Human 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase

CONSERVED DOMAINS RESPONSIBLE FOR CATALYTIC ACTIVITY AND STEROL-REGULATED DEGRADATION*

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A full length cDNA for human 3-hydroxy-3-methylglutaryl coenzyme A reductase, the membrane-bound glycoprotein that regulates cholesterol synthesis, was isolated from a human fetal adrenal cDNA library. The nucleotide sequence of this cDNA shows that the human reductase is 888 amino acids long and shares a high degree of homology with the hamster enzyme. The amino-terminal membrane-bound domain is the most conserved region between the two species (7 substitutions out of 339 amino acids). This region, which is predicted to span the endoplasmic reticulum membrane seven times, mediates accelerated degradation of reductase in the presence of sterols. The carboxyl-terminal catalytic domain is also highly conserved (22 substitutions out of 439 amino acids). However, the linker region between these two domains has diverged (32 substitutions out of 110 amino acids). Conservation of the structure of the membrane-bound domain in HMG-CoA reductase supports the hypothesis that sterol-regulated degradation is an important mechanism for suppression of reductase activity and for regulation of cholesterol metabolism in humans as well as in hamsters.

HMG-CoA reductase is the rate-limiting enzyme for cholesterol synthesis. Its activity is regulated via a negative feedback mechanism mediated by sterols and non-sterol metabolites derived from mevalonate, the product of the reaction catalyzed by reductase (1). Normally in mammalian cells this enzyme is suppressed by cholesterol derived from the internalization and degradation of low density lipoprotein via the LDL receptor. Competitive inhibitors of the reductase induce the expression of LDL receptors in the liver, which in turn increases the catabolism of plasma LDL and lowers the plasma concentration of cholesterol, an important determinant of atherosclerosis (2, 3).

Recent studies of the HMG-CoA reductase in hamster cells have shown that this enzyme is encoded by a family of mRNAs of about 4.7 kb, which are transcribed from a 25-kb gene (4, 5). Full length cDNA clones have been obtained and this has revealed the complete amino acid sequence of the enzyme (6). The hamster reductase is a 97-kDa glycoprotein bound to the membrane of the endoplasmic reticulum by a hydrophobic amino-terminal region that is predicted to span the endoplasmic reticulum membrane seven times (7, 8). The catalytic site of the reductase is in the carboxyl-terminal half of the enzyme, which projects into the cytoplasm of the cell (8).

The genomic segments comprising this gene have also been isolated and the sequence of the promoter region has been determined (5). The reductase gene from hamster cells lacks a TATA or CCAAT box; transcription initiates from multiple sites spread out over 100 bp in the genome. The 5′ untranslated region contains an intron of about 3.5 kb. Variable splicing at the 5′ end of this intron produces mRNA molecules with 5′ untranslated regions ranging between 68 and 670 nucleotides in length. In cultured cells, reductase activity can be suppressed either by cholesterol derived from LDL or by oxygenated derivatives of cholesterol solubilized in solvents and added to the culture medium (1). This suppression is accomplished by two mechanisms, 1) decreased transcription of the gene (9) and 2) enhanced degradation of the protein (10–13). Inasmuch as both the mRNA and protein turn over with short half-lives, both of these mechanisms are efficient means of regulating reductase activity.

Only limited information is available about HMG-CoA reductase in man, primarily due to the lack of availability of tissues that express high levels of the enzyme. Reductase from human liver is immunologically cross-reactive with the rat enzyme (14, 15) and can be inactivated by phosphorylation, just as the rat enzyme (15). Purification of reductase from human liver yields a single species of 52 kDa (14, 16). Purification of reductase from rat liver by a similar protocol also yields a species of 50–55 kDa. However, subsequent studies have shown that the purified enzyme represents a proteolytic fragment of the native protein whose true molecular weight is 97 kDa (6, 11, 17).

In the current studies, we examine the structure and function of human reductase. For this purpose, we have used the cloned hamster cDNA to select a full length cDNA for the human enzyme. The human cDNA was then sequenced to determine the primary structure of the human enzyme. Comparison of the human amino acid sequence with that of the hamster enzyme has identified two highly conserved regions of the molecule that are separated by a less conserved region. The functional implications of these three domains are discussed.

