Endocytosis of the low density lipoprotein (LDL) receptor (LDLR) in coated pits employs the clathrin adaptor protein ARH. Similarly, agonist-dependent endocytosis of heptahelical receptors in coated pits employs the clathrin adaptor β-arrestin proteins. In mice fed a high fat diet, we found that homozygous deficiency of β-arrestin2 increased total and LDL plus intermediate-density lipoprotein cholesterol levels by 23 and 53%, respectively ($p < 0.05$), but had no effect on high density lipoprotein cholesterol levels. We therefore tested whether β-arrestins could affect the constitutive endocytosis of the LDLR. When overexpressed in cells, β-arrestin1 and β-arrestin2 each associated with the LDLR, as judged by co-immunoprecipitation, and augmented fluorescent LDL. However, physiologic expression levels of only β-arrestin2, and not β-arrestin1, enhanced endogenous LDLR endocytosis (by 65%) in stably transfected β-arrestin1/β-arrestin2 double-knockout mouse embryonic fibroblasts (MEFs). Concordantly, when RNA interference was used to suppress expression of β-arrestin2, but not β-arrestin1, LDLR endocytosis was reduced. Moreover, β-arrestin2$^{-/-}$ MEFs demonstrated LDLR endocytosis that was 50% less than cognate wild type MEFs. In fusion protein pull-down assays, β-arrestin2 bound to the LDLR cytoplasmic tail stoichiometrically, and binding was abolished by mutation of LDLR Tyr to Ala. Mutation of LDLR cytoplasmic tail Ser to Asp enhanced both the affinity of LDLR fusion protein binding to β-arrestin2, and the efficiency of LDLR endocytosis in cells expressing β-arrestin2 physiologically. We conclude that β-arrestin2 can bind to and enhance endocytosis of the LDLR, both in vitro and in vivo, and may thereby influence lipoprotein metabolism.

In human beings, supraphysiologic levels of plasma low density lipoprotein (LDL)$^3$ cholesterol are associated with virtually all cases of atherosclerosis (1). Approximately 75% of plasma LDL clearance occurs through endocytosis of the low density lipoprotein receptor (LDLR), predominately in the liver (2). The well-characterized endocytosis of the LDLR through clathrin-coated pits depends upon association of the 50-amino acid cytoplasmic tail of the LDLR with components of the cellular endocytic machinery. Although mutagenesis studies of the LDLR have delineated cytoplasmic tail domain residues necessary for LDLR endocytosis (3, 4), proteins with which the LDLR cytoplasmic tail interacts have only recently been identified. The clathrin heavy chain terminal domain itself can interact with peptides from the LDLR cytoplasmic tail but with relatively low affinity (5). The N-terminal domain of the autosomal recessive hypercholesterolemia (ARH) protein also binds to the LDLR cytoplasmic domain, whereas the C-terminal domain of ARH binds to both clathrin and AP-2 (6). Thus, ARH appears to link the LDLR to the endocytic machinery, in a process that seems to be required for endocytosis of the LDLR in hepatocytes and lymphocytes (6, 7). Whether other adaptor-type proteins may be involved in LDLR endocytosis remains to be determined.

Candidate clathrin adaptor proteins for the LDLR could include the β-arrestins, which play important roles in the endocytosis of heptahelical G protein-coupled receptors. β-Arrestin1 and β-arrestin2 were initially characterized as ubiquitously expressed proteins involved in heptahelial G protein-coupled receptor desensitization (8). With their N-terminal domains (9), the β-arrestins bind stoichiometrically to agonist-activated heptahelial receptors, and they do so with greater affinity after the receptors have been phosphorylated by G protein-coupled receptor kinases (10). The binding of a β-arrestin to the receptor inhibits receptor/heterotrimeric G protein interaction (8) but can also initiate signaling via c-Src and other kinases (11). To mediate G protein-coupled receptor internalization, β-arrestins link receptors to clathrin (12) and AP-2 (13), both of which are bound by the β-arrestin C-terminal domain. Recent data generated in β-arrestin-deficient cells suggest that particular receptors can interact preferentially with specific β-arrestin isoforms (14).

