Molecular mechanisms of autosomal recessive hypercholesterolemia

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Received July 11, 2002; Revised September 12, 2002; Accepted September 16, 2002

Mutations in the phosphotyrosine-binding domain protein ARH cause autosomal recessive hypercholesterolemia (ARH), an inherited form of hypercholesterolemia due to a tissue-specific defect in the removal of low density lipoproteins (LDL) from the circulation. LDL uptake by the LDL receptor (LDLR) is markedly reduced in the liver but is normal or only moderately impaired in cultured fibroblasts of ARH patients. To define the molecular mechanism underlying ARH we examined ARH mRNA and protein in fibroblasts and lymphocytes from six probands with different ARH mutations. None of the probands had detectable full-length ARH protein in fibroblasts or lymphoblasts. Five probands were homozygous for mutations that introduced premature termination codons. No relationship was apparent between the site of the mutation in ARH and the amount of mRNA. The only mutation identified in the remaining proband was a SINE VNTR Alu (SVA) retroposon insertion in intron 1, which was associated with no detectable ARH mRNA. 125I-LDL degradation was normal in ARH fibroblasts, as previously reported. In contrast, LDLR function was markedly reduced in ARH lymphoblasts, despite a 2-fold increase in LDL cell surface binding in these cells. These data indicate that all ARH mutations characterized to date preclude the synthesis of full-length ARH and that ARH is required for normal LDLR function in lymphocytes and hepatocytes, but not in fibroblasts. Residual LDLR function in cells that do not require ARH may explain why ARH patients have lower plasma LDL levels than do patients with homozygous familial hypercholesterolemia who have no functional LDLRs.

INTRODUCTION

Low density lipoproteins (LDL) are the major cholesteryl ester transport particles in the blood. Elevated plasma levels of LDL-cholesterol (C) are a key risk factor for the development of coronary artery disease. Between 60 and 70% of circulating LDL is removed from the circulation by LDL receptor (LDLR)-mediated endocytosis in the liver (1). Reductions in LDLR activity result in elevations in plasma LDL-C and deposition of cholesteryl in body tissues (1). The critical importance of the LDLR pathway in preventing the accumulation of LDL is illustrated by the very high plasma levels of LDL and severe premature atherosclerosis that characterizes the two known autosomal dominant forms of severe hypercholesterolemia, familial hypercholesterolemia (FH) and familial defective apolipoprotein B-100 (FDB).

FH is caused by mutations in LDLR and is the most common and most severe of the single gene disorders of LDL metabolism (1). Approximately 1 in 500 individuals in the general population is a FH heterozygote, having one mutant LDLR allele. FH heterozygotes have 2–4-fold elevations in plasma LDL-cholesterol levels. If these patients are not effectively treated with lipid-lowering agents, ~50% of the men will have a myocardial infarction before age 60 (2). Individuals with two defective LDLR alleles, FH homozygotes, have a markedly delayed rate of clearance of LDL from the circulation, resulting in a 6-fold increase in plasma LDL-C levels accompanied by cutaneous xanthomas and frequently by myocardial infarction in the first or second decade of life. Fibroblasts cultured from patients with homozygous FH fail to take up and degrade LDL (3). FDB patients have a clinical picture similar to FH and delayed clearance of circulating LDL (4). The metabolic defect in this disorder is caused by a missense mutation in the region of apolipoproteinB-100 that binds the LDLR (5). Fibroblasts from these patients have normal LDLR function (6).
Two autosomal recessive forms of severe hypercholesterolemia have been described. The first is sitosterolemia which is distinguished from other monogenic forms of hypercholesterolemia by the marked elevation in plasma levels of plant sterols in affected individuals (7). This disease is caused by mutations in either of two ATP-binding cassette transporters, ABCG5 or ABCG8 (8). The second autosomal recessive disorder has been named autosomal recessive hypercholesterolemia (ARH) (9). Patients with this disease, which was first described by Khachadurian in 1973 (10), have plasma cholesterol levels and clinical symptoms that are intermediate between those of FH heterozygotes and FH homozygotes (9,11). The age of onset of symptomatic coronary artery disease in these patients is later and the xanthomas tend to be large and bulky (9,11). The reduction in the rate of clearance of plasma LDL in these patients is later and the xanthomas tend to be large and bulky (9,11). The reduction in the rate of clearance of plasma LDL in these patients is later and the xanthomas tend to be large and bulky (9,11). The reduction in the rate of clearance of plasma LDL in these patients is later and the xanthomas tend to be large and bulky (9,11). The reduction in the rate of clearance of plasma LDL in these patients is later and the xanthomas tend to be large and bulky (9,11).