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† The abbreviations used are: HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL, low density lipoprotein; SDS, sodium dodecyl sulfate; bp, base pairs; kb, kilobase pairs; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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EXPERIMENTAL PROCEDURES

MATERIALS—A human genomic DNA library (18) was kindly provided by Tom Maniatis of the Department of Biological Chemistry, Harvard Medical School, Boston, MA. A human fetal adrenal cDNA library (19) was kindly provided by Tokuo Yamamoto of the Department of Molecular Genetics, University of Texas Health Science Center at Dallas, Dallas, TX. Other materials were obtained from previously described sources.

Isolation and Sequencing of Genomic and cDNA Clones—Recombinant bacteriophage were screened by the method of Benton and Davis (20). Hybridization was performed in buffer A (buffer A = 0.75 M NaCl, 75 mM sodium citrate, 50 mM sodium phosphate, pH 7.0, 0.2% bovine serum albumin). 0.2% polyvinyl pyroliodine, 100 µg/ml denatured salmon sperm DNA, and 0.1% SDS plus 30% formamide with ~2 x 10^5 cpm/ml of 32P-labeled DNA probe at 42 °C for 16 h. 32P-labeled DNA probes were prepared by random hexanucleotide-primed labeling (21). Filters were washed in 0.3 M NaCl, 30 mM sodium citrate (pH 7.0), and 0.1% SDS at 55 °C.

The cDNA library was first enriched for full length cDNAs by linearizing plasmid DNA from the total human fetal adrenal cDNA library with the restriction endonuclease SalI, fractionating the digest on low melting temperature agarose, recovering plasmid DNA greater than 6.5 kb in length, religating the DNA, and transforming competent E. coli HB101 (22). The resulting clones were screened by colony hybridization as described by Grunstein and Hogness (23). Hybridization-positive clones were performed in buffer A plus 50% formamide at 42 °C for 16 h. Filters were washed in 15 mM NaCl, 1.5 mM sodium citrate (pH 7.0), and 0.1% SDS at 55 °C.

Hybridization-positive genomic and cDNA clones were isolated and plating or plaque lifting was performed and characterized by standard techniques (24). DNA sequencing was performed by the dideoxy chain termination method after subcloning DNA fragments into bacteriophage M13 vectors (25, 26). Specific oligonucleotide primers were synthesized by the phoshoramidite method (27) using the Applied Biosystems 380A oligonucleotide synthesizer. Computer analysis of DNA sequences was done on an IBM personal computer using the Beckman Microgenie DNA analysis program (28).

Cell Culture—Cells were grown in monolayer culture at 37 °C in an atmosphere of 5-7% CO₂. Stock cultures of Chinese hamster ovary cells were grown in medium A (Ham’s F-12 containing 25 mM Hepes (pH 7.4), 100 units/ml of penicillin, 100 µg/ml streptomycin, and 2 µM glutamine) supplemented with 10% lipoprotein-deficient serum. Stock cultures of UT-2 cells (29) were grown in medium A supplemented with 10% fetal calf serum and 0.2 mM methionol. Stock cultures of TR-74 cells (see below) were grown in medium A supplemented with 10% lipoprotein-deficient serum. For growth experiments, on day 0 cells in 60 mm dishes were dissociated with trypsin/EDTA and 1.5 x 10⁵ cells were seeded into a 35-mm well in 2 ml of medium A supplemented with 10% fetal calf serum with 0.2 mM methionol. On days 1 and 3, the cells were fed medium A supplemented with 10% fetal calf serum either with or without 0.2 mM methionol. On day 5, the dishes were fixed with 95% ethanol and stained with 1% crystal violet. For experiments in which reductase activity was measured, on day 0, 3.5 x 10⁵ Chinese hamster ovary cells or 6 x 10⁴ TR-74 cells were seeded in 60-mm Petri dishes in 3 ml of medium A supplemented with 10% fetal calf serum. The cells were fed on days 2 and 3 with 3 ml of medium A supplemented with 10% lipoprotein-deficient serum. Additions of steroids were made on days 3 and 4.

