Functional features and genomic organization of mouse NaCT, a sodium-coupled transporter for tricarboxylic acid cycle intermediates

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In the present study, we report on the molecular cloning and functional characterization of mouse NaCT (Na⁺-coupled citrate transporter), the mouse orthologue of *Drosophila* Indy. Mouse NaCT consists of 572 amino acids and is highly similar to rat and human NaCTs in primary sequence. The mouse *nact* gene coding for the transporter is approx. 23 kb long and consists of 12 exons. When expressed in mammalian cells, the cloned transporter mediates the Na⁺-coupled transport of citrate and succinate. Competition experiments reveal that mouse NaCT also recognizes other tricarboxylic acid cycle intermediates such as malate, fumarate and 2-oxo-glutarate as excellent substrates. The Michaelis–Menten constant for the transport process is $38 \pm 5 \mu\text{M}$ for citrate and $37 \pm 6 \mu\text{M}$ for succinate at pH 7.5. The transport process is electrogenic and exhibits an obligatory requirement for Na⁺. Na⁺-activation kinetics indicates that multiple Na⁺ ions are involved in the activation process. Extracellular pH has a differential

effect on the transport function of mouse NaCT depending on whether the transported substrate is citrate or succinate. The Michaelis–Menten constants for these substrates are also influenced markedly by pH. When examined in the *Xenopus laevis* oocyte expression system with the two-microelectrode voltage-clamp technique, the transport process mediated by mouse NaCT is electrogenic. The charge-to-substrate ratio is 1 for citrate and 2 for succinate. The most probable transport mechanism predicted by these studies involves the transport of citrate as a trivalent anion and succinate as a bivalent anion with a fixed Na⁺/substrate stoichiometry of 4:1. The present study provides the first unequivocal evidence for the electrogenic nature of mammalian NaCT.

Key words: charge-to-substrate ratio, citrate transport, electrogenicity, NaCT, sodium coupling, succinate transport.

INTRODUCTION

We have recently cloned and characterized a sodium-coupled transporter for tricarboxylic acid cycle intermediates from rat and human tissues [1,2]. This transporter, known as NaCT (Na⁺-coupled citrate transporter), recognizes the tricarboxylate citrate with higher affinity when compared with the dicarboxylates such as succinate, malate, fumarate and 2-oxo-glutarate. Both in the rat and human, NaCT is expressed primarily in the liver and, to a lesser extent, in the brain and testes. NaCT is the mammalian orthologue of *Drosophila* Indy, a plasma membrane transporter for tricarboxylic acid cycle intermediates [3–5]. Interestingly, the mammalian NaCT is a Na⁺-coupled transporter for tricarboxylic acid cycle intermediates, whereas *Drosophila* Indy is Na⁺-independent even though it transports tricarboxylic acid cycle intermediates similar to mammalian NaCT [6,7]. The tissue distribution pattern of *Drosophila* Indy and mammalian NaCT is similar when the metabolic function of the organs involved is taken into consideration. In *Drosophila*, Indy is expressed predominantly in fat body which represents the metabolic equivalent of the liver in mammals [3–5,7]. Interest in NaCT as the mammalian orthologue of Indy stems from the findings that partial disruption of the function of Indy in *Drosophila* leads to a significant increase in the lifespan of the organism [3–5]. This raises the possibility that mammalian NaCT may have therapeutic potential as a drug target to enhance the lifespan in mammals. It has been argued that disruption of the transport function of Indy causes decreased delivery of tricarboxylic acid cycle intermediates into cells for energy production, a condition similar to caloric restriction [3–5,7]. Since caloric restriction in rodents

and primates is known to prolong the lifespan [4,5,8], decreased availability of tricarboxylic acid cycle intermediates to various tissues associated with the disruption of Indy function provides the probable molecular basis for the extension of lifespan. We have shown recently that NaCT in the human liver cell line HepG2 is involved in the utilization of extracellular citrate for the synthesis of fatty acids and cholesterol [9]. We have also shown that NaCT is a target for lithium action [9]. However, the action of lithium on NaCT varies depending on the animal species. Whereas human NaCT is activated by lithium, rodent NaCTs are inhibited by lithium. In HepG2 cells, lithium activates the transport function of NaCT and thus promotes the transporter-mediated utilization of extracellular citrate in lipid synthesis [9]. These results suggest that NaCT may also have potential as a drug target for the treatment of obesity, hyperlipidaemia and hypercholesterolaemia in humans.