We recently showed that ARH is caused by mutations in a gene (eponymously designated \( ARH \)) that encodes a putative adaptor protein which we called ARH (13). ARH is a 308 amino acid protein that contains a phosphothreonine binding (PTB) domain. PTB domains have been found in several adaptor proteins involved in cell signaling and transport and bind to the amino acid motif NPxY found in the cytoplasmic tail of the LDLR as well as many other membrane receptors (14). The integrity of the NPxY sequence is required for LDLR internalization (15,16). Previously we identified six mutations, including five nonsense mutations and one missense mutation, in probands with ARH (13). In this report we show that the missense mutation, which was previously thought to be the disease-causing mutation in this patient (ARH4), is not responsible for the functional inactivation of ARH in this patient; this patient, like all the other patients with ARH described to date, produces no full-length ARH protein due to a premature stop codon. We examined the relationship between the site of the mutation and the levels of ARH mRNA and protein in this patient and in four other ARH patients with different premature termination mutations. We show that premature termination codons are associated with widely different levels of ARH mRNA, although all preclude the expression of full-length ARH protein.

In patients with ARH, LDL uptake in the liver is reduced to the same extent as it is in patients with homozygous FH, suggesting that ARH is required for normal LDLR function in the liver (9). In contrast to the liver, LDLR function in fibroblasts from the same patients was shown to be normal or only moderately impaired (9,12,13). In this paper we show that LDLR activity is markedly reduced in immortalized lymphocytes from ARH subjects. These data indicate that ARH acts to facilitate internalization of LDLRs and is required for efficient LDL uptake in some cell types, such as lymphocytes and hepatocytes, but not in other cell types, such as fibroblasts.

RESULTS

A map of \( ARH \) indicating the sites of the mutations in the six probands included in this study is shown in Figure 1. Mutations in five of these probands (ARH1–ARH4 and ARH6) have been reported previously (13). One of these patients (ARH4) had a missense mutation (c.605C > A, P202H) that was not detected in 20 normolipemic, ethnically matched controls (13). However, when the mutation was recreated and the recombinant ARH protein expressed in HEK293 cells, normal amounts of ARH protein were detected by immunoblotting (data not shown). In contrast, no immunodetectable ARH protein was apparent in fibroblasts from this same patient (see later). Therefore, we PCR amplified and sequenced the ARH cDNA from this patient’s fibroblasts. When oligonucleotides from exons 4 and 9 were used for amplification, the amplified product was 195 nucleotides larger than expected (Fig. 1B). Sequence analysis of the cDNA revealed the insertion of 195 bp immediately downstream of exon 7. The 195 bp corresponded to nucleotides 774–969 in intron 7 (Fig. 1B). The genomic sequence from ARH4 revealed a G to A substitution in intron 7 (IVS7 + 773) that created a new AG splice acceptor site. The sequence context of the AG dinucleotide created by the mutation (TTAATTTTAAAAATCTAGGT) matches the canonical splice acceptor sequence (NYAG/GU); at least two sequences (TTAAT and TTAAC) that are between 11 and 40 nt upstream of the splice site match the branch site consensus sequence (YURAY; data not shown). The 3’ end of the insert is defined by a novel GC splice donor site at position 968 in intron 7 of the wild-type ARH. The sequence context of this donor site shows an almost perfect match to the consensus sequence for GC splice donor sites reported by Thanaraj and Clark (17). The usual splice acceptor site at the 3’ end of intron 7 is maintained, so exons 8 and 9 are correctly spliced in the ARH4 mRNA. The 195 bp insert encodes an open reading frame of 62 amino acids followed by a stop codon. The expected length of the protein is 311 amino acids.