Isolation of TR-74 Cells—Plasmid pHRed-102 was introduced in the UT-2 cell line by co-transfection with pSV3-Neo using the calcium phosphate precipitation method (30). A co-precipitate of pHRed-102 (0.5 µg) and pSV3-Neo (0.5 µg) was added to 5 x 10⁴ UT-2 cells in a 100-mm dish in medium B (Dulbecco’s modified Eagle’s medium containing 100 units/ml of penicillin, 100 µg/ml streptomycin, and 34.5 µg/ml of proline) supplemented with 10% fetal calf serum and 0.2 mM methionol. After 5 h, the medium was removed, the cells were exposed to medium B plus 10% glycerol for 4 min at 24 °C, and the medium was replaced with medium B supplemented with 10% fetal calf serum plus 0.2 mM methionol. The following day the medium was changed to medium B supplemented with 10% fetal calf serum and 700 µg/ml G418 lacking methionol. This medium was changed every 3 to 4 days until individual colonies could be picked and grown in medium B supplemented with 10% fetal calf serum and 350 µg/ml G418. One of these clones, designated TR-74, was subsequently adapted to grow in medium A supplemented with 10% lipoprotein-deficient serum.

Other Assays—Protein was measured by a modified Lowry procedure (31). Enzymatic activity of HMG-CoA reductase was measured in detergent-solubilized cell extracts as described (32). One unit of reductase activity represents the formation of 1 nmol of [14C]mevalonate per min at 37 °C.

RESULTS

The following strategy was used to isolate a full length cDNA for the human HMG-CoA reductase. First, a human genomic clone was selected with a probe derived from the hamster cDNA. This provided a homologous probe to the 5’ end of the human reductase mRNA that was used to screen a lambda DNA library from the fetal adrenal, a tissue that expresses high levels of HMG-CoA reductase. The use of a human specific probe allowed the screening of the cDNA library to be done at higher stringency with a lower background. Inasmuch as the probe was confined to the 5’ end of the coding region, only full length cDNA clones would be detected.

A human fetal liver genomic library was screened under reduced stringency with the 1.5-kb Pst I fragment from the hamster cDNA for HMG-CoA reductase, pRed-227 (6). This fragment includes 163 bp of 5’ untranslated sequence and 1347 bp of the coding region. Southern blots of human genomic DNA indicated that this fragment recognized a unique sequence in the human genome (data not shown). Approximately 1 x 10⁶ recombinant phage were screened and one clone, designated AHRed-1, was isolated and DNA was prepared for further characterization. Studies of AHRed-1 DNA showed that the 0.9-kb BamHI fragment contained sequences that hybridized with the most 5’ 500 bp of the hamster cDNA (Fig. 1A). When smaller fragments of this region were sequenced, it was found that the 149-bp BamHI/BglII fragment plus 56-bp 3’ of the BglII site was 90% homologous with residues 23 to 165 of the hamster cDNA and corresponded to the presumed second exon of the human reductase gene.
(data not shown). The 430-bp EcoRI/BglII fragment that included a portion of the first intron and 150 bp of the second exon was then used to isolate a full length cDNA for the human HMG-CoA reductase.

A size-fractionated human fetal adrenal library (19) was screened using the EcoRI/BglII genomic fragment from pHRed-1. Approximately 3 x 10^4 colonies were screened and one positive clone, designated pHRed-102, was identified. This plasmid contained a 4.3-kb insert with the restriction map indicated in Fig. 1B. Restriction fragments of pHRed-102 were subcloned into M13 bacteriophage vectors and subjected to DNA sequencing as indicated in Fig. 1B.

The nucleotide sequence of the human reductase cDNA is shown in Fig. 2. The sequence contains an open reading frame of 2664 nucleotides that would encode an 888-amino acid protein. The first nucleotide of the ATG that begins this open region with the next are also highly conserved the parts of the human gene is located at position -669 (5). Transcripts that include a portion of the 5′ untranslated sequence are present in this cDNA clone. Within this region, the sequence of 187 bp 3′ of the coding region was determined.

The nucleotide sequences of the human and hamster reductase mRNAs show a high degree of homology throughout. Within the translated region, the sequence of 187 bp 3′ of the coding region was determined. The 3′ untranslated region is approximately 1.6 kb long. Within this region, the sequence of 187 bp 3′ of the coding region was determined.

