Mutations in the Bile Acid Biosynthetic Enzyme Sterol 27-Hydroxylase Underlie Cerebrotendinous Xanthomatosis*

(Received for publication, December 13, 1990)

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The sterol storage disorder cerebrotendinous xanthomatosis (CTX) is characterized by abnormal deposition of cholesterol and cholestanol in multiple tissues. Deposition in the central nervous system leads to neurological dysfunction marked by dementia, spinal cord paresis, and cerebellar ataxia. Deposition in other tissues causes tendon xanthomas, premature atherosclerosis, and cataracts. In two unrelated patients with CTX, we have identified different point mutations in the gene (CYP27) encoding sterol 27-hydroxylase, a key enzyme in the bile acid biosynthesis pathway. Transfection of mutant cDNAs into cultured cells results in the synthesis of immunoreactive sterol 27-hydroxylase protein with greatly diminished enzyme activity. We have localized the CYP27 gene to the q33-qter interval of human chromosome 2, and to mouse chromosome 1, in agreement with the autosomal recessive inheritance pattern of CTX. These findings underscore the essential role played by sterols in the central nervous system and suggest that mutations in other sterol metabolizing enzymes may contribute to diseases with neurological manifestations.

Approximately one-fourth of the cholesterol present in an adult mammal is located in the nervous system (1), where it is a major constituent of the myelin sheath that both insulates and facilitates axonal conduction (2). The central role played by cholesterol in the nervous system is made readily apparent in certain genetic diseases in which alterations in genes involved in sterol metabolism have profound effects on neural tissues.

One such disease is cerebrotendinous xanthomatosis (CTX),1 a rare sterol storage disorder first described in 1937 in individuals with neurological dysfunction and tendon xanthomas (3, 4). Subsequent studies demonstrated that cholesterol and the 5α-reduced form of cholesterol, cholestanol, accumulate in neural and other tissues of CTX subjects (5), and that these individuals have a reduced capacity to synthesize bile acids, the normal end products of cholesterol catabolism (6). The metabolic defect underlying the failure to synthesize bile acids is controversial and has been postulated to involve either a sterol 27-hydroxylase (7) or a sterol 24-hydroxylase (8). The mechanism by which a defect in hepatic bile acid synthesis leads to the diverse symptoms observed in CTX is at present not known (4); however, the pleiotropic nature of the disease does not appear to be related to hypercholesterolemia as a majority of CTX patients have normal serum cholesterol levels (4).

To gain insight into this disease and to explore the link between bile acids, sterols, and the nervous system, we recently isolated cDNA clones for the rabbit (9) and human sterol 27-hydroxylase mRNAs (10). The enzyme encoded by these cDNAs is a mitochondrial cytochrome P-450 that together with two protein cofactors, adrenodoxin and adrenodoxin reductase, hydroxylates a variety of sterols at the C-27 position (9-11). In the bile acid synthesis pathway, sterol 27-hydroxylase catalyzes the first step in the oxidation of the side chain of sterol intermediates (12, 13). Hybridization studies using the cloned rabbit cDNA revealed that sterol 27-hydroxylase mRNA was present in many different tissues and that the mRNA was encoded by a single copy gene (9). In this paper, we describe the use of the human sterol 27-hydroxylase cDNA to determine a molecular basis for CTX and to map the chromosomal location of the gene.

EXPERIMENTAL PROCEDURES

Materials—Enzymes used in cloning, DNA sequencing, and polymerase chain reactions were obtained from New England Biolabs, Gibco, U.S. Biochemicals, and Perkin-Elmer Cetus Instruments. Nylon filters (Biotrans) for blot hybridization experiments were obtained from ICN Pharmaceuticals. Oligonucleotides for DNA sequence analysis and polymerase chain reactions were synthesized on an Applied Biosystems Model 380A DNA synthesizer. Genomic DNA was extracted from human diploid fibroblasts with an Applied Biosystems Model 340A extractor.

Fibroblast Culture—The normal and CTX fibroblasts used in these studies were obtained from Drs. M. S. Brown and J. L. Goldstein, University of Texas Southwestern Medical Center, Dallas, TX. The clinical features of the CTX subjects from which the fibroblasts were biopsied have been described previously. Subject CTX1 was a Canadian female, designated patient SC in Pastershank et al. (14). Subject CTX2 was a black American, originally described as patient JC in Harlan and Still (15), and characterized extensively with respect to sterol and bile acid metabolism by Selten and colleagues (6). CTX and normal fibroblasts were cultured as described previously (16). Genomic DNA and total RNA were isolated from these cells and analyzed by blot hybridization as described by Lehman et al. (17, 18).