No mutation was identified in either the coding region or in the splice junctions of a new patient (ARH7) with a clinical picture highly suggestive of ARH (severe hypercholesterolemia, normocholesterolemic parents, large xanthomas and premature coronary artery disease) (18). This patient also had no immunodetectable ARH protein in her cultured fibroblasts (see below). Therefore, the entire gene was sequenced. The sequences of all nine exons and introns 2–8 were normal. However, a small region of intron 1 could not be amplified. Genomic blotting using either an exon 1-specific probe (data not shown) or a probe from intron 1 revealed a 2.6 kb larger fragment (Fig. 2). The insert was amplified using 30 base oligonucleotides and sequenced. The novel sequence was flanked by direct repeats (GAAAACCTGTTTTCTC) and contained an \( Alu \) sequence at the 5’ end and a long terminal repeat (LTR) at the 3’ end (Fig. 2B). The insert contained multiple tandem copies of a 40–50 bp sequence characteristic of a variable number tandem repeat (VNTR) sequence (19). The insert resembles a class of retroelements referred to as SINE VNTR \( Alu \) (SVA) (20). This insert was not detected in 35 normal subjects. Molecular characterization of this sequence and the mechanism by which it entered this gene is being investigated.

Immunoblot analysis using a polyclonal antibody to the C-terminal 15 amino acids of ARH was performed on fibroblast cell lysates to determine if any of these mutations were associated with protein. Cultured fibroblasts were not available from ARH2. No protein was detected in any of the
cell lines (Fig. 3). The amount of ARH in fibroblasts from a subject with homozygous FH did not differ from that observed in normal fibroblasts. The amount of ARH mRNA differed between the cell lines and no clear relationship was apparent between the site of the premature stop codon and the amount of mRNA (Fig. 3). Fibroblasts from ARH1 and ARH3 had only trace amounts of ARH mRNA, indicating that transcripts from these alleles are recognized by the surveillance mechanism that degrades mRNAs containing premature termination codons (21). ARH1 and ARH3 were previously shown to be homozygous for mutations that introduce premature termination signals at codons 170 and 136, respectively (13). Southern blotting indicated that ARH7 is homozygous for a 2.6 kb insertion in intron 1 (Fig. 2). Although no mRNA was detectable by Northern blotting, small amounts of cDNA could be amplified using RT–PCR. Sequencing of the amplified fragment revealed no mutations, insertions or deletions (data not shown). Surprisingly, ARH6 had a normal amount of ARH mRNA despite having a mutation (71delG) that creates a stop codon near the N-terminus at position 55. The mRNA for ARH in proband 4 was estimated to be 195 bp larger than the normal mRNA, which is consistent with the inclusion of sequence from intron 7, as described above.

Epstein–Barr virus (EBV)-transformed lymphocytes were available from ARH2 as well as ARH6. Northern blot and immunoblot analysis was performed in these two cell lines. The sizes and levels of ARH mRNA in these two ARH cell lines were similar to those seen in cells from controls. Immunoblot analysis using the C-terminal antibody revealed a band at ~32 kDa (Fig. 4) that was present in normal lymphocytes and in lymphocytes from ARH2, who is homozygous for a nonsense mutation at codon 22, but not in lymphocytes from ARH6 whose ARH alleles both have a premature termination signal at codon 55. The origin of this smaller protein, which

