Molecular Cloning and Characterization of a Full-length Complementary DNA Encoding Human Acid Ceramidase

IDENTIFICATION OF THE FIRST MOLECULAR LESION CAUSING FARBER DISEASE*

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Jürgen Koch‡, Sabine Gärtnert§§, Chi-Ming Li¶, Lothar E. Quintern‡, Katussevani Bernardo***, Orna Levran‡‡, Doris Schnabel§§§, Robert J. Desnick¶¶, Edward H. Schuchman¶, and Konrad Sandhoff†††

From the ‡Institut für Organische Chemie und Biochemie, D-53121 Bonn, Federal Republic of Germany and the ¶Department of Human Genetics, Mount Sinai School of Medicine, New York, New York 10029

Human acid ceramidase ((AC)1 N-acylsphingosine amidohydrolase, EC 3.5.1.23) hydrolyzes the sphingolipid ceramide into sphingosine and free fatty acid. Ceramide is an essential component of all sphingolipids and an important cell-signaling molecule. Moreover, an inherited deficiency of AC activity leads to the lysosomal storage disorder known as Farber disease. Human AC was purified from urine, and 117 amino acid residues were determined by microsequencing. Degenerative oligonucleotide probes were then constructed and used to screen for human fibroblast and pituitary cDNA libraries. Several partial cDNA clones were obtained, and two of these were combined to construct a full-length cDNA containing a 17-base pair (bp) 5'-untranslated sequence, a 1185-bp open reading frame encoding 395 amino acids, a 1110-bp 3'-untranslated sequence, and an 18-bp poly(A) tail. Transient expression of the full-length cDNA in COS-1 cells led to a 10-fold increase in AC activity. In addition, biosynthetic studies carried out in the transfected cells demonstrated that 13-kDa (α) and 40-kDa (β) AB subunits were derived from a common 55-kDa precursor encoded by the full-length cDNA. This protein pattern was identical to that seen in normal human skin fibroblasts. A homoallelic point mutation (T222K) was also identified in the AC gene of a patient suffering from Farber disease, further confirming the authenticity of the full-length cDNA.

Human acid ceramidase ((AC)1 N-acylsphingosine amidohydrolase, EC 3.5.1.23) catalyzes the hydrolysis of ceramide to free fatty acid and sphingosine (1). An inherited deficiency of AC activity leads to the lysosomal storage disorder known as Farber disease (FD), also called Farber lipogranulomatosis (2). Patients with FD accumulate ceramide in most tissues, leading to painful swelling of the joints and tendons, pulmonary insufficiency, and a shortened life-span. The clinical diagnosis of FD is usually confirmed by biochemical methods, including the determination of lysosomal ceramide accumulation and/or the deficiency of AC activity. To date, seven FD subtypes have been described with varying degrees of clinical involvement; notably, a direct correlation between the amount of ceramide accumulation and the clinical severity of FD patients has recently been demonstrated (3).

In addition to its central role in disease pathogenesis, sphingolipid biosynthesis, and membrane formation, ceramide is an important cell-signaling molecule involved in a variety of diverse processes such as neurite growth, monocyte differentiation, and Fas (Apo1/CD95)-induced apoptosis (for reviews, see Refs. 4 and 5). Moreover, sphingosine (the catabolic product of ceramide degradation) has been shown to inhibit protein kinase C activity and can exert a variety of effects on cell growth and differentiation (6–8). Since ceramide degradation is the only catabolic source of intracellular sphingosine (9, 10), AC activity may be the rate-limiting step in determining the intracellular levels of this compound.

About 2 years ago, AC was purified to apparent homogeneity from human urine (11). The final preparation contained a single polypeptide of ~50 kDa that could be resolved into two subunits (designated α (13 kDa) and β (40 kDa)) after treatment with sulfhydryl-reducing reagents. Treatment of the purified enzyme with N-glycanase F reduced the molecular mass of the β subunit to ~29 kDa, whereas the molecular mass of the α subunit remained unchanged. Polyclonal antibodies were raised against the purified urinary enzyme, and the biosynthesis of AC was studied in human skin fibroblasts. Metabolic labeling and immunoprecipitation analyses indicated that both subunits arose from a single precursor of ~55 kDa by proteolytic processing.

