Autosomal recessive hypercholesterolemia is characterized by a cell type-specific defect in low density lipoprotein receptor (LDLR) endocytosis. LDLR-mediated uptake of LDL is impaired in the liver, but not in fibroblasts of subjects with this disorder. The disease is caused by mutations in ARH, which encodes a putative adaptor protein that interacts with the cytoplasmic tail of the LDLR, phospholipids, and two components of the clathrin endocytic machinery, clathrin and adaptor protein-2 (AP-2) in vitro. To determine the physiological relevance of these interactions, we examined the effect of mutations in the ARH on LDLR location and function in polarized hepatocytes (WIF-B). The integrity of the FDNPVY sequence in the LDLR cytoplasmic tail was required for ARH-associated LDLR clustering into clathrin-coated pits. The phosphotyrosine binding domain of ARH plus either the clathrin box or the AP-2 binding region were required for both clustering and internalization of the LDLR. Parallel studies performed in vivo with the same recombinant forms of ARH in livers of Arh+/− mice confirmed the relevance of the cell culture findings. These results demonstrate that ARH must bind the LDLR tail and either clathrin or AP-2 to promote receptor clustering and internalization of LDL.

Approximately 70% of plasma cholesterol circulates as a constituent of human low density lipoproteins (LDL).1 The primary route of clearance of LDL from the plasma is by LDL receptor (LDLR)-mediated endocytosis in the liver. The critical role of the LDLR pathway is illustrated by the very high plasma levels of LDL that characterize three genetic disorders in which LDLR-mediated endocytosis is disrupted: familial hypercholesterolemia (FH), familial defective apolipoprotein B-100 (FDB), and autosomal recessive hypercholesterolemia. FH is an autosomal co-dominant disorder caused by mutations in the LDLR gene (1). Individuals with homozygous FH inherit two defective LDLR alleles and have markedly reduced LDLR clearance, resulting in a ~6-fold increase in plasma LDL-cholesterol levels. Fibroblasts and lymphocytes cultured from patients with homozygous FH also fail to internalize and degrade LDL. FDB is an autosomal dominant phenocopy of FH caused by missense mutations in apolipoprotein B-100 that impair binding of LDL to the LDLR (2). Autosomal recessive hypercholesterolemia is caused by mutations in ARH, a putative adaptor protein that is required for normal clearance of LDL from the circulation (3). Subjects with autosomal recessive hypercholesterolemia, like those with FH and FDB, are hypercholesterolemic and have markedly reduced rates of clearance of circulating LDL (4, 5); however, unlike FH, the defect in LDLR endocytosis is cell type-specific. Although LDL degradation is reduced in the liver (5) and in immortalized lymphocytes (6, 7), cultured fibroblasts from patients with autosomal recessive hypercholesterolemia internalize and degrade LDL at normal or only modestly reduced rates (8).

In wild-type mice, approximately two-thirds of the hepatic LDLRs are intracellular, whereas in Arh−/− mice the majority of immunodetectable LDLRs are located on the sinusoidal membrane (9). In lymphocytes of subjects with ARH, a similar redistribution of LDLRs to the plasma membrane occurs, resulting in a 16-fold increase in cell surface receptor number (10). These observations indicate that ARH is required for the normal transit of LDLR from the cell surface to endosomes. One possibility is that ARH may serve to escort LDLR to clathrin-coated pits. Alternatively ARH may stabilize the association of the LDLR within coated pits as a prerequisite to efficient internalization, or play a role in the early events of endocytosis (10–12).

Data from in vitro pull-down experiments are consistent with the hypothesis that ARH functions as an endocytic adaptor that links the LDLR to structural components of the coated pit (11, 13). ARH has at least four highly conserved regions beginning with a sequence of ~40 amino acids at the N terminus of the protein that has no known function. Amino acids 44–178 encode a phosphotyrosine binding (PTB) domain that binds the internalization sequence (FDNPVY) in the LDLR cytoplasmic tail (11, 13) and phosphoinositides (11). Residues 212–216 comprise a canonical clathrin box sequence (LLDLE) that mediates high affinity binding to the terminal domain of the heavy chain of clathrin (11, 13, 14). Following the clathrin box motif, the protein has a highly conserved amino acid sequence (residues 252–268) that binds the β2-adaptin subunit of the adaptor protein-2 (AP-2) (13, 15). Together these findings suggest that ARH couples the LDLR to the endocytic machinery, but the physiological significance of these interactions have not been confirmed in cultured hepatocytes or in vivo.