Figure 1. Gene structure of ARH, showing location of insertion and premature stop codons in six unrelated ARH probands (A) and RT–PCR amplification of ARH cDNA in ARH4 (B). Oppositely oriented 25 base oligonucleotides complimentary to sequences in exons 4 and 9 of ARH were used to amplify the intervening sequence from 2 μg of total RNA from cultured fibroblasts. The amplification products were size-fractionated on a 2% agarose gel, stained with ethidium bromide (0.01%) and then subjected to UV illumination. Sequence comparison of the PCR-amplified fragments revealed a G to A substitution at nucleotide 773 in intron 7 of ARH4, creating an AG splice acceptor site for a new exon, designated exon 7a, and predicting the introduction of a premature termination codon (TAA) just prior to the GC splice donor site. Solid or shaded boxes, exons; dotted box, additional exon in ARH4; lines, introns.
Figure 2. Sequence analysis of the inserted sequence at intron 1 of ARH in ARH7. Southern blot analysis of intron 1 of ARH in ARH7 and two normal individuals (A). Genomic DNA (5 μg) was digested with HindIII and BglII, size-fractionated on a 0.8% agarose gel and Southern blotting was performed using a 792 bp probe from intron 1. The restriction fragments for ARH7 were ~2.6 kb larger than the bands seen in the normal controls. The insert was PCR-amplified, subcloned and sequenced as described in the Materials and Methods. Sequence of the insert in the ARH gene of ARH7 (B). Sequence analysis revealed a 2.6 kb insert containing a composite SVA retroposon that includes an Alu element, 32 copies of a VNTR and an LTR from the human endogenous retrovirus HERV-K18, flanked by a 15 bp direct repeat. Dotted line, Alu element; black arrows, ~40–50 bp VNTRs; boxed sequences, 6 bp direct repeats flanking the VNTRs (20); dashed black line, sequence gap; dashed gray line, long terminal repeat; and boxed and underlined sequence, flanking direct repeats. The copy number of VNTRs is predicted to be ~32 based on the size of the SVA insert. An unusual long polyA tract (n = 57) is located at the 3′ end of the SVA.
was not detected in fibroblasts (Fig. 3), is not clear. The mutation in ARH2 introduces a stop codon at residue 22. The next codons encoding methionines are at positions 46 and 55 (Fig. 5). Both of these codons are in an appropriate sequence context for translation initiation (22). Further studies will be required to determine if either of these potential initiation sites are utilized in vivo.

The amount of total LDLR in lymphoblasts from ARH6 was similar to that measured in normal lymphoblasts grown under identical conditions. The amount of LDLR protein was higher in ARH6 cells cultured in human lipoprotein-poor serum (HLPPS) than in cells in which LDL (20 μg/ml) was added to the medium (Fig. 6A). Thus, the total amount and regulation of the LDLR in response to sterols did not differ between normal and ARH lymphoblasts. The FH patient used in this experiment is homozygous for a serine to leucine substitution at residue 156, which results in the production of a 240 kDa precursor form of the LDLR that fails to undergo maturation (23). To determine if the distribution of LDLR was altered in the ARH lymphoblasts, fluorescence-activated cell sorting (FACS) was performed after staining the cells with anti-LDLR antibodies (Fig. 6B). Cells from the ARH patient had higher levels of LDLR on the cell surface relative to cells from normal individuals (Fig. 6B).

Next, we examined the amount of cell surface binding and degradation of radiolabeled LDL in the ARH cells. The amount of LDL that bound to the cell surface was increased 2-fold in lymphocytes from ARH patients (ARH2 and ARH6), but LDL degradation was only 25% of normal in these cells (Fig. 7). In fibroblasts from ARH6, both the binding and the degradation of 125I-labeled LDL were comparable with the values obtained from normal fibroblasts (Fig. 7). To examine if there was a more global effect on receptor-mediated endocytosis, we examined transferrin receptor function by comparing the binding, internalization and secretion of transferrin in lymphocytes from a normal, an ARH and an FH subject. No significant differences in transferrin trafficking were detected between the three cell lines (Fig. 8).

**DISCUSSION**

All six of the ARH alleles characterized in this paper contained either a premature termination codon or a structural rearrangement in the ARH gene. No relationship was seen between the relative position of the premature stop codon and the amount of ARH mRNA. A genomic rearrangement identified in one patient due to the insertion of a SVA retroposon into intron 1 of the ARH gene; this allele was associated with no mRNA. All six mutant alleles were associated with no detectable full-length ARH protein. LDL receptor function was normal in ARH fibroblasts but was markedly impaired in transformed lymphocytes despite a 2-fold increase in cell surface LDL binding in these cells. LDL degradation was reduced 75% in the ARH lymphocytes. These data indicate that ARH acts at the cell membrane to facilitate the internalization of LDLRs in lymphocytes but not in fibroblasts.

A total of eight mutations have been identified in patients with ARH: the six mutations described in this paper, an additional frameshift mutation in exon 1 (72insG) that results in a premature stop codon in amino acid 33 (13) and a transversion in the splice acceptor site of intron 1 (24).
Although no cells were available from the latter two patients, it is likely that no full-length ARH protein is produced from these alleles. Seven of the eight ARH mutations are predicted to produce transcripts containing premature termination codons. Transcripts containing premature termination codons are usually recognized by a specific surveillance mechanism and degraded at a more rapid rate than are wild-type mRNAs (25). This process, called nonsense-mediated mRNA decay, allows cells to remove the mutant mRNAs from the pool of translatable mRNAs, thereby preventing the formation of truncated polypeptides that may be disruptive to cells. Levels of ARH mRNA were barely detectable by northern blotting in cells from two of the patients (ARH1 and 3) analysed in this study and were significantly reduced in a third patient (ARH4). This result suggests that transcripts from these alleles are recognized by the mRNA surveillance mechanism and targeted for rapid decay.