Polymerase Chain Reactions—Amplification of sterol 27-hydroxylase mRNA from fibroblasts was accomplished as described by Saiki et al. (19). Total RNA (1 μg) was first converted into cDNA in a volume of 50 μl using a cDNA synthesis kit from Gibco. Primers for the polymerase chain reaction (see below) were added directly to the reverse transcription reaction, and amplification was carried out after a 10-min incubation at 100 °C and Taq enzyme addition for 35 cycles of 1 min/95 °C and 5 min/68 °C. A final cycle of 1 min/95 °C...

* This research was supported by Grants HL-20948 (to D. W. R.) and GM-26105 (to U. F.) from the National Institutes of Health and Grant I-871 (to D. W. R.) from the Robert A. Welch Foundation of Texas. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶¶ The abbreviation used is: CTX, cerebrotendinous xanthomatosis.

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and 10 min at 68°C was then carried out prior to purification of the
amplified cDNA by Centricon 30 (Amicon Corp.) column filtration.

The coding region of the sterol 27-hydroxylase mRNA was amplified
in three overlapping fragments. One pair of oligonucleotides
representing nucleotides 170 to 194 (dTCCGGCCGCCCAACGGA-
GACGAGA) and nucleotides 1250 to 1274 (dAAGGAAAGGCAT-
CAACTTCACATTTT) was used to amplify mRNA spanning nucleo-
tides 195 to 1249. A second pair derived from nucleotides 1066 to
1090 (dTACCACCTCTAAAGGACCCCTGAGA) and nucleotides
1728 to 1752 (dGCAAGGGGTGTCCTCCACCTCTCGA) was used to
amplify the 3' region of the cDNA encompassing nucleotides 1091 to
1728.

We were unsuccessful in amplifying the extreme 5' end of the
mRNA using a variety of different primers and conditions. It did
prove possible to amplify the 5' region of the sterol 27-hydroxylase
gene. To this end, a genomic clone harboring the gene was isolated,
and DNA of the 5'-flanking region and intron 1 were subjected to
DNA sequence analysis. Two primers were synthesized from this
information, representing a portion of the 5'-flanking region
(dACTCAGGACTCGACCCAAAGGTGCA) and a portion of intron 1
d(TCTCCTTCCCTCGTCCACCTCAT), and used for amplifica-
tion as described above except that 1 μg of genomic DNA was the
starting template. DNA sequence analysis of the amplified fragments was accom-
plished by the methods of Sanger et al. (20), using bacteriophage T7
dNA polymerase (Sequenase, U.S. Biochemicals). For each CTX
mutation, RNA and DNA from two separate aliquots of cells were
amplified and sequenced.

Transfection Analysis of CTX Mutations—Two mutations detected in
the CTX subjects were recreated in an expressible cDNA encoding
human sterol 27-hydroxylase (10)..Site-directed mutagenesis of the
cDNA was accomplished in bacteriophage M13 vectors (21) with
oligonucleotides 23 bases in length as described previously (22). The
vector used to drive expression of both the normal and mutated
cDNAs in simian COS-M6 cells was a pCMV derivative (9). COS
cells were cultivated as described by Esser et al. (22) and transfected
with the sterol 27-hydroxylase expression vectors as described by
Andersson et al. (9) and Cali et al. (10), except that a bovine adreno-
donin cDNA was not employed. Sterol 27-hydroxylase enzyme
activity in transfected cells was detected by thin layer chromatography
assay (9). The substrate was [3H]-cholestanoic acid (10). Cellular
protein content was determined as described by Bradford (23).

Immunochromatography—An antibody peptide matrix was raised
against an amino acid sequence (Cys-Pro-Gly-Val-Arg-Arg-Arg-Cys~
Arg-Ser-Leu-Glu-Glu-Ile-Pro) corresponding to residues 15 to 28 of
the mature sterol 27-hydroxylase protein (10) and containing an extra
cysteine at the amino terminus for coupling purposes. The peptide
was synthesized by Multisysteme Systems (San Diego, CA). Antibody-
es were raised in New Zealand White rabbits following a regimen
described previously (24). Immunoblotting was as described (24),
except that an alkaline phosphatase detection system (Bio-Rad) was
used to visualize immune complexes.