Endocytosis of G protein-coupled receptors in clathrin-coated pits is agonist-dependent (15), like the binding of β-arrestins to G protein-coupled receptors (16). In contrast, coated pit-mediated endocytosis of the LDLR is constitutive in fibroblasts (17). However, because ARH serves a clathrin adaptor function for one S-transferase; LDLRet, LDLR cytoplasmic tail domain; siRNA, small interfering RNA; RNAi, RNA interference; LDPS, lipoprotein-deficient serum; DII, 1.1-iodoacetadecyl-3,3,3'3'-tetrachloroacarboxyanine; [125I]sarile, 125I-[Sar1,Ile8]angiotensin II; FPLC, fast protein liquid chromatography; CHO, Chinese hamster ovary; HEK, human embryonic kidney; PBS, phosphate-buffered saline; IDL, intermediate-density lipoprotein.
the LDLR much like that served by the β-arrestins for hepta-
helical receptors (6), we tested the hypothesis that β-arrestins could act as LDLR adaptor proteins, and thereby enhance endocytosis of the LDLR.

MATERIALS AND METHODS

Lipoprotein Metabolism in Mice—All animal care conformed to the “Guide for the Care and Use of Laboratory Animals” issued by the National Institutes of Health. The generation of β-arrestin2−/− mice has been described previously (18). Derived from matings between β-arrestin2−/− founder mice, the β-arrestin2−/− and +/+ mice used in this study (n = 30) were littermates and were hybrids of the C57Bl/6J and C3H/HeJ genetic backgrounds. Serum cholesterol measurements were performed on ~200-μl blood samples, obtained from tail cuts performed under methoxyflurane anesthesia. Blood samples were first obtained after 4 weeks on a Paigen diet (by weight: 15% fat, 1.25% cholesterol, and 0.5% choline acid) (19), Dynets, Inc., #610308). After initial blood sampling, mice were fed a normal, low fat diet (Purina Breeder Chow), and blood was sampled again after 8 weeks. Thus, our serum lipid studies constitute a dietary crossover study. The mice were supplied with only water for 12 h prior to blood sampling (19).

Blood was allowed to clot for 2 h at room temperature. The thrombus was pelleted at 4 °C, and serum was removed. All mice were bled on the same day, and each batch of 30 samples (high and low fat diet) was assayed separately. Lipid analyses were performed with FPLC-PAGE and immunoblotting, which were performed as described previously (20).

Plasmid Constructs—The LDLR cDNA was obtained from the American Type Culture Collection (21). This construct was epitope-tagged at its N terminus with the FLAG® octapeptide, as described previously (22). From the first amino acid of the mature LDLR (Ala) onward (21), the remainder of the LDLR sequence was left intact. The resulting LDLR construct behaved identically to the native LDLR in LDL uptake experiments (data not shown), and is referred to as “LDLR” throughout the text. To create S833D and S833A mutations in the full-length LDLR, oligonucleotides encoding the indicated mutations. Plasmid lipoprotein fractionation by FPLC was performed on 100-μl samples of mouse plasma as described previously (20).

Immunoprecipitations—Immunoblotting—Cell Culture and Transfection—CHO 11a cells, which lack functional LDLRs, were generously provided by Monty Krieger and grown as described before (25). Transfections were performed with LipofectAMINE®, in Opti-MEM™ medium (Invitrogen), according to the manufacturer’s instructions. Human embryonic kidney (HEK) 293 cells were either passaged until confluent (26), or subcultured into T75 flasks in DMEM 10% FBS 100 μg/ml streptomycin. To compare MEFs expressing no β-arrestin isoform with genetically identical MEFs expressing only β-arrestin1 or only β-arrestin2, we used stable transfections in a β-arrestin1−/−/β-arrestin2−/− cell line. These cells were transfected with LipofectAMINE® (Invitrogen) and either pcDNA3.1/Hygro (Invitrogen), FLAG-tagged β-arrestin1/pcDNA3.1/Hygro, or FLAG-tagged β-arrestin2/pcDNA3.1/Hygro. Stably transfected lines were selected with and maintained in growth medium containing hygromycin at 250 μg/ml and screened by immuno-

