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cDNA CLONING AND FUNCTIONAL CHARACTERIZATION OF RAT INTESTINAL MONOCARBOXYLATE TRANSPORTER

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SUMMARY : A cDNA clone which encodes a monocarboxylate transporter (ratMCT1) was isolated from a rat small intestinal cDNA library, which was screened by using full-length MCT1 cDNA of Chinese hamster ovary cells. The ratMCT1 cDNA was sequenced and predicted a protein of 494 amino acids with twelve potential transmembrane domains. The amino acid sequence showed 93.1% and 84.6% identity to the hamster and human monocarboxylate transporters, respectively. When expressed in *Xenopus laevis* oocytes, the ratMCT1 cRNA caused a significant increase in the uptake of radiolabeled lactic acid. Poly(A)$^+$ RNA transcripts hybridizing to the ratMCT1 cDNA were detected in rat brain, heart, kidney, lung, muscle and brain capillaries. These results indicate that MCT1 contributes to pH-dependent and carrier-mediated transport of monocarboxylic acids in many tissues, not just in the small intestine.

Intestinal epithelial cells are well known to have several transport systems to regulate the influx and efflux of nutrients for the maintenance of homeostasis. The microenvironmental pH in the close vicinity of the brush-border membranes is acidic compared with the luminal bulk pH. The resultant proton gradient across the brush-border membrane has been suggested to play an important role as the driving force for active transport processes in the small intestine on the basis of several membrane physiological studies (1-5). However, it is sometimes difficult to discriminate passive diffusion according to pH-partition theory from a pH-dependent carrier-mediated transport of organic weak electrolytes by means of membrane physiological studies, since both of them show

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ABBREVIATIONS: CHO, Chinese hamster ovary; MCT, monocarboxylate transporter; BBMVs, brush-border membrane vesicles; RT-PCR; reverse transcriptase-polymerase chain reaction.

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apparently the same pH-dependent nature. Thus, molecular identification of the transporters is desirable.

A proton-coupled transport was demonstrated as the major mechanism for the absorption of lactic acid by the brush-border membrane vesicles (BBMVs), rather than the previously reported sodium-dependent transport (2,6). Lactic acid transport across the plasma membrane is of fundamental importance to mammalian cells, because lactic acid is produced in a large quantity as an end product of glycolysis or is used as an energy source and so must be expelled from or taken up into the cells (7). Based on studies using BBMVs (3,4,8), cultured intestinal cell line, Caco-2 cells (9,10) and primary cultured brain capillary endothelial cells (11,12), we have suggested the existence of a family of proton-gradient-dependent monocarboxylate transporters which mediate transport of lactic acid as well as other monocarboxylic acid compounds.

Recently, a proton/monocarboxylate cotransporter, MCT1, which mediates transport of lactic acid and pyruvic acid was isolated from Chinese hamster ovary (CHO) cells (13) and it seems to show functional similarities with the transporter that was observed in the intestinal brush-border membrane (3-5,9,10) and in the blood-brain barrier (11,12). Our previous study shows that a protein, which is homologous with monocarboxylate transporter MCT1 isolated from CHO cells, exists and functions in the intestinal epithelial cells as an intestinal proton/monocarboxylate cotransporter (14). Northern blot analysis with the cDNA probe encoding choMCT1 shows that MCT1-isoforms exist in the rat and rabbit intestinal enterocytes and Caco-2 cells (14). The purpose of the present study was to establish whether a choMCT1-related carrier-protein exists in rat intestinal epithelial cells by cloning the MCT1 homologue from rat intestinal cDNA library and to clarify whether or not it functions as the monocarboxylate transporter.

MATERIALS AND METHODS

Total RNA was isolated from small intestinal mucosal cells of a Wistar rat (Japan SLC, Inc., Hamamatsu, Japan) by CsCl gradient centrifugation. Poly(A)$^+$ RNA from the cells was isolated by affinity chromatography on oligo(dT)-cellulose (Pharmacia LKB, Piscataway, NJ, U.S.A.). The oligo(dT)-primed cDNA library constructed by λZAPII (Stratagene, La Jolla, CA, U.S.A.) consisted of 50,000 plaques on 10 agar plates, and plaques were transferred to a Hybond -C extra membrane (Amersham, Buckinghamshire, England). DNA was fixed on the membrane by UV cross-linking and screened using the full length MCT1 cDNA of CHO cells (13), obtained from American Type Culture Collection (Rockville, MD, U.S.A.), under the following high stringency conditions. Membranes were prehybridized for 2 hr at 65°C in 3 x standard saline citrate solution (SSC), 10 x Denhardt's solution, 1.0% sodium dodecylsulfate (SDS), and 100

