Expression and functional features of NaCT, a sodium-coupled citrate transporter, in human and rat livers and cell lines

Elangovan Gopal,1 Seiji Miyauchi,1 Pamela M. Martin,1 Sudha Ananth,1 Sonne R. Srinivas,1 Sylvia B. Smith,2 Puttur D. Prasad,1 and Vadivel Ganapathy1

Departments of 1Biochemistry and Molecular Biology and 2Cellular Biology and Anatomy, Medical College of Georgia, Augusta, Georgia

Submitted 9 August 2006; accepted in final form 6 September 2006

A SODIUM-COUPLED TRANSPORTER FOR CITRATE, NaCT has been cloned from the rat brain (8), mouse brain (6), and human liver cell line HepG2 (7). It belongs to the SLC13 gene family, whose other members include sodium-coupled dicarboxylate transporters NaDC1 and NaDC3 and sodium-coupled sulfate transporters NaSi-1 and SUT-1 (15, 17). According to Human Genome Organization nomenclature, human NaCT is referred to as SLC13A5. NaCT is the mammalian ortholog of the “I’m not dead yet” (Indy) gene (5, 12), a critical determinant of lifespan in Drosophila melanogaster (19). However, there are important differences between mammalian and Drosophila transporters. Mammalian NaCT transports citrate and other dicarboxylic acid cycle intermediates in a Na+ coupled and electrogenic manner (6–8). In contrast, even though the substrate specificity is similar, the Drosophila counterpart transports its substrates in a Na+ independent and electroneutral manner (5, 12). There are important functional differences even among mammalian NaCTs. Rat and mouse NaCTs exhibit a higher affinity for citrate [Michaelis constant (Km): 20–40 μM] (6, 8) than does human NaCT (Km: ~600 μM) (7). In addition, human NaCT is stimulated by Li+, whereas rodent NaCTs are inhibited by Li+ (9). On the other hand, Caenorhabditis elegans NaCT transports citrate in a Na+-coupled and electrogenic manner (2) but is unaffected by Li+ (9).

To date, the brain is the only mammalian tissue from which NaCT has been cloned (6, 8). Subsequent studies (22, 23) have shown that the expression of the transporter in the brain is restricted to neurons. The human ortholog was cloned from HepG2 cells rather than from any native tissue (7). NaCT mRNA is most abundant in the liver and testis in the human, rat, and mouse (6–8). It has been postulated that the transporter plays a critical role in the liver by mediating the entry of citrate from the circulation (plasma concentration of citrate: ~150 μM) into liver cells, where it may function in a variety of metabolic pathways including the citric acid cycle and synthesis of fatty acids and cholesterol and also as a key regulator of glycolysis (3). Despite this hypothesized role of NaCT in hepatic metabolism, neither the expression nor the function of the transporter has been investigated in hepatocytes or in liver cell lines. The functional differences between human and rodent NaCTs, observed with the cloned transporters, have not been confirmed in native cells. Furthermore, the postulate that NaCT functions in the entry of citrate from the circulation into liver cells necessitates its expression in the sinusoidal membrane of hepatocytes, but the localization of the transporter in the liver has not yet been established. Therefore, we undertook the present study to investigate the expression and function of NaCT in human and rat liver cell lines and in rat primary hepatocytes and to localize the transporter by immunofluorescence in human and rat livers.

MATERIALS AND METHODS

Materials. [14C]Citrate (specific radioactivity: 55 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA). Human liver cell lines HepG2 and Huh-7 and rat liver cell line MH1C1 were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Cell culture media were obtained from Mediatech (Herndon, VA). Mouse monoclonal antibody against canalicular multispecific organic anion transporter (cMOAT)/multidrug resistance-related protein 2 (MRP2) was from Kamiya Biomedical (Seattle, WA). Chicken polyclonal antibody against monocarboxylate transporter 1 (MCT1) was from Chemicon (Temecula, CA). Secondary antibodies (raised in goats) against rabbit IgG, mouse IgG, and chicken IgG, conjugated to red (Alexa568) or green (Alexa488) fluorophores, were from Invitrogen/Molecular Probes (Carlsbad, CA).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Cell culture. HepG2 cells were cultured in DMEM supplemented with 1% nonessential amino acids, 1 mM pyruvate, 0.15% sodium bicarbonate, and 10% FBS. Huh-7 cells were cultured in a DMEM-F-12 mixture (1:1) supplemented with 2 mM glutamine and 10% FBS. MH1C1 cells were cultured in F12K nutrient mixture supplemented with 2.5% FBS and 15% horse serum. All media contained 100 U/ml penicillin and 100 μg/ml streptomycin.

