A Chinese Hamster Mutant Cell Line with a Defect in the Integral Membrane Protein CII-3 of Complex II of the Mitochondrial Electron Transport Chain

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In this study, a respiration-deficient Chinese hamster cell line with a defect in succinate dehydrogenase activity is shown to result from a single base change in a codon in the coding sequence for the membrane anchor protein CII-3 (also referred to as QPs-1). A premature translation stop results in the truncation of 33 amino acids from the C terminus. Bovine cDNA encoding this peptide complements the mutation. There is about 82% identity between these two mammalian proteins. The gene for CII-3 was mapped on human chromosome 1, and because it is also found on minichromosomes characterized by our laboratory, we can localize it on the short arm within 1-2 megabases from the centromere.

A series of respiration-deficient Chinese hamster cell mutants isolated by our laboratory can grow normally in tissue culture as long as an adequate supply of glucose is available for glycolysis (1–3). Depletion of glucose leads to rapid cessation of growth and cell death. The mutants were grouped into seven complementation groups by somatic cell fusions (1), and one, represented by mutant CCL16-B9, was characterized to be almost completely deficient in succinate dehydrogenase activity (SDH)1 (4, 5). This enzyme, which links the reactions of the Krebs cycle to the electron transport chain, is part of a complex of four polypeptides (complex II) in the inner mitochondrial membrane. The active site for the substrate is on the flavoprotein (Ip). The iron-sulfur protein (Ip, 27 kDa) is believed to link it to two small integral membrane proteins (CII-3 and CII-4, 15 and 7–9 kDa, respectively). A B-type heme group is associated with the membrane proteins. Electrons from the oxidation of the substrate in the mitochondrial matrix pass from the Fp via three non-heme iron-sulfur centers in the Ip [([2Fe-2S], [4Fe-4S], [3Fe-4S])] to the integral membrane proteins and from there to ubiquinone. The function of the heme group associated with the membrane proteins is not completely clear (see Refs. 6–9 for reviews).

All four peptides are encoded by nuclear genes in eukaryotic organisms. Thus, the precursor polypeptides are synthesized in the cytosol and subsequently or concurrently imported into mitochondria. Following processing to their mature forms, the biogenesis of a functional complex II requires covalent attachment of flavin to the largest subunit (Fp) (10), formation of the three non-heme iron-sulfur clusters in the Ip subunit, and assembly of the heme with the membrane anchor proteins. Although this complex is the smallest and least intricate of the electron transport complexes in the inner mitochondrial membrane, much remains to be learned about the mechanism or pathway of its assembly.

With the Fp-Ip complex dissociated from the membrane by chaotropic ions, SDH activity can be assayed with artificial electron acceptors such as tetrazolium ions, phenazine methosulfate and dichlorophenol-indophenol, or ferricyanide. Based on the absence of activity in the mutant CCL16-B9, we hypothesized that the defective protein was either the Ip or the Fp subunit (5). It was not known at the time whether these two subunits can assemble independently of the membrane anchors. Somatic cell hybridization experiments had shown that CCL16-B9 is complemented by a gene on human chromosome 1 (11). With probes for the human Ip gene (SDH-2) the Ip gene was mapped to the location Ip36.1-2 (12, 13). However, the complete bovine Ip cDNA failed to complement the SDH-deficient Chinese hamster cell line.2 The somatic cell hybridization experiments also yielded two independent hybrids in which the only human DNA present was in the form of a minichromosome containing the centromere of human chromosome 1, a few megabases of the short arm of chromosome 1, and a portion of the pericentric heterochromatic region on the long arm of chromosome 1 (11, 14–16). The Ip gene was not present on these minichromosomes (12).

Two reports described cloning of cDNA for the membrane anchor protein CII-3 (also referred to as QPs-1) (17, 18). Here, we show that the cDNA from bovine heart complements the defect in the Chinese hamster mutant. The gene for the CII-3 subunit is on human chromosome 1 and on the smallest minichromosome of the somatic hybrid Chinese hamster cell line containing an estimated 1–2 megabases of the proximal short arm. Finally, cDNAs were cloned by RT-PCR from both wild type and mutant hamster cell lines, and the sequence comparison shows that a single base substitution in the mutant creates a premature stop codon.