μg/ml of denatured salmon sperm DNA. Hybridization was performed for 24 hr at 65°C in the same medium after prehybridization containing 50 ng of choMCT1 fragments that had been labeled with [α-^{32}P]dCTP to a specific activity of $1x10^9$ cpm/μg using a BcaBEST labeling kit (Takara, Kyoto, Japan). Membrane was washed first for 30 min at 65°C in 2 x SSC containing 1.0% SDS, then for 30 min at 65°C in 0.5 x SSC containing 1.0% SDS, and finally for 60 min at 65°C in 0.1 x SSC containing 1.0% SDS. The membrane was subjected to autoradiography. pBluescript SK(-) was excised from the positive-plaque purified phage using an *in vivo* excision protocol (Stratagene). DNA was purified by using a Qiagen plasmid midi kit (Qiagen GmbH, Hilden, Germany). The complete sequence of double-stranded DNA was determined by the dideoxy chain termination method.

Total RNAs were isolated from rat tissues (small intestine, brain, heart, kidney, liver, lung and muscle) using the acid guanidium thiocyanate-phenol-chloroform method (15) and the respective poly(A)$^+$ RNAs were isolated by using oligo(dT)-latex beads (Takara). All RNA samples were checked for degradation by visualization of ribosomal RNA bands with ethidium bromide after agarose electrophoresis in the presence of formaldehyde. Poly(A)$^+$ RNA was size-fractionated on a denaturing formaldehyde-agarose gel and transferred to a nylon membrane (Hybond -N, Amersham). The blots were probed with [α-^{32}P]dCTP-labeled rat intestinal MCT1 cDNA under high stringency conditions. To ensure the intactness of blotted RNA and to normalize the signals concerned, the same blots were subsequently probed for β-actin mRNA as an internal control. Rat brain capillaries were isolated as described previously (11). Poly(A)$^+$ RNA was isolated from rat brain capillaries by a one-step procedure as described previously (16). For RT-PCR, a set of PCR primers was designed based on the nucleotide sequences of the cloned rat intestinal ratMCT1 cDNA. One μg of poly(A)$^+$ RNAs was used as the template for RT and the PCR conditions used were 94°C for 1 min, then 30 cycles of 98°C for 20 sec, 68°C for 5 min, followed by 72°C for 10 min. The specificity of the pair of primers was established by PCR using cDNA as the template. Poly(A)$^+$ RNA derived from rat brain capillaries was subjected to RT-PCR and the products were analyzed by agarose gel electrophoresis. The sequence of the PCR product was as expected.

The cRNA was functionally expressed in *Xenopus laevis* oocytes as described previously (14,17). Two oocytes were incubated in 50 μl of HEPES-MES buffered saline containing 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes/Tris (pH 7.5) or 10 mM Mes/Tris (pH 6.0) and a radio-labelled compound at 25°C. The uptake was terminated by washing the oocytes three times with 15 ml of ice-cold HEPES-MES buffered saline at pH 7.5. Washed oocytes were transferred to vials containing 0.5 ml of 5% SDS to be solubilized and the associated radioactivity was measured with liquid scintillation counting.

RESULTS AND DISCUSSION

The rat monocarboxylate transporter (ratMCT1) cDNA isolated from the small intestinal cDNA library is 3,320 bp long with putative polyadenylation signal at the terminal region (Fig. 1). From the similarity with previously cloned choMCT1, coding region was expected to start from ATG at 206 and end at 1825. This cDNA was presumed to encode