Cell Culture LDLR Assessment—For cells transfected with the N-terminal FLAGTM-tagged LDLR construct, cell surface LDLRs were quantitated by cell surface immunofluorescence and flow cytometry, as described (22). For MEFs, which expressed only endogenous LDLRs, cell surface receptor number was assessed at 4 °C by ligand binding, using a rotating cell assay (22). MEFs were detached as described (22), Mouse monoclonal anti-β-arrestin2 (Invitrogen), pelleted, and washed in ice-cold minimal essential medium. Next, cells were resuspended in 200 μl of ice-cold Dil-LDL labeling medium lacking ("total binding") or containing unlabeled LDL at 500 μg of protein/ml ("nonspecific binding"), and incubated at 4 °C for 3 h.
Subsequently, cells were pelleted and washed with 1.5 ml of ice-cold HEPES-buffered saline (with 2.5 mM CaCl₂), then fixed in this solution with 3.6% formaldehyde. Specific cell surface Dil-LDL-binding was then assessed as described above and was used to estimate the relative cell surface LDLR number within each experiment. The intra-assay coefficient of variation was <7%, and mean nonspecific binding constituted 35–60% of mean total binding. Within a given experiment, cells were used only if cell surface LDLR density was within 30% (transfected cells) to 50% (MEFs) of control cells.

**LDL and LPDS Preparation**—LDL (1.019 < d < 1.055) from normal human plasma and newborn calf lipoprotein-deficient serum (d > 1.21 g/ml) were prepared by vertical spin density gradient ultracentrifugation, as described (27, 29). LDL purity was assessed by Coomassie Blue staining of SDS-PAGE samples. LDL (1.019–1.055 g/ml) were made in the Schericheria coli strain BL21 by standard methods (32).

For elution from glutathione-agarose with reduced glutathione, fusion proteins were concentrated and dialyzed in Centriprep 10 units (Amicon) and 10% of total binding. Within a given experiment, cells were used only if cell surface LDLR density was within 30% (transfected cells) to 50% (MEFs) of control cells.

**Angiotensin II Receptor Internalization**—Fibroblasts in 35-mm wells were incubated with serum-free medium (Dulbecco’s modified Eagle’s medium:1% bovine serum albumin/20 mM HEPES, pH 7.4) containing either vehicle (“naïve”) or 100 nM angiotensin II for 30 min (37 °C), and then washed with 40 mM sodium acetate/150 mM NaCl, pH 5.0 (10 min, 25 °C) to remove cell surface-bound angiotensin II (30). After three washes with PBS, cells were incubated for 3 h at 4 °C in serum-free medium containing 1 nM [125I]sarile (an angiotensin II receptor antagonist, PerkinElmer Life Sciences), to assess cell surface angiotensin II receptor binding in the absence (total binding) or presence (nonspecific binding) of 500 nM angiotensin II (31). Cells were then washed thrice, solubilized in 0.1 M NaOH, and aliquoted to gamma counting and Lowry protein assay (31). Specific binding (total – nonspecific) was used to assess receptor internalization, as: 100 × (cpm bound to angiotensin II-challenged cells)/[cpm bound to naïve cells]. Total binding in each well constituted <10% of the CPM used in each binding assay well, and nonspecific binding averaged 40 ± 10% of total binding.

**Fusion Protein Production**—GST and the GST/LDLRct proteins were made in the Escherichia coli strain BL21 by standard methods (33). After elution from glutathione-agarose with reduced glutathione, fusion proteins were concentrated and dialyzed in Centriprep 10 units (Amicon). Purity of the preparations was ~90%, as determined by SDS-PAGE and Coomassie Blue staining.

**β-Arrestin2 Purification**—The FLAG™-tagged rat β-arrestin2 cDNA (24) in pVL3939 (Invitrogen) was used to make a recombinant baculovirus, with BaculoGold DNA (BD Pharrmingen). Spodoptera frugiperda-9 cells infected with the β-arrestin2FL baculovirus were harvested 72 h after infection and lysed in buffer C (50 mM HEPES, pH 7.4, 0.5% Nonidet P-40, 250 mM NaCl, 10% glycerol, 2 mM EDTA, and protease inhibitors). Insoluble debris was pelleted, and the supernatant was mixed with anti-FLAG M2-agarose (Sigma) at 4 °C, 16 h. Beads were loaded into a column and washed extensively with buffer C. β-Arrestin2FL was eluted with 0.1 M glycine, pH 3.5, into a 0.1 volume of 0.5 M Tris, pH 7.5. Fractions were analyzed by SDS-PAGE and Coomassie Blue staining. Purity of the preparation was ~95%.