Primary hepatocytes from the rat liver. Hepatocytes were isolated from the livers of 6-wk-old male Wistar rats according to the procedures described by Baur et al. (1) and Iga et al. (4). Rats were anesthetized by an intraperitoneal injection of Nembutal (1 mg/kg). Heparin (5,000 IU/ml, 0.3 ml) was injected slowly into the caudal vena cava over 2 min, and the portal vein was cannulated after the vessel was tied off below the cannulation site. The liver was perfused with 250 ml of buffer, at a rate of 20 ml/min, of the following composition (in mM): 137 NaCl, 5.4 KCl, 0.5 NaH2PO4, 0.4 NaHPO4, 4.2 NaHCO3, 0.5 EGTA, 5 glucose, and 20 HEPES (pH 7.4). This was followed by a perfusion with the same buffer (300 ml), but now containing collagenase [0.05% (wt/vol)], trypsin inhibitor (0.005%, wt/vol), 1 mg/ml CaCl2, 0.8 mM MgSO4, and 5 glucose. The liver was then removed, and cells were dispersed in Krebs-Ringer bicarbonate, and 10% FBS. All media contained 100 U/ml penicillin and 100 μg/ml streptomycin.

Animals. Anesthetized by an intraperitoneal injection of Nembutal (1 mg/kg). Anesthesia was maintained with 2.5% FBS and 15% horse serum. All media contained 100 U/ml penicillin and 100 μg/ml streptomycin. The cell viability, determined by trypan blue exclusion, was 95–98%. Cells were then suspended in William’s E medium (5 × 10⁶ cells/ml) supplemented with dexamethasone (1 mM), insulin (1 mM), 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell suspension was then used for seeding in 24-well culture plates for uptake measurements. Culture medium was changed with fresh medium after 4 h following the initial seeding. Plates were incubated for an additional 16 h at 37°C in the incubator with 5% CO₂-95% air and then used for measurements of citrate uptake. The experimental protocol for the use of the animals was approved by the Institutional Animal Care and Use Committee.

Uptake measurements. Uptake of citrate in liver cell lines and primary hepatocytes was measured as described previously (6–8). The uptake buffer was 25 mM HEPES-Tris (pH 7.5) containing (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgSO₄, and 5 glucose. NaCT-specific transport was determined by subtracting transport values measured in the absence of Na⁺ from transport values measured in the presence of Na⁺. Na⁺-free uptake buffer was prepared by substituting NaCl with an equimolar concentration of N-methyl-D-glucamine (NMDG) chloride. Substrate saturation kinetics were analyzed by fitting NaCT-specific transport data to the Michaelis-Menten equation. Kinetic constants (Kᵣ and V_max) were calculated by using nonlinear as well as linear regression methods. The dependence of NaCT-mediated transport of citrate on Na⁺ was determined by comparing transport values measured in the presence of varying concentrations of Na⁺ where NaCl was replaced isosmotically with NMDG chloride. Na⁺ activation kinetics were analyzed by fitting NaCT-specific transport data to the Hill equation to determine the Hill coefficient (n_H, the number of Na⁺ involved in the activation process). When the effect of Li⁺ was investigated, Na⁺-containing uptake buffer was used. As a control for the Li⁺ effect, an equal concentration of NMDG chloride was added to maintain the osmolality of the uptake buffer.

Uptake measurements were made in duplicate or triplicate, and experiments were repeated with at least three independent cell cultures. Results are expressed as means ± SE of these replicative values.