NaCT can transport not only citrate, a tricarboxylate, but also succinate, a dicarboxylate. For NaDC1 and NaDC3, which are structurally and functionally related to NaCT and also transport citrate as well as succinate, it has been shown that these transporters recognize their substrates only in their bivalent form [10,11]. It is not known whether this is the case with NaCT. NaDC1 and NaDC3 transport their substrates with a Na⁺/substrate stoichiometry of 3:1 and consequently the transport process is electrogenic [12–14]. There is no information available for NaCT on Na⁺/substrate stoichiometry and on the electrogenic nature of the transport process. Furthermore, since NaCT transports citrate, an important metabolic intermediate with a plethora of metabolic functions closely related to lipid and cholesterol synthesis, and also since the transporter may play a role as a determinant of

Abbreviations used: HRPE, human retinal pigment epithelial; NaCT, Na⁺-coupled citrate transporter.

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lifespan, a knockout animal model would prove to be very useful to understand the physiological functions of this transporter. Information on the functional features and genomic organization of the mouse NaCT would become relevant to the generation of such an animal model. In the present study, we report on the cloning, functional features and genomic organization of mouse NaCT. We also provide evidence for the electrogenic nature of the transport process mediated by the rodent NaCT, irrespective of whether the transported substrate is a tricarboxylate or a dicarboxylate. This is the first direct evidence that NaCT can transport its substrates in trivalent as well as in bivalent forms with a fixed Na^+ /substrate stoichiometry of 4:1.

MATERIALS AND METHODS

Cloning of mouse NaCT cDNA

A search of GenBank® database using the rat and human NaCT amino acid sequences as queries identified a sequence (accession no. XM.137672), predicted from the NCBI (National Center for Biotechnology Information) contig NT_039520 by automated computational analysis using the gene prediction method GenomeScan, which is most probably the mouse orthologue of NaCT. This 1947 bp long (open reading frame plus the termination codon) sequence codes for a putative protein consisting of 648 amino acids. This putative protein is 76 amino acids longer than the cloned rat NaCT and 80 amino acids longer than the cloned human NaCT. Since this is a theoretically predicted sequence of the coding region by computational analysis, it does not necessarily represent the sequence of the actual mRNA. A comparison of the nucleotide sequence of this putative mRNA with that of the mouse gene (contig NT_039520) allowed us in the identification of the exons that code for the sequences containing the translational start codon and the translational-termination codon. In addition, a search of GenBank® database revealed that the sequence containing the translational-termination codon is located in two expressed sequence tags (gi10645928 and gi6101350). This information allowed us to design primers suitable for amplification of the entire coding region of mouse NaCT using mouse brain mRNA as the template. The upstream primer, containing the translational start codon (shown in bold-face letters), was 5'-GTCTCCCTTTACGCGATGG-3' and the downstream primer, located just two nucleotides downstream of the translational-termination codon, was 5'-TCGTCTAGAGCTTGCTCTTGC GGCTCT-3'. The underlined sequence in the downstream primer is an *Xba*I site, added to the 5'-end of the primer for cloning purpose. Reverse transcriptase-PCR with these primers and mouse brain mRNA as the template yielded an approx. 1.8 kb product. This product was subcloned into pGEM-T Easy vector. The insert was then released from the plasmid by digestion with *Eco*RI and *Xba*I and subcloned into the vector pGH19 at the *Eco*RI-*Xba*I site. The pGH19 vector (provided by Dr P. S. Aronson, Yale University School of Medicine, New Haven, CT, U.S.A.) contains the 3'-untranslated region of the *Xenopus* β -globin gene downstream of the cloning site and has been shown to increase the expression levels of heterologous genes in oocytes [15,16]. This vector also contains the T7 promoter upstream of the cloning site and thus is suitable for functional expression in mammalian cells using the vaccinia virus expression technique [1,2]. The sense as well as antisense strands of the cDNA insert were sequenced by the *Taq* Dye Dioxymy terminator cycle method using an automated PerkinElmer Applied Biosystems 377 Prism DNA sequencer. The sequence was analysed using the NCBI server (<http://www.ncbi.nlm.nih.gov/>).

