Cloning and Identification of Hepatocellular Carcinoma Down-regulated Mitochondrial Carrier Protein, a Novel Liver-specific Uncoupling Protein*

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We report the identification of a novel cDNA fragment that shows significantly reduced expression in cancerous tissue compared with paired non-cancerous liver tissue in patients with hepatocellular carcinoma (HCC). The full-length transcript of 1733 bp encodes a protein of 308 amino acids that has all the hallmark features of mitochondrial carrier proteins. We designate the novel protein as HDMCP (HCC-down-regulated mitochondrial carrier protein). The HDMCP orthologs in human, mouse, and rat are found to exhibit close similarity in protein sequence and gene organization, as well as exclusive expression in the liver. Moreover, conserved syntenic regions have been demonstrated at the HDMCP gene locus in the human, mouse, and rat genome. Taken together, we suggest that HDMCP might have a conserved and unique biological function in the liver. Overexpression of HDMCP in transiently transfected cancer cells results in the loss of staining by MitoTracker dye, indicating that HDMCP could induce the dissipation of mitochondrial membrane potential (ΔΨm). However, HDMCP-mediated disruption of ΔΨm is not related to mitochondrial permeability transition or apoptosis. In addition, we further demonstrate that the dissipation of ΔΨm is accompanied by significant reduction of cellular ATP in 293T cells overexpressing HDMCP or uncoupling protein 2 (UCP2). Our present findings suggest that HDMCP might be one of the long postulated uncoupling proteins that catalyze the physiological “proton leak” in the liver. The down-regulation of HDMCP in HCC cancer cells might result in the elevation of ΔΨm, a common phenomenon found in cancer cells.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY569438, AY570298, and AY603424.

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† The abbreviations used are: HCC, hepatocellular carcinoma; HD-MCP, HCC down-regulated mitochondrial carrier protein; UTR, untranslated regions; ΔΨm, mitochondrial membrane potential; PTP, permeability transition pore complex; UCP, uncoupling protein; ROS, reactive oxygen species; DLCs, delocalized lipophilic cations; nt, nucleotide; aa, amino acids; RT, reverse transcription; mAb, monoclonal antibody; PBS, phosphate-buffered saline; TUNEL, terminal dUTP nick-end labeling; RACE, rapid amplification of cDNA ends; pAb, polyclonal antibody.

Hepatocellular carcinoma (HCC) is one of the most frequent neoplasms worldwide. It has been suggested that chronic hepatitis B and C virus infection, dietary aflatoxin, alcohol consumption, and hepatic iron overload are the etiological factors for HCC development. However, the pathogenic mechanisms responsible for HCC are still not well defined.

Mitochondrial defects caused by altered expression of respiratory chain subunits and glycolytic enzymes, decreased oxidation of NADH-linked substrates, as well as mitochondrial DNA mutations have been proposed to contribute to the development and progression of various cancer types including HCC (1–5). It has long been recognized that the most well known function of mitochondria is the production of ATP through oxidative phosphorylation. During mitochondrial respiration, the transfer of electrons along the respiratory chain in the inner mitochondrial membrane is coupled to the translocation of protons (H+) from the mitochondrial matrix into the intermembrane space. This process generates a proton electrochemical gradient across the inner mitochondrial membrane, which is known as the proton-motive force. This proton-motive force is used to drive the protons back into the matrix through F0F1-ATP synthase resulting in the synthesis of ATP (6, 7). Alternatively, the proton-motive force can also be dissipated by “proton leak” catalyzed by multiple parameters, such as classical uncouplers of oxidative phosphorylation (8, 9), fatty acids (10), and the uncoupling protein 1 (UCP1) of brown adipose tissue (11, 12). The mitochondrial membrane potential (ΔΨm) arises from the net movement of positive charge across the inner mitochondrial membrane, reflecting the balance between processes that contribute to the generation of the proton gradient and those that consume it (7).

In the early 1980s, Chen and co-workers (13–17) discovered that relative to the mitochondria in normal cells, those in cancer cells displayed a greater uptake and retention of rhodamine 123, a ΔΨm-dependent staining dye, suggesting that cancer cells generate higher ΔΨm compared with normal cells. This is in agreement with a later report showing that ΔΨm of carcinoma cells is ~ 60 mV higher than that of control epithelial cells (18). However, to date, no real understanding of the biochemical basis has been proposed for this well defined observation for cancer cells.

We report here the isolation and characterization of a novel protein that might be one of the long postulated liver tissue-specific uncoupling proteins. It bears all the hallmark features of the mitochondrial carrier proteins and is significantly down-regulated during the development of HCC. We designate this novel protein as HCC-down-regulated mitochondrial carrier protein (HDMCP).
Liver-specific Uncoupling Protein

EXPERIMENTAL PROCEDURES

**Tissue Specimens**—Paired samples of cancerous and non-cancerous liver tissue of a total of 52 patients undergoing hepatic resection as treatment for HCC were retrieved from the National Cancer Centre Tissue Repository. Resected tissue was immediately snap-frozen in liquid nitrogen and samples were written informed consent from the patients. Among the 52 pairs of HCC samples, 18 were randomly selected for spotted array analysis, 7 for real time reverse transcription (RT)-PCR analysis, and 27 for Affymetrix analysis.