This manuscript reports the isolation and characterization of a full-length cDNA encoding human AC and the identification of the first molecular lesion causing FD. The availability of this cDNA should facilitate further molecular genetic analysis of FD patients and stimulate additional studies defining the role of AC in ceramide metabolism, cell differentiation, and mammalian development.

EXPERIMENTAL PROCEDURES

Materials—[3-32P]dATP (>1000 Ci/mmol), [α-32P]dATP (>1000 Ci/mmol), [35S]methionine (>1000 Ci/mmol), [35S]dATP (>3000 Ci/mmol), Amplify®, DNA modifying enzymes, restriction enzymes, Tαq DNA polymerase, DNA polymerase I (Klenow fragment), Sequenase®-ver-
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Aldolase C from Sigma, Dulbecco’s modified Eagle’s medium, fetal calf serum, and the house strain XLI-blue MRF was purchased from Stratagene (Heidelberg, FRG), and additional human cDNA libraries were kindly provided by Drs. H. Okayama (Bethesda, MD), E. Bause (Bonn, FRG), R. Suzuki (Chapel Hill, NC), and W. Stoffel (Cologne, FRG). Reagents and protocols for the synthesis of oligonucleotide probes were from Biosearch and New Brunswick Scientific. All other reagents were of the highest purity available from commercial sources.

Concentration of AC and Amino Acid Microsequencing—The methods used to purify AC from human urine have been previously described (11). To isolate tryptic peptides from urinary AC, 10 mg of the homogenous enzyme were treated with 40 mM dithiothreitol and alkylated using a 4% (v/v) solution of iodoacetamide. This preparation was then hydrolyzed with 10 mg of trypsin, and the resulting peptides were isolated using a HIBAR Lichrosphere, 500 CH-8 (10 μM) HPLC column. Initial separation of the AC tryptic peptides was achieved using a linear 0–70% gradient of acetonitrile in 0.1% trifluoroacetic acid. Individual peptide peaks were collected and further resolved by reversed-phase chromatography in a linear 0–70% gradient of acetonitrile in 25 mM ammonium acetate buffer (pH 6.0). Selected peptides were sequenced by automated Edman degradation using an Applied Biosystems gas phase amino acid sequencer. In addition, a small quantity of each AC subunit was individually isolated by preparative isoelectric focusing and subjected to quencher. In addition, a small quantity of each AC subunit was individually isolated by preparative isoelectric focusing and subjected to quencher.

Oligonucleotide and cDNA Probes—Oligonucleotide mixtures corresponding to amino acid sequences of minimal N-glycosylation were synthesized on an Applied Biosystems DNA synthesizer, analyzed on a Capil-Agate, derivatized with 0.2% PAB ATP and T4 polynucleotide kinase according to the manufacturer’s instructions. cDNA probes were labeled with [32P]PAB ATP using random hexanucleotides and DNA polymerase I (Klenow fragment).

cDNA Library Screenings—About 1014 individual colonies from a human fibroblast cDNA library were initially screened with the AC-specific oligonucleotide mixtures according to the methods described in Sambrook et al. (12). Hybridization was carried out for 16 h at 6 °C below the predicted melting temperature of the respective probes. The membranes were then washed in 0.1 SSC containing 0.1% SDS at 2 °C below the predicted melting temperatures. Partial cDNAs obtained from this screening were authenticated by DNA sequencing and used to screen ~106 individual clones from a human pituitary cDNA library. Hybridization was carried out for 16 h at 50 °C, and the filters were washed in 0.2 × SSC containing 0.1% SDS at 68 °C.

Construction of a Full-length AC cDNA—To construct a full-length AC cDNA, a partial cDNA isolated from the human fibroblast library was used as a template for PCR amplification using sense and antisense AC-specific primers containing KpnI and Sall restriction sites, respectively. The resulting 1131-bp fragment was then cloned into the pSV-Sport 1 mammalian expression vector. Next, a 107-bp 5′-specific AC fragment was obtained by PstI digestion of a partial cDNA obtained from the pituitary library screening and ligated to the pSV-Sport 1 vector containing the fibroblast sequence. DNA sequencing was carried out on the reconstructed full-length cDNA by the dyeoxy chain termination method (13) to confirm the authenticity of the combined sequence.