Functional expression of mouse NaCT cDNA in HRPE (human retinal pigment epithelial) cells

The cloned mouse NaCT cDNA was expressed functionally in HRPE cells using the vaccinia virus expression technique [1,2]. Cells transfected with vector alone served as control for the determination of endogenous transport activities. The transport of [^{14}C]citrate (specific radioactivity, 55 mCi/mmol) and [^3H]succinate (specific radioactivity, 40 Ci/mmol), both from Moravsek Biochemicals (Brea, CA, U.S.A.) was measured in control cells and in cDNA-transfected cells in parallel. On the basis of time-course studies, initial transport rates for citrate and succinate were measured using 30 and 15 min incubation respectively. The transport buffer was 25 mM Hepes/Tris (pH 7.5) containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 and 5 mM glucose. Interaction of various tricarboxylic acid cycle intermediates and related compounds with the transporter was assessed by monitoring the ability of these compounds to compete with citrate and succinate for NaCT-mediated transport. The NaCT-specific transport was determined by subtracting the transport values measured in vector-transfected cells from the transport values measured in cDNA-transfected cells. Substrate saturation kinetics was analysed by fitting the NaCT-specific transport data to the Michaelis-Menten equation. The Michaelis-Menten constant K_m and the maximal velocity $V_{\max}$ were calculated by using non-linear as well as linear regression methods. The dependence of NaCT-mediated transport of citrate and succinate on Na^+ was determined by comparing the transport values measured in the presence of various concentrations of Na^+ , where NaCl was replaced isosmotically with *N*-methyl-D-glucamine chloride. The Na^+ -activation kinetics was analysed by fitting the NaCT-specific transport data to the Hill equation. The Hill coefficient h (the number of Na^+ involved in the activation process) was determined by using non-linear as well as linear regression methods.

Functional expression of mouse and rat NaCTs in *Xenopus laevis* oocytes

Capped cRNA from the cloned mouse NaCT cDNA was synthesized using the mMESSAGE mMACHINE kit (Ambion, Austin, TX, U.S.A.). In some experiments, rat NaCT [1] was used for functional expression. Mature oocytes from *X. laevis* were isolated by treatment with collagenase A (1.6 mg/ml), manually defolliculated and maintained at 18 °C in the modified Barth medium supplemented with 10 mg/ml gentamicin as described previously [17,18]. On the following day, oocytes were injected with 50 ng of cRNA. Water-injected oocytes served as controls. Oocytes were used for electrophysiological studies 4–6 days after cRNA injection. Electrophysiological studies were performed by the two-microelectrode voltage-clamp method [17,18]. Oocytes were perfused with a NaCl-containing buffer (100 mM NaCl/2 mM KCl/1 mM MgCl_2 /1 mM CaCl_2 /3 mM Hepes/3 mM Mes/3 mM Tris, pH 7.5), followed by the same buffer containing citrate and succinate. The membrane potential was clamped at -50 mV. The charge-to-substrate ratio was determined for citrate and succinate in three different oocytes. Oocytes were perfused with 50 μM [^{14}C]citrate or 50 μM succinate (unlabelled plus radiolabelled succinate) and inward currents were monitored over a period of 10 min. At the end of the experiment, the amount of citrate and succinate transported into the oocytes was calculated by measuring the radioactivity associated with the oocytes. The area within the curve describing the relationship between the time and inward current was integrated to calculate the charge transferred into the oocyte during incubation.