To address the physiological role of ARH, we compared LDLR localization and internalization in polarized hepatocytes (WIF-B cells) (16) overexpressing wild-type or one of several mutant forms of ARH with impaired binding to the LDLR, to clathrin or to AP-2. The ability of these ARH proteins to support LDL clearance in vivo was also examined by expressing these proteins in mice lacking ARH (Arh−/− mice) (9). The results indicate that the PTB domain, the clathrin box, and the AP-2
binding motif of ARH interact in a combinatorial fashion to promote the localization of LDLR to clathrin-coated pits and the clearance of circulating LDL by the liver.

**EXPERIMENTAL PROCEDURES**

**Materials**—All cell culture reagents used in these studies were purchased from Sigma. The rabbit anti-LDLR polyclonal antibody used for immunofluorescence and immunoelectron microscopy was obtained from Maine Biotechnology Services, Inc. (Portland, ME), and the mouse monoclonal antibody to the LDLR (IgG-C7) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) (17). Two rabbit polyclonal anti-ARH antibodies were used in these studies: an antibody directed against the 15 C-terminal amino acids in ARH, which does not recognize mouse or rat ARH (7), and an antibody raised against the full-length recombinant human protein that cross-reacts with rat ARH. The mouse monoclonal anti-α-adaptin (AP.6) was from BD Transduction Laboratories and mouse monoclonal anti-clathrin heavy chain (X22) was kindly provided by Dr. Richard Anderson (UT Southwestern, Dallas, TX). Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 568-conjugated goat anti-mouse IgG were purchased from Molecular Probes (Eugene, OR) and 10-nm gold-labeled goat anti-rabbit IgG were from Amersham Biosciences. Cells were fixed using paraformaldehyde purchased from Electron Microscopy Sciences (Hatfield, PA). Cholesterol measurements were determined enzymatically using an assay kit from Roche Applied Science.

**Cell Culture**—WIF-B cells represent a fusion cell line between human fibroblasts and rat hepatocytes (16); the cells were a generous gift from Dr. Ann Hubbard (Johns Hopkins University). Cells were cultured at 37 °C with 5% CO2/air in modified F12 medium supplemented with 5% fetal bovine serum and HAT (10 mM hypoxanthine, 4 mM aminopterin, 1.6 mM thymidine) as described (18). Cultures were split and passaged using standard trypsinization procedures and used between passages 7 and 15. Cells were grown on glass coverslips and used at maximal density and polarity, typically after 8–10 days of culture.

**Generation of Recombinant Adenoviruses**—Recombinant adenoviral vectors containing the cDNA of the wild-type human LDLR and the mutant human LDLR-Y807A were generated by *in vitro* Cre-lox recombination (19). Recombinant tetracycline-inducible viruses containing either wild-type or mutant ARH were constructed using pACreCMV and pBHGCMVrtTA plasmids kindly provided by Dr. Christopher Newgard (Duke University, Durham, NC). Homologous recombination in 911 cells (20) was used to construct recombinant tet-inducible viruses, which were cloned, amplified, and purified as previously described (21).

**Infection and Immunofluorescence**—Eight days after plating cells on coverslips, the WIF-B cells were washed with PBS (0.15 M NaCl, 10 mM phosphate buffer, pH 7.3) and then incubated for 1 h at 37 °C in serum-free medium with 6 × 10^6 viral particles. The cells were washed again and refed with complete medium. The following day, the cells were incubated with doxycycline (20 ng/ml) for 6 h. The coverslips were then rinsed with cold PBS and fixed with 4% (w/v) paraformaldehyde (PFA) diluted in PBS at 4 °C for 5 min. The non-permeabilized cells were allowed to bind IgG-C7 in Buffer A for 45 min before washing and incubating at 37 °C in antibody-free medium for 20 min. The cells were then fixed, permeabilized, and processed for immunofluorescence.

**Immunoblot Analysis**—WIF-B cells were plated and grown to confluence in 6-well plates, as described previously. After infection with adenoviruses, the cells were harvested, and lysates were subjected to immunoblot analysis using rabbit polyclonal anti-human LDLR or rabbit polyclonal anti-human ARH antibodies (7). Immunoblot analysis on liver cell extracts from *Arh*−/− mice was performed as described previously (9).