NaCT antibody. An antibody specific for NaCT was generated in rabbits against the peptide sequence LGQMELFDPDSKDVVMN, which corresponds to the amino acid sequence 237–252 in rat NaCT (8). This sequence shows 81% identity and 100% similarity with the corresponding sequence in human NaCT (amino acid position 234–249, LGQMELFDPDSKDVLMN) (7). The antibody was affinity purified with immobilized antigenic peptide prior to use in immunofluorescence experiments. The specificity and cross-reactivity of the antibody were examined as follows. Human retinal pigment epithelial cells, which do not express NaCT, were transfected with either vector alone or human NaCT CDNA, and the antibody was used to detect heterologously expressed NaCT protein. Detection of positive immunofluorescence signals in cDNA-transfected cells but not in vector-transfected cells was taken as evidence for the specificity and cross-reactivity of the antibody. In some experiments, antibody neutralized with excess antigenic peptide was used to confirm its specificity.

Immunofluorescence localization. Immunofluorescence methods were used to localize NaCT in human and rat liver sections using protocols described previously (16, 20). Cryosections of the liver were fixed in ice-cold acetone for 5 min, washed with PBS (pH 7.4), and blocked with 1% Power Block for 10 min at room temperature. For double-labeling experiments, sections were then incubated overnight at 4°C with the primary rabbit polyclonal antibody against NaCT and mouse monoclonal antibody against MRP2, also known as cMOAT, a marker for the liver canalicular membrane (13, 21). A similar procedure was used for double labeling with NaCT antibody and MCT1 antibody. MCT1 is a marker for the liver sinusoidal membrane (11). Sections were rinsed and incubated for 1 h with goat anti-rabbit IgG coupled to Alexa488 (green fluorescence) and either goat anti-mouse IgG or goat anti-chicken IgG coupled to Alexa568 (red fluorescence). Coverslips were mounted with Vectashield Hardset mounting medium, and sections were examined by epifluorescence using a Zeiss Axioplan 2 microscope.

RESULTS

Characterization of citrate uptake in human and rat liver cell lines. We first examined HepG2 cells, a model for human hepatocytes, for the expression of NaCT by monitoring citrate uptake. These cells were able to take up citrate in a time- and Na⁺-dependent manner (Fig. 1A). The uptake was linear for at least up to 45 min and obligatorily dependent on Na⁺. Citrate (10 μM) uptake was about 15-fold greater in the presence of Na⁺ than in its absence. Na⁺ activation kinetics of citrate uptake provided evidence of cooperativity with respect to Na⁺ interaction, suggesting that more than one Na⁺ is involved in the activation process (Fig. 1B). There was, however, no saturation of the activation process even with 140 mM Na⁺, making it difficult to determine n_H. Competition experiments revealed that unlabeled citrate (5 mM) was able to inhibit [¹⁴C]citrate uptake only by ~40% (Fig. 1C), hinting that the transport system has a low affinity for this substrate (Kᵣ > 5 mM). The inhibitory potentials of other intermediates of the citric acid cycle were even lower. The lack of robust inhibition of [¹⁴C]citrate (10 μM) uptake by 5 mM citrate was surprising because NaCT cloned from the same cell line showed a Kᵣ of ~0.6 mM in a heterologous expression system (7). This raised doubts as to whether or not citrate uptake in these cells is mediated by NaCT. To address this issue, we examined the effect of Li⁺ on citrate uptake. One of the unique features of human NaCT is the marked stimulation of its transport function by Li⁺ (9). This characteristic is not shared by rodent orthologs or by any other member of the SLC13 gene family. We found that citrate uptake in HepG2 cells, when measured in the presence of Na⁺, was stimulated markedly by Li⁺ (Fig. 2A). There was a significant stimulation (~2-fold) even at a Li⁺ concentration as low as 1 mM, and the stimulatory effect increased with increasing concentrations of Li⁺ (~8-fold stimulation at 20 mM Li⁺).