**In Vitro Binding of β-Arrestin2 to the LDLRct**—[35S]β-arrestin2 was synthesized by in vitro translation, using the Tn7® Quick Coupled Transcription/Translation System (Promega), [35S]methionine/[35S]cysteine (11 μCi/ml, PerkinElmer Life Sciences), and the rat β-arrestin2 expression plasmid (23), according to the manufacturer’s instructions. Serially diluted reticulocyte lysate and purified β-arrestin2 were immunoblotted to quantitate [β-arrestin2] in the lysate preparations (~5.4 fmol/μl). The indicated concentration of β-arrestin2 was incubated with 0.5 μg of purified GST, GST/LDLRct, or GST/LDLRct-S833D and 10 μl of glutathione-agarose (Sigma) in KOAc buffer (mM; K+ acetate 100, HEPES 50, MgSO4 0.5, DTT 0.2, 0.2% bovine serum albumin and protease inhibitors, pH 7.4), in a total volume of 240 μl. After mixing for 2 h at 4 °C, beads were pelleted, washed thrice in KOAc buffer, and dried. Proteins were then desorbed by heating in Laemmli buffer at 65 °C for 10 min and subjected to SDS-PAGE on 10% gels. Gels were stained with Coomassie Blue, and dried for autoradiography. Radiactivity in β-arrestin2 bands was measured with a PhosphorImager (Molecular Dynamics, Amersham Biosciences). For each [β-arrestin2] nonspecific binding was defined as the β-arrestin2 cpm pulled down by GST alone and was subtracted from the β-arrestin2 cpm bound to the GST/LDLRct construct, to obtain “specific binding.” Nonspecific binding constituted 40% of total binding for assays with the GST/LDLRct-S833D, and 60% of total binding for assays with the GST-LDLRct. Assays were performed in triplicate.

**Statistical Analyses**—For saturation binding experiments, nonlinear regression was performed with Prism 2™ software (GraphPad, Inc.), using a variable Hill slope. LDL uptake in various cell lines and mouse serum lipids values obtained on low and high fat diets were compared using one-way analysis of variance, with Tukey’s multiple comparison post test. All p values are two-tailed.
Fig. 2. β-Arrestins associate with the LDL receptor in intact cells. CHO IdIA cells were transfected with the indicated plasmids, encoding the LDL receptor and either β-arrestin1 (A, “βarr1”), β-arrestin2 (B, “βarr2”), or no protein (empty vector, “−”). Two days after transfection, membrane fractions from disrupted cells were prepared, solubilized, and subjected to LDLR immunoprecipitation (IP). Immunoprecipitates and corresponding samples of solubilized membranes (Lysate) underwent SDS-PAGE and immunoblotting for either β-arrestin1 (A) or β-arrestin2 (B), as described under “Materials and Methods.” Shown are individual experiments, representative of three performed for each β-arrestin.

restins with the LDLR. As Fig. 2 demonstrates, β-arrestin2 and β-arrestin1 do indeed associate with the LDLR in intact cells, whether or not the receptor is bound to LDL (data not shown). Thus, the β-arrestin/LDLR interaction seems to differ from the β-arrestin/heptahelical receptor interaction: whereas association with the LDLR appears ligand-independent, association with heptahelical G protein-coupled receptors appears to be agonist-dependent (16). Interestingly, the ligand dependence of the β-arrestin/receptor interaction mirrors the ligand dependence of receptor endocytosis. LDLR endocytosis appears to be ligand-independent (17), whereas heptahelical receptor endocytosis appears to be agonist-dependent (15).

Functional Effects of β-Arrestins on LDLR Endocytosis—To test whether the observed association of β-arrestins with the LDLR in cells affected LDLR function, we assessed LDL uptake in the same transfected cell system used for co-immunoprecipitation. Overexpression of either β-arrestin1 or β-arrestin2 augmented LDLR endocytosis, by ~70% (p < 0.05), compared with cells expressing only endogenous levels of β-arrestins (Fig. 3). Thus, as with endocytosis of the β2-adrenergic receptor (33), endocytosis of the LDLR, too, can be enhanced by β-arrestin overexpression.

If β-arrestins interacted with the LDLR as they do with heptahelical receptors, mutations in the N-terminal domain of the β-arrestins should impair the β-arrestin/LDLR interaction (9). To examine this possibility, we employed a V53D mutant of β-arrestin1. The binding of this β-arrestin1 mutant to heptahelical receptors is impaired (9), but its binding to clathrin and AP-2 is intact (13, 34). For these reasons, this mutant has been used to inhibit heptahelical receptor endocytosis in HEK 293 cells, which express relatively high levels of endogenous β-arrestin isoforms (33). Just as it inhibits agonist-induced G protein-coupled receptor endocytosis, the β-arrestin1 V53D mutant inhibited constitutive endocytosis of the LDLR (Fig. 4). Moreover, the degree of inhibition seen with β-arrestin1 V53D was comparable to that observed with the K44A (dominant-inhibitory) mutant of dynamin (Fig. 4). Thus, it appears that β-arrestins do bind the LDLR as they bind to heptahelical receptors via their N-terminal domain.