Sequential Expression of the Full-length AC cDNA in COS-1 Cells—For the transient expression studies, COS-1 cells were grown in 60-mm tissue culture dishes to ~50% confluency. 3 h prior to transfection, Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum was removed, and the cells were supplemented with serum-free Opti-MEM medium. The cells were then transfected with 7 μg of the full-length AC construct per dish using the N-[1-(2,3-Dioleoyloxy)propyl- N,N,N-trimethylammonium methylsulfate lipoethanol reagent according to the manufacturer’s instructions. About 1 h after the liposomes containing DNA, the cells were washed briefly in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. The cells were grown for an additional 48 h and then used for either metabolic labeling studies or harvested by trypsinization and used for the determination of AC activity (see below).

AC Activity and Protein Assays—The methods used for determining AC activity with radioactive ceramide and the quantification of free sphingosine have been previously described (10). Briefly, after incubation of the cell extracts with the substrate, the cellular lipids were extracted and dissolved in methanol, and the free amino groups were derivatized with ortho-phtalaldehyde. After high pressure liquid chromatography elution, the total amount of free sphingosine was determined using a fluorescence detector. For quantification, C16-sphingine was used as an internal standard. Protein was determined according to the method of Lowry et al. (14).

Metabolic Labeling, Immunoprecipitation, and Glycosylation Studies—Prior to metabolic labeling, the transfected COS-1 cells were grown for 3 h in methionine-free minimum essential medium containing 4% dialyzed fetal calf serum. Metabolic labeling was achieved by the addition of 0.1 μCi/ml of [1,5-35S]methionine to the culture medium for 3 h. The labeled cells were then washed once with phosphate-buffered saline, harvested with a rubber policeman, and lysed in 0.5 ml of Triton-buffered saline (pH 7.2) containing 0.5% bovine serum albumin, Nonidet P-40 (1% w/v), EDTA (10 mM), phenylmethylsulfonyl fluoride (2.5 μg), EDTA (1 mM), and leupeptin (1 μg). Cell extracts and media were preadsorbed for 4 h with 20 μl of rabbit preimmune serum and 40 μl of a 50% suspension (w/v) of protein A-Sepharose, and the immune complexes were sedimented by centrifugation (5 min at 500 × g). The supernatants were then carefully removed, 0.8 ml of a crude anti-AC antisera IgG fraction was added, and the mixtures were incubated for an additional 4 h at 4 °C. Immunoprecipitations were carried out in the presence of 15 μl of protein A-Sepharose (50% suspension) for 2 h. For SDS-PAGE, the precipitate was incubated for 3 min at 100 °C in Laemmli sample buffer containing β-mercaptoethanol (10%) (15). Electrophoresis was performed on 10% polyacrylamide gels according to the method of Schagger and Jagow (16). Radioactivity was detected by fluorography. To investigate the glycosylation state of the recombinant AC expressed in the COS-1 cells, the immunoprecipitated enzyme was digested with protein N-glycanase F (300 units) for 24 h at 37 °C in 50 mM sodium acetate buffer (pH 6.0) containing 0.5% (w/v) β-mercaptoethanol.

FD Patient History—Cultured skin fibroblasts from a juvenile FD patient (designated BF1) were kindly supplied by Dr. E. Vamos (Hospital Universitaria Brugmann, Brussels, Belgium). BF1 was a female infant of Belgian origin who was born to consanguineous parents (first cousins). Her detailed clinical history has been reported in Toppet et al. (17). Briefly, BF1 presented with typical FD joint lesions containing subcutaneous nodules, hoarseness, hepatosplenomegaly, and tracheal compression, death occurred at 22 months of age. AC activity in the cultured skin fibroblasts from this patient was ~5% of normal levels (17).

RNA Isolation and Northern Blot Analysis—RNA was isolated using the isothiocyante/cesium chloride method (18). Poly(A) RNA was obtained using type 7 oligo(T)–cellulose, subjected to agarose gel electrophoresis, and transferred to nylon membranes for Northern blot analysis (19). Hybridizations were carried out using the partial fibroblast AC cDNA as a probe.

Isolation of Genomic DNA—Confluent skin fibroblasts from BF1 were washed with phosphate-buffered saline, harvested with a rubber policeman, and then washed again three times with phosphate-buffered saline. After centrifugation (800 × g), the cells were resuspended in 300 μl of phosphate-buffered saline and lysed by repeated freezing and thawing. The cell homogenates were then incubated at 37 °C for 16 h in the presence of proteinase K (0.5 mg/ml final concentration) containing 10% SDS. Genomic DNA was extracted with phenol/chloroform, precipitated with ethanol in the presence of 7.5 μg ammonium acetate, and washed with 70% ethanol. The DNA was then dissolved in water and used for PCR amplification.

