closed arrows indicate multivesicular bodies characteristic of late endosomes and early lysosomes.
Role of ARH in LDL Receptor Function

Table II

| Cell line | Temperature °C | Cell number | Non-coated pit membrane | Coated pit membrane | Coated pit enrichment a |
|-----------|---------------|-------------|-------------------------|---------------------|------------------------|
|           |               |             | Membrane length µm | Gold particles | Particles per µm | Membrane length µm | Gold particles | Particles per µm |                     |
| Normal    | 4             | 26          | 1243                  | 283               | 0.21          | 11               | 32             | 2.82             | 13.4                |
| ARH−/−    | 4             | 26          | 1143                  | 511               | 0.45          | 9                | 67             | 7.34             | 16.3                |
| LDLR−/−   | 4             | 25          | 945                   | 0                 | 0             | 13               | 0              | 0                | 12.1                |
| Normal    | 37            | 25          | 1146                  | 522               | 0.45          | 9                | 48             | 5.47             | 12.7                |
| ARH−/−    | 37            | 27          | 1334                  | 896               | 0.67          | 12               | 100            | 8.51             |                     |
| LDLR−/−   | 37            | 25          | 804                   | 0                 | 0             | 9                | 0              | 0                |                     |

a Coated pit enrichment = coated pit particles per µm/normal coated pit particles per µm. Gold particles on the cell surface of normal, ARH−/−, and LDLR−/− cells were quantified from 25 random photographic images and binned into either coated pit or non-coated pit categories based upon the presence of an electron-dense coat under the membrane and an invaginated morphology typical of coated pits.

Fig. 7. Receptors from normal and ARH−/− cells are intrinsically equivalent. Triton X-100 extracts from LDLR−/−, ARH−/−, and normal cells were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The upper panel shows 125I-LDL binding, while the lower panel shows an immunoblot for the presence of LDLR (see "Experimental Procedures").

Fig. 8. Prewashing with acid or EDTA does not affect the LDL binding activity of LDLR in normal and ARH−/− cells. Cells were washed with buffer at pH 7.5 (Control), buffer at pH 5.0 (Acid-washed), or buffer containing 10 mM EDTA (EDTA-washed) prior to incubation with 15 µg/ml 125I-LDL for 90 min at 4 °C. Bound 125I-LDL was assayed as described under "Experimental Procedures." expelled from the invaginating portion of coated pits during pit budding (36), suggesting that poorly anchored receptors are not readily captured by the budding portion of the coated pit. ARH may stabilize the association of the receptor with the invaginating portion of the budding pit, thereby increasing the efficiency of LDLR internalization.

The second major finding of this study is that the absence of ARH aboliised the ability of more than 90% of cell surface LDLR to bind LDL. In coated pits, the ratio of LDL to LDLR was similar in normal and ARH−/− cells. Comparison of the ratio of LDL-gold to LDLR-gold in the non-coated pit membranes of normal and ARH−/− cells indicated that the receptors that failed to bind LDL on ARH−/− cells were predominantly located outside coated pits. The ratio of LDL to LDLR also indicated that in normal cells LDL bound to the LDLR with lower efficiency inside coated pits than outside. This lower efficiency can be explained by receptor occlusion resulting from binding of LDL to adjacent LDLR when the receptors are at high density as they are in coated pits (37). In contrast, ARH−/− cells exhibited less efficient binding of LDL to receptors outside of coated pits than inside pits. These observations strongly suggest that ARH plays a role in the LDL binding activity of receptors outside coated pits.

It is currently unclear how ARH affects LDL binding by the LDLR. Scatchard plots of LDL binding to ARH−/− cells indicated that the small proportion of LDLR that bound to LDL did so with normal affinity (Fig. 5C). The receptors from ARH−/− and normal cells did not appear to have different covalent modifications since the LDLR had equivalent electrophoretic mobility mobility on denaturing SDS-polyacrylamide gels and bound equivalent amounts of 125I-LDL in ligand blots. Non-covalent associations of the LDLR with alternative ligands also did not appear to be the cause of the LDL binding defect since washing ARH−/− cells with acidic or EDTA-containing buffers did not improve their ability to bind LDL (Fig. 8). We also did not see any difference in the electrophoretic mobility of LDLR from ARH−/− cells on denaturing blue native gels nor did we detect any difference in chemical cross-linking behavior of LDLR from the two cell types. Thus, the failure of most LDLRs in ARH−/− cells to bind LDL was not an intrinsic property of the LDLR nor were the LDLRs blocked by an alternative ligand. How then might ARH affect LDL binding?

We propose the following model. In the absence of ARH, the LDLR located outside the pits associates with the cell surface in such a manner that the ligand binding domains are partially occluded. The apoB-100 binding site on LDLR encompasses a large surface (38), requiring cysteine-rich repeats 3–7 and the first epidermal growth factor repeat. In contrast, the apoE binding site only requires cysteine-rich repeat 5 (39, 40). Thus, partial occlusion may be sufficient to prevent binding to apoB-100 but not apoE binding. ARH binding to the cytoplasmic tail of LDLR, perhaps in combination with ARH binding to phosphatidylinositol 4,5-bisphosphate, may relieve the partial occlusion, allowing the full exposure of the ligand binding domains of LDLR and thereby making them accessible to LDL. Since LDL is able to bind to the LDLR in the coated pits of the ARH-deficient cells, other LDLR binding components can promote the availability of the ligand binding domains. The finding that ARH is required for both ligand binding and internalization of LDLR suggests that ARH plays a more complex role in LDL metabolism than originally envisioned.
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Peter Michaely, Wei-Ping Li, Richard G. W. Anderson, Jonathan C. Cohen and Helen H. Hobbs

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