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A third human carnitine/organic cation transporter (*OCTN3*) as a candidate for the 5q31 Crohn's disease locus (*IBD5*)

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Abstract

Organic cation transporters function primarily in the elimination of cationic drugs in kidney, intestine, and liver [1–3]. The murine organic cation/carnitine (*Octn*) transporter family, *Octn1*, *Octn2*, and *Octn3* is clustered on mouse chromosome 11 (NCBI Accession No. NW_000039). The human *OCTN1* and *OCTN2* orthologs map to the syntenic *IBD5* locus at 5q31 [1], which has been shown to confer susceptibility to Crohn's disease [4]. We show that the human OCTN3 protein, whose corresponding gene is not yet cloned or annotated in the human reference DNA sequence, does indeed exist and is uniquely involved in carnitine-dependent transport in peroxisomes. Its functional properties and inferred chromosomal location implicate it for involvement in Crohn's disease.

Keywords: Organic cation transporter; Carnitine transporter; *OCTN3* gene; Crohn's disease; Peroxisomes

Organic cation transporters function primarily in the elimination of cationic drugs and other xenobiotics in intestine and other organs using distinct mechanisms that can be dependent on sodium-gradients, membrane potential, or pH [5]. L-Carnitine is an organic cation that, in conjunction with carnitine acyltransferases, contributes to the transport of activated long-chain fatty acids into mitochondria for β-oxidation and energy metabolism, as well as into peroxisomes and endoplasmic reticulum [6]. This cell-wide carnitine system consists of at least six proteins, three of which are encoded by the *Octn* transporter subfamily. The known *Octn* mouse genes encode proteins that share >87% amino acid identity [2] and they are oriented tandemly within a 161 kb region (Fig. 1). Although being highly homologous to each other, we have generated antibodies specific for each murine protein (Fig. 2A). Ours and other studies [1,7–9] led to the identification of human *OCTN1* and *OCTN2,* but to date *OCTN3* has been resilient to our cloning attempts, and it is not represented in any DNA sequence database. Using biochemical and confocal fluorescent microscopy analysis, we now demonstrate the subcellular localization of the human OCTN3 protein in peroxisomes, suggesting a unique role for OCTN3 in the maintenance of intracellular carnitine homeostasis.

Materials and methods

Cell culture. Studies were performed with the approval of the Institutional Review Board of the Hospital for Sick Children, Toronto. The cell lines were cultured human skin fibroblasts from an individual homozygous for an OCTN2 mutation and the human hepatoma cell line HepG2 (from ATCC). Cells were grown in 100-mm^2 dishes containing α-MEM + 10% fetal calf serum in the presence of 5% CO_2 at 37 °C.

RNA. Total RNA from mouse C57bl/6J testis was isolated using Trizol Reagent (Gibco-BRL).

cDNA cloning. cDNA for mouse OCTN3 was isolated by PCR after oligo(dT)15—primed RT of mouse testis total RNA, cloned into the TA-cloning vector pCR2.1 (Invitrogen Canada, Burlington, Ontario, Canada), and sequenced. The primers used to amplify the

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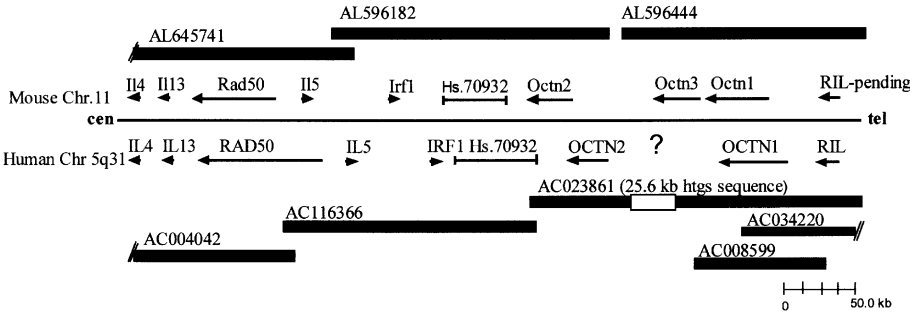

Fig. 1. Physical map of the *OCTN* locus on human and mouse chromosome 5q31 and 11, respectively. Synteny is maintained but the human gene corresponding to mouse *Octn3* is missing. Its expected position coincides with 25.6 kb of high-throughput genomic sequence (htgs) from clone AC023861 that is not represented in other clones (e.g., AC 118464; not shown) or in Celera scaffold GA_x5HB7VCVS5L, spanning the region. Some of the htgs sequences hit smaller unassembled Celera scaffolds (e.g., GA_x5HB7VCVQ85 and GA_x5HB7VCVS3L) that are localized to chromosome 5. Our fluorescence in situ hybridization data place clone AC023861 uniquely at chromosome 5q31.