**Cell Culture**—HepG2, Hep3B, WRL68, SNU-182, SNU-398, SNU-449, and SKHeP1 were obtained from the American Type Culture Collection. HuH7, HLE, PLC/PRF/5 and HuH6, clone 5 (HuH6-C5), were derived from the Japanese Liver Research Resources Center (a nasopharyngeal carcinoma cell line) has been described earlier (19). Human embryonic kidney 293T cells were obtained from Dr. Paula Lam (National Cancer Centre, Singapore). All cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin/streptomycin (100 units/100 μg/ml), 2 mm L-glutamate, 0.1 mm nonessential amino acids, and 1 mm sodium pyruvate in a 37 °C humidified incubator with 5% CO₂.

**Subtracted cDNA Library Construction and Screening by Micro-arrays**—Two reciprocal subtracted cDNA libraries were generated between cancerous and non-cancerous liver tissue from an HCC patient using a cDNA synthesis and plasmid cloning kit (Invitrogen) according to the recommended protocol with some modifications. Briefly, tracerc DNA was hybridized with biotinylated driver mRNA prepared by PhotoProbe Blot Labeling/ Detection kit (Vector Laboratories). The removed RNA and hybrid was concentrated by extraction twice with VECTREX Avidin D (Vector Laboratories). Second strand DNA of the subtracted cDNA was then generated by random priming, ligated to pSP6 T1 and transformed into Epicurian Col XI-2-blue ultracompotent cells (Stratagene).

A total of 789 cDNA clones, including clone C78, the gene focus in the present study, were derived from these two libraries. These clones were PCR-amplified, purified, and spotted in duplicate onto glass slides using the GMS 417 microarrayer (Genetic Microsystems). 10 μg of total RNA were labeled and hybridized to the arrays by using the two-step 3DNA assay expression array detection kit (Genisphere Inc). RNA from a normal liver sample is served as a reference in each hybridization. The arrays were scanned using the GMS 418 array scanner (Genetic Microsystem) and analyzed using ImageGene 4.0 software (Biodiscovery, Los Angeles). Signal intensities of Cy5 (test samples) and Cy3 (reference) were normalized by applying a scale factor to all intensities measured for the Cy5 channel to generate a Cy5/Cy3 intensity ratio of 1 in the majority of the spot elements. The calibrated Cy5/Cy3 ratios represented relative gene expression.

**RNA Isolation, Real Time RT-PCR, and Northern Blot Analysis**—Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized with random primers (Invitrogen) and oligo(dT)₁₂₋₁₇ primers (Invitrogen) using SuperScript II reverse transcriptase (Invitrogen). Quantification of C78 and 18 S rRNA expression was performed on a RotorGene real time PCR machine (Cobett Research, Australia) using QuantiTech™ SYBR Green PCR kit (Qiagen). The primer sequences were as follows: C78-RF and C78-R (Fig. 3) for C78 and 5'-CCCTGCGCCCTATTTGACTC-3' and 5'-CCCTGAGGAGCTGATGAG-3' for 18 S RNA. All PCRs were performed in duplicate. Standard curves of C78 and 18 S rRNA were generated independently by 10X serial dilution of template DNA. The relative copy number of each sample was calculated according to the corresponding standard curve using RotorGene version 4.6 software. Normalization was performed in each sample by dividing the copy number of C78 to that of 18 S RNA. The relative expression levels were calculated by arbitrarily designating the lowest normalized value to 1.

A human multiple tissue Northern blot (Clontech) was hybridized with probes prepared by purified C78 and β-actin PCR product and labeled using Strip EZ™ DNA kit (Ambion) in Ultragene (Ambion). Autoradiography was conducted on Kodak BioMax MR film (Eastman Kodak) at −80 °C.

**GeneChips Analysis**—5 μg of total RNA was used to generate biotinylated cRNA, followed by hybridizing to Affymetrix HG-U133A and -B GeneChips according to the manufacturer’s recommendations. After scanning, the average intensity for the genes in total was normalized to 100. Probe ID representing HDMCP was selected. After scanning, the average intensity for the genes in total was normalized to 100. Probe ID representing HDMCP was selected.