**Electron Microscopy**—WIF-B cells were cultured in 100-mm dishes to 95% confluence and infected with adenoviruses (see above). After 12 h, the cells were harvested in PBS, washed in Hank’s buffered salt solution (HBSS), and processed for immunoelectron microscopy as described (10). For LDL internalization colloidal gold-conjugated LDL was produced as described previously (22). The colloidal gold-labeled LDL was used within 5 days of synthesis. Infected WIF-B cells were incubated at 4 °C in serum-free medium prior to addition of 10 µg/ml colloidal gold-labeled LDL. The cells were maintained at 37 °C for 90 min and then washed three times using 50 mM Tris-HCl, 150 mM NaCl, 2 mg/ml BSA, pH 7.4. Cells were fixed, embedded, sectioned, and placed on nickel grids as described (23, 24). Electron micrographs were taken using a JEOL 1200 electron microscope operating at 80 kV.

**Quantification of Gold Labeling**—Electron microscope images were obtained by taking 25 photographs of each cell type. The length of the non-coated pit membranes, the diameter of the coated pits, and the number of gold particles associated with each region were determined. The labeling intensity was expressed as the number of gold particles per micrometer length of the different regions of the plasma membrane.

**In Vivo Complementation of ARH Function in Mice**—Mice with a targeted disruption of *Arh* (9) were maintained on a hybrid background of C57Bl/6 and 129sv strains. The mice were housed on a 12-h dark/12-h light cycle and maintained on standard chow (number 7002: Harlan Teklad, Madison, WI) and water *ad libitum*. To induce hypercholesterolemia, the *Arh*−/− mice (aged 12–16 weeks) were fed normal chow diets supplemented with 2% cholesterol and 21% anhydrous milk fat for 3–5 weeks. On Day 0 the mice were anesthetized by intraperitoneal injection of sodium pentobarbital (100 µg/g body weight) and ~100 µl of blood was obtained via retro-orbital puncture. Recombinant adenovirus was then injected into the external jugular vein as a single dose (2 × 10^11 pfu in 200 µl of 10% Tritr-HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl2, 2 mM CaCl2, 0.2% (w/v) BSA) (25). After recovery from anesthesia, mice were maintained on the 2% cholesterol diet until Day 4 post-injection, when the animals were sacrificed. Because ARH expression from the recombinant adenovirus was sufficient to restore LDLR endocytosis in the *Arh*−/− mice without addition of doxycycline, no doxycycline was supplied to the mice. Blood samples were drawn from the inferior vena cava for measurement of plasma cholesterol levels. The livers were removed, and immunoblot analysis of ARH was performed on tissue lysates to confirm ARH expression. Animal experiments were approved by the University of Texas Southwestern Medical Center Animal Care and Use Committee (IACUC) and were performed according to all federal animal welfare policies and regulations.
RESULTS

Immunolocalization of Endogenous LDLR and ARH—WIF-B cells are an immortalized cell line derived from the fusion of rat hepatocytes with human fibroblasts (16). The cells polarize in culture, providing a model system for the study of membrane protein trafficking (18) including the LDLR (26). Human chromosomes 19 and 1 are not retained in this cell line (16) so these cells do not synthesize human LDLR or ARH. To determine the cell surface location of the endogenous LDLR in polarized WIF-B cells, we performed immunofluorescence microscopy in non-permeabilized cells, using an anti-LDLR antibody that interacts with the rat LDLR. The cells were then permeabilized and stained with antibodies against the α-subunit of AP-2 and the heavy chain of clathrin (clathrin-HC). The plasma membrane-associated LDLR was located on the basolateral surfaces and only partially colocalized with AP-2 or with clathrin-HC (Fig. 1A).

To determine the location of endogenous ARH in WIF-B cells, permeabilized WIF-B cells were stained with antibodies to ARH and to either AP-2 or clathrin-HC. With all three antibodies, staining was present in the cytoplasm and associated with the basolateral membrane. The majority of ARH staining on the plasma membrane colocalized with AP-2 and clathrin-HC, suggesting that the ARH associated with the plasma membrane was predominantly, though not exclusively, localized to clathrin-coated pits in these cells (Fig. 1B).