Figure 1 Amino acid sequence of mouse NaCT and the exon-intron organization of mouse *nact* gene

(A) Comparison of the primary structure of mouse NaCT with that of rat and human NaCTs. Identical amino acids are indicated by dark shading and conserved amino acid substitutions are indicated by light shading. (B) Exons, identified by boxes, are numbered in the gene and numbers above the exon boxes indicate the number of base pairs in respective exons. The numbers associated with the introns indicate the size of the respective introns in kilobase pairs. The shaded region in exon 12 denotes the 3'-untranslated region.

with citrate or succinate. The values for substrate transport and charge transfer were used to determine the charge-to-substrate ratio.

Data analysis

Experiments with HRPE cells were repeated three times with three independent transfections, and transport measurements were made in duplicate in each experiment. Electrophysiological measurements of substrate-induced currents were repeated at least three times with different sets of oocytes. The data are presented as means $\pm$ S.E.M. for these replicates. The kinetic parameters were calculated using the commercially available computer program Sigma Plot, version 6.0 (SPSS, Chicago, IL, U.S.A.).

RESULTS

Structural features of mouse NaCT

The coding region of mouse NaCT cDNA is 1719 bp long (including the termination codon) and the predicted protein coded by this cDNA consists of 572 amino acids (Figure 1A). This

protein is 76 amino acids shorter than the protein predicted by computational analysis (GenBank[®] accession no. XM.137672). A comparison of the nucleotide sequence of this predicted mRNA with that of the mouse *nact* gene located in the contig NT_039520 shows that the extra 76 amino acids arise from intron 1 using alternative splice junctions as predicted by computational analysis. However, this sequence is not found in the mRNA isolated from mouse brain. The mouse NaCT protein cloned from the brain shows 86% sequence identity (93% similarity) with rat NaCT and 74% sequence identity (85% similarity) with human NaCT (Figure 1A). The amino acid sequence identity is much lower with other members of the gene family (SLC13), namely the low-affinity Na⁺-coupled dicarboxylate transporter NaDC1 (mouse NaCT versus mouse NaDC1, 50% identity), the high-affinity Na⁺-coupled dicarboxylate transporter NaDC3 (mouse NaCT versus mouse NaDC3, 44% identity), the Na⁺-coupled sulphate transporter NaSi1 (mouse NaCT versus mouse NaSi1, 40% identity) and the sulphate transporter SUT1 (mouse NaCT versus mouse SUT1, 39% identity). NaCT represents the mammalian orthologue of *Drosophila* Indy with which the cloned mouse NaCT shares 33% identity and 57% similarity in amino acid sequence. Hydropathy analysis, according to the

Table 1 Exon-intron organization of mouse *nact* gene

5'-Exon			Intron				3'-Exon		
No.	Size (bp)	Sequence	Donor	Size (bp)	No.	Acceptor	Sequence	No.	
1	> 118	...GACAAG	gtcagt...	4312	1	...cttcag	TTTGCC...	2	
2	129	...AAGCAG	gtgagt...	273	2	...ctccag	GTATGT...	3	
3	137	...CTCAGC	gtaaac...	1230	3	...ctgcag	GCTGAT...	4	
4	188	...TGCCAG	gtgcac...	1068	4	...ccacag	GAAGCC...	5	
5	169	...GCAGGA	gtgagt...	1475	5	...tttcag	ATTGTT...	6	
6	123	...ACACAA	gtaagt...	3957	6	...gtctag	TTTAAA...	7	
7	219	...CACCGT	gtaagt...	825	7	...tatcag	TCATAT...	8	
8	101	...AGGAAG	gtaagg...	1413	8	...tcgcag	AAAGGA...	9	
9	119	...TGTGAG	gtactt...	2897	9	...tcctag	ACGTCA...	10	
10	162	...TCCATG	gtaagt...	2403	10	...ctctag	GCTCGT...	11	
11	138	...GACATG	gtaaca...	1489	11	...ccctag	ATGAAA...	12	
12	> 132	...ACTTAG...							