PCR Amplification of Genomic DNA and Mutation Identification—To confirm the mutation causing FD in BF1, the genomic AC sequence from exons 8–10 was amplified using the sense and antisense PCR primers 5′-ctgtctttatatgTGAGAAAAT-3′ and 5′-cactttacctTTTTG- TATTTTTC-3′, respectively (lower case and upper case letters indicate intronic and exonic sequences within the intron/exon boundaries). PCR amplification was carried out for 30 cycles (each consisting of denaturation at 94 °C for 5 min, annealing for 2 min at 45 °C, and extension at 72 °C for 13 min) using Taq polymerase. The amplified DNA was

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TABLE I

Synthetic oligonucleotides used for cDNA library screenings derived from tryptic peptides of the purified AC

Letters in parentheses indicate wobble position nucleotides included in the probe mixtures. I, inosine residues; N, each of the four nucleotides.

ACO 1 5′-AA(T/C) ATG ATT AA(T/C) TT(T/C) GTT CC-3′
ACO 2 5′-GGC AAC ACG CTC GGC CCC TTT GAG GAG ATG-3′
ACO 3 5′-GTC GCT GTG GA(A/G) GA(T/C) AA(A/G) AA(T/C) GGT-3′
ACO 4 5′-TAT TA(T/C) GTN CAA ADN AA(T/C) TA-3′
ACO 5 5′-GA(T/C) TG(T/C) CCN GA(T/C) CCN TG(T/C) AT-3′

Concentrated using a Centricron 30 microcentrator and sequenced by the method of Wong et al. (20).

RESULTS

Amino Acid Microsequencing—Seven tryptic peptides were obtained from purified human AC and subjected to amino acid microsequencing. N-terminal sequencing also was carried out on each intact AC subunit. From these efforts, a total of 117 amino acid residues was determined, including the N-terminal region of the β-subunit. Based on regions of minimal codon redundancy, five synthetic oligonucleotides (ACOs) were constructed for cDNA screening (Table I). ACOs 1 and 3 contained inosines in selected wobble positions, while ACO 2 was a unique sequence based on codon usage.

cDNA Library Screenings—About 10⁴ independent clones from a human fibroblast cDNA library were initially screened with ACO 4, and 106 clones were isolated and analyzed. One of these clones hybridized to all five oligonucleotide probes and was therefore selected for further characterization. DNA sequence analysis revealed that the predicted amino acid sequence of this cDNA (designated pACORF1) was colinear with all of the AC amino acid residues determined by microsequencing and that the N-terminal amino acid codon of the β-subunit corresponded to an internal region of the cDNA.

However, despite these observations, analysis of the 5′ region revealed that only 85 amino acid residues were encoded from the first in-frame ATG to the N-terminal codon of the β-subunit. Based on previous analysis of the α-subunit polypeptide (11), which revealed that it was ~13 kDa and non-glycosylated, this appeared to be too short to account for the entire α-subunit sequence. In addition, there were no cysteine residues present in this region (which would be required to form disulfide bonds with the β-subunit), nor was there a signal peptide sequence following the ATG (21). Thus, it was reasoned that this cDNA was missing an authentic 5′ end.

To isolate additional AC cDNAs, an AC-specific probe was generated by PCR amplification of the pACORF1 cDNA (see under “Experimental Procedures” for details), and 10⁶ clones from a human pituitary cDNA library were screened. One of the clones obtained from this screening was identical to pACORF1, except for the 5′ end. In this cDNA (designated pACORF2), the 5′ end encoded an α-subunit containing three cysteine residues and an authentic signal peptide sequence immediately following the first in-frame ATG. In addition, the predicted size of the α-subunit encoded by the pituitary cDNA (~13 kDa) was in good agreement with the previous protein data.

Comparison of the AC fibroblast and pituitary cDNAs with other known sequences in the GenBank and EBI data bases revealed >98% identities with eight partial sequences (accession numbers Z46047, Z19164, Z25930, T68915, T57743, T60639, T53836, and T53857). No known functions had been previously attributed to these sequences, the longest of which was 492 bp.