Physiologic Levels of β-Arrestin2 Enhance LDLR Endocytosis—If β-arrestins play a role in LDLR endocytosis, then cells lacking β-arrestin isoforms would be expected to demonstrate impaired LDL uptake. We tested this possibility with fibroblasts from mouse embryos deficient in both β-arrestin1 and β-arrestin2. To minimize the effects of genetic variability on comparisons between β-arrestin-expressing and β-arrestin1/2 double-knockout cells, we stably transfected the double-knockout cells to express either β-arrestin1 alone or β-arrestin2 alone, each at levels equivalent to those prevailing in wild type fibroblasts (Fig. 5A). Either in the absence of both β-arrestins or in the presence of physiologic levels of β-arrestin1, LDL uptake via endogenous LDLRs was equivalent in these fibroblasts. In contrast, physiologic β-arrestin2 levels enhanced LDL uptake via endogenous LDLRs by 65% (p < 0.05). Although physiologic levels of β-arrestin1 failed to augment LDLR internalization, they succeeded in increasing angiotensin II AT1 receptor (35) internalization (Fig. 5B), as we have shown previously (14). In these fibroblasts, therefore, physiologic levels of β-arrestin2, but not β-arrestin1, enhanced LDLR endocytosis, and thereby recapitulated results obtained with
Loss of β-arrestin isoform selectivity in overexpression systems characterizes not only the LDLR (Figs. 3 and 5) but also the β₂-adrenergic receptor (14, 33).

That physiologic levels of β-arrestin2 expression enhanced LDLR endocytosis was corroborated by two complementary approaches. First, using a genetic strategy, we compared LDL uptake in β-arrestin2⁻/⁻ MEFs with that in littermate wild type MEFs. In these cells, physiologic expression of β-arrestin2 enhanced LDLR endocytosis by ~100% (Fig. 5C). Second, using an RNAi strategy, we assessed 293 cell LDL uptake after we suppressed expression of either β-arrestin1 or β-arrestin2 by ~65% (Fig. 6). Whereas suppression of β-arrestin1 expression failed to affect LDLR endocytosis, suppression of β-arrestin2 expression reduced LDLR endocytosis by 29% (p < 0.05, Fig. 6). Thus, whether assessed in stably transfected β-arrestin1/2 double-knockout MEFs, β-arrestin2 knockout and cognate wild type MEFs, or siRNA-treated 293 cells, physiologic levels of β-arrestin2 expression enhanced LDLR endocytosis.

β-Arrestin2 Binds the LDLR Stoichiometrically—Functionally significant interaction between β-arrestins and phosphorylated heptahedral receptors occurs with a receptor:β-arrestin stoichiometry of ~1 (10). We therefore sought to determine whether the augmentation of LDLR endocytosis by β-arrestin2 could correspond to stoichiometric binding of the LDLR with β-arrestin2. For this purpose, we employed a GST fusion protein encompassing the 50 amino acids of the LDLR cytoplasmic tail domain (LDLRct). In addition, we constructed a S833D mutant LDLRct fusion protein, intended to mimic phosphorylation of Ser⁴⁵⁰ by the LDLR kinase, a process believed to occur in adrenocortical cells (36, 37). We used these fusion proteins in glutathione-agarose pull-down assays with in vitro translated, [⁴⁵⁰S]β-arrestin2. Although the native LDLRct did bind β-arrestin2, the S833D LDLRct mutant did so with ~2-fold greater

the β₂-adrenergic receptor (14). This selectivity of the LDLR for β-arrestin2 expressed at physiologic levels, in fibroblasts, contrasted with the lack of β-arrestin selectivity observed when we overexpressed β-arrestins and LDLRs in CHO cells (Fig. 3).
affinity (Fig. 7), much as phosphorylated heptahelical receptors bind β-arrestin2 with higher affinity than their non-phosphorylated counterparts (10). Saturation binding experiments with these fusion proteins demonstrated that, at saturation, ~1.5 mol of β-arrestin2 was bound per mole of either the wild type or the S833D mutant LDLRct (Fig. 7B). This stoichiometric binding of β-arrestin2 to the LDLRct further substantiated the potential physiologic importance of their interaction.