computer program TMPred (http://www.ch.embnet.org/cgi-bin/TMPRED_form_parser), predicts two alternative models for mouse NaCT. According to the strongly preferred model, mouse NaCT possesses 13 transmembrane domains with its N-terminus facing the cytoplasmic side of the membrane and C-terminus facing the exoplasmic side of the membrane. The alternative model predicts mouse NaCT as possessing 12 transmembrane domains with its N- and C-termini facing the exoplasmic side of the membrane. There are three putative N-glycosylation sites, Asn-179, Asn-382 and Asn-566. Of these, only Asn-566 is preserved in rat and human NaCT. In the topology model with 13 transmembrane domains, Asn-179 and Asn-382 are located in the cytoplasmic loops and Asn-566 lies on the exoplasmic side. In the topology model with 12 transmembrane domains, all three asparagine residues are located on the exoplasmic side of the membrane.

Exon-intron organization of mouse *nact* gene

The mouse gene coding for NaCT is located on chromosome 11 and consists of 12 exons (Figure 1B). Exon 1 codes for the translational start site and exon 12 codes for the translational termination site. The size of the gene is approx. 23 kb, excluding the promoter region. The exact lengths of individual exons and introns and the identity of the splice junctions are described in Table 1.

Functional features of mouse NaCT as assessed in a mammalian cell expression system

Figure 2 shows the transport of succinate and citrate at pH 7.5 by mouse NaCT when expressed heterologously in HRPE cells. The transport of 50 nM succinate is 7.8-fold higher in cells transfected with mouse NaCT cDNA when compared with that in cells transfected with vector alone [42.7 ± 4.6 fmol $\cdot$ (10⁶ cells)⁻¹ $\cdot$ min⁻¹ in cDNA-transfected cells and 5.5 ± 0.7 fmol $\cdot$ (10⁶ cells)⁻¹ $\cdot$ min⁻¹ in vector-transfected cells]. The transport of 20 μ M citrate is 20.2-fold higher in cells transfected with mouse NaCT cDNA when compared with that in cells transfected with vector alone [48.4 ± 1.0 pmol $\cdot$ (10⁶ cells)⁻¹ $\cdot$ min⁻¹ in cDNA-transfected cells and 2.4 ± 0.1 pmol $\cdot$ (10⁶ cells)⁻¹ $\cdot$ min⁻¹ in vector-transfected cells]. These results show that mouse NaCT is capable of transporting succinate as well as citrate and that the magnitude of citrate transport is much higher when compared with that of succinate transport in terms of stimulation of transport induced by the cloned transporter.

Figure 2 Uptake of succinate and citrate via mouse NaCT

HRPE cells were transfected with either vector alone (C) or mouse NaCT cDNA (NaCT). Uptake of 50 nM [³H]succinate and 20 μ M [¹⁴C]citrate was measured at pH 7.5 in transfected cells. The uptake of each substrate measured in control cells was taken as 1 and the corresponding uptake in cDNA-transfected cells is given as a ratio (fold increase) in comparison with this control uptake.

Figure 3 Influence of extracellular pH on mouse NaCT-mediated uptake of citrate and succinate

HRPE cells were transfected with either vector alone or mouse NaCT cDNA and uptake of 10 μ M citrate (●) and 10 μ M succinate (○) was measured in these cells. The pH of the uptake buffer was varied by appropriately adjusting the concentrations of Mes, Hepes and Tris. The uptake measured in vector-transfected cells was subtracted from the corresponding uptake measured in cDNA-transfected cells to determine the cDNA-specific uptake. Results represent only the cDNA-specific uptake.

Citrate is a tricarboxylate with pK values of 3.1, 4.8 and 6.4 [19]. Succinate is a dicarboxylate with pK values of 4.2 and 5.6 [19]. At pH 7.5, citrate exists approx. 90% as a tervalent anion and approx. 10% as a bivalent anion. Succinate, on the other hand, exists almost completely as a bivalent anion at pH 7.5. Therefore it would be of interest to compare the influence of pH on the NaCT-mediated transport of citrate and succinate under identical conditions. The results shown in Figure 3 indicate that pH has differential influence on the transport of 10 μ M citrate and 10 μ M succinate via mouse NaCT. The transport of citrate exhibits a clear optimum pH at 7. The transport rate decreases significantly when pH is made more alkaline or more acidic than 7. In contrast, the transport of succinate is maximal at pH 7.5 and decreases significantly when pH is < 7.5.