Immunolocalization of Recombinant LDLR and ARH in WIF-B Cells—To examine the molecular basis for the interactions between ARH and the LDLR, we infected WIF-B cells with a recombinant adenovirus expressing the human LDLR (AdLDLR). The cells were incubated with a monoclonal anti-human LDLR antibody (IgG-C7) before permeabilization. The cells were then permeabilized and incubated with antibodies to aminopeptidase-N (APN), AP-2, clathrin-HC, and ARH, and processed for visualization using immunofluorescence confocal microscopy. The human LDLR was expressed only on the basolateral membrane (Fig. 2). No co-localization with APN, an apical membrane protein (Fig. 2A) was seen, which is consistent with previous observations (26). Anti-LDLR staining was uniformly distributed along the basolateral membrane, whereas the staining patterns of AP-2, clathrin, and ARH were more punctate (Fig. 2B). Thus, the distribution of the recombinant LDLR on the cell surface resembled that of the endogenous LDLR protein (Fig. 2B).

Expression of Recombinant ARH Does Not Alter the Cellular Localization of AP-2 or Clathrin-HC—High level expression of the adaptor proteins epsin 2, synaptojanin, and Dab2 disrupts AP-2 localization (27–29). In contrast to these findings, we did not observe changes in the distribution of AP-2 or clathrin with the overexpression of recombinant human ARH in WIF-B cells (AdARH) (Fig. 3). The recombinant ARH was predominantly cytoplasmic but some signal was also apparent at the plasma membrane. The membrane-associated recombinant ARH appeared to co-localize extensively with AP-2 and clathrin-HC (Fig. 3).

Expression of Recombinant ARH Results in Cell Surface Clustering of LDLR—First we examined the effect of ARH overexpression on the cell surface localization of the endogenous LDLR. WIF-B cells were infected with AdARH or an adenovirus expressing β-galactosidase (AdβGal). No changes in the cell surface or intracellular distribution of the LDLR were seen (data not shown). Next we examined the LDLR localization in WIF-B cells expressing both human ARH and LDLR by infecting the WIF-B cells with AdLDLR and either AdARH or AdβGal. Expression of LDLR with the control virus resulted in diffuse LDLR staining on the basolateral surface. In contrast, co-expression of ARH with LDLR...
the wild-type LDLR or LDLR-Y807A. Co-expression of ARH resulted in clustering of the LDLR in cells expressing wild-type LDLR but not in the cells expressing LDLR-Y807A (Fig. 4A). The expression of ARH and LDLR was similar in the two sets of cells (Fig. 4B). Thus, the ability of ARH to drive LDLR clustering in the WIF-B cells required the integrity of the FDNPVY internalization sequence in the LDLR cytoplasmic tail.

Expression of ARH Promotes Clustering of the LDLR in Clathrin-coated Pits and LDLR Internalization—Immunofluorescence microscopy did not provide sufficient resolution to determine if the punctuate pattern of anti-LDLR staining in the WIF-B cells expressing ARH and LDLR reflected clustering of LDLR in coated pits. Therefore, we performed immunoelectron microscopy to localize the LDLR on the cell surface. In WIF-B cells infected with AdLDLR and AdβGal, the immunogold labeling of LDLR showed an even distribution along the plasma membrane with most of the staining outside the clathrin-coated pits (Fig. 5). In contrast to these findings, the immunogold particles clustered into coated pits, in cells co-expressing LDLR and ARH. The quantification of immunogold particles on the cell surface revealed a ~33-fold increase in the number of LDLRs/μm of coated pit membrane (TABLE ONE). Thus, the expression of ARH resulted in a redistribution of LDLR from the non-coated pit portion of the plasma membrane to coated pits. The number of LDLRs per micrometer of plasma membrane was reduced ~9-fold in cells expressing both ARH and the LDLR. These results are consistent with ARH increasing internalization of LDLR, because overexpression of ARH did not change the total amount of LDLR in cells (Fig. 4B).

To confirm that the LDLR clustering induced by overexpression of ARH resulted in greater internalization of LDLRs, we compared the intracellular immunogold staining of WIF-B cells expressing LDLR and β-galactosidase to those expressing LDLR and ARH. In the WIF-B cells expressing LDLR and β-galactosidase the immunogold particles were almost exclusively localized on the cell surface (Fig. 6A), whereas in cells co-expressing ARH, a large fraction of the LDLR was intracellular (Fig. 6B). Next we examined the effect of ARH expression on gold-LDL internalization. In cells expressing the LDLR but no ARH, the gold particles were mainly localized on the cell surface (Fig. 7A). The LDL-gold was found predominantly inside the cells co-expressing ARH and LDLR (Fig. 7B). Thus, ARH expression resulted in the clustering of LDLR in coated pits and promoted LDL internalization.