Construction of a Full-length AC cDNA—To construct a full-length AC cDNA for subsequent expression analysis, 107 nucleotides from the 5′ end of the pituitary sequence were used to replace the 5′ end of the fibroblast sequence (see under “Experimental Procedures” for details). This hybrid cDNA (designated pACFL) was 1205 bp long and contained an open reading frame of 1158 bp encoding 395 amino acid residues (Fig. 1). This corresponded to a protein with a calculated molecular mass of ~44 kDa. There were six potential N-glycosylation sites in the encoded sequence, and the codon representing the N terminus of the β-subunit was located at nucleotide position 427. As noted above, the sequence from the first ATG to the N-terminal codon of the β-subunit encoded a peptide of ~13 kDa, in good agreement with the previous SDS-PAGE data for the α-subunit.

Transient Expression Studies—The reconstructed full-length AC was cloned into the pSV-Sport 1 expression vector and lipofected into COS-1 cells for transient expression. As shown in Table II, cells transfected with the full-length construct had up to 10-fold higher AC activities as compared with untransfected or mock-transfected cells.

Metabolic Labeling of Transiently Transfected COS-1 Cells—COS-1 cells transfected with the pACFL cDNA also were metabolically labeled with [35S]methionine. The labeled cellular proteins were solubilized with detergent and immunoprecipitated with anti-AC antibodies raised against the native enzyme purified from human urine (11). Denaturing SDS-PAGE performed under reducing conditions revealed a 55-kDa precursor form that was processed into a 40-kDa β-subunit and a 13-kDa α-subunit (Fig. 2). Deglycosylation with N-glycanase F reduced the molecular masses of the precursor and β-subunits to ~40 and 29 kDa, respectively, but did not alter the migration of the α-subunit. These findings are identical to those reported previously for human skin fibroblasts (11) and provided additional evidence that the full-length cDNA encoded an authentic AC polypeptide. Compared to metabolically labeled non-transfected COS-1 cells, the amount of immunoprecipitated AC in pACFL transfectants was drastically increased.

Mutation Analysis—Northern blot analysis using poly(A) RNA isolated from normal skin fibroblasts and a patient (BF1) with the juvenile form of Farber disease revealed AC mRNAs of about the same size and hybridization intensity (not shown). Thus, reverse transcriptase PCR and DNA sequence analyses were carried out to identify potential point mutations within the BF1 AC gene. These studies revealed three nucleotide differences in the patient's cDNA as compared with the wild type sequence: 1) the normal A at nucleotide position 214 was substituted by a G in BF1's cDNA, resulting in the predicted substitution of a normal methionine at codon 72 for a valine (M72V); 2) a normal G at nucleotide position 277 was substituted by an A in BF1, resulting in an isoleucine for valine substitution at codon 93 (V93I); and 3) an A at nucleotide position 665 was found in BF1 instead of the normal C, predicting the substitution of a lysine for the normal threonine at codon 222 (T222K).

Analysis of genomic DNA obtained from BF1 and her parents revealed that she was homoallelic for the T222K mutation and that the parents were heterozygous (Fig. 3). In addition, analysis of the genomic DNA from 14 unrelated normal individuals revealed that the M72V and V93I alterations were polymorphisms (not shown).

DISCUSSION

Ceramide plays a critical role in normal cell function and disease pathogenesis. Thus, understanding the metabolism of this lipid is of critical importance to both basic biologists and physicians alike. Toward this end, we report the isolation and expression of a full-length cDNA encoding AC, the enzyme responsible for the hydrolysis of ceramide into free fatty acid.
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Fig. 1. Nucleotide and predicted amino acid sequence of the full-length AC cDNA. The first 107 nucleotides (indicated in **bold**) were derived from the pituitary sequence, while the remaining residues were from the fibroblast sequence. Underlined amino acids indicate N-linked glycosylation sites. The double underlined cysteine residue indicates the N-terminus of the AC β-subunit. The double underlined nucleotides in the 3' untranslated region indicate the three polyadenylation sites.

and sphingosine. Authenticity of this cDNA was based on co-linearity with 117 amino acid residues derived from purified AC, as well as transient expression studies in COS-1 cells. Moreover, a homoallelic point mutation was identified within the corresponding AC cDNA and genomic sequences from a patient with FD, which is the lysosomal storage disorder due to the deficiency of AC enzymatic activity.