Kinetic analysis indicates that the transport of citrate and succinate mediated by mouse NaCT is saturable at all three pH values studied (8.5, 7.5 and 6.5; Figure 4). However, the values for K_m and V_{max} are influenced markedly by pH for both substrates (Tables 2 and 3). For citrate transport, the V_{max} value is maximal at pH 7.5. The K_m value for total citrate remains the same at pH 7.5 and 8.5 (38 ± 5 μ M) but decreases appreciably at pH 6.5 (17 ± 1 μ M). In contrast, the V_{max} value for succinate transport

Figure 4 Kinetics of mouse NaCT-mediated citrate and succinate uptake at different pH values

HRPE cells were transfected with either vector alone or mouse NaCT cDNA and uptake of citrate (A) and succinate (B) was measured in these cells at three different pH values (▼, pH 8.5; ○, pH 7.5; ●, pH 6.5). The concentration range was 5–250 μM for citrate and 10–500 μM for succinate. The uptake measured in vector-transfected cells was subtracted from the corresponding uptake measured in cDNA-transfected cells to determine the cDNA-specific uptake. Only the uptake values that are specific for mouse NaCT were used in kinetic analysis. Results are presented as Eadie–Hosftee plots: V/S (uptake rate/substrate concentration) versus V (uptake rate).

does not differ significantly over the pH range 6.5–8.5. However, the K_m value for total succinate remains the same at pH 8.5 and 6.5 (62 ± 9 and $71 \pm 18 \mu\text{M}$ respectively) but decreases significantly at pH 7.5 ($37 \pm 6 \mu\text{M}$). Based on the percentages of individual ionic forms of citrate and succinate at different pH values, we could calculate the apparent K_m values for each of the ionic forms at all three pH values (Tables 2 and 3). For citrate, the only two ionic forms that are quantitatively relevant over the pH range 6.5–8.5 are the bivalent citrate (i.e. H-citrate^{2-}) and the tervalent citrate (i.e. citrate^{3-}). In contrast, for succinate, there is only one ionic form that is quantitatively relevant in this pH range and it is the bivalent succinate (i.e. succinate^{2-}).

The transport of citrate and succinate via mouse NaCT is obligatorily dependent on the presence of Na^+ . Figure 5 describes the Na^+ -activation kinetics for the transport of citrate and succinate. The relationship between the transport rate and Na^+ concentration is sigmoidal for both substrates. Interestingly, the transport of citrate reaches saturation with an Na^+ concentration of 120 mM at pH 7.5, whereas the transport of succinate does not reach saturation within the concentration range of Na^+ tested (up to 140 mM) at pH 7.5 or 6.5. Analysis of the data according to Hill equation shows that the Hill coefficient (h) is 3.3 ± 0.6 for citrate. The corresponding value for succinate is 2.3 ± 0.3 at pH 7.5 and 2.1 ± 0.2 at pH 6.5.

The substrate specificity of mouse NaCT was investigated by assessing the ability of various tricarboxylic acid cycle intermediates and related compounds to compete with [^{14}C]citrate and [^3H]succinate for transport via mouse NaCT. These studies have shown that citrate, succinate, fumarate, malate and 2-oxoglutarate are recognized by the transporter as substrates. These five compounds effectively competed with radiolabelled citrate and succinate for transport via mouse NaCT (Table 4). The monocarboxylates pyruvate and lactate exhibit no or little inhibitory activity for the transport of citrate and succinate. Similarly, maleate, the *cis*-isomer of fumarate, is also not effective as an inhibitor. When compared with citrate, the tricarboxylates isocitrate and *cis*-aconitate also do not interact effectively with the transporter.