Because the S833D mutant LDLRct binds β-arrestin2 with higher affinity than the wild type LDLRct, and because physiologically expressed β-arrestin2 appears to enhance LDLR endocytosis, we asked whether the S833D mutation of the full-length LDLR would enhance LDLR endocytosis in intact cells (Fig. 7C). We found that LDLR endocytosis was augmented 36% when Ser833 was mutated to Asp. By contrast, as previously reported (3), LDLR endocytosis was not affected when Ser833 was mutated to Ala. Thus, a mutation that increases the affinity of LDLRct/β-arrestin2 binding also enhances LDLR endocytosis in cells expressing physiologic levels of β-arrestin2.

A Role for LDLR Tyr807 in the β-Arrestin2/LDLRct Association—Endocytosis of the LDLR depends critically upon the reverse-turn conformation of the receptor’s cytoplasmic tail, conferred by the tetrapeptide sequence 804NPVY807 (38). Mutation of Tyr807 to any but aromatic residues reduced LDLR internalization by ~80% in transfected CHO cells (3). Because the Y807A LDLR mutation reduced LDLR endocytosis in fibroblasts (3), and because β-arrestin2 appears to enhance LDLR endocytosis in fibroblasts (Fig. 5), we asked whether the LDLR Y807A mutation would affect the association of β-arrestin2 with the LDLR. To address this question, we employed glutathione-agarose pull-down assays with the wild type and S833D LDLRct constructs used in Fig. 7. Mutation of Tyr807 to Ala abrogated binding of β-arrestin2 to the LDLRct constructs (Fig. 8). Thus, the association of the LDLR with β-arrestin2, as with the ARH protein (6) and clathrin itself (5), appears to require the tetrapeptide sequence 804NPVY807.

**Fig. 8.** Binding of β-arrestin2 to the LDLRct involves LDLR Tyr807. Glutathione-agarose pull-down assays were performed as in Fig. 7, but with 75 nm [35S]β-arrestin2 and 3 μg of either GST itself, the GST-LDLRct (Wild Type), GST-LDLRct-S833D mutant protein (S833D), or each of these GST-LDLRct constructs modified to contain a Y807A mutation (Y807A). After glutathione-agarose pull-down, specimens were subjected to SDS-PAGE, Coomassie Blue staining, and autoradiography. Upper panel, a PhosphoImager record from individual experiments performed with wild type and S833D mutant LDLRct constructs. Lower panel, the gel depicted above was stained with Coomassie Blue and photographed before drying and autoradiography. Results from these experiments are representative of three experiments performed in triplicate with each LDLRct construct.

**DISCUSSION**

The LDLR represents a previously unrecognized, functionally significant binding partner for β-arrestin2. By showing that physiologic levels of β-arrestin2 can augment LDLR endocytosis in cultured fibroblasts and 293 cells, these studies demonstrate a novel mechanism by which LDLR endocytosis may be facilitated. Although β-arrestin2 is clearly not necessary for LDLR internalization, β-arrestin2 can clearly enhance LDLR internalization. Indeed, β-arrestin2-mediated enhancement of hepatic LDLR endocytosis could very plausibly explain the results of our in vivo lipoprotein metabolism studies.
Lastly, at physiologic levels of expression, receptor regulation. Phosphorylation of the LDLR on Ser833, a residue conserved from frog to human (43), occurs in adrenocortical cells but not in fibroblasts (36, 37). Over more than a decade, potential physiologic purposes for this phosphorylation event have remained obscure. Mutation of Ser833 to Ala failed to affect either LDLR endocytosis in CHO fibroblasts (3), the basolateral targeting of LDLRs in transgenic mouse livers, or the extent to which hepatocyte LDLRs reside outside of coated pits (44). However, modeling Ser833 phosphorylation with an LDLRct S833D mutation increased both the affinity of –arrestin2/LDLRct binding and the efficiency of LDLR endocytosis in cells expressing physiologic levels of –arrestin2 (Fig. 7). In adrenocortical cells, Ser833 phosphorylation conceivably could, by enhancing LDLR/–arrestin2 interaction, enhance LDLR endocytosis. However, even in the absence of Ser833 phosphorylation, –arrestin2 may still enhance LDLR endocytosis, as suggested by our data from β-arrestin1/2 double-knockout MEFs (Fig. 5) and siRNA-treated 293 cells (Fig. 6).