**Cloning of a Full-length HDMCP cDNA and mRNA Sequencing**—C78 sequence was searched against the EST data base by the BLAST program and was found to be highly homologous to EST clone (AA677293). The EST sequence was found to be located at position 137,866 to 138,343 in BAC clone R-63812 (AL157871) using the BLAST program. The GENSCAN web server at the Massachusetts Institute of Technology (genes.mit.edu/GENSCAN.html) was employed to predict putative genes in an ~13 kb of DNA sequence encompassing the EST sequence from BAC clone R-63812. A putative gene was predicted to encode a protein of 323 amino acids (aa). cdna was generated from total RNA derived from a normal liver sample by random priming using SuperScript II reverse transcriptase (Invitrogen), as well as Thermoscript reverse transcriptase (Invitrogen), according to the manufacturer’s instructions. RT-PCR product generated by C78-F and C78-R primers (Fig. 3), designed according to GENSCAN-predicted exons, was confirmed by sequencing. In order to get the full-length sequence of C78, 5’-rapid amplification of cDNA ends (5’-RACE) was performed using the FirstChoice™ RLM-RACE kit (Ambion) according to the manufacturer’s instructions. cDNA was first amplified with a 5’-RACE primer (provided in kit) and C78RC-OR primer (Fig. 3), followed by a second PCR using a nested 5’-RACE inner primer (provided) and C78RC-IR primer (Fig. 3). The transcription initiation site was mapped by sequencing the PCR products that were cloned into the pCR2.1-TOPO vector (Invitrogen). DNA sequencing was performed on an ABI Prism 377XL automated DNA sequencer using ABI PRISM BigDye Terminator cycle sequencing Ready Reaction kit (Applied Biosystem).

**In Silico Cloning of HDMCP Orthologous Gene in Mouse and Rat, as Well as Determining Gene Organization and Conserved Synteny Regions**—HomoloGene data base from the National Center for Biotechnology Information (NCBI) shows possible homologous genes of HD-MCP in the mouse and rat species. First, the cDNA and protein sequences of HDMCP orthologous genes derived from AY569438 (human), BAF15527 (mouse), andXM_245451 (rat) were analyzed by multiple sequence alignment using ClustalW version 1.8 available at GeneNet service at Kyoto University Bioinformatics Center (clustalw.genome.ad.jp). Based on the conserved protein sequences in HD-MCP orthologs, and later confirmed by PCR and autosequencing, we concluded that the first 9 aa of protein sequence encoded by exon 1 in the rat ortholog was replaced by a stretch of unrelated 17 aa in XM_245451 (rat), the mouse ortholog was used as a template to align an ~7-kb rat genomic DNA sequence encompassing the sequence of XM_245451, to assist in determining exon 1 with a start codon, and assembling the 5’- and 3’-untranslated regions (UTR). Furthermore, gene organization of each ortholog was determined by BLAST 2 sequence algorithm using cdna sequences and their corresponding genomic DNA sequences, together with the consensi rule for splice sites, which are dinucleotides GT and AG next to the 5’ and 3’ intron boundaries.

In order to determine the syntetic regions at the HD-MCP gene locus in human, mouse, and rat genomes, the map location of human, mouse, and rat HD-MCP orthologs was first identified. The gene content of the genomic region flanking the HD-MCP gene was determined by using the map viewer from the NCBI Genome browser and compared with mouse chromosomes. The regions of synteny between human, mouse, and rat chromosome were then determined by analyzing and comparing each gene using HomoloGene data base from the NCBI.

**Construction of HDMCP-FLAG and Uncoupling Protein 2 (UCP2)-FLAG Protein**—HD-MCP was first amplified from normal liver RNA with specific primers HD-MCP-F and HD-MCP-R (Fig. 3). The PCR product was then used as a template to generate the HD-MCP-FLAG protein using two additional steps of PCR. HD-MCP-F was the forward primer for both PCRs. A FLAG peptide tag (KDDDKDYK) at the C terminus of the fusion protein was first generated by the reverse primer containing the 3’-end of the HD-MCP coding sequence and part of the FLAG sequence (5’-GTGGGTCACTTGGTAACTCTGTGGACGACCGCCGGC-3’; the FLAG sequence is underlined). The second PCR was then performed using the reverse primer containing a stop codon and part of the FLAG sequence that overlapped with the reverse primer from the first PCR (C-FLAG, 5’-GCCCCATTTTATGTCCGTCACCTT-3’; the stop codon is italic, and the FLAG sequence is underlined). The UCP2-FLAG protein was generated by a similar approach from cdna of human skeletal muscle. Forward primer UCP2-F (5’-ATCATGGTTCCAGACGATGTGAGCCCAAGACGACCGCCGGC-3’) was used together with a reverse primer (5’- GTGGGTCACTTGGTAACTCTGTGGACGACCGCCGGC-3’; the FLAG sequence is underlined) and second PCR reverse primer (C-FLAG, same as above). Platinum Pfx polymerase (Invitrogen) was used for all amplifications. The first five cycles of amplification were programmed as 94 °C for 3 min, 60 °C for 1 min, and 72 °C for 1 min. The remaining 25 cycles were programmed as 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. A single deoxyadenosine (dA) was

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added to the 3’-end of the final PCR products by Taq polymerase (Promega) and cloned into pcCR2.1-TOPO vector (Invitrogen). Positive clones were confirmed by sequencing. Subcloning into pcDNA3 (Invitrogen) was performed by restriction enzyme digestion and standard ligation protocols. The gene orientation was verified by restriction enzyme digestion.