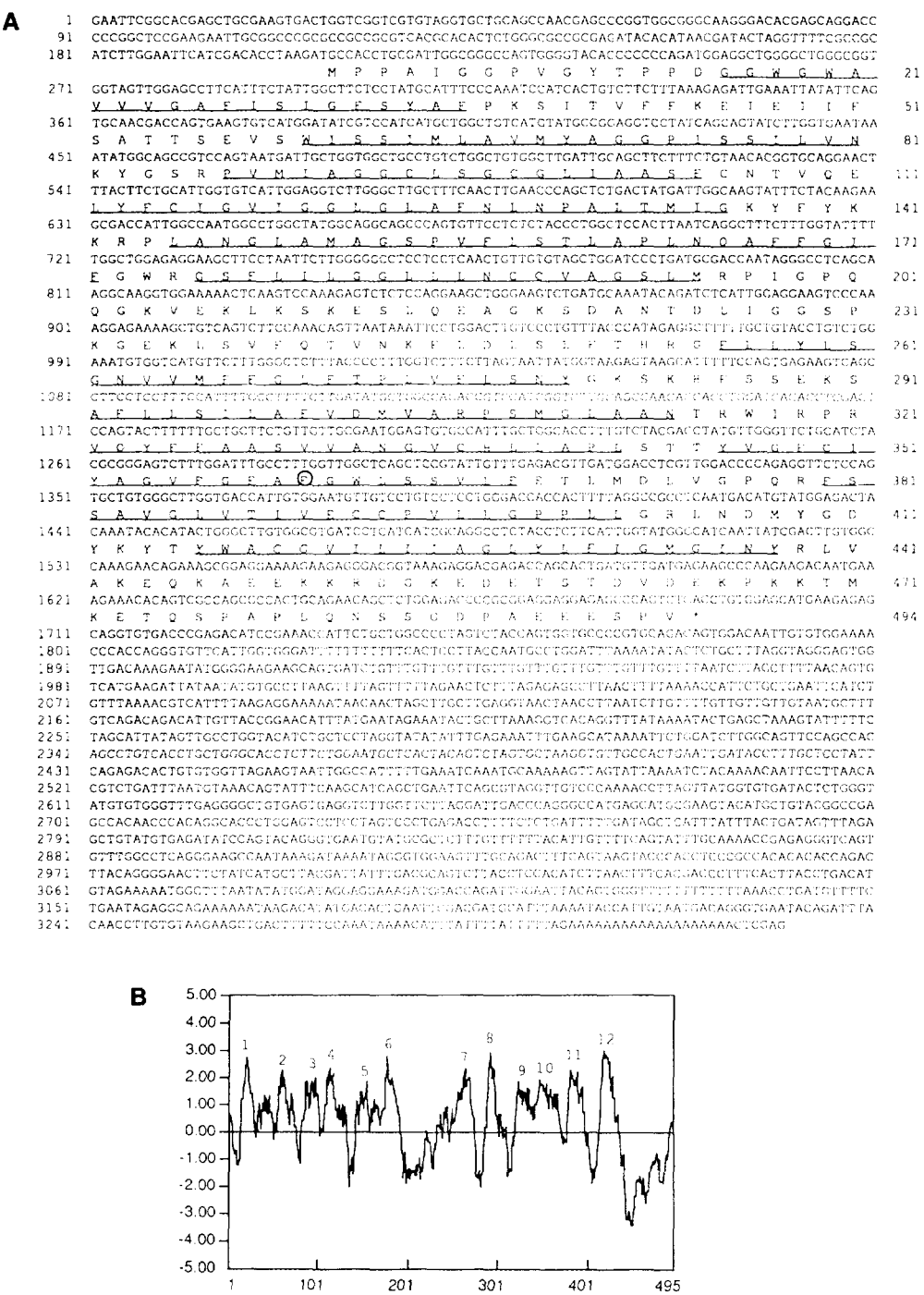

Figure 1. (A) Nucleotide sequence of the rat intestinal monocarboxylate transporter (ratMCT1) cDNA and the predicted amino acid sequence. Putative transmembrane domains are underlined. Circle indicates the position of the phenylalanine, of which mutation to cysteine yields a protein that enhances mevalonate uptake in choMCT1 (20). (B) Hydropathy plot for deduced ratMCT1 protein. Negative scores indicate the degree of hydrophobicity, and positive ones indicate that of hydrophilicity. The putative transmembrane regions are numbered from 1 to 12.

a protein of 494 amino acids with an estimated core molecular mass of 53,235. Hydropathy analysis according to the algorythm of Kyte and Doolittle (18) with a window of 9 residues showed that the ratMCT1 protein possesses 12 relatively distinct hydrophobic domains with a single large hydrophilic loop between transmembrane domains 6 and 7 (Fig. 1B). The amino acid sequence of the ratMCT1 was homologous with those of hamster (13) and human (19) MCT1, with 93.1% and 84.6% identities, respectively. The homology among the three proteins is significantly higher in transmembrane domains than in intervening regions (data not shown). Interestingly, in the 10th membrane-spanning region, the three proteins commonly have Phe, a change of which to Cys converts MCT1 to a mevalonate transporter in chinese hamster (20) (see circle in Fig. 1A).