MATERIALS AND METHODS

Cell Lines and Hybrids—The cell lines used in this study and somatic hybrids derived from the fusion of the Chinese hamster mutant cells with human cells have been described previously (11, 14, 15). CCL16-B1 is a Chinese hamster lung fibroblast from which the SDH-deficient mutant cell line CCL16-B9 was selected (2, 5). Hybrid cell lines XJ M5.1.1(r+) and XJ M12.1.3 were derived by fusing CCL16-B9 cells

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2 P. J. Meijer, unpublished observations.
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with human lymphocytes or HT1080 fibrosarcoma cells (19), followed by selection of respiration-competent cells. Hybrid cell line XJ X5 M5.1.1(−) was isolated as a respiration-deficient segregant, which had lost human chromosome 1. XJ M12.1.3 is one of two independent primary hybrids with a human minichromosome. Secondary hybrids XEW8.2.3 and XEW9.10.4 were isolated after fusing irradiated XJ M12.1.3 hybrids with CCL16-B9 cells and selecting for SDH-positive hybrids. Hela and HT1080 cells served as a source of human genomic DNA.

All cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 5 mM glucose and 10% fetal calf serum. The same medium with galactose instead of glucose (DMEM-GAL) was used to select and maintain respiration-competent cells or hybrids (2, 3).

cDNA and Expression Vectors—A partial cDNA for the CII-{3} subunit of complex II was originally described and generously provided by Dr. Yu (Oklahoma State University) (17). Dr. B. Ackrell (University of California, San Francisco) provided the complete bovine cDNA for the CII-{3} subunit cloned into pUC118 (18). It was excised as an EcoRI fragment and recloned into the mammalian expression vector pcDNA3 (Invitrogen) for complementation analyses.

Transfection Experiments—Cells were grown in DMEM to 50% confluency and transfected using LipofectAmine reagent (Life Technologies, Inc.). The expression vector, containing the CII-{3} cDNA insert and as controls, the same vector with an unrelated cDNA insert or no vector, were used. The conditions for optimal transfection efficiency of the CII-{3} cells were established with the eukaryotic expression vector pCH110 containing the β-galactosidase gene (Pharmacia Biotech Inc.). Selective media, either DMEM with 800 μg/ml G418 or DMEM-GAL, were added 2 or 4 days after the transfection. After 8 days, some plates selected with G418 were switched to medium with DMEM-GAL. Stable transfectants were maintained in DMEM-GAL with 400 μg/ml G418.

RT-PCR and cDNA Cloning—A computer search identified two di- gonucleotide sequences within the coding sequence of the bovine CII-{3} cDNA suitable for PCR and RT-PCR. The sequences for these primers are 9-TGGCAGCCTACAGAGGACACAC-9(QPS1(r)) and 5-CTG-GAGTGAAGACACTTTTATACCGTCC-3(QPS1(f)). Subsequently, only the primer corresponding to the 3′-end of the coding sequence (QPS1(r)) was useful to clone a large portion of the CII-{3} cDNA from wild type and mutant hamster cells by the 5′-RACE protocol (20). PCR products were cloned into the pGEM-T vector (Promega) for direct sequencing. With the sequence information for the partial hamster cDNAs, a complete hamster cDNA was cloned.

Assay of Succinate Dehydrogenase Activity—The assay measuring the succinate-dependent reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride was adapted from Green and Nara-hara (21). The reaction product was quantitated spectrophotometrically after extraction with ethanol. Mitochondria were isolated by differential centrifugation as described previously (2, 5).

Molecular Genetic Techniques—DNA sequencing was performed using the Sequenase 2.0 kit (U. S. Biochemical Corp.) and the SP6 and T7 sequencing primers. Southern and Northern analyses were carried out by standard protocols (22), using probes labeled by the random primer method.

Restriction Enzymes, Isotopes, and other Reagents—Restriction enzymes were obtained from New England Biolabs and used according to the manufacturer’s instructions. [α-32P]CTP was from ICN. All other chemicals were of the highest grade available.

RESULTS

Southern Analysis of Human, Hamster, and Hybrid Genomic DNA with the CII-{3} cDNA Probe—Bovine CII-{3} cDNA was used as a probe in Southern analyses with genomic DNA from hamster and human cell lines and from various human-hamster hybrids. The selection and analyses of the hybrids are as described under "Materials and Methods." In addition to hamster chromosomes, the hybrids contain a small number of human chromosomes. Hybrid XJ X5 M5.1.1(+) contains an intact human chromosome 1, while the hybrid XJ X5 M5.1.1(−) lost the entire chromosome 1 during subsequent culture in nonselective medium (11). Hybrids XJ X12.1.3 and XJ X12.2.2 contain a human minichromosome with a few megabases from the short arm of chromosome 1; the secondary hybrids XEW8.2.3 and XEW9.10.4 were derived from XJ X12.1.3 after irradiation and contain a human minichromosome with 1–2 megabases of the short arm.