Chromosomal Localization Studies—A panel of 11 Chinese hamster
× human hybrid cell clones from five independent fusion experiments
between Chinese hamster and human cell lines (25) was used for
primary chromosomal assignment of the human sterol 27-hydroxylase
gene (CYP27, Ref. 26). Regional mapping of the human CYP27 gene
was carried out with four hybrids from series X,XVIII, and XIX
containing different regions of human chromosome 2. For localization
of the murine CYP27 gene, a panel of 13 rodent × mouse hybrid
clones or subclones derived from four fusion experiments were
studied. The origin and characterization of hybrids in both human and mouse
mapping panels have been described (27).

Southern blotting experiments were carried out as detailed previ-
ously (28) using radiolabeled probes derived from the 5' or 3' regions
of the human sterol 27-hydroxylase cDNA (10). Radiolabeling with
32P was done by the random priming method (29).

RESULTS

The sterol 27-hydroxylase gene in CTX subjects was first
examined in Southern blotting experiments with genomic DNA isolated from the fibroblasts of two unrelated affected
individuals. As shown in Fig. 1, these studies did not reveal
any gross abnormalities in the gene structure: digestion of the
CTX DNAs with three different restriction enzymes yielded
a simple hybridization pattern identical with that obtained
with the DNA of a normal individual. The relative intensities
of the normal and CTX hybridizing fragments in the autoradi-
ograms were similar, suggesting that both copies of the sterol 27-hydroxylase were present in the CTX subjects
(Fig. 1).

Total RNA was isolated from the fibroblasts of subjects
CTX1 and CTX2 and analyzed by blot hybridization. As
shown in Fig. 2, a single 2.0-kilobase species of sterol 27-
hydroxylase mRNA was detected that was present in near
equal amounts in both normal and CTX fibroblasts. These
studies suggested that if mutations in sterol 27-hydroxylase
were present in these CTX subjects, they would most likely
be either missense or nonsense mutations.

The presence of sterol 27-hydroxylase mRNA in the CTX
fibroblasts allowed the use of the polymerase chain reaction
to amplify and then sequence this mRNA. The data from
these experiments are summarized in Fig. 3 and illustrate
the locations of two different mutations that were present in
subjects CTX1 and CTX2. Both mutations involved single
base pair substitutions that convert arginone codons (CGP)
to cysteine codons (TGP). No other nucleotide changes were

FIG. 1. Southern blot hybridization of genomic DNA iso-
lated from subjects CTX1 and CTX2. Genomic DNA (10 μg)
from normal and CTX fibroblasts was digested with the indicated
restriction enzyme and subjected to Southern blot analysis as
described by Lehman et al. (17). Radioactive single-stranded DNA
probes (45) corresponding to approximately nucleotides 500 to 710,
711 to 914, and 1400 to 1594 of the human sterol 27-hydroxylase
cDNA (10) were used in the hybridization reactions. The positions
to which HindIII fragments of bacteriophage λ-DNA migrated in the
agarose gel and their sizes in kilobases (kb) are indicated on the left
side of the figure. Filters were exposed to Kodak XAR-5 x-ray film for
120 h in the presence of an intensifying screen.

FIG. 2. Blot hybridization analysis of normal and CTX
sterol 27-hydroxylase mRNAs. Different amounts of total cellular
RNA isolated from normal and CTX fibroblasts were analyzed by
blot hybridization after electrophoresis through 1.5% (w/v) agarose
gels. Hybridization probes labeled with 32P were derived from nucleo-
tides 510 to 710, 711 to 914, and 1400 to 1594 of the sterol 27-
hydroxylase cDNA. After washing, the filters were exposed to Kodak
XR-5 x-ray film for 96 h in the presence of an intensifying screen.
The positions to which RNAs of known size (kilobases, kb) migrated
to in the gel are shown on the left of the figure.
The locations of the amino acid changes detected in subjects CTX1 and CTX2 after amplification and DNA sequencing are indicated above the line. Two protein domains of this mitochondrial cytochrome P-450 that may be affected by these changes are indicated below the schematic.

**Fig. 4.** Expression of normal and CTX sterol 27-hydroxylase cDNAs. COS cells were transfected with the indicated cDNA in the pCMVZ vector. Forty-eight hours after transfection, the medium was made 2.5 μM in substrate (5β-[7β-3H]cholesterol-3α,7α,12α-triol) as outlined under "Experimental Procedures" and left on the cells for the indicated time periods. The conversion of substrate into products (5β-[7β-3H]cholestan-3α,7α,12α,27-tetrol plus 3α,7α,12α-trihydroxy-5β-[7β-3H]cholestanolic acid) was determined by thin layer chromatography analysis of media sterols. The results shown are representative of two separate transfection experiments.

detected in the two CTX mRNAs, which together with the Southern blotting data of Fig. 1, suggests that both individuals were homozygous at this locus. In addition, the CTX2 mutation was not present in the sterol 27-hydroxylase mRNAs of five unrelated individuals of the same ethnic background analyzed in a similar manner (data not shown).