Contribution of ARH Modules to LDLR Clustering—In vitro pull-down studies defined sequences within ARH that interact with the LDLR, the terminal domain of the heavy chain of clathrin, and β2-adaptin, a component of AP-2 (11, 13). To determine if these sequences are required for ARH-mediated clustering and internalization of LDLR in hepatocytes, we developed a series of recombinant adenoviruses expressing mutant ARH proteins that disrupt the ability of ARH to bind LDLR, clathrin, and AP-2 (Fig. 8A). We first tested the effect of substituting alanine for phenylalanine at residue 165 (F165A) in the PTB domain of ARH, that abolishes binding of ARH to the LDLR in vitro (13). Expression of ARH-F165A in WIF-B cells was associated with clustering of LDLR (Fig. 8B). Previously, Stolt et al. (32, 33) showed that the corresponding mutation in the PTB domain-containing adaptor protein, Dab-1, reduced the binding affinity of Dab1 for another member of the LDLR family (ApoER2), by 10-fold. An even greater reduction in affinity (70-fold) was achieved by substituting tyrosine for serine at amino acid 114. Because the PTB domain of Dab1 strongly interacts in affinity (70-fold) was achieved by substituting tyrosine for serine at amino acid 114. Because the PTB domain of Dab1 strongly interacts...
PTB domain of ARH and the cytoplasmic tail of LDLR being essential for ARH to promote clustering of the LDLR.

In addition to the PTB domain, what other sequences in ARH are required to support clustering and internalization of the LDLR? The expression of a truncated form of ARH (ARH-N187X), containing the PTB domain but not the clathrin box or the AP-2 binding region, failed to promote LDLR clustering (Fig. 8B). Thus, sequences in the C-terminal half of ARH are required for ARH-mediated clustering of LDLR in coated pits. To determine the relative importance of the clathrin box and the AP-2 binding regions of ARH, we replaced the first two leucine residues of the clathrin box (Leu212 and Leu213) with alanines (AdARH-L212A/L213A); previously this mutant form of ARH did not bind the heavy chain of clathrin in vitro (13). Unlike the truncated ARH construct (ARH-N187X), the clathrin box mutant supported clustering of the LDLR in the WIF-B cells (Fig. 8B). A similar result was obtained (Fig. 8B) when we substituted alanine for arginine at position 266 (AdARH-R266A) to abolish interactions between ARH and AP-2 (13). Thus, disruption of either the clathrin box or the AP-2 motif did not abolish localization of the LDLR in clathrin-coated pits. Next we expressed recombinant ARH with mutations in both the clathrin box and in the AP-2 binding site (AdARH-LL/AA/R266A) in WIF-B cells. No LDLR clustering was seen (Fig. 8B). Therefore the integrity of either the clathrin box or the AP-2 binding region is sufficient to support clustering, but inactivation of both sites prevents ARH-mediated clustering of the LDLR.