To demonstrate that the cloned small intestinal ratMCT1 cDNA codes for a functional monocarboxylate transporter, the cDNA was inserted in Bluescript SK(-), oriented for sense transcription under the control of the T3 promoter for the synthesis of cRNA. Then the resultant cRNA was microinjected into *Xenopus laevis* oocytes to express the protein and transport activity was assayed. As shown in Fig. 2, an injection of ratMCT1 cRNA led to a significant stimulation of uptake of L-[^{14}C]lactic acid compared with water injection. The transport activity of ratMCT1 expressed in oocytes was higher at pH 6.0 than that at pH 7.5. Higher transport activity was observed for L-lactic acid compared with that for the D-isomer. These results clearly demonstrate that the cloned

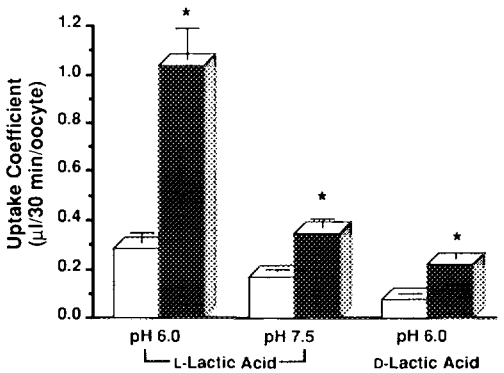

Figure 2. Transport activity of ratMCT1 expressed in *Xenopus laevis* oocytes. Uptakes of 3 μM L-[^{14}C]lactic acid and D-[^{14}C]lactic acid were measured by incubating *Xenopus laevis* oocytes injected with ratMCT1 cRNA (closed columns) or water (open columns) in HEPES-MES-buffered saline (pH 6.0 or pH 7.5) for 30 min. Each value represents the mean ± S.E. of 6 determinations.
* Significantly different from the uptake by water-injected control oocytes by the Student's *t* test at $p<0.05$.

ratMCT1 cDNA codes for a functional monocarboxylate transporter which can be characterized as a stereo-selective proton-coupled transporter.

Northern blot analysis of poly(A)$^+$ RNA from several rat tissues revealed that ratMCT1 is expressed in small intestine, brain, lung, heart, liver, kidney, and skeletal muscle (Fig. 3). The principal transcript recognized by the ratMCT1 cDNA is 3.3 kb in size. When normalized with β-actin signal, the rank order of MCT1 mRNA abundance was heart >> skeletal muscle > whole brain, kidney > small intestine, liver > lung.

Previous studies have demonstrated that the monocarboxylic acid transport system plays an important role in the transport of short-chain monocarboxylic acids and ketone bodies at the plasma membrane, because those compounds are essential for brain metabolism in the normal state and in pathologic situations such as ischemia and hypoglycemia (21). Moreover, we have reported that the monocarboxylic acid transport system functions as a transporter of drugs having a monocarboxylic acid moiety in the molecule at the blood-brain barrier (11,12). Accordingly, we examined the expression of MCT1 in the blood-brain barrier by trying to isolate a poly(A)$^+$ RNA from rat brain capillaries. Since it was rather difficult to obtain a sufficient amount of poly(A)$^+$ RNA for Northern blot analysis, so we used RT-PCR to clarify the expression of the ratMCT1 gene