Transfection, Immunostaining, and $\Delta F_m$ Detection—Cells grown on coverslips in a 6-well tissue culture plate were transfected with 2 µg of pcDNAs/HDMCP-FLAG plasmid using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. For time course analysis of HDMCP expression, cells were stained at 4, 6, and 24 h after transfection. For $\Delta F_m$ restoration studies in HDMCP-overexpressed cells, fresh media containing test compound (either 5 µM bongkrekic acid, or 50 µM bongkrekic acid, or 5 µg/ml oligomycin (Sigma)) were added after the cells were transfected for 2 h and incubated for another 22 h. To determine the $\Delta F_m$, cells were incubated in media containing 250 nm Mitotracker Green (Molecular Probes) at 37 °C for 30 min. Cells were then fixed with 3.7% formaldehyde in culture media at 37 °C for 10 min and permeabilized in 0.1% Triton X-100 at room temperature for 5 min. After washing with PBS, nonspecific staining was blocked with 10% normal goat serum in PBS at room temperature for 1 h. The cells were then incubated overnight with anti-FLAG monoclonal antibody (mAb) (Stratagene) at 4 °C. After incubation at room temperature for 1 h in Alexa Fluor 488-conjugated goat anti-mouse antibody (Ab) (Molecular Probes), coverslips were washed in PBS and mounted onto slides with 4.6-diamidino-2-phenylindole containing VECTASHIELD (Vector Laboratories). Slides were examined using the LSM-510 laser-scanning confocal microscope (Carl Zeiss). In addition, cells were incubated with anti-FLAG polyclonal antibody (mAb) (Sigma) and anti-mitochondrial mAb (marker) or anti-cytochrome c mAb (Promega) 24 h post-transfection. Alexa Fluor 488-conjugated goat anti-rabbit Ab and Alexa Fluor 546- or Alexa Fluor 633-conjugated goat anti-mouse Ab were used for primary Ab detection.

TUNEL Assay—In situ cell death detection kit (Roche Applied Science) was used to detect apoptotic cells at day 3 after transfection in Hep3B cells. Briefly, cells were fixed with 3.7% formaldehyde for 15 min at room temperature, rinsed with PBS, and permeabilized in 0.1% Triton X-100, 0.1% sodium citrate for 2 min on ice. Cells were then treated with TUNEL reaction mixture in a humidified chamber at 37 °C for 1 h in the dark, followed by rinsing three times with PBS. To visualize HDMCP protein, cells were incubated with anti-FLAG mAb followed by incubation of Alexa Fluor 488-conjugated goat anti-mouse Ab.

Measurement of ATP and ADP/ATP Ratio—293T cells in 6-well plates were transfected with pcDNA3/HDMCP-FLAG, pcDNAs/UCP2-FLAG, or pcDNA3 vector alone, using Lipofectamine with PLUS reagent according to the manufacturer’s instructions. Cell pellets were collected 24 h after transfection and resuspended in Nucleotide Releasing Buffer (NRB). The ATP and ADP were assayed by a luminometric method using ApoSENSOR™ ADP/ATP ratio assay kit (Biovision). To measure the ATP level, 1 µl of ATP-monitoring enzyme diluted in 50 µl of NRB was added to 100 µl of cell lysate and read immediately with a 10-s integration on a LumatLB 9507 (Berthold technologies) (data A). To measure the ADP level, the sample was read again after 10–15 min (data B). 1 µl of ADP-converting enzyme diluted in 50 µl of NRB was then added and read immediately (data C). The ADP level was calculated as data C – data B. The background-subtracted luminescence values (relative light units) were normalized for total protein in each sample. In order to compare the results of eight independent transfections, the relative light units/µg of protein of vector control was set as 100% to compare the changes of ATP, ADP, and ADP/ATP ratio in HDMCP-overexpressed cells.

Statistical Analysis—The statistical differences between groups were analyzed using Wilcoxon Signed-Ranks test for paired samples. Two-tailed $p$ values of <0.05 were considered to be statistically significant.