Cells from two other patients (ARH2 and ARH6) had normal levels of ARH mRNA, despite the presence of premature termination codons in exons 1 and 2 of the ARH gene. The mechanism by which these transcripts evade nonsense-mediated decay is not known. Previous studies have noted that different nonsense mutations in the same gene can be associated with wide differences in susceptibility to nonsense-mediated decay (26–28). Studies of nonsense mutations in the major urinary protein (MUP) (29), triose phosphate isomerase (TIM) (30) and β-globin (30) gene demonstrate that nonsense-mediated mRNA decay requires at least one exon 3' of the premature termination codon and a minimum interval of 50–55 bp between the nonsense codon and the last intron–exon boundary. Transcripts from ARH2 and ARH6 fulfill both of these criteria. In the HLA-A gene, which contains eight exons, premature termination codons in exons 2, 3, 4 and in the 5' region of exon 5 trigger nonsense-mediated decay whereas those in the 3' region of exon 5 and in exons 6 and 7 do not (28).

Transient transfection experiments using HLA-A minigenes indicate that the failure of premature termination codons in exons 6 and 7 to trigger nonsense-mediated decay is due to the unusually small size of these exons (33 and 48 nt, respectively) rather than to their position in the gene. The mutations in ARH2 and ARH6 both introduce premature termination codons towards the 5' end of the transcript (at codons 22 and 55, respectively) in exons 1 and 2, which are both longer than 55 bp, the minimum footprint required for detection by the surveillance machinery. An alternative explanation for the normal mRNA levels in ARH2 and ARH6 is that translation may initiate after the premature stop codon either through leaky scanning or by reinitiation of translation, which abrogates nonsense-mediated mRNA decay in mammalian cells (31). Small amounts of a ~32 kDa protein was detected in the lymphoblasts from ARH2, suggesting that translation may initiate from one of two downstream AUG codons (M46 and M55). Both residues are in-frame and in an adequate context for initiation (22). A trace amount of immunoreactive protein of size similar to that seen in ARH2 cells was also present in normal lymphocytes. No evidence of any ARH protein was found in cells from ARH6, or from any other ARH patients. These data suggest that re-initiation of translation may contribute to the preservation of ARH2 transcripts. Further studies will be required to elucidate the mechanism by which transcripts from ARH6, which contain a nonsense mutation in residue 55, evade the mRNA surveillance mechanism and escape nonsense-mediated mRNA decay.

One allele (ARH7) that produces no detectable ARH mRNA had an insertion of an unusual class of composite retroposon referred to as a SVA. The entire ARH gene was sequenced in ARH7 and no other alterations were found. SVA elements were first identified in the fourth intron of the RPI gene (20) and contain a series of different repetitive sequences, including an Alu sequence, a VNTR and a long terminal repeat (LTR) from

![Figure 4. Northern blot and immunoblot of ARH in EBV-transformed lymphocytes from a normal subject, two homozygous FH patients and probands from ARH2 and ARH6. Northern and immunoblotting were performed as described in Figure 3 and in the Materials and Methods.](image-url)
the human endogenous retrovirus HERV-K18. The mechanism by which the SVA insert interferes with ARH expression is not known. The SVA is located ∼700 bp downstream of exon 1 and may act as a transcriptional silencer (32), although the SVA sequence in the RP1 gene is not associated with changes in gene expression (20). Additional studies will be required to determine the causal link between the presence of the SVA sequence and the lack of any detectable ARH mRNA in cells from this patient.