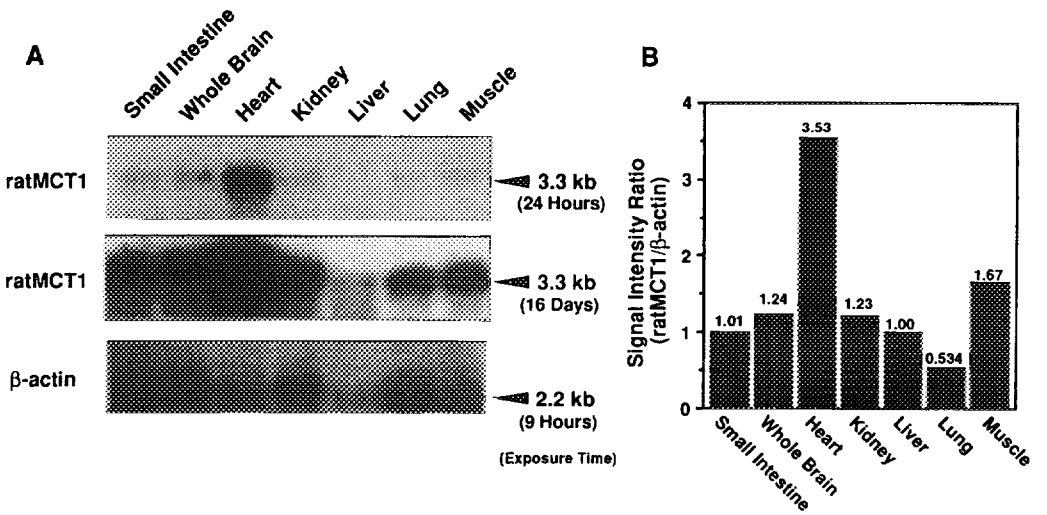

Figure 3. Northern blot analysis of poly(A)$^+$ RNA transcripts from various rat tissues. (A) Each lane contained 5 μg of poly(A)$^+$ RNA. Lanes 1, 2, 3, 4, 5, 6 and 7 represent small intestine, brain, heart, kidney, liver, lung and muscle, respectively. Arrowhead indicates the major transcript 3.3 kb. After probing with the ratMCT1 cDNA, the same blot was probed with β-actin cDNA. (B) Relative MCT1 poly(A)$^+$ RNA levels in rat tissues standardized with β-actin signals.

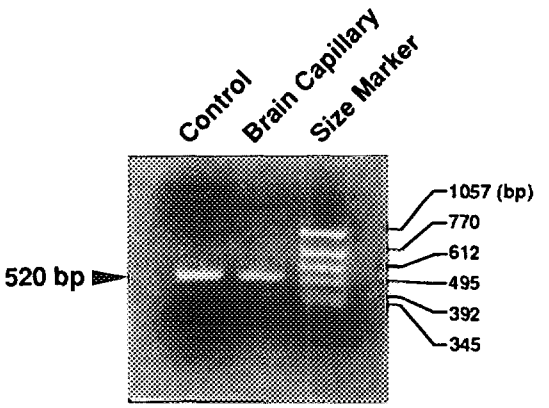

Figure 4. Detection of rat MCT1 transcript in rat brain microvessels. Poly(A)$^+$ RNA from rat brain capillaries and ratMCT1 cRNA (control) were subjected to RT-PCR using primer pairs specific for ratMCT1 cDNA. The RT-PCR products were analyzed by agarose gel electrophoresis.

at the blood-brain barrier. The primers for the PCR had oligonucleotide sequences corresponding to nucleotides 190 to 209 and 690 to 709 of ratMCT1. The PCR product had a size of 520 bp (Fig. 4), which was the precisely expected size from the designed PCR primers. As the negative control study, PCR was performed in the absence of RNA, and no band was detected (data not shown). Furthermore, the nucleotide sequence of the PCR product was confirmed to be the same as that of a part of ratMCT1 by the dideoxy chain termination method. Therefore ti is thought that MCT1 is present in brain capillary endothelial cells functions for the transport of lactic acid between brain and blood.

A proton gradient provides the driving force for active absorption of monocarboxylic acids from the intestinal lumen, so the direction of net transport is strongly dependent on the pH gradient across the cell membrane. It has been reported that lactic acid is absorbed from the intestinal lumen or generated within the epithelium, crosses the basolateral membrane of the enterocyte and enters the blood stream via a facilitated diffusion (22) or an anion exchang (23) mechanism in the studies using rat intestinal BBMVs, but not by a proton cotransport mechanism. The present study has established for the first time the existence of the transporter in the intestinal epithelial cells, and available evidence indicates that this membrane protein may participate in the transport of monocarboxylic acids.

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