RESULTS

Identification of a Significantly Down-regulated cDNA Fragment in Cancerous Liver Tissue Compared with Paired Non-cancerous Liver Tissue from HCC Patients—We employed spotted microarrays to screen a total of 789 cDNA clones derived from two reciprocal subtracted cDNA libraries generated from cancerous and paired non-cancerous liver tissue from an HCC patient. Among the differentially expressed cDNA clones, C78 was found to be the most significantly and consistently down-regulated novel cDNA fragment in the cancerous tissue compared with paired non-cancerous liver tissue in a total of 18 pairs of HCC samples. Fig. 1A shows the significant down-regulation of C78 in cancerous tissue of 18 pairs of HCC samples using spotted microarrays for differential cDNA library screening. Real time RT-PCR using seven independent pairs of HCC samples demonstrated 3–460-fold reduction of C78 gene expression in the cancerous tissue. This represents relative expression levels ranging from 115 to 725 copies in non-cancerous tissue compared with 1–40 copies observed in cancerous tissue (Fig. 1B). These results were further confirmed when 27 independent pairs of HCC samples were studied using Affymetrix GeneChips showing the median expression level in cancerous tissue was significantly lower than that in non-cancerous liver tissue (101 versus 1800 intensity unit; $p = 0.001$, Wilcoxon Signed-Ranks test) (Fig. 1C).

We investigated the endogenous expression of HDMCP in HCC cell lines compared with a normal liver sample using real time RT-PCR. C78 was shown to be significantly down-regulated in the HCC cell lines compared with that of the normal liver sample. After normalization to 18 S rRNA for an equal amount of template loading, the relative expression levels were shown as follows: normal liver (~50,000 copies); HepSB, HuH6-C5, and HepG2 (~230–265 copies); SNU-499 (~160 copies); HuH7 and WRL68 (~25–40 copies); PLC/PRF/5, HLE, SNU398, SKHepl, and SNU186 (~1–10 copies).

Northern Blot Analysis Revealed the Exclusive Expression of C78 in Liver Tissue—C78 cDNA fragment probe was employed to poly (A)⁺ RNA isolated from 12 different human tissue types in order to determine the tissue distribution of C78. Fig. 2 shows that the C78 mRNA transcript of ~1.8 kb could be detected predominantly in the liver. No signal could be detected in the other tissues examined.
Liver-specific Uncoupling Protein

Cloning of Full-length C78 and the Detection of an Alternatively Spliced C78 Transcript—The initially isolated C78 cDNA fragment contains 197 nucleotides (nt) plus a poly(A) tail. We first extended the sequence of C78 to 477 nt by identifying a highly homologous EST clone, IMAGE clone 454792 (AA677293). By using the GENSCAN program, a putative gene could be predicted within the stretch of an ~13-kb DNA sequence encompassing the 477-nt DNA sequence from the BAC clone R-63812 (AI157871). As predicted, a 1.5-kb DNA fragment was generated by RT-PCR by using primers designed from the GENSCAN-predicted gene.

5′-RACE using cDNA generated by both SuperScript II and ThermoScript reverse transcriptase amplified a consistent product of ~350 bp. However, the latter template generated an additional band of ~420 bp (data not shown). According to the sequences of both DNA fragments, they shared identical transcription initiation sites, and their 3′-end sequence overlapped with the 1.5-kb fragment mentioned above. After comparing to the human genomic DNA sequence, the shorter fragment was subsequently found to comprise exons 1–4, whereas the longer fragment was found to splice at a different 3′-splice acceptor in intron 2 which therefore generated an additional stretch of 61 nt between exons 2 and 3 (Fig. 3). We concluded that besides obtaining the full-length C78 cDNA of 1733 bp (AY569438), we had also identified an alternatively spliced transcript (AY570298). The predicted full-length product is consistent with the 1.8-kb transcript identified in the liver mRNA (Fig. 2).

Nucleotide sequence analysis revealed that the full-length C78 cDNA comprised an open reading frame for a polypeptide of 308 aa and the 5′- and 3′-UTR of 73 and 733 nt in length, respectively (Fig. 3). The alternative splice form, however, was found to encode a peptide of only 30 aa in length due to a premature termination codon present within the additional 61-nt stretch (AY570298).

Several cDNA sequences in UniGene Cluster Hs.108268 (chromosome 14 open reading frame 68, C14orf68) were found to have a coding region identical to the C78 cDNA sequence but with minor variations of DNA sequences at the UTR. The C78 cDNA identified in the present study (AY569438) has longer 5′- and 3′-UTR in comparison to the reported cDNA sequence (BX248260). The other two cDNA sequences (XM208731 and AK009201) both have a stretch of 97 nt missing at the 3′-UTR.