The similar phenotypes associated with ARH and FH suggest that the ARH protein plays a role in LDLR-mediated endocytosis, but the specific role of ARH in this process has not been defined. Studies in cultured skin fibroblasts, which provided critical insights in the molecular mechanisms underlying FH (33), have failed to reveal a defect in LDL uptake or degradation in ARH. Whereas fibroblasts from patients with homozygous FH take up radiolabeled LDL at less than 10% of the rate observed in fibroblasts from normal individuals (1), LDL uptake in ARH fibroblasts is between 30 and 100% of normal (11). In the present study, LDL binding and degradation were essentially normal in fibroblasts from a patient with ARH (ARH6). Therefore, although ARH is expressed in fibroblasts, it is not required for LDLR function in these cells.

Norman et al. (34) identified two unrelated subjects with an autosomal recessive form of hypercholesterolemia that clinically resembled FH. FACS analysis using BODIPY-labeled LDL indicated increased cell-surface LDL binding in lymphocytes from these patients but the rate of LDL degradation in their lymphocytes was as low as in a patient with FH (34). Accordingly, Norman et al. proposed that the patients had a defect in a protein that was required for LDLR internalization (34). In the present study, FACS analysis using an anti-LDLR antibody indicated that lymphocytes from ARH patients had an increase in cell surface LDLRs compared with lymphocytes from normal individuals. A comparable increase in 125I-LDL binding was observed in these cells, indicating that the LDLRs at the cell surface were capable of binding to LDL. Despite the increased cell surface binding, the amount of LDL degradation was ∼75% lower in the lymphocytes from ARH patients. These data are consistent with the participation of ARH in the internalization of the LDLR–LDL complex. The accumulation of LDLRs on the surface of ARH cells indicates that ARH is not required for trafficking of LDLRs to the plasma membrane. The observation that LDLR internalization is markedly reduced but not abolished in lymphocytes from patients with ARH, and that incubation with LDL leads to down-regulation of the LDLR in lymphocytes from these patients indicates that ARH facilitates but is not absolutely required for LDLR internalization. Residual LDLR function may explain why plasma LDL levels are lower in patients with ARH than in patients with classical FH. The specific function of ARH in LDLR internalization remains to be defined. One possibility is that ARH is required to cluster LDLRs in coated pits. Alternatively, ARH may anchor the receptor to the endocytic machinery during internalization.

**MATERIALS AND METHODS**

**Subjects**

Cultured fibroblasts derived from skin biopsy specimens and/or lymphocytes immortalized by transformation with the EBV (35) were obtained from five previously described
patients with autosomal recessive hypercholesterolemia (ARH1, 2, 3, 4 and 6) (11,13) and from an Italian woman (ARH7) with severe hypercholesterolemia (600–700 mg/dl), large xanthomas that developed at puberty, aortic stenosis and severe coronary and peripheral vascular disease. LDL uptake and degradation was 75% of normal in her cultured fibroblasts and both of her parents had normal plasma cholesterol levels.

DNA sequencing

Genomic DNA was prepared from cultured fibroblasts and from EBV-transformed lymphocytes using a commercial kit (Puregene, Gentra Systems, Minneapolis, MN, USA). RNA was extracted from fibroblasts and lymphocytes using RNA STAT-60 (Tel-Test Inc., Friendswood, TX, USA) according to the manufacturer's protocol and reverse-transcribed using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). Genomic and cDNA samples were PCR-amplified and sequenced using ABI PRISM BigDye Terminators version 3 on an ABI3100 automated sequencer using the protocol provided by the manufacturer (Applied Biosystems).

Northern blotting

Total cell RNA (20 μg) was size-fractionated by electrophoresis on 1% (w/v) agarose, 2% (v/v) formaldehyde gels and transferred to nylon membranes (Zetaprobe, BioRad). Hybridizations were performed using 32P-labeled cDNA probes (Megaprime DNA Labeling System, Piscataway, NJ, USA) for human ARH and mouse cyclophilin.