We then studied the influence of membrane potential on the transport of citrate and succinate via mouse NaCT by assessing the effect of K^+ -induced depolarization on the transport processes. In these experiments, the cells were preincubated for

Table 2 Influence of pH on kinetic parameters for citrate transport via mouse NaCT

pH	$V_{\max}$ [$\text{pmol} \cdot (10^6 \text{ cells})^{-1} \cdot \text{min}^{-1}$]	Apparent K_m (μM)				
		Citrate _{total}	H ₃ -citrate	H ₂ -citrate ⁻	H-citrate ²⁻	Citrate ³⁻
8.5	68 ± 6	38 ± 5	0	0	0.3	37.7
7.5	182 ± 13	38 ± 5	0	0	2.8	35.2
6.5	78 ± 3	17 ± 1	0	0.1	7.4	9.4

Table 3 Influence of pH on kinetic parameters for succinate transport via mouse NaCT

pH	$V_{\max}$ [$\text{pmol} \cdot (10^6 \text{ cells})^{-1} \cdot \text{min}^{-1}$]	Apparent K_m (μM)			
		Succinate _{total}	H ₂ -succinate	H-succinate ⁻	Succinate ²⁻
8.5	83 ± 5	62 ± 9	0	0.1	61.9
7.5	85 ± 7	37 ± 6	0	0.5	36.5
6.5	72 ± 11	71 ± 18	0	7.9	63.0

Figure 5 Na^+ -activation kinetics of citrate and succinate uptake mediated by mouse NaCT

HRPE cells were transfected with either vector alone or mouse NaCT cDNA and uptake of 20 μM citrate (**A**) and 2.5 μM succinate (**B**) was measured in these cells. Concentration of Na^+ was varied over the range of 10–140 mM by adjusting the concentrations of NaCl and *N*-methyl-D-glucamine chloride appropriately to maintain the osmolality. The uptake measured in vector-transfected cells was subtracted from the corresponding uptake measured in cDNA-transfected cells to determine the cDNA-specific uptake. Only the uptake values that are specific for mouse NaCT were used in kinetic analysis. Citrate uptake was measured at pH 7.5 whereas succinate uptake was measured at two different pH values ($\circ$, pH 7.5; $\bullet$, pH 6.5).

10 min with a buffer containing either 90 mM NaCl, 50 mM *N*-methyl-D-glucamine chloride and 5.4 mM KCl (pH 7.5) or 90 mM NaCl and 55.4 mM KCl (pH 7.5). After preincubation, transport of 10 μM citrate and 10 μM succinate was measured in the same buffers as in preincubation. The rationale for these studies is that exposure of the cells to high K^+ (55.4 mM instead of 5.4 mM) would depolarize the cells and any observed difference in transport between these two experimental conditions would indicate the influence of membrane potential on the transport process. These studies have shown that K^+ -induced depolarization leads to a significant decrease in the transport of citrate as well as succinate. The transport of citrate under normal conditions is $18.5 \pm 2.2 \text{ pmol} \cdot (10^6 \text{ cells})^{-1} \cdot \text{min}^{-1}$ and this value is decreased by 40% [$10.6 \pm 0.8 \text{ pmol} \cdot (10^6 \text{ cells})^{-1} \cdot \text{min}^{-1}$] under depolarized conditions. The transport of succinate under normal conditions is $7.8 \pm 0.4 \text{ pmol} \cdot (10^6 \text{ cells})^{-1} \cdot \text{min}^{-1}$ and this value is decreased by 30% [$5.4 \pm 0.2 \text{ pmol} \cdot (10^6 \text{ cells})^{-1} \cdot \text{min}^{-1}$] under depolarized conditions. Since membrane depolarization suppresses the transport activity, these results show that the transport process mediated by mouse NaCT is electrogenic and that the transport of citrate as well as succinate via the transporter results in the transfer of positive charge into the cells.