The bovine CII-{3} cDNA probe hybridizes with both hamster and human DNA restriction fragments even at relatively high stringency (Fig. 1). Multiple bands are revealed, particularly from the human genome. In the absence of detailed knowledge about the structure of the gene, the existence of exons and introns might account for this observation, but the existence of multiple genes or pseudogenes cannot be discounted. Restriction enzymes were used in these investigations, which do not cut the bovine cDNA (XbaI, EcoRI, PstI). Genomic DNAs from human cells show bands not present in DNA from hybrids containing a human minichromosome, but clearly, there are bands shared between total human DNA and the minichromosome. As expected, all hybrids always contain the DNA fragment of the hamster genome. Results with hybrids XJ M12.1.3, XJ M12.2.2, and XEW9.10.4 (not shown) were identical to those obtained with the XEW8.2.3 hybrid. The data strongly suggest that the gene for the CII-{3} subunit is on the human minichromosome.

Complementation of B9 Mutants with CII-{3} cDNA in Expression Vector pcDNA3—The full-length bovine cDNA for CII-{3} (18) was cloned into the poly-linker of the eukaryotic expression vector pcDNA3 with a strong cytomegalovirus promoter and the bovine growth hormone polyadenylation signal. The vector also contains the neomycin resistance gene with its own promoter. Transfection of this construct into the mutant CCL16-B9 cells was achieved as described under "Materials and Methods." In one selection strategy, cells were exposed to G418 2 days after transfection and maintained in glucose-containing DMEM for the first 8 days. The medium was then changed to DMEM-GAL, medium in which glucose is replaced with galactose. In the other protocol, direct selection of complemented cells was accomplished by transfecting to DMEM-GAL either 2 or 4 days after the transfection.

DMEM-GAL medium selects for respiration-competent cells. Past experience with hybrid selections involving these phenotypes had shown that complementation of the defective mitochondrial function is not instantaneous, since new functional complexes have to be assembled and accumulated in the mitochondria before complex II levels are adequate to support respiration and oxidative phosphorylation (11, 15). Thus, a lag
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FIG. 2. Sequence comparisons of two each (out of four total) CII-3 cDNAs from wild type and mutant Chinese hamster cells. The right nucleotide sequence shows the segment including the C → T transition in the antisense strand. In the sense strand, the corresponding UGG codon (Trp) is converted to the UGA stop codon.

The failure to obtain the 3′-RACE product with the QPS1(f) primer from the bovine cDNA sequence can also be explained because bovine and hamster sequences differ at the nucleotide that happened to be chosen as the 3′-end of this primer.

Restoration of SDH Activity in the Complemented Mutants—Crude mitochondria were isolated from wild type and mutant cells and from cells derived from several individual complemented clones. SDH assays showed that the activity measured with wild type mitochondria was almost totally inhibitable with malonate, indicative of the specificity of the assay for SDH (Fig. 4). As shown previously, CCL16-B9 mitochondria had less than 5% of the activity of wild type mitochondria. In the complemented mutants, the activity was restored to levels ranging from 30 to 50% of wild type.

DISCUSSION

In this study, the respiratory defect in a Chinese hamster mutant cell line lacking succinate dehydrogenase activity has been characterized at the nucleotide sequence level. Second, the corresponding human gene has been mapped near the centromere on human chromosome 1.

A single nucleotide substitution in the coding sequence for one of the membrane anchor proteins of complex II (CII-3, or QPs-1) creates a premature stop codon and hence caused the production of a truncated peptide in CCL16-B9 cells. The mutant peptide is missing 33 amino acids at the C terminus. A structure of the CII-3 membrane anchor protein has been proposed by Yu et al. (17). Its predominant features are three transmembrane segments and a long N-terminal tail extending into the mitochondrial matrix. The N-terminal domain and possibly a loop of 19 amino acids between the second and third transmembrane segment could interact with the Ip subunit and could be responsible for the association of the SDH enzyme with the membrane. The last 20 amino acids have been postulated to form the third transmembrane segment of this integral membrane protein (17). It is also possible that the entire C-terminal domain interacts with the Ip subunit.