Contribution of ARH Modules to LDLR Internalization—To determine if the ARH modules required for LDLR clustering were also required for receptor internalization, we infected polarized WIF-B cells with recombinant adenoviruses expressing wild-type and mutant forms of LDLR and ARH. Cell surface receptors were labeled by incubating the infected cells with the IgG-C7 at 4 °C, and then the cells were warmed to 37 °C to allow receptor internalization. In cells expressing wild-type LDLR, almost all the receptors remained on the cell surface (Fig. 9). In contrast, most receptors were internalized in cells expressing both LDLR and wild-type ARH. Interaction of ARH with the LDLR was required, because cells expressing LDLR-Y807A and ARH failed to internalize. Moreover the mutant ARH protein that failed to support LDLR clustering (S117Y, LL/AA/R266A) also failed to support receptor internalization. The mutant forms of ARH that promoted receptor clustering also promoted internalization (Fig. 9). These data are consistent with ARH-mediated clustering of LDLRs being required for receptor internalization in WIF-B cells and that both clustering and internalization require the conserved tyrosine at position 807 in the cytoplasmic tail of the LDLR.
To examine the role of the various ARH interaction sites on LDLR function in vivo, we used tetracycline-inducible recombinant adenoviruses to reconstitute hepatic ARH expression in Arh/H11002/H11002 mice (9). Because chow-fed Arh/H11002/H11002 mice are only mildly hypercholesterolemic (9), the mice were fed a high-fat-high cholesterol diet (2% cholesterol, 21% milk fat) for 3–5 weeks, resulting in a 4–7-fold increase in plasma cholesterol (375–700 mg/dl). Expression of wild-type ARH in these mice was associated with a 50% reduction in circulating levels of plasma cholesterol, whereas no significant change in cholesterol levels occurred in mice expressing a control adenovirus (Fig. 10, A and B). Next, we tested the same series of recombinant ARH adenoviruses used for the studies in the WIF-B cells. Expression of recombinant ARH constructs containing either the ARH-F165A or the ARH-S117Y mutations in the PTB domain failed to lower LDL-C levels, perhaps because of a dominant-negative effect since the plasma level of LDL-C actually increased. In contrast to these results, recombinant ARH with mutations in the clathrin box (AdARH-L212A/L213A) or in the AP-2 binding site (ARH-R266A) decreased plasma cholesterol to levels similar to those of mice infected with the wild-type ARH adenovirus. However, no reduction in cholesterol level was seen in association with expression of the recombinant ARH adenovirus expressing an ARH containing both mutations (AdARH-LL/AA/R266A) (Fig. 10, A and B). Thus, with the exception of the F165A mutation, which prevented ARH function in vivo but not in WIF-B cells, the effects of ARH on plasma cholesterol levels in mice paralleled those obtained in WIF-B cells.

### Functional Evaluation of Mutant Forms of ARH in Vivo

To examine the role of the various ARH interaction sites on LDLR function in vivo, we used tetracycline-inducible recombinant adenoviruses to reconstitute hepatic ARH expression in Arh mice (9). Because chow-fed Arh mice are only mildly hypercholesterolemic (9), the mice were fed a high-fat-high cholesterol diet (2% cholesterol, 21% milk fat) for 3–5 weeks, resulting in a 4–7-fold increase in plasma cholesterol (375–700 mg/dl). Expression of wild-type ARH in these mice was associated with a 50% reduction in circulating levels of plasma cholesterol, whereas no significant change in cholesterol levels occurred in mice expressing a control adenovirus (Fig. 10, A and B). Next, we tested the same series of recombinant ARH adenoviruses used for the studies in the WIF-B cells. Expression of recombinant ARH constructs containing either the ARH-F165A or the ARH-S117Y mutations in the PTB domain failed to lower LDL-C levels, perhaps because of a dominant-negative effect since the plasma level of LDL-C actually increased. In contrast to these results, recombinant ARH with mutations in the clathrin box (AdARH-L212A/L213A) or in the AP-2 binding site (ARH-R266A) decreased plasma cholesterol to levels similar to those of mice infected with the wild-type ARH adenovirus. However, no reduction in cholesterol level was seen in association with expression of the recombinant ARH adenovirus expressing an ARH containing both mutations (AdARH-LL/AA/R266A) (Fig. 10, A and B). Thus, with the exception of the F165A mutation, which prevented ARH function in vivo but not in WIF-B cells, the effects of ARH on plasma cholesterol levels in mice paralleled those obtained in WIF-B cells.

### Table ONE

**Quantification of gold LDLRs on the surface of WIF-B cells using immunoelectron microscopy**

| Adenovirus   | n | Membrane length | Gold particles | Gold/μm | Membrane length | Gold particles | Gold/μm |
|--------------|---|-----------------|----------------|---------|-----------------|----------------|---------|
| LDLR         | 25| 634             | 1128           | 1.78    | 14              | 6              | 0.43    |
| LDLR+ARH     | 25| 502             | 50             | 0.10    | 25              | 84             | 3.36    |

* n is the number of independent negative images screened and counted.
ARH Promotes Clustering of LDLR into Clathrin-coated Pits