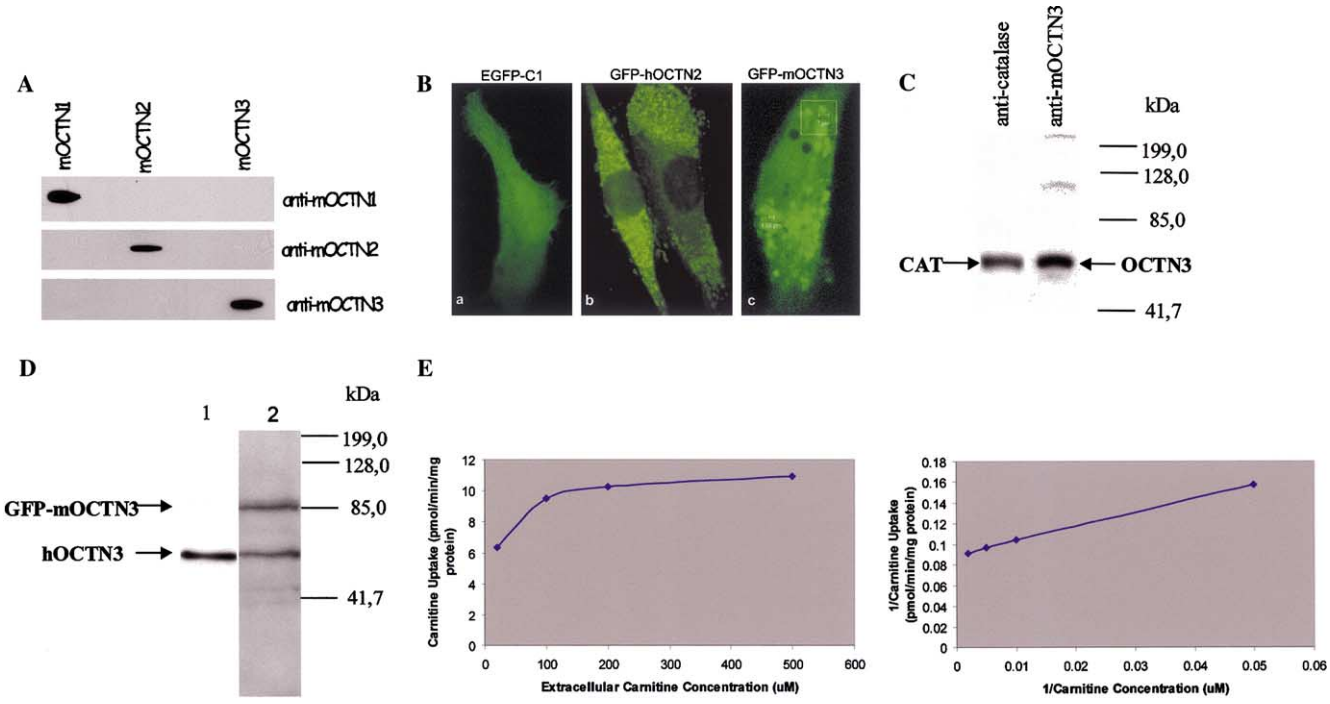

Fig. 2. Panel A is the slot blot analysis demonstrating specificity of each OCTN antibody. Panel B is the expression of (a) GFP vector in HepG2 cells, (b) GFP-hOCTN2 in human OCTN2 mutant fibroblasts, and (c) GFP-mOctn3 in HepG2 cells. Panel C is a Western blot showing expression of mOctn3 and catalase (CAT) in isolated peroxisomes from mouse liver. Panel D is a Western blot of HepG2 using the specific antibody to mouse Octn3 peptide (lane 1: untransfected cells, lane 2: HepG2 transfected with GFP-mOctn3 construct), demonstrating expression of a putative hOCTN3 protein. Panel E shows the kinetics of L-[^{3}H]carnitine uptake of the intermediate-affinity carnitine transporter mOctn3 in HepG2 cells (the left panel demonstrates the saturable carnitine uptake and the right panel is the corresponding Lineweaver–Burk plot (K_m was estimated to be 20 μM and the V_{max} was 11.32 pmol/min/mg protein in mock-transfected cells. There was a 3–5-fold increase of L-[^{3}H]carnitine uptake in mOctn3 transfected cells).

full-length coding mOCTN3 cDNA were designed from GenBank AB018436 sequence and the forward primer XS-171, 5′-TCTCGAG CTATGGCTTGACTACGACGAGGTG-3′ and the reverse primer A1-1883, 5′-CAGCTTCTGGACCAGGTGT-3′.