The stimulatory effect of Li⁺ on the transport function of cloned human NaCT was associated with an increase in Kᵣ and
a decrease in $V_{\text{max}}$ (9). Similar changes were seen in the case of citrate uptake in HepG2 cells (Fig. 2B). In the absence of Li$^+$, citrate uptake was saturable with a $K_t$ of 5.1 ± 0.5 mM and a $V_{\text{max}}$ of 1.8 ± 0.1 nmol·mg protein$^{-1}$·min$^{-1}$. However, in the presence of Li$^+$ (10 mM), $K_t$ decreased almost threefold (1.8 ± 0.4 mM) and $V_{\text{max}}$ decreased by threefold (0.58 ± 0.03 nmol·mg protein$^{-1}$·min$^{-1}$). We then asked whether the increase in affinity seen in the presence of Li$^+$ was specific for citrate or if similar changes occurred in the case of other substrates as well. To answer this question, we compared the potencies of 2.5 mM citrate and other substrates of NaCT to compete with [14C]citrate (10 μM) for uptake in the absence and presence of Li$^+$. However, in the absence of Li$^+$, none of the known substrates of NaCT caused >20% inhibition of [14C]citrate uptake (Fig. 3A). In the presence of Li$^+$, the magnitude of inhibition increased markedly for all compounds tested with the exceptions of isocitrate, lactate, and pyruvate, which did not interact with NaCT (Fig. 3B). These data show that Li$^+$ enhances the affinity of the transport system not only for citrate but also for other substrates. We then examined the effect of Li$^+$ on the Na$^+$ activation kinetics of citrate uptake (Fig. 4). As was seen previously (Fig. 1B), when there was no Li$^+$, citrate uptake was activated by increasing concentrations of Na$^+$ in a cooperative manner with little sign of saturation. In contrast, when Li$^+$ was present, citrate uptake was stimulated at all concentrations of Na$^+$ examined, and the kinetics of Na$^+$ activation of citrate uptake were clearly different. The activation was still cooperative, but the process showed evidence of saturation with increasing concentrations of Na$^+$, making it possible to analyze the data according to the Hill equation. This analysis gave a value of 1.9 ± 0.1 for $n_H$. Thus, Li$^+$ influenced not only the substrate affinity but also Na$^+$ activation kinetics of the citrate uptake system in HepG2 cells.

The characteristics of citrate uptake in HepG2 cells were similar to those of human NaCT cloned from the same cells.
with a notable exception. The similarities included Na$^+$ dependence, the involvement of multiple Na$^+$, a preference for citrate over other citric acid cycle intermediates, and stimulation by Li$^+$. These data suggest that citrate uptake in these cells is mediated by NaCT. The difference between citrate uptake in these cells and cloned human NaCT was in substrate affinity. The affinity of the uptake system for citrate was lower in these cells and cloned human NaCT was in substrate affinity.

Characterization of citrate uptake in primary hepatocytes from the rat liver. To characterize the features of NaCT in native liver cells, we used primary hepatocytes prepared from the rat liver. Freshly isolated hepatocytes showed robust Na$^+$-dependent citrate uptake activity, but the activity decreased significantly as the cells were cultured with multiple passages (data not shown). Therefore, we used hepatocytes only for two passages in the present study. Even with the second passage, the citrate uptake activity decreased by 25% compared with freshly isolated cells, but the general functional features of the uptake system were not altered. The uptake was clearly Na$^+$ dependent and linear for at least up to 30 min. Unlabeled citrate and other intermediates of the citric acid cycle inhibited the uptake of [14C]citrate markedly (data not shown). Unlabeled citrate (5 mM) inhibited [14C]citrate (10 μM) uptake by ~90%. The inhibitory potencies of the other compounds tested were as follows: succinate > fumarate > α-ketoglutarate = isocitrate = cis-aconitate. The uptake was saturable with a $K_v$ of 30 ± 8 μM. We compared the affinities of the uptake system for citrate and succinate by assessing the dose-response relationship for the inhibition of [14C]citrate (20 μM) uptake (Fig. 6A). Unlabeled citrate inhibited the uptake with an IC$_{50}$ value (i.e., the concentration necessary to cause 50% inhibition) of 52 ± 6 μM. The corresponding value for succinate was 399 ± 77 μM. The $K_i$ values, calculated from these IC$_{50}$ values [$K_i = IC_{50}(1 + I/K_v)$, where I represents the concentration of radiolabeled citrate], were 31 ± 4 μM for citrate and 239 ± 46 μM for succinate. The inhibition of the transport activity by Li$^+$ was evident in primary hepatocytes (Fig. 6B). These charac-

Fig. 3. Influence of Li$^+$ on the substrate affinity of the Na$^+$-coupled citrate uptake system in HepG2 cells. The uptake of [14C]citrate (10 μM) was measured for 30 min in the presence of 130 mM NaCl with either 10 mM NMDG chloride (−Li$^+$; A) or 10 mM LiCl (+Li$^+$; B) in the absence (control) or presence of 2.5 mM of unlabeled citrate and other citric acid cycle intermediates as well as monocarboxylates.