A major finding of this study is that ARH promotes clustering of LDLR into clathrin-coated pits on the basolateral membrane of hepatocytes. In the absence of ARH, LDLRs were diffusely distributed on the sinusoidal surfaces of polarized WIF-B cells. When ARH and LDLR were co-expressed in these cells, the LDLR clustered in coated pits. ARH-mediated LDLR clustering was accompanied by an increase in receptor internalization. The ARH-induced changes in the cellular distribution of LDLR required a functional internalization sequence (FDNVPY) in the cytoplasmic tail of the LDLR and an intact PTB domain together with either (but not both) a clathrin box or an AP-2 binding motif in ARH. The structural requirements for ARH function observed in hepatocytes were corroborated for the first time by reconstitution studies in Arh<sup>−/−</sup> mice. The hypercholesterolemia of Arh<sup>−/−</sup> mice was ameliorated by adenoviral-mediated hepatic expression of wild-type ARH, and the binding modules required for receptor clustering and internalization in WIF-B cells were also required to clear lipoproteins in vivo. Taken together, these data indicate that ARH promotes the clustering of LDLRs on the cell surface of hepatocytes by coupling the cytoplasmic tail of the receptor to structural components of the clathrin-coated pit (clathrin and AP-2), and that ARH-mediated clustering of the receptor is required for efficient clearance of LDL by the liver.

Adenovirus-mediated expression of recombinant LDLR in WIF-B cells recapitulated the cell surface pattern of expression of the endogenous receptors in these cells. Plasma membrane associated LDLR were located on the basolateral (sinusoidal) membrane as previously observed (26) and a subset appeared to co-localize with AP-2 or clathrin (Fig. 2). Immunoelectron microscopy confirmed that most of the receptors were located outside of coated pits, which is similar to the distribution of LDLR in the livers of mice expressing a human LDLR transgene (22). The pattern of expression of recombinant ARH in WIF-B cells also appeared similar to that of the endogenous ARH.

Expression of recombinant ARH in WIF-B cells did not affect the distribution of endogenous LDLR, clathrin or AP-2, but markedly altered the distribution of recombinant LDLR (Figs. 3 and 4). Only co-expression of ARH with LDLR in the WIF-B cells resulted in clustering of receptors into coated pits, as observed by electron microscopy (Fig. 5). These findings indicated that endogenous ARH in WIF-B cells failed to promote clustering of recombinant LDLRs, either because the level of expression was too low, or because of species differences between endogenous ARH and the recombinant human LDLR.

Previous studies have reported that high level expression of PTB domain-containing proteins such as ARH and Dab-2 have a dominant negative effect on LDLR clustering and internalization in HeLa cells (11, 15). High level expression of ARH in WIF-B cells did not interfere with LDLR clustering or internalization. The apparent discrepancy between our results and prior studies may be caused by the differences in cell types used or to differences in the relative levels of expression of ARH and LDLR. When cell surface LDLR are located predominantly in coated pits, as in fibroblasts or epithelial-like cell lines, such as Hela cells, other adaptor proteins like Dab-2 or β-arrestin may assist with internalization of LDLRs (22, 27) and high levels of ARH may interrupt these interactions. In cell types such as hepatocytes, where most of the LDLRs reside outside coated pits, ARH may be required to stabilize the association with other elements of coated pits.

Because of the static morphological techniques employed in this study, we were unable to define the temporal sequence of the interactions between ARH, the LDLR and proteins/lipids in the coated pits. ARH may promote the clustering of LDLR in existing coated pits, either by facilitating the migration of receptors to coated pits, or by anchoring incoming receptors in pits to prevent their egress. Time-lapse fluorescence imaging studies in COS-1 cells suggest that coated pits form at specific sites on the membrane (35). Moreover, evidence from studies in HeLa cells is consistent with other adaptor proteins, such as β-arrestin, recruiting receptors to preexisting coated pits (36). Other studies support an alternative scenario in which clathrin lattices form at random sites on the cell membrane but only progress to mature coated pits if sufficient components, including adaptor proteins and cargo, are present (37). Live cell imaging of green fluorescent-tagged clathrin suggests that most nucleation events are transient and abort prior to invagination and scission (37). The subset of nascent pits that become associated with cargo, including the LDLR, almost always proceeds to completion (38). ARH may hold the LDLR in the nascent coated pit, promoting assembly and budding and preventing egress of the receptor to non-coated pit membrane during endocytosis.

The integrity of the FDNVPY sequence in the cytoplasmic tail of the LDLR was required for ARH-mediated clustering and internalization of the LDLR in WIF-B cells. Substitution of the tyrosine in the FDNVPY motif (Tyr<sup>367</sup>) with alanine, which markedly reduced the interaction between ARH and the LDLR tail in vitro (11, 13), also resulted in a failure of ARH to promote clustering of LDLRs in the WIF-B cells. This finding confirms that interaction between the PTB domain of ARH and the internalization sequence in the LDLR is required for clustering.