Initially, a putative full-length AC cDNA was obtained from...
COS-1 cells were labeled with \[^{35}S\]methionine, and immunoprecipitated proteins after reduction with \(b\)-mercaptoethanol; these were obtained from the cell lysates using anti-AC antibodies. lane 1, immunoprecipitated proteins after reduction with \(b\)-mercaptoethanol; lane 2, the same as in lane 1, except the immunoprecipitated proteins were also treated with \(N\)-glycosidase F.

a human skin fibroblast library. However, several features of this cDNA indicated that it may have been artifactual, including the facts that the first in-frame ATG was not followed by a signal peptide coding region, that there were no cysteine residues encoded within the 5′ region, which presumably contained the \(\alpha\)-subunit sequences (11), and that the molecular mass of the putative peptide encoded by this region did not agree with the known molecular mass (−13 kDa) of the authentic \(\alpha\)-subunit. Analysis of a different AC cDNA isolated from a human pituitary library revealed that the 5′ end was different from that encoded by the fibroblast sequence; this cDNA had an extended 5′ open reading frame including three cysteine residues, an authentic signal coding region, and a 17-bp 5′-untranslated sequence. Except for these 95 nucleotides, the fibroblast and pituitary sequences were identical.

Based on these findings, a hybrid AC cDNA was constructed that contained the 5′ pituitary sequence and the remaining fibroblast sequence. This full-length cDNA consisted of a 17-bp 5′-untranslated sequence, a 1185-bp open reading frame encoding 395 amino acids, a 1110-bp 3′-untranslated sequence containing three polyadenylation sites, and a 17-bp poly(A) tail (Fig. 1). The region from nucleotide 427 (i.e. the N-terminal codon of the \(\beta\)-subunit) to nucleotide 1185 encoded a peptide with a calculated mass of 29 kDa that contained six potential \(N\)-glycosylation sites. The upstream region encoded a putative \(\alpha\)-subunit with a predicted molecular mass of 13 kDa containing three cysteine residues but no \(N\)-glycosylation sites.

Previous studies using purified AC have shown that the human enzyme was a heterodimeric protein consisting of an unglycosylated \(\alpha\)-subunit (−13 kDa) and an \(N\)-glycosylated \(\beta\)-subunit (−40 kDa) (10). Complete deglycosylation resulted in the reduction of the molecular mass of the \(\beta\)-subunit by about 11 kDa, which corresponds to three or four \(N\)-linked oligosaccharide side chains. Biosynthetic studies carried out in cultured skin fibroblasts indicated that both subunits arose from a single glycosylated precursor protein of about 55 kDa by proteolytic processing (11).

After transfection of the full-length AC cDNA into COS-1 cells, metabolic labeling and immunoprecipitation studies using monospecific anti-AC antibodies raised against the purified enzyme revealed a protein pattern identical to that of human fibroblasts. Denaturing SDS gel electrophoresis showed that in the transfected COS cells, a 55-kDa precursor protein was processed into the 40-kDa \(\beta\)- and 13-kDa \(\alpha\)-subunits. Endoglycosidase F treatment resulted in a reduced molecular mass for the precursor and the \(\beta\)-subunit (from 55 to 40 kDa and from 40 to 29 kDa, respectively), whereas the size of the \(\alpha\)-subunit remained unchanged. Furthermore, COS-1 cells transfected with the full-length AC cDNA had up to 10-fold higher activities over the endogenous level of non-transfected and mock-transfected cells. The maximum of AC activity peaked between pH 4 and pH 5 and was not detectable at neutral or alkaline pH values (data not shown). These studies provided additional proof that the protein encoded by the full-length cDNA was AC.

Further evidence was provided by the analysis of the AC sequence obtained from a patient with FD. Three nucleotide changes (M72V, I92V, and T222K) were identified in this patient as compared to the wild type sequence. Analysis of 14 unrelated normal individuals demonstrated that M72V and I92V were normal polymorphisms, whereas T222K was only found in the FD patient. Furthermore, analysis of the consanguineous parents revealed that both were heterozygous for the T222K mutation.

This manuscript reports the first isolation of a full-length cDNA encoding AC and the first identification of a mutation causing FD. The availability of this cDNA allows us to generate the respective animal model and should help us to define a possible role of AC in signal transduction, as well as the identification of various molecular lesions underlying the different clinical subtypes of FD.

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