The insertion of this short hydrophobic peptide into the inner mitochondrial membrane is not a trivial aspect of the assembly problem. It remains to be established whether the truncated peptide of the mutant is also inserted into the membrane, and if so, whether the topology of the first two transmembrane domains is preserved. From the hydrophobicity plot alone, this major N-terminal domain should still be available for interaction with the Ip peptide. Another question is how membrane insertion of CII-3 may be coupled to the assembly of a heme protein. The membrane anchor protein may interact with the Ip peptide and assist its folding and formation of the iron-sulfur clusters. Iron ions and labile sulfide must associate with the Ip peptide, possibly in a stepwise fashion during formation of the three iron-sulfur clusters. Assembly of a complete complex II apparently is required for the formation of an active Ip-Fp complex with three iron-sulfur centers in the Ip subunit ([Fe-2S], [3Fe-4S], [4Fe-4S]) and a covalently linked flavin on the Fp subunit. After maturation of complex II, the Ip-Fp complex can be dissociated in an active form from the integral membrane proteins by chaotropic ions (6, 25). Attempts to dissociate the Ip and Fp peptides and reconstitute an active SDH activity have been unsuccessful.

We have argued before from studies with yeast mutants that
The availability of a mammalian mutant cell line, which can be complemented with \( C_{II-3} \) cDNA should enable systematic studies of site-directed mutations. Bovine and hamster amino acid sequences differ by almost 18%, but the substitutions are mostly conservative and scattered. We observe 30–50% restoration of SDH activity in complemented cells, with some variation. Differences in the amino acids are indicated in the bovine sequence. The hamster cDNA is missing the two penultimate codons and the stop codon. The boxed segments represent the three putative transmembrane regions predicted by the TMAP algorithm (23).

The cytomegalovirus promoter is a relatively strong promoter, and we detect a transcript in complemented cells that is more abundant than the endogenous transcript (results not shown). Overexpression of the very hydrophobic \( C_{II-3} \) peptide might be deleterious to the cells indirectly causing reduced SDH activity.

The gene for the \( C_{II-3} \) subunit of complex II maps on the short arm of human chromosome 1. The selection of two independently derived primary hybrid cell lines (X M12.1.3, X M12.2.2) with a human minichromosome containing the centromere from human chromosome 1 suggested a close linkage between the complementing gene and the centromere. Even shorter minichromosomes with the complementing gene could be selected in radiation hybrids. Southern analyses of human genomic DNA suggest that there are multiple \( C_{II-3} \) genes or pseudogenes (Fig. 1). The detection of multiple bands with the bovine cDNA probe could be explained by the existence of introns in this gene or by restriction sites present in the human but not the bovine coding sequence. However, the presence of bands in genomic DNA, which are missing from the minichromosome, suggests that more than one gene or pseudogene exist in the human genome. We believe that the locus on the minichromosome (1p12–13) represents an active gene, since the minichromosome has been repeatedly selected in somatic cell hybridization and complementation experiments.

Genomic DNA sequences corresponding to the \( C_{II-3} \) gene must be analyzed to clarify the structure and the nature of these genes. Intron-specific probes and comparisons of whole human genomic DNA with the minichromosome distinguish multiple loci. If more than one active gene exists, one has to address the question of the expression of these genes in various tissues (isozymes), and their expression may be related to the regulation of the capacity for oxidative phosphorylation in different cell types.

Finally, new questions can be raised about the expression of the recessive phenotype in the pseudodiploid Chinese hamster cells in culture. A priori one expects two alleles. One of these may be deleted or silenced by hypermethylation, as demonstrated by us for ornithine decarboxylase-deficient Chinese hamster ovary cells (28), and also in the case of another respiratory-deficient Chinese hamster mutant cell line defective in mitochondrial protein synthesis.3 However, hypermethylated alleles can be de-repressed by treatment of the cells with 5-aza-cytidine, and such attempts have failed with the CCL16-B9 cells. CCL16-B9 cells make normal amounts of \( C_{II-3} \) transcript when compared to their wild type parents (results not shown). All four RT-PCR products derived from mutant RNA had the same mutation, arguing against the presence of two different mutated transcripts. Therefore, the second allele in these pseudodiploid hamster cells must be deleted or permanently inactivated.

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