C78 Is a Mitochondrial Carrier Protein and Is Tentatively Designated as HDMCP—Sequence analysis reveals that the 308-aa polypeptide encoded by the full-length C78 clone is highly similar to mitochondrial carrier proteins. Fig. 4 shows the comparative alignment of the deduced amino acid sequence of C78 and three human mitochondrial carrier proteins including C14orf69 (Q8N8R3), mitochondrial carnitine/acylcarnitine carrier protein (CAC) (O43772), and mitochondrial glutamate carrier 1 (GHC1) (Q9H3J6). Like the other mitochondrial carrier proteins, C78 comprises three highly conserved homologous tandem repeats of ~100 aa each with the typical mitochondrial carrier signature GXXGXXP(D/E)XII(IV)/X(1-3) RXKXQGXXA. Each repeated element is made of two anti-parallel transmembrane hydrophobic α-helices (Fig. 4, open boxes) linked by an extensive hydrophilic region (Fig. 4, solid line). The three tandem repeats are linked by short extramembranous hydrophilic loops (Fig. 4, dashed line). Thus a total of six hydrophobic segments (Fig. 4, denoted as TM1 to TM6) are capable of being folded into membrane-spanning α-helices with the three long hydrophilic segments connecting the two α-helices in each repeat protruding into the mitochondrial matrix (21–23). The tripartite structure and the similarity of its hydrophobic profile to those of the other mitochondrial carrier proteins clearly indicate that C78 belongs to this superfamily. Tentatively, we designated C78 as HDMCP (Hepatocellular carcinoma-down-regulated mitochondrial carrier protein).

Within the mitochondrial carrier protein family, HDMCP shows the highest similarity (37%) to a putative mitochondrial carrier protein encoded by the C14orf69 gene. Similar to C14orf69 protein but unlike most of the other family members, the protruding N terminus of the polypeptide chain is absent in HDMCP (Fig. 4), indicating that the N terminus is embedded in the inner membrane of the mitochondria.

It was also noticed that the hydrophobic loop between TM3 and TM4 in the HDMCP protein was of comparable length to the glutamate/H+ symporter carrier but was extraordinarily long when compared with most of the mitochondrial carrier proteins (Fig. 4). A unique stretch of protein sequence in the hydrophilic loop of HDMCP (Fig. 4, shown in italics) was found to have the consensus cAMP- and cGMP-dependent protein kinase phosphorylation site at Ser137 (Fig. 4, shown in bold) when analyzed with the ScanProsite software at the ExPASy server (www.expasy.ch/cgi-bin/scanprosite).

Gene Conservation between the Human HDMCP Genes and Their Mouse and Rat Orthologs—Based on NCBI data bases, UniGene clusters, and HomoloGene orthologs, the putative HDMCP orthologs were found in the mouse species (UniGene Cluster Mm.202653, expressed sequence AJ876593) and in the rat species (LocusLink LOC299316, expressed sequence AI876593) and in the UniGene clusters, and HomoloGene orthologs, the putative mouse and rat orthologs are highly homologous to those encoded by the human HDMCP cDNA sequence (87.3% identity).

On the other hand, based on the conserved protein sequences in HDMCP orthologs, we concluded that the first 17 aa of protein sequence encoded by rat cDNA sequence (XM_234551) in LOC299316 was an unrelated amino acid sequence. Further investigation revealed the wrong prediction of exon 1 in this sequence entry. We have identified the putative exon 1 with a start codon and coding region encoding the first 9 aa, which was identical to that of the mouse ortholog. In addition, 579 bp of 3′-UTR in exon 6 is missing in this rat cDNA sequence (AY003424).

Both the mouse and rat ortholog encoded 310 aa. Fig. 5A showed the conserved protein sequence in human, mouse, and rat orthologs of HDMCP. The polypeptide encoded by human HDMCP is highly homologous to the mouse ortholog (87.3% identity) and the rat ortholog (86.0% identity). Moreover, the
similarity is even higher between mouse and rat orthologs (94.8% identity).

A comparison of the gene organization of the HDMCP ortholog from human ([H11011]7 kb), mouse, and rat species (both [H11011]5.7 kb) revealed the remarkable conservation of splice sites in all the six exons (data not shown). Identical lengths of exons at the coding regions were observed in all orthologs except for exon 5 of the human ortholog (Fig. 5B).

The fact that 100% of 26 human EST clones in Hs.108268 as well as 73% of the 68 mouse EST clones in Mm.202653 were derived from liver tissue suggests that both human and mouse HDMCP orthologs are liver tissue-specific. This observation is consistent with our Northern blot analysis of human tissues and demonstrated the exclusive expression of HDMCP in liver tissue (Fig. 2).

Synteny between the Human HDMCP Genes and Its Mouse and Rat Orthologs—We have characterized a large region of the human, mouse, and rat genomes flanking the HDMCP gene locus, and we revealed conserved syntenic regions at human chromosome 14q32.31, mouse chromosome 12F1, and rat chromosome 6q32. The order of genes that map proximal or distal to the HDMCP gene locus is highly conserved (Fig. 6). The human chromosomal region comprises 10 genes in the order EVL-C14orf66-YY1-C14orf69-HDMCP-WARS-MGC4645-KIAA1446DLK1-MEG3 and appears to exhibit conserved synteny homology with the corresponding mouse and rat regions as shown in Fig. 6.