Expression of GFP-fusion protein. For the mammalian expressible mOctn3 cDNA construct, a *Xho*I–*Hin*dIII insert was subcloned in frame downstream of the GFP sequence using pEGFP-C1 vector (Clontech). Transfection of the GFP-mOctn3 in the human hepatoma cell line HepG2 was done as previously described for the transfection of GFP-hOCTN2 in the cultured skin fibroblasts of an individual homozyous for an OCTN2 mutation [9].

Subcellular localization of the GFP-mOctn3 fusion protein. HepG2 cells were seeded in the Lab-Tek Tissue Culture Chamber Slide one day prior to the transfection experiment. Plasmid DNA (5 μg) and LipoTAXI (Stratagene) were used in the transfection experiment. Thirty-six hours post-transfection, the cells were fixed in 4% paraformaldehyde for 10 min at room temperature and the cells were overlaid with Prolong anti-fade (Molecular Probes, Oregon, USA) and a glass coverslip. Confocal laser scanning fluorescent microscopy analysis was done with a Zeiss Axiovert 100 M microscope and the LSM software. The images were also analyzed in 3-D using the Volocity software.

Slot blot analysis demonstrating the specificity of the polyclonal antibodies to the three different carnitine transporters. Synthetic peptide (1 μg) to mouse Octn1, Octn2, and Octn3 was loaded onto a PVDF BioTrace membrane (Pall) using the Minifold II Slot Blot system (Mandel) and the blot was probed successively with a polyclonal rabbit antibody to either mOctn1, mOctn2, or mOctn3 peptide. HRP-protein A and the ECL kit (Amersham Pharmacia) were used.

Isolation of peroxisomes from mouse liver. Liver cells were obtained from a 2 g (wet weight) liver from a C57bl/6J mouse, pelleted in 0.25 M sucrose, 1 mM EDTA, 0.1% (v/v) ethanol, 10 mM MOPS-NaOH, pH 7.4, and homogenized with 6 strokes. The homogenate was centrifuged at 3000*g* (5500 rpm) for 10 min at 4 °C using rotor inner SM-24 in a Sorvall RC-5B Superspeed centrifuge to pellet the nuclei and heavy mitochondria. The supernatant (light mitochondrial fraction) was centrifuged at 17,000*g* (13,000 rpm) using rotor inner SM-24 in the Sorvall RC-5B centrifuge for 10 min at 4 °C. The translucent pellet was resuspended in a homogenization sucrose buffer and homogenized with 3 strokes of the pestle and the volume was adjusted to 5 ml. The light mitochondrial fraction was diluted with an equal volume of 55% Optiprep (Cedarlane). 5 ml of the suspension was transferred to Ultraclear tubes and centrifuged in the Beckman Optima LE-8 DA ultracentrifuge using rotor SW55 Ti at 250,000*g* (51,350 rpm) for 4 h at 4 °C. This method allowed for the separation of pure isolates of peroxisomes which are denser and are at the bottom of the tubes.

Protein determination. The kit using the Bradford method (BioRad) was used.

Western blotting analysis. Twenty-five μg of isolated peroxisomes from mouse liver cells and 100 μg of total protein from HepG2 lysate were loaded onto a 10% SDS–PAGE. Following electrophoresis, the protein was transferred onto a PVDF BioTrace membrane. The blot was successively probed with the polyclonal rabbit antibodies raised against mouse Octn3 and bovine catalase (specific marker for peroxisomes). HRP-protein A and the ECL kit (Amersham Pharmacia) were used for the detection system. The blot was then exposed to BioMax X-ray film (Kodak).

Carnitine uptake assay in HepG2 transfected with the GFP-mOctn3 construct and GFP vector. HepG2 cells were seeded in P-6 wells. Experiments were done in duplicate sets. HepG2 were transfected with either the pEGFP-C1 vector (5 μg) or the GFP-mOctn3 cDNA (5 μg). The transfection reagent was LipoTAXI. Thirty-six hours post-transfection, the cells were washed with 10mM phosphate buffer, pH 7.4 (PBS), twice and 950 μl RPMI minus serum was added to each well. Fifty microliters L-[^{3}H]carnitine (1 μCi/ml) with varying concentrations of cold carnitine (1, 2, 5, 10, 20, 50, 100, 200, and 500 μM, and 100 mM for non-specific uptake) was added and the cells were incubated for 4 h at 37 °C in the presence of 5% CO_2. The medium was then removed and discarded in the radioactive waste. The cells were washed twice with PBS and 1 ml of 0.5 M NaOH was added to lyse the cells. To determine the L-[^{3}H]carnitine uptake in the transfected HepG2 cells, 800 μl of the cell lysate was transferred to a scintillation vial and 10 ml of scintillation liquid cocktail (Beckman–Coulter) was added to each vial and mixed. The samples were then counted after overnight incubation in the cold room. Protein determination was measured by the Bradford method.