Fig. 4. Influence of Li$^+$ on Na$^+$ activation kinetics of the citrate uptake system in HepG2 cells. The uptake of [14C]citrate (10 μM) was measured for 30 min at increasing concentrations of Na$^+$ with either 10 mM NMDG chloride (−Li$^+$) or 10 mM LiCl (+Li$^+$). The concentration of Na$^+$ was varied by the addition of appropriate concentrations of NaCl, and osmolality of the uptake buffer was maintained by the addition of appropriate concentrations of NMDG chloride. A: concentration of Na$^+$ versus Na$^+$-dependent citrate uptake. B: Hill plot in the presence of Li$^+$.
teristics were comparable with those obtained with cloned rat NaCT in a heterologous expression system (8).

**Immunolocalization of NaCT in the liver.** To test the specificity of the anti-NaCT antibody, we first examined the immunofluorescence in human retinal pigment epithelial cells that had been transfected with either vector alone or human NaCT cDNA (Fig. 7A). The antibody did not yield positive signals in vector-transfected cells. In contrast, red fluorescence, indicative of an immunopositive reaction, was evident in cells transfected with cloned human NaCT. We then examined the immunofluorescence in HepG2 cells with the antibody (Fig. 7B). Immunopositive signals (red fluorescence) were observed in these cells. Signals were absent when the antibody that had been neutralized with excess amounts of the antigenic peptide was used. We then examined the localization of the transporter protein in the human and rat liver (Fig. 7, C–E). We used MRP2 as a marker for the canalicular membrane and MCT1 as a marker for the sinusoidal membrane. In both human and rat liver sections, NaCT expression was evident (green fluorescence) and MRP2 expression was also evident (red fluorescence). However, the two signals did not overlap. On the contrary, NaCT-specific signals overlapped with MCT1-specific signals, indicating that NaCT is expressed exclusively in the sinusoidal membrane.

**DISCUSSION**

The experiments reported in this study describe, for the first time, the expression and function of NaCT in intact liver cells. We investigated the characteristics of the transporter in human and rat liver cell lines and in primary hepatocytes from the rat liver. Comparative experiments with human and rodent cell lines were necessitated because of the marked differences observed in substrate affinity and the Li\(^{+}\) effect between cloned human and rodent NaCTs. These experiments showed that NaCT is expressed and functional in human and rat liver cell lines and in rat primary hepatocytes. The characteristics of NaCT, examined in human liver cell lines, are similar to those of the cloned human NaCT for the most part, with a notable exception. The transporter in liver cell lines is Na\(^{+}\) dependent, interacts with two or more Na\(^{+}\) per transport cycle, shows a preference for citrate over other intermediates of the citric acid cycle, and is stimulated by Li\(^{+}\). The Li\(^{+}\)-induced activation of the transport process is associated with an increase in \(K_t\) and a
decrease in $V_{\text{max}}$. These features are also seen with cloned NaCT. The notable difference is in $K_t$. Cloned NaCT exhibited a $K_t$ of $\sim 0.6$ mM for citrate. In contrast, the transporter expressed constitutively in HepG2 cells and in Huh-7 cells exhibited a $K_t$ of $\sim 6$ mM. The reasons for this difference are not known, but we believe that posttranslational modifications may play a role. When cloned NaCT was expressed heterologously in mammalian cells, we measured the transport function within 12–15 h following transfection (6–8). There may not be sufficient time for posttranslational modifications of the newly synthesized transporter protein under these conditions. In contrast, the transporter in HepG2 and Huh-7 cells was expressed constitutively, and therefore posttranslational modifications of the transporter protein are likely to occur under these conditions.