In order to determine the effects of the observed amino acid substitutions on sterol 27-hydroxylase enzyme activity, each of the mutations shown in Fig. 3 was recreated by site-directed mutagenesis in an expressible cDNA and analyzed by transfection into simian COS cells. As substrate, we used 5β-[7β-3H]cholestan-3α,7α,12α-triol, an intermediate in the bile acid synthesis pathway that is converted to 5β-[7β-3H]cholestan-3α,7α,12α,27-tetrol and further oxidized to 3α,7α,12α-trihydroxy-5β-[7β-3H]cholestanolic acid by the action of sterol 27-hydroxylase (10). As shown in Fig. 4 (upper left panel), mock transfection of COS cells with the expression vector alone did not give rise to sterol 27-hydroxylase activity. However, transfection of cells with a vector containing the normal sterol 27-hydroxylase cDNA (upper right panel), led to a 50% conversion of substrate into product over an 18-h time period. In contrast, transfection with a cDNA containing the CTX1 mutation led to the detection of a low, but demonstrable level of activity (lower left panel), whereas transfection with a CTX2 cDNA did not result in detectable enzyme activity (lower right panel). Reversion of the CTX2 mutation by site-directed mutagenesis restored the capacity to synthesize a fully active enzyme (data not shown).

To confirm that the transfected cells did indeed express a sterol 27-hydroxylase protein, cell extracts were subjected to immunoblot analysis using an antiserum against a peptide from the predicted protein sequence. In this analysis (Fig. 5), mock transfected cells did not produce any immunoreactive protein (lane 1), whereas cells transfected with the normal cDNA (lanes 2 and 6) or the CTX2-revertant cDNA (lane 4) expressed two immunoreactive proteins corresponding to the precursor (upper band) and mature (lower band) forms of the mitochondrial sterol 27-hydroxylase protein. Both CTX2 (lane 3) and CTX1 (lane 5) cDNAs expressed comparable levels of these two proteins; however, the amount of the mature form appeared somewhat lower in both cases.

The assignment of precursor and mature protein labels to the upper and lower bands, respectively, is based on the observation that a radiolabeled preparation of sterol 27-hydroxylase translated in vitro from synthetic RNA in a rabbit reticulocyte lysate co-migrates with the upper of the two proteins visualized by immunoblotting (data not shown), and on the predicted size of the mitochondrial signal sequence (53 residues) that is removed to generate the mature human enzyme (10). Although not tested formally, we believe that the excess of the precursor form of sterol 27-hydroxylase (Fig. 5) is a consequence of overexpression of the cDNA in the transfected COS-M6 cells. Regardless, the data of Fig. 5 indicate that the low to absent enzyme activity in COS cells transfected with the CTX cDNAs cannot be attributed to lack of expression.

The inheritance pattern of CTX suggests that the defective gene should map to one of the autosomal chromosomes (4). Thus, we next determined the chromosomal location of the sterol 27-hydroxylase gene (CYP27) by Southern blotting analysis of a panel of Chinese hamster × human somatic cell hybrids. The data in Table I, derived with a near full length cDNA probe, indicate that the presence or absence of human chromosome 2 in hybrid cell lines was in perfect concordance with human HindIII (14.5, 13.0, and 6.5 kilobases) and BglII (10.5 and 5.0 kilobases) fragments hybridizing with this probe. All other human chromosomes were excluded by 2 to 7 discordant hybrids.

Regional localization of the CYP27 gene was accomplished by Southern blot analysis of four hybrid cell lines containing
Table I

Correlation of human CYP27 sequences with human chromosomes in rodent x human somatic cell hybrids

The numbers of hybrids that are concordant (+/+) or discordant (+/- or +/-) with the human CYP27 sequence are given for each chromosome. Hybrids in which a particular chromosome was structurally rearranged or present in fewer than 10% of cells were excluded.