Results and discussion

To test whether the human OCTN3 protein indeed exists and what its function might be, we first expressed a green fluorescent protein (GFP)-mouse Octn3 construct in human cells (HepG2) and determined that it was localized to peroxisomes, spherical organelles measuring approximately 1 μm in diameter as demonstrated by 3-D projection of the captured images using the Zeiss LSM510 and Volocity software (Fig. 2B). This localization was confirmed by Western blot analysis of isolated mouse liver peroxisomes using our anti-mouse Octn3 and anti-catalase antibodies (Fig. 2C). Importantly, we were also able to show that the Octn3 antibody specifically detected a human protein of the expected size (63 kDa) in HepG2 cells, confirming the existence of a gene in the human genome capable of encoding this protein (Fig. 2D). We further studied the uptake of L-[^{3}H]carnitine in HepG2 cells transfected with the GFP-mOctn3 construct and determined that the Octn3 protein had a K_m of 20 μM for carnitine indicating that it belongs to the intermediate-affinity carnitine transporter class (Fig. 2E).

A low-affinity carnitine transporter with $K_m \geqslant 500$ μM has been suggested by kinetic studies in small intestine, liver, brain, epididymis, etc. [10–12] and an intermediate-affinity carnitine transporter with K_m of 20–200 μM has been suggested by kinetic studies in intestinal epithelial cells, testis, muscle culture, and renal tubular cells [13–16]. Contrary to Octn3, Octn1 has been shown to be a low-affinity carnitine transporter [2]. Moreover, we had shown previously that a GFP-human OCTN2 protein localized to the plasma membrane and that it is a high-affinity carnitine transporter with a K_m of 5 μM [9,17]. Our observations demonstrate an interspecies functionality of the organic cation transporters in human and mouse, and that each has a specific role in the maintenance of intracellular carnitine homeostasis.

High-resolution linkage disequilibrium mapping identified a region about 500 kb in size at 5q31 (named *IBD5*) encompassing the *OCTN* genes, to confer susceptibility to Crohn's disease [4]. This locus has been scrutinized by several groups, but no pathogenic sequence variant has been found in *OCTN1*, *OCTN2*, or any other candidate genes including the nearby cytokine genes. We hypothesize that the human *OCTN3* gene, which has not been examined in these studies, is located between *OCTN2* and *OCTN1* coincident with a segment of DNA that is not properly represented in human genome sequence assemblies (Fig. 1). Problems have likely occurred because of clone instability or sequence misassembly arising due to the nearly identical DNA sequences at each gene locus.

The clinical target tissues of the human high-affinity carnitine transporter defect (hOCTN2 defect) are heart, muscle, and kidney with no prominent involvement of the bowel [17,18]. Recently, an intermediate-affinity carnitine transporter (CT1) with a K_m of 25 μM for carnitine has been cloned from rat intestine [19] and has been shown to share a high-amino acid homology with mOctn3 [2]. Given the fact that the functionally important carnitine transporters in bowel appear to be of intermediate- or low-affinity [11,15,19] and that carnitine has an essential role in β-oxidation in intestinal bioenergetic metabolism [20–22], we propose that

OCTN3 is a potential susceptibility gene for *IBD5*. Its dysregulation could lead to impaired fatty acid oxidation in colonic epithelial cells. Metabolic inhibition of β-oxidation in the epithelium of rat colonic mucosa has been shown to produce ulceration, mucus cell depletion, vessel dilatation, and increases of inflammatory cells consistent with acute colitis [20]. Presence of undefined bacterial metabolites in the colonic lumen causing specific breakdown of fatty acid oxidation in colonic epithelial cells has thus been suggested to be an initiating event of the disease process that leads to an immune response and eicosanoid response which could perpetuate further epithelial cell damage [23]. We suggest, therefore, that it will be vitally important to revisit the sequence analysis of the *IBD5* locus and further test *OCTN3* for involvement in Crohn's disease. Furthermore, identification of mutations in *OCTN3* may elucidate an underlying potentially treatable pathophysiological risk factor in inflammatory bowel disease which may be responsive to carnitine therapy.

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Competing interests statement

The authors declare that they have no competing financial interests.

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