Southern blotting and PCR-amplification of intron 1 insertion

A total of 5 μg of genomic DNA were digested with HindIII and BgIII, size-fractionated on a 0.8% agarose gel and genomic blotting was performed using a 32P-labeled 792 bp fragment from intron 1 of ARH. The fragment was generated using PCR with the following oligonucleotides: 5'-ACAGACTCGGC-GCCACCTCTC-3' and 5'-CTTCCACGTCCCCAGATTCA-GAGA-3'. The 2.6 kb insert in ARH7 was amplified from 500 ng of genomic DNA using the Takara LA TaqTM system (Panvera, WI, USA) supplemented with 200 nM of each oligonucleotide (5' primer, 5'-GGAGGCGAGGCTCGTGTCGTTGCTCTAG-3' and the 3' primer, 5'-TGGCTAGGCTGGTGCTCTTGGCTTCAAGG-3'), 400 μM of each deoxynucleotide and

Figure 6. Immunoblot analysis of LDLR in lymphocytes from a normal subject, ARH6, and an FH patient (A). Cells were grown in medium containing 0 μg/ml or 20 μg/ml LDL for 48 h. Cell lysates were fractionated on a 4–15% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane and blotted with a peptide antibody to the terminal 15 amino acids of the human LDLR tail. The filter was washed and the signal was detected as described in the Materials and Methods.

FACS analysis comparing the relative amount of immunoreactive LDLR on the cell surface of lymphocytes from a normal subject, an FH patient and a proband homozygous for ARH6 (B). Lymphocytes grown in medium containing 10% HLPPS for 48 h were incubated with a rabbit anti-bovine LDLR antibody, washed in PBS and stained with Alexa 488-labeled secondary antibodies for 30 min. The dotted line corresponds to samples stained with secondary antibody only. The dark solid lines correspond to cells stained with human LDLR antibody.

DNA sequencing

Northern blotting

Southern blotting and PCR-amplification of intron 1 insertion
2.5 U Takara *LA Taq*™ polymerase in a 50 μl reaction volume. The PCR amplification sequence was: 94°C for 1 min; 35 cycles at 98°C for 20 s, 70°C for 4 min; 72°C for 10 min. The PCR fragment was purified, subcloned into pGEM™-T Easy Vector System (Promega, WI, USA) and sequenced.

**Immunoblot analysis of ARH**

Cultured fibroblasts were plated at a density of 150 000 cells per 100 mm dish and grown in DMEM containing 10% fetal calf serum (FCS). The medium was changed on days 3 and 5. On day 7, the cells were harvested in PBS and collected by centrifugation at 500 g for 5 min. The cell pellet was lysed in buffer A (1% (v/v) Triton, 50 mM Tris (pH 8.0), 2 mM CaCl₂, 80 mM NaCl), supplemented with protease inhibitors (Complete Mini, Roche Molecular Diagnostic Corp, Indianapolis, IN, USA). Lysates were centrifuged for 10 min at top speed in a microfuge at 4°C and the supernatant was collected. A total of 10 μg/ml ¹²⁵I-LDL (158 cpm/ng) was then added to the cells for 5 h. The medium was removed and the amount of ¹²⁵I-labeled acid soluble material (degraded LDL) was determined as described in the Materials and Methods.
a nitrocellulose membrane (Hybond C, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and visualized by immunoblotting using serum (1:1000) from a rabbit immunized with a peptide containing the C-terminal 15 amino acids of human ARH and a rabbit anti-rat BIP polyclonal antibody (Stressgen Biotechnologies, Victoria, BC, Canada). HRP-conjugated donkey anti-rabbit IgG and the Enhanced Chemiluminescence Western Blotting Detection Kit (Amersham, Piscataway, NJ, USA) were used for signal detection.

EBV-transformed lymphoblasts were seeded at 1.0 × 10⁶ cells/ml in 5 ml of RPMI 1640 (Mediatech Cellgro) + 10% HLPPS at 37°C for 48 h. The lymphocytes were harvested in PBS, collected by centrifugation, lysed in buffer A and immunoblotted using a rabbit polyclonal antibody (3143) against the C-terminal 15 amino acids of the human LDLR, and anti-ARH and BIP antibodies.

**ARH cDNA expression**

A fragment of the human ARH cDNA containing the entire coding sequence was amplified by PCR and cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). A point mutation (c.605C>A) that changes residue 202 from proline to histidine was introduced using PCR-based site-specific mutagenesis procedure (QuikChange, Stratagene, La Jolla, CA, USA). The constructs were transfected into HEK293 cells using SuperFect Reagent (Qiagen, Valencia, CA, USA).