Another point of interest with regard to activation of the transport system by Li$^+$ in human liver cell lines are the apparent anomalous substrate saturation kinetics. In the absence of Li$^+$, the transport system follows Michaelis-Menten kinetics describing a single saturable process. This is evident from the linearity of the Eadie-Hofstee plots. In contrast, the plots were clearly curvilinear in the presence of Li$^+$ as if two or more transport systems with varying substrate affinities participated in the transport process. It is interesting that this phenomenon was seen only in the presence of Li$^+$. We speculate that the concentration of Li$^+$ used in the experiment in relation to the affinity of the transport system for Li$^+$ might play a role in this phenomenon.

Comparative experiments of the transporter function in human and rat liver cell lines confirmed the functional differences between cloned human and rodent NaCTs observed in heterologous expression systems. The transporter in human liver cell lines is stimulated by Li$^+$, whereas the transporter in the rat liver cell line is inhibited by Li$^+$. The transporter in the rat liver cell line exhibits higher affinity for citrate than the transporter in human liver cell lines. Further studies are nec-

Fig. 7. Immunolocalization of the Na$^+$-coupled transporter for citrate, NaCT, in the liver. A: immunolabeling of human retinal pigment epithelial cells with rabbit polyclonal anti-NaCT antibody. Cells were transfected with either vector alone or human NaCT cDNA. The secondary antibody was goat anti-rabbit IgG conjugated to Alexa568 (red fluorescence). B: immunolabeling of HepG2 cells with either rabbit polyclonal anti-NaCT antibody or antibody that had been neutralized with excess antigenic peptide. C–E: double immunolabeling of human and rat liver sections was carried out with rabbit polyclonal anti-NaCT antibody and either mouse monoclonal anti-multidrug resistance-related protein 2 (MRP2) antibody or chicken anti-monocarboxylate transporter 1 (MCT1) antibody. The appropriate secondary antibodies, conjugated to either Alexa488 or Alexa568, were used. Insets show higher magnification.
necessary to elucidate the molecular events underlying these species-dependent differences. We also investigated the expression and function of this transporter in primary hepatocytes from the rat liver. The functional features of the transporter in primary hepatocytes agreed for most part with those of cloned rat NaCT. One interesting difference was in \( K_t \). The \( K_t \) for citrate in the rat liver cell line MH1C1 was about fourfold lower than that in primary rat hepatocytes. It remains to be seen if posttranslational modifications of the transporter protein have any relevance to this functional difference.

The present study showed that NaCT is expressed exclusively in the sinusoidal membrane of hepatocytes in the intact liver. This is not all that surprising because Na\(^+\)-coupled transporters are not usually found in the canalicular membrane (10, 14). However, this finding is of functional significance in relation to the physiological role of this transporter. Citrate is present in the circulation at significant concentrations (~150 \( \mu \)M). Citrate in the blood could arise from dietary sources. It is also possible that citrate is released from the cells into the blood via mediated or nonmediated processes. The transporter present in the sinusoidal membrane may facilitate the concentrative entry of citrate from the blood into hepatocytes. This may have important biological implications. Citrate plays a critical role in the hepatic synthesis of fatty acids and cholesterol. In addition to the citrate that is produced in the citric acid cycle and then exported out of the mitochondria into the cytoplasm via the mitochondrial citrate transporter, circulating citrate may also serve as the carbon source for fatty acid and cholesterol synthesis via NaCT-mediated entry into the cytoplasm. The mitochondrial citrate transporter has been cloned (18), and this transporter is distinct from NaCT. Citrate is also an important regulator of glycolysis by serving as an allosteric inhibitor of the regulatory enzyme phosphofructokinase-1. Therefore, NaCT may play a role in the control of glycolysis and glucose metabolism in the liver as well.

REFERENCES

5. Inoue K, Fei YJ, Huang W, Zhuang L, Chen Z, Ganapathy V. Functional identity of Drosophila melanogaster Indy as a cation-indepen-
carboxylate cotransporter 1 (MCT1) in the liver of pre-ruminant and adult bovines. Vet J. In press.

Downloaded from http://ajpgi.physiology.org/ by 10.220.33.1 on July 6, 2017