Another potential role for –arrestin2 in LDLR endocytosis is suggested by the human disease autosomal recessive hypercholesterolemia (ARH). The ARH protein, like β-arrestin2, can link the LDLR to clathrin and AP-2 (6). LDLR endocytosis is dramatically reduced in lymphocytes and hepatocytes of ARH subjects. However, there is little, if any, detectable abnormality of LDLR endocytosis in ARH fibroblasts (6, 7). Could β-arrestin2 compensate for ARH deficiency in fibroblasts but not in hepatocytes or lymphocytes? This possibility is suggested by our immunoblot analysis of mouse liver and fibroblasts, in Fig. 5, and LDL uptake studies. In mice, fibroblasts appear to have ~2-fold more –arrestin2 than liver. In LDL uptake studies with β-arrestin1/2 double-knockout fibroblasts stably transfected with β-arrestin2, expression of β-arrestin2 failed to augment LDLR endocytosis when β-arrestin2 levels were ~0.5 times those obtaining in wild type fibroblasts (data not shown).

Our in vivo studies demonstrate that, unlike ARH in humans (7), –arrestin2 is not necessary for normal lipoprotein metabolism in the low-fat fed mouse. Furthermore, on a high fat diet that down-regulates LDLRs (45), –arrestin2 deficiency fails to cause hypcholesterolemia. Increases in LDL and LDL associated with –arrestin2 deficiency are consonant with a role for –arrestin2 in hepatic LDLR endocytosis, but they do not exclude an alternative or concomitant role for –arrestin2 in the endocytosis of other receptors, including the hepatic LDLR-related protein (46). Whether –arrestins may affect endocytosis of the LDLR-related protein or other members of the LDLR family remains to be explored.

Acknowledgments—We express our most profound gratitude to Robert J. Lefkowitz, a quintessential mentor, for his endless generosity with animals, reagents, and invaluable advice. We also thank Nancy Krieger, Richard T. Premont, and Larry S. Barak for invaluable discussions, and Michael K. Altenburg for assistance with FPLC of mouse plasma.