<table>
<thead>
<tr>
<th>Hybrids</th>
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<tr>
<td></td>
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<tr>
<td>+/+</td>
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<td>7 9 5 5 6 6 5 5 6 7 4 5 7 5 3 3 6 7 4 5 5 4 2 1</td>
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<tr>
<td>Discordant hybrids</td>
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</tr>
<tr>
<td>Informative hybrids</td>
<td>3 0 4 2 4 4 4 4 3 4 4 2 5 4 7 3 4 5 5 6 4 7 3</td>
</tr>
</tbody>
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Fig. 6. Regional mapping of sterol 27-hydroxylase gene (CYP27) on human chromosome 2. The regions present in four analyzed hybrid cell lines are indicated by vertical bars next to an idiogram of chromosome 2. Southern blot analysis of hybrid cell line DNA using a sterol 27-hydroxylase cDNA probe revealed the presence (+) or absence (−) of the human CYP27 fragments as indicated. The bracket demarks region q33-qter that contains the CYP27 locus.

Defined portions of human chromosome 2 in a Chinese hamster or rat background. The results are summarized in Fig. 6 and indicate that the CYP27 gene maps to the distal portion (q33-qter) of the long arm of chromosome 2. Thus CYP27 is asyntenic with genes for 12 other subfamilies of cytochrome P-450s and with the adrenodoxin and adrenodoxin reductase genes (26, 30, 31). In experiments not shown, the CYP27 gene was similarly mapped to chromosome 1 of the mouse within a large syntenic group of genes that have homologs on human chromosome 2 (32).

Discussion

We report different point mutations in the gene for sterol 27-hydroxylase in two unrelated subjects with CTX and the autosomal localization of this gene to chromosome 2 in the human and chromosome 1 in the mouse.

In patient CTX1, a mutation encoding a cysteine in place of an arginine was found (Fig. 3). The novel cysteine is located at position 446 and is two amino acids away from a ubiquitously conserved cysteine of cytochrome P-450 proteins that serves as a ligand for the heme cofactor (33). In patient CTX2, another arginine to cysteine encoding mutation was found (Fig. 3). In this case, the novel cysteine occurs at position 362 in a sequence that is highly conserved among mitochondrial cytochrome P-450s and is thought to bind the adrenodoxin cofactor (34). Thus, in both cases, the observed mutations are present in functional domains of the sterol 27-hydroxylase and would be expected to disrupt enzyme activity. This expectation was met in expression studies involving cDNAs harboring the CTX mutations (Fig. 4). Interestingly, both CTX mutations occurred in CG dinucleotides, which are thought to be hypermutable in the human genome (35).

Although it is not yet certain that all forms of CTX are caused by mutations in the CYP27 gene, the results presented here, together with previous physiological (36), biochemical (7, 37), and immunochemical results (38) obtained with other CTX subjects, suggest that this conclusion is warranted. On the basis of our data, molecular assays can now be developed for early diagnosis and carrier detection of CTX in populations like Sephardic Jews of Moroccan descent in whom the frequency of this disease is very high (39, 40). Such assays should prove especially valuable in CTX, since early treatment with bile acids can retard or even reverse the course of the disease (41).

The presence of sterol 27-hydroxylase mRNA in a variety of tissues (9) suggests that this enzyme plays a multifaceted role in sterol metabolism that extends beyond the synthesis of bile acids in the liver. The deposition of sterols in the neural tissues of CTX patients together with the occurrence of a progressive neurological dysfunction (4) suggest that this enzyme performs a vital function in the nervous system. Evidence for such a role is bolstered by the observation that other sterol storage disorders that do not involve disruption of the sterol 27-hydroxylase gene, such as sitosterolemia (4), are not characterized by central nervous system defects (42).

Sterol 27-hydroxylase may facilitate reverse sterol transport (43) in neural tissues or participate in the redistribution of sterols among cells of the central nervous system (44). Alternatively, the enzyme may play a key regulatory role in maintaining sterol homeostasis, disruption of which may lead to sterol accumulation (10). Data to support these hypotheses may come from the future analysis of sterol 27-hydroxylase expression in extrahepatic tissues and in other disease states with neurological manifestations.
Acknowledgments—We thank Daphne Davis, Lisa Beatty, and Edith Womack for technical assistance, Helena Dahlback, Henry Danielsson, and Kjell Wikvall for radiolabeled steroids, and S. Anderson, M. S. Brown, J. L. Goldstein, H. H. Hobbs, D. F. Jelinek, and T. C. Sudhof for advice and critical reading of the manuscript.

REFERENCES

43. Glomset, J. A. (1968) J. Lipid Res. 9, 155–167