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The nonrandom distribution of amino acid substitutions is likely attributable to selective constraint on different regions of the protein during evolution. To rule out the alternate possibility (i.e. a difference in the mutation rate at the nucleotide level), we compared the rate of silent substitutions with that of replacement substitutions in the three domains of the reductase. The per cent corrected divergence for the human and hamster reductase sequences was calculated according to the method of Perler et al. (35) and are listed in Table I. The rates of replacement substitutions were 1.2%, 17.8%, and 2.8% in the membrane-bound, linker, and catalytic domains, respectively. In contrast to this large variation, the rates of silent site substitutions were quite similar—70%, 51%, and 61% in the respective domains. These data suggest that the DNA has evolved at similar rates in these three domains except when constrained by replacement amino acid substitutions.

To evaluate the functional behavior of the human reductase cDNA, we introduced the plasmid containing the cDNA into hamster cells by calcium phosphate-mediated transfection. The vector in which the human cDNA was cloned contains the SV40 early promoter region with an intron donor and acceptor splice site upstream from the cDNA insert, which allows expression of the gene in hamster cells (22). As recipients we used UT-2 cells, a mutant line of Chinese hamster ovary cells that lacks HMG-CoA reductase activity and requires exogenous mevalonate for growth (29). The plasmid containing the human reductase cDNA (pHRed-102) was transfected into UT-2 cells together with pSV3-Neo, a plasmid that contains a bacterial gene conferring resistance to
Structural Domains of HMG-CoA Reductase

Fig. 2. Nucleotide sequence and deduced amino acid sequence of pHRed-102 and comparison with the sequence of the hamster reductase. The nucleotide sequence derived from sequencing pHRed-102 as described in Fig. 1 is shown. The ATG that initiates translation of the reductase protein is labeled +1. The 5' untranslated sequence is numbered -50 to -1. The translated 888-amino-acid-residue sequence of the human reductase is listed beneath residues +1 to 2667. Asterisks above the nucleotide sequence indicate bases that differ in the human and hamster nucleotide sequences. The lines of the diagram above the nucleotide sequence indicate the equivalent positions in the deduced amino acid sequence.
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Fig. 3. Schematic representation of the secondary structure of HMG-CoA reductase. The proposed structure of hamster reductase is drawn as described by Liscum et al. (8). The membrane of the endoplasmic reticulum is shaded with the seven transmembrane segments of reductase traversing this region. The sites of amino acid substitutions in the human reductase relative to the hamster enzyme are indicated. Each replacement is marked as to whether it is conservative (*) or nonconservative (▲).

![Diagram of secondary structure](image)

Fig. 4. Mevalonate-dependent growth of Chinese hamster ovary (CHO), UT-2, and TR-74 cells. The indicated cells were seeded on day 0 in medium A supplemented with 10% fetal calf serum and 0.2 mM mevalonate. On day 1, the cells were fed medium A plus 10% fetal calf serum either with or without the added mevalonate and refed on day 3 with the same medium. On day 5, the cells were fixed and stained as previously described (29).

and protein when cells are exposed to sterols, reductase activity is suppressed 50–60% within 4–6 h (13). This suppression of reductase activity is mediated by a 2-fold increase in the rate of degradation of the protein (13). Fig. 5 shows that reductase activity in TR-74 cells also decreases by about 50% following the addition of sterols. This moderate level of suppression contrasts with the greater than 95% suppression seen with Chinese hamster ovary cells in which transcription is also suppressed. The similar behavior of the transfected human reductase and the transfected hamster reductase (13) following their introduction and expression in UT-2 cells suggests that the portion of the reductase protein responsible for regulated degradation is functionally conserved between the human and hamster enzyme.

DISCUSSION

In the current paper we describe the isolation and characterization of a full length cDNA for human HMG-CoA reductase. Sequence analysis of this cDNA has allowed us to analyze the protein structure of the human reductase and to compare it with the hamster enzyme. In addition, we have studied the expression of the human reductase by introducing pHRed-102 into UT-2 cells, a mutant cell line that does not normally express functional reductase activity, and selecting a cell line, designated TR-74, now capable of growing under conditions in which reductase activity is required for survival. These

![Diagram of cell lines](image)