REFERENCES

1. Brown, M. S., and Goldstein, J. L. (1986) Science 232, 34–47
2. Kesaniemi, Y. A., Witzum, J. L., and Steinbrecher, U. P. (1983) J. Clin. Invest. 72, 950–959
3. Davis, C. G., van Driel, I. R., Russell, D. W., Brown, M. S., and Goldstein, J. L. (1987) J. Biol. Chem. 262, 4075–4082
4. Chen, W. J., Goldstein, J. L., and Brown, M. S. (1990) J. Biol. Chem. 265, 3116–3123
5. Kibbe, R. G., Rizo, J., Giersch, L. M., and Anderson, R. G. (1998) J. Cell Biol. 142, 69–77
6. He, G., Gupta, S. Y., Mi, M., Michaela, P., Hobbs, H. H., and Cohen, J. C. (2002) J. Biol. Chem. 277, 44444–44449
7. Garcia, C. K., Wulkan, R. A., Zuliani, G., Fellin, R., Maidi, M., Calandra, S., Bertoloni, S., Cosso, F., Grisdi, N., Barnes, R., Cohen, J. C., and Hobbs, H. H. (2002) Science 293, 1394–1398
8. Attramadal, H., Arriza, J., Auki, C., Dawson, T., Cudina, J., Kwatra, M. M., Snyder, S., Caron, M., and Lefkowitz, R. K. (1992) J. Biol. Chem. 267, 17882–17890
9. Krupnick, J. G., Santini, F., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1997) J. Biol. Chem. 272, 32507–32511
10. Gurevich, V. V., Dion, S. R., Sarto, J. J. C., Pasiakiewicz, J. K., Kim, C. M., Sterne-Marr, R., Hoyer, M. M., and Benovic, J. L. (1995) J. Biol. Chem. 270, 720–731
11. Miller, W. E., and Lefkowitz, R. J. (2001)Curr. Opin. Cell Biol. 13, 139–145
12. Goodrich, D. F. Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) Nature 383, 447–450
13. Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S., and Caron, M. G. (2000) J. Biol. Chem. 275, 23120–23126
14. Kiehout, T. A., Lin, F. S., Perry, S. J., Conner, D. A., and Lefkowitz, R. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1601–1606
15. von Zastrow, M., and Chilika, B. K. (1992) J. Biol. Chem. 267, 3530–3538
16. Barak, L. S., Ferguson, S. S., Zhang, J., and Caron, M. G. (1997) J. Biol. Chem. 272, 27497–27500
17. Anderson, R. G., Brown, M. S., Beisiegel, U., and Goldstein, J. L. (1982) J. Cell Biol. 93, 523–531
18. Bohn, L. M., Lefkowitz, R. J., Gainetdinov, R. R., Peppel, K., Caron, M. G., and Lefkowitz, R. J. (1999) Science 286, 2495–2498
19. Paigen, B., Morrow, A., Holmes, P., Mitchell, D., and Williams, R. (1987) Atherosclerosis 68, 231–240
20. Kattoff, I., Hindsdale, M. E., Moxon, H., Altenburg, M. K., Watanabe, M., Quartaroli, S. H., Sullivan, P. M., and Maeda, N. (1999) J. Clin. Invest. 103, 1579–1586
21. Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L., and Russell, D. W. (1984) Cell 39, 27–38
22. Freedman, N. J., Ament, A. S., Oppermann, M., Stoffel, R. H., Exm, S. T., and Lefkowitz, R. J. (1997) J. Biol. Chem. 272, 17734–17743
23. Freedman, N. J., Liggitt, S. B., Drachman, D. E., Pei, G., Caron, M. G., and Lefkowitz, R. J. (1995) J. Biol. Chem. 270, 17953–17961
24. Miller, W. E., Maudsley, S., Amin, S., Khan, D. F., Luttrel, L. M., and Lefkowitz, R. J. (2000) J. Biol. Chem. 275, 11312–11319
25. Kingsley, D. M., and Krieger, M. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 5454–5458
26. Amin, S., Nelson, C. D., Garrison, T. R., Miller, W. E., and Lefkowitz, R. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 1740–1744
27. Goldstein, J. L., Basu, S. K., and Brown, M. S. (1983) Methods Enzymol. 98, 241–260
28. Innesarity, T., Pittas, R., and Mahley, R. (1986) Methods Enzymol. 129, 542–565
29. Chung, B., Segrest, J., Ray, M., Brunsell, J., Hokanson, J., Krauss, R., Beau-
drie, K., and Cone, J. (1986) *Methods Enzymol.* **128**, 181–209
30. Haddad, G., Amiri, F., and Garcia, R. (1997) *Regul. Pept.* **68**, 111–117
31. Oppermann, M., Freedman, N. J., Alexander, R. W., and Lefkowitz, R. J. (1996) *J. Biol. Chem.* **271**, 13266–13272
32. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor
33. Menard, L., Ferguson, S. S., Zhang, J., Lin, F. T., Lefkowitz, R. J., Caron, M. G., and Barak, L. S. (1997) *Mol. Endocrinol.* **11**, 1266–1277
34. Kushimoto, A., Brown, M. S., Slaughter, C. A., and Goldstein, J. L. (1987) *J. Biol. Chem.* **262**, 1344–1351
35. Kushimoto, A., Goldstein, J. L., and Brown, M. S. (1987) *J. Biol. Chem.* **262**, 9367–9373
36. Bansal, A., and Gierasch, L. M. (1991) *Cell* **67**, 1195–1201
37. Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G., and Barak, L. S. (2000) *J. Biol. Chem.* **275**, 17201–17210
38. Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G., and Barak, L. S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 3712–3717
39. Lin, F. T., Krueger, K. M., Kendall, H. E., Daaka, Y., Fredericks, Z. L., Pitcher, J. A., and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272**, 31051–31057
40. Lin, F. T., Chen, W., Shenoy, S., Cong, M., Exum, S. T., and Lefkowitz, R. J. (2002) *Biochemistry* **41**, 10692–10699
41. Mehta, K. D., Chen, W. J., Goldstein, J. L., and Brown, M. S. (1991) *J. Biol. Chem.* **266**, 10406–10414
42. Yokode, M., Pathak, R. K., Hammer, R. E., Brown, M. S., Goldstein, J. L., and Anderson, R. G. (1992) *J. Cell Biol.* **117**, 39–46
43. Dueland, S., Drisko, J., Graf, L., Machleder, D., Luus, A. J., and Davis, R. A. (1993) *J. Lipid Res.* **34**, 923–931
44. Rohlmann, A., Gotthardt, M., Hammer, R. E., and Herz, J. (1998) *J. Clin. Invest.* **101**, 689–695