HDMCP Localized in Mitochondria—To demonstrate the subcellular localization of HDMCP, we transiently transfected an expression plasmid pcDNA3/HDMCP-FLAG into Hep3B cells. Confocal microscopy revealed the punctate distribution of the FLAG-tagged HDMCP protein in the cytoplasm of the transfected cells (Fig. 7A, green cells, indicated by arrows). As expected, the FLAG-tagged products (green) colocalized with a mitochondrial marker (Fig. 7B, blue) that showed a cyan color in the merged image (Fig. 7D), confirming that expression of HDMCP is in the mitochondria.

Overexpression of HDMCP Induced the Loss of ΔΨm—To address the function of HDMCP, Hep3B cells transfected with pcDNA3/HDMCP-FLAG were stained with MitoTracker OrangeTM, a ΔΨm-sensitive dye (24). We showed that all mitochondria of the nontransfected cells stained red, whereas transfected cells overexpressing HDMCP-FLAG were totally devoid of MitoTracker staining (Fig. 7C, indicated by arrows). The merged confocal images of FLAG (Fig. 7C, green), mitochondria (blue), and MitoTracker (red) revealed that HDMCP-FLAG colocalized with mitochondria but not with MitoTracker in the transfected cells (Fig. 7D, cyan). In nontransfected cells, mitochondria colocalized with MitoTracker but not FLAG (Fig. 7D, magenta). These data suggested that HDMCP could induce the dissipation of ΔΨm in the transfected cells.

Consistent with the above observations, overexpression of HDMCP in the transfected HepG2 cells and CNE-2 cells also...
showed the exclusion of MitoTracker staining in the mitochondria (data not shown). For cells transfected with vector control or with unrelated genes tagged with FLAG, all showed the staining of mitochondria with MitoTracker as shown by the green staining (Fig. 9, C, arrows). However, the majority of the mitochondria still retained cytochrome c staining as shown by the red staining (Fig. 9, D, arrows). The HDMCP-overexpressed cells showed the exclusion of MitoTracker staining as an indication of the loss of \( \Delta \Psi_m \) in the mitochondria as shown by the green staining (Fig. 9, A, green). Colocalizations of the staining of HDMCP (Fig. 9C, arrows) and cytochrome c (Fig. 9A, green) appeared as yellow in the superimposed image (Fig. 9C, arrows). The HDMCP-overexpressed cells showed the exclusion of MitoTracker staining as an indication of the loss of \( \Delta \Psi_m \) (Fig. 9E, arrows). However, the majority of the mitochondria still retained cytochrome c in the mitochondria as shown by the green staining (Fig. 9, D and E, arrows).

To investigate whether the destabilization of \( \Delta \Psi_m \) was directly related to the opening of the permeability transition pore complex (PTP) that resulted in mitochondrial permeability transition, the HDMCP-overexpressed cells were treated with the two PTP inhibitors, cyclosporin A and bongkrekic acid. Both PTP inhibitors were not able to prevent the exclusion of MitoTracker in HDMCP-overexpressed cells (data not shown), suggesting that the loss of \( \Delta \Psi_m \) was not related to the opening of the PTP complex.

The Loss of \( \Delta \Psi_m \) in Most of the HDMCP-overexpressed Cells Could Not Be Restored by Oligomycin—It was observed that
most of the HDMCP-overexpressed cells maintained a loss of \( \Delta \Psi_m \) following treatment of oligomycin, a \( \Delta \Psi_m \) stabilizer that inhibits the action of F0F1-ATPase (data not shown). At the same time, we also observed that the \( \Delta \Psi_m \) of a small number of HDMCP-overexpressed cells could be partially restored in the presence of oligomycin as shown by the colocalization of HD-MCP and MitoTracker staining (data not shown).

HDMCP and UCP2 Similarly Induced the Dissipation of \( \Delta \Psi_m \) Accompanied by a Significant Drop in the Level of Cellular ATP—In order to study the uncoupling activity of HDMCP, we tried extensively to establish cell lines that stably expressed HDMCP by using a Tet-Off system, but we were unsuccessful. To overcome this difficulty, we have employed 293T cells that are known to have a high efficiency of transfection. UCP2 was cloned and employed as a positive control throughout our study. Transfected 293T cells overexpressing HDMCP and UCP2 that were stained with MitoTracker Orange and anti-FLAG antibody showed the dissipation of \( \Delta \Psi_m \) (Fig. 10, A and B, respectively). Moreover, the levels of total cellular ATP measured 24 h after transfection dropped to 57.3 ± 18.1% (mean ± S.D.) \((p = 0.012)\) in HDMCP-overexpressed cells and 60.5 ± 16.4% \((p = 0.012)\) in UCP2-overexpressed cells when compared with vector control (100%) (Fig. 10C). Although the ADP level was not significantly different in HDMCP- and UCP2-overexpressed cells compared with vector control (Fig. 10C), the ADP/ATP ratio was significantly increased in cells overexpressing HDMCP \((p = 0.012)\) and UCP2 \((p = 0.017)\) (Fig. 10C).