Table 1

<table>
<thead>
<tr>
<th>Domain</th>
<th>Amino acid residues</th>
<th>Amino acid substitutions</th>
<th>Percent corrected divergence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane-bound</td>
<td>1–339</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Linker</td>
<td>340–449</td>
<td>32</td>
<td>29</td>
</tr>
<tr>
<td>Catalytic</td>
<td>450–888</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>Whole protein</td>
<td>1–888</td>
<td>61</td>
<td>7</td>
</tr>
</tbody>
</table>

from the corresponding nucleotide sequence of the hamster reductase. Amino acid residues that differ from the hamster protein are enclosed in boxes and the corresponding amino acids in the hamster protein are indicated beneath the human sequence. A gap of 3 nucleotides has been inserted in the hamster sequence after position 1260 to align both sequences throughout the coding region. A single nucleotide gap has been inserted after position 2739 in the hamster sequence to align the sequences in the 3’ untranslated region.
The finding of strong conservation between the membrane domains of the hamster and human reductase supports previous speculation about the functional importance of this region (8). The complex arrangement of seven transmembrane portions with short connecting segments on the luminal and cytoplasmic sides of the membrane of the ER as well as the site for \(N\)-linked glycosylation has been maintained in the two species. Even conservative substitutions of one hydrophobic amino acid for another hydrophobic residue seem not to be tolerated.

How does the conservation within this domain of the reductase compare with that of other proteins? Soluble proteins vary widely in their rates of evolution depending on the functions of the proteins and the evolutionary constraints placed on their sequences. Histone IV has had only 2 amino acid substitutions out of 102 residues from plants to mammals (34), whereas the fibrinopeptides have diverged greatly over a short period of evolutionary time (34). Even different parts of the same protein can diverge at different rates depending on the functional importance of the residues. Within hemoglobin, those residues involved in heme binding are conserved much greater than residues on the surface of the protein (40). Membrane proteins display the same diversity of evolutionary rates as soluble proteins. Proteins that span the membrane only once and probably serve primarily just to anchor the protein to the membrane display a high rate of amino acid substitutions within the hydrophobic stretch of the protein. For example, the membrane-spanning region of the LDL receptor is only 68% conserved between the bovine and human sequences (19, 41). Similarly the membrane-spanning portion of glycoporin is only 55–70% conserved among the horse, human, and pig sequences (42).

In contrast to proteins anchored to the membrane by a single transmembrane sequence, those proteins that have complex hydrophobic regions that span the membrane several times are believed to perform functions within the membrane. The acetylcholine receptor is such a protein (43). It is a complex of \(\alpha\), \(\beta\), \(\gamma\), and \(\delta\) subunits in the ratio of 2:1:1:1. Each of these subunits contains four stretches of hydrophobic residues that span the membrane. The intact receptor binds acetylcholine and forms an ion channel through the membrane. Within this complex receptor, different rates of evolution are found between the membrane-spanning portions of different subunits and different transmembrane regions of each subunit. In the \(\alpha\) subunit, the first three membrane-spanning regions (M1, M2, and M3) are very strongly conserved among species as diverse as humans and the fish, *Torpedo californica* (91–100% conservation) (43). On the other hand, other hydrophobic membrane-spanning regions in the same receptor complex are less conserved. For example, the fourth membrane-spanning region (M4) of the \(\beta\) subunit is only 53% conserved between calf and *Torpedo* (44). Comparison of the human and bovine sequence of rhodopsin, a protein in the rod outer segment that spans the rod disk membrane seven times and is responsible for light reception, also illustrates the high degree of conservation of transmembrane sequences of complex membrane proteins. The seven transmembrane stretches are conserved 96, 91, 100, 96, 89, 92, and 88% (45).

The striking amount of conservation within the membrane-associated portions of proteins that span a membrane several times indicates the degree of evolutionary constraint placed on these regions. Why are conservative substitutions of hydrophobic amino acids not tolerated in these regions as easily as in proteins that only span the membrane once? Although
the three-dimensional intramembranous structure of these proteins is not known, it is possible that packing of neighboring helical regions requires that the side chains fit precisely together. Replacement of a small hydrophobic side chain with a longer one might not allow the strong hydrophobic packing necessary for the protein to fold within the membrane. Charged residues are also found in these transmembrane regions and the charges are presumably neutralized by interactions with other charged elements within the lipid bilayer. Substitutions might hinder the interaction that is necessary to neutralize the charged elements within the lipid bilayer. Substitutions might affect degradation of the protein. Such a mechanism of control could represent a unique mechanism for metabolic regulation of membrane-bound proteins within mammalian cells.

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REFERENCES