**LDL activity in fibroblasts and EBV transformed lymphocytes**

LDLR cell-surface binding, degradation and internalization was determined in fibroblasts (36) and in EBV-transformed

**Figure 8.** Surface binding, internalization and secretion of transferrin in normal, ARH and FH lymphoblasts. Lymphoblasts (∼5 × 10⁶ cells/ml) were incubated with 0.2 µg/ml ¹²⁵I-transferrin (199 cpm/ng) at 4°C for 60 min, washed three times in ice-cold PBS, then resuspended in culture medium containing 0.5% BSA + 10 µg/ml unlabeled transferrin at 37°C for 0, 5, 15 or 30 min. At each time point, cells were cooled to 4°C, the medium was removed following centrifugation, the cells were washed twice with 0.5 ml ice-cold buffer C (0.2 M acetic acid, 0.5 M NaCl, pH 2.4), then solubilized overnight in 1.0 N NaOH. The radioactivity in the medium (secreted transferrin), the combined acid washes (surface bound transferrin), and in the cell pellet (internalized transferrin) was determined and expressed as the percentage of the total radioactivity at time zero.
lymphoblasts (37) as described previously. For the EBV-transformed lymphoblasts, the cells were seeded at 1.0 × 10^6 cells/ml in 75 ml culture flasks and incubated for 48 h in RPMI 1640 (Mediatech Cellgro, Herndon, VA, USA) + 10% HLPPS prior to adding the ^125^I-LDL.

**FACS analysis of transformed lymphocytes**

EBV-transformed lymphoblasts were seeded at ~1.0 × 10^6 cells/ml in 2 ml of RPMI 1640 (Mediatech Cellgro) + 10% HLPPS in 60 mm culture dishes for 48 h and stained with unconjugated rabbit polyclonal anti-bovine LDLR antibody (638) for 30 min on ice. Cells were washed twice with ice-cold PBS and stained with a secondary antibody (goat anti-rabbit antibody conjugated to Alexa 488) for 30 min. Flow cytometry analysis was performed using a FACS Calibur Flow Cytometer (Beckton Dickinson, San Jose, CA, USA) and the data were analysed using the CellQuest software package (Becton Dickinson). For each sample 50 000 live events were collected.

**^125^I-transferrin internalization**

Human transferrin (Research Diagnostics Inc., Flanders, NJ, USA) was labeled with Na^125^I (100 μg transferrin/mCi Na^125^I) using iodogen (Pierce Chemical Co., Rockford, IL, USA). The binding, uptake and secretion of transferrin were determined as described (38,39). Lymphoblasts (~6 × 10^5) from a normal individual, an FH homoyzote (S156L) and ARH6 were harvested, washed in PBS, resuspended in 12 ml of buffer B (RPMI 1640 + 25 mM HEPES, 0.5% BSA) and aliquoted into microfuge tubes (~5 million cells/tube). The cells were cooled to 4°C for 30 min prior to the addition of 0.2 μg/ml ^125^I-transferrin (199 cpm/ng) and then incubated for 1 h. The cells were pelleted in a microfuge, washed three times in ice-cold PBS, resuspended in buffer B (warmed to 37°C) supplemented with 10 μg/ml unlabeled transferrin, and incubated for 0, 5, 15 or 30 min at 37°C. At each time point, three tubes from each cell line were cooled to 4°C and harvested by centrifugation at 500 g for 5 min. The medium was removed, and the cell pellets were washed twice with 0.5 ml of buffer C (0.2 M acetic acid, 0.5 M NaOH, pH 2.4). The medium (secreted transferrin), the combined acid washes (surface-bound transferrin), and the cell pellets (internalized transferrin), were counted in a gamma counter. The cell pellets were then dissolved in 0.5 ml of 1.0 M NaOH (overnight incubation at room temperature) and the protein content was determined using the BCA method (Pierce Chemical Co., Rockford, IL, USA).

**ACKNOWLEDGEMENTS**

We wish to thank Tommy Hyatt, Yuanlan Liao and Angela Padgett-Thomas for excellent technical assistance, Drs Michael Brown and Joseph Goldstein for helpful discussions and Dr Cesar Sirtori for fibroblasts from an ARH patient. This work was supported by HL 20948, HL53917, AI39416 the W.M. Keck Foundation and the Donald W. Reynolds Cardiovascular Clinical Research Center at Dallas. Helen Hobbs is an investigator of the Howard Hughes Medical Institute.

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