ARH-F165A supported receptor clustering in WIF-B cells but failed to rescue ARH activity in the knock-out mice (Fig. 10). This mutation was chosen based on modeling of the PTB domain of ARH using the known crystal structure of X11, which also binds nonphosphorylated target sequences (13). Based on this modeling we predicted that two conserved aromatic residues (Tyr<sup>117</sup> and Phe<sup>165</sup>) form a hydrophobic channel that cradles the peptide backbone of the FDNVPY sequence.
Substitution of valine or alanine at position 165 interrupted the physical interaction between ARH and the LDLR tail in vitro (13) but did not prevent clustering and internalization of the LDLR in WIF-B cells. Perhaps high level of expression of ARH-Y165A in WIF-B cells compensated for the reduced affinity of this mutant for the LDLR tail, and thereby supported LDLR clustering. The ability of the ARH-F165A to support internalization does not indicate that the PTB domain is not required since substitution of tyrosine for serine at amino acid 117 in the PTB domain of ARH prevented clustering and internalization of the LDLR in WIF-B cells and in livers of Arh−/− mice.

ARH-induced clustering of LDLR into clathrin-coated pits failed to occur unless the PTB domain was present with at least one of two protein interacting motifs in the C-terminal portion of the protein, the clathrin box or the AP-2 binding sequence. Disruption of either the clathrin box or the AP-2 binding sites alone did not prevent association of the LDLR with coated pits. This finding suggests that ARH-mediated clustering of LDLR in clathrin-coated pits is not dependent on access to any single binding site in the coated pit. Rather, the presence of multiple binding modules in ARH allows efficient clustering of LDLR through interaction with different components of the endocytic machinery. These observations are consistent with the findings of McMahon and co-workers (39) that the assembly and internalization of clathrin-coated vesicles involves a series of low-affinity interactions with components of the coated pit, several of which interact with multiple partners. The low affinities of the interactions necessitate multiple interactions among network components to provide stability, but also allow the pit to form in diverse ways depending on the composition of its environment.

The results of the present study reiterate the different contributions of ARH to LDLR endocytosis in different cells types. Previous studies have demonstrated that ARH is essential for normal LDLR endocytosis in transformed lymphocytes, but not in fibroblasts (6, 7). These differences presumably reflect differences in the kinetics of LDLR trafficking in different cell types. In fibroblasts, LDLRs are located predominantly on the cell surface in clathrin-coated pits (1), indicating that internalization of receptors from clathrin-coated pits to endosomes is the slowest step in LDLR trafficking in these cells. LDLR endocytosis is not impaired in ARH-deficient fibroblasts (6–8) suggesting that ARH is not required for translocation of LDLRs from coated-pits to endosomes. In contrast the vast majority of LDLRs in lymphocytes are located in intracellular, EEA1-positive vesicles (10–12). Thus the return of LDLRs from the endosomes to the cell surface appears to be rate limiting in lymphocytes. In ARH-deficient lymphocytes, LDLRs accumulate on the plasma membrane, primarily outside of coated pits (10), which is similar to what we observed in WIF-B cells.

In contrast to our findings in hepatocytes, the density of LDLRs in coated pit membranes is similar in normal and in ARH-deficient lymphocytes (10). These differences may reflect cell-type specific differences in the relative rates at which LDLRs are transported from non-coated membranes to coated pits and from coated pits to endosomes. ARH may promote movement of LDLRs from non-coated membranes to coated pits in both hepatocytes and lymphocytes. In the absence of ARH, the large numbers LDLRs on the plasma membranes may result in translocation of LDLRs to clathrin-coated pits by less efficient mechanisms. These mechanisms may be adequate to compensate for ARH deficiency in lymphocytes, but not in hepatocytes.

Co-expression of ARH and the LDLR in the WIF-B cells not only...
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resulted in LDL clustering but also in a major redistribution of the LDLR from the cell surface to internal vesicular compartments. Because we have yet to identify a mutation in ARH or in the LDLR that uncouples receptor clustering from receptor internalization, we cannot determine whether the ARH-mediated increase in LDLR internalization is simply as consequence of increased receptor clustering in clathrin-coated pits or whether ARH also has a direct effect on the internalization of the receptor once it reaches coated pits in hepatocytes.

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