DISCUSSION

The high similarity of protein sequences and gene organization detected within HDMCP orthologs (Fig. 5) as well as their predominant expression in liver tissue indicate that they are structurally and functionally conserved throughout evolution. Furthermore, the presence of syntenic regions at the HDMCP gene loci in human, mouse, and rat genomes (Fig. 6) indicate that the HDMCP orthologous genes are most likely to be de-
Our finding is in agreement with the reported observations that a large number of syntenic segments are present and conserved between mice and humans (29).

Through homology searching of the GenBank data base, HDMCP is highly homologous to a putative protein encoded by C14orf69 (37%), a gene located directly upstream of the HD-MCP gene (Fig. 6). Unlike HDMCP, the EST clones identical to C14orf69 are detected in multiple tissues and not specifically expressed in the liver. Therefore, it appears that these two mitochondrial carrier proteins might not be functionally redundant.

Based on the hallmark features of mitochondrial carrier proteins (Fig. 4) and the specific cellular localization at mitochondria (Fig. 7D), HDMCP is suggested to be a mitochondrial carrier protein. We have demonstrated that transient expression of HDMCP in cancer cells leads to a dramatic dissipation of $\Delta\Psi_m$ (Fig. 7C). By using UCP2 as a positive control, we further confirmed that the expression of HDMCP resulted in the loss of $\Delta\Psi_m$, which was accompanied by a significant reduction of cellular ATP and an increase in the ADP/ATP ratio (Fig. 10C). This is consistent with the fact that uncoupling of oxidative phosphorylation results in decreased $\Delta\Psi_m$ and cellular ATP. The reduction of ATP synthesis likely results from the loss of intact $\Delta\Psi_m$. Although HDMCP has relatively low amino acid homology to UCP1 and its homologs (25–26%), our data suggest that HDMCP, like UCP2, is a functional uncoupling protein.

The liver is a major contributor to energy expenditure (30). It is known that up to 25% of oxygen consumption is used to drive the proton leak in resting hepatocytes (31, 32). Yet there has been no report on the identification of a putative molecule that mediates the uncoupling of oxidative phosphorylation in normal mammalian hepatocytes. The specific expression of HDMCP in liver tissue and its ability to induce the potent dissipation of $\Delta\Psi_m$ in Hep3B cells (Fig. 7B, 10B) suggests that HDMCP may play a role in the regulation of energy metabolism in the liver.
pressed cells. 100% in order to compare the changes in HDMCP- and UCP2-overexpressed cells partially restore $\Delta \Psi_m$. This could suggest that in the presence of oligomycin, the retention of protons could delay the dissipation of $\Delta \Psi_m$ induced by HDMCP.

It has been shown that reactive oxygen species (ROS) are involved in pathogenesis of various liver diseases (36–38). Mild uncoupling of respiration has been proposed as the mechanism to prevent the accumulation of ROS generated by mitochondria (39), to increase the sensitivity and rate of response of oxidative phosphorylation to effectors (40), and to regulate metabolic pathways by adjusting the NAD$^+$/NADH ratio (39, 41). HDMCP might function to prevent the accumulation of ROS, thereby protecting hepatocytes from oxidative stress associated with various liver diseases. Mitochondrial carrier proteins are known to catalyze the transport of a variety of metabolites, nucleotides, and cofactors into and out of the mitochondrial matrix (21, 22, 42). Like most of the carrier proteins, HDMCP could also play a role in determining the availability of some unknown metabolites present in the cytoplasm and mitochondrial matrix. Further investigations are required to clarify the involvement of HDMCP in more specific metabolic pathways in the liver.

The down-regulation of HDMCP in cancerous tissue of HCC patients might allow the bypass of the physiological proton leak that results in the elevation of $\Delta \Psi_m$ in the HCC cancer cells. This is in agreement with the observation that cancer cells generally have an elevated $\Delta \Psi_m$ compared with normal epithelial cells (18). The inherent differences in the $\Delta \Psi_m$ between cancer cells and normal cells have been shown to contribute to the selective accumulation of rhodacyanine dyes in the mitochondria of cancer cells (17, 18, 43). Delocalized lipophilic cations (DLCs) are a new class of putative anti-cancer agents that have been employed for photochemotherapy and photodynamic therapy (44–46). DLCs exploit the differences of $\Delta \Psi_m$ between normal and cancer cells with the goal to achieve selective cytotoxicity for cancer cells. The identification of HDMCP might therefore also allow the discovery of new DLCs for HCC-specific photodynamic therapy.

Moreover, the identification of the bioenergetic function of HDMCP in liver mitochondria should prove valuable for further understanding the mechanism of hepatocarcinogenesis, as well as liver diseases that are associated with energy metabolism. Functional studies through gene knockout or overexpression could help to dissect the physiological role of HDMCP in liver cell metabolism.

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