We also examined the effects of Ca^{2+} and Mg^{2+} on the transport of 10 μM citrate because citrate may form complexes with these metal ions. The normal transport buffer contains 1.8 mM CaCl_2 and 0.8 mM MgSO_4 . Therefore we modified the transport buffer such that it lacked CaCl_2 or MgSO_4 or both. These studies have shown that the presence or absence of Mg^{2+} does not affect the

Table 4 Substrate specificity of mouse NaCT

Transport of 10 μM [^{14}C]citrate and 0.1 μM [^3H]succinate were measured in parallel in vector-transfected cells and in cells transfected with mouse NaCT cDNA in the absence or presence of 2 mM concentration of various substrate analogues. NaCT-specific transport was calculated by subtracting the transport in vector-transfected cells from the transport in cDNA-transfected cells. Data (means $\pm$ S.E.M.) represent only NaCT-specific transport.

Substrate analogue	[^{14}C]citrate transport		[^3H]succinate transport	
	[pmol $\cdot$ (10 ⁶ cells) ⁻¹ $\cdot$ min ⁻¹]	(%)	[pmol $\cdot$ (10 ⁶ cells) ⁻¹ $\cdot$ min ⁻¹]	(%)
Control	38.4 $\pm$ 6.0	100	0.145 $\pm$ 0.010	100
Citrate	0.7 $\pm$ 0.1	2	0.004 $\pm$ 0.001	3
Succinate	0.1 $\pm$ 0.1	1	0.007 $\pm$ 0.003	5
Fumarate	1.6 $\pm$ 0.4	4	0.007 $\pm$ 0.002	5
Malate	0.2 $\pm$ 0.1	1	0.004 $\pm$ 0.001	3
2-Oxoglutarate	4.6 $\pm$ 0.5	12	0.018 $\pm$ 0.001	12
Maleate	31.2 $\pm$ 2.5	82	0.165 $\pm$ 0.014	113
Isocitrate	26.3 $\pm$ 1.1	69	0.125 $\pm$ 0.003	86
<i>cis</i> -Aconitate	18.1 $\pm$ 1.8	47	0.062 $\pm$ 0.005	43
Pyruvate	32.3 $\pm$ 1.5	84	0.151 $\pm$ 0.013	104
Lactate	29.8 $\pm$ 3.4	78	0.162 $\pm$ 0.008	111

transport activity. The absence of Ca^{2+} , however, leads to approx. 20% decrease in citrate transport.

Functional features of mouse NaCT as assessed in the *X. laevis* oocyte expression system

We have reported previously on the cloning and functional characteristics of rat and human NaCT [1,2], but all of these studies were done with a mammalian cell expression system. These studies indicated that mammalian NaCT is electrogenic, based on the influence of membrane depolarization of the transport activity. However, the electrogenic nature of the transporter has not yet been established unequivocally. The present studies with mouse NaCT show that both succinate and citrate are transported via this transporter. Even though the results from the Na^+ -activation kinetics have shown that multiple Na^+ ions are involved in the transport process, the participation of two Na^+ ions in the activation of succinate transport and three Na^+ ions in the activation of citrate transport does not necessarily predict the electrogenic nature of these transport processes. Therefore we have chosen to use the *Xenopus* oocyte expression system to analyse the electrogenicity of mouse NaCT.

Expression of mouse NaCT in oocytes led to a marked increase in the uptake of [^{14}C]citrate and [^3H]succinate (results not shown), indicating functional expression of the transporter. We then used these oocytes for electrophysiological studies to determine if the perfusion of the oocytes in the presence of substrates leads to inward currents when monitored by the two-microelectrode voltage-clamp method. These studies have shown that exposure of the NaCT-expressing oocytes to citrate induces measurable inward currents ($30 \pm 7 \text{ nA}$ at 0.5 mM citrate in three different sets of oocytes; Figure 6). The citrate-induced current is obligatorily dependent on the presence of Na^+ . Iso-osmotic replacement of Na^+ with *N*-methyl-D-glucamine abolishes the citrate-induced currents almost completely. These currents are not dependent on Cl^- as iso-osmotic replacement of Cl^- with gluconate does not affect the citrate-induced currents (Figure 6). Exposure of the oocytes to succinate also induces inward currents in a Na^+ -dependent manner (results not shown).

We then analysed the charge-to-substrate ratio for the transport process. This was motivated by the findings that the transport of citrate as well as succinate occurs via an electrogenic process.

Figure 6 Electrogenicity of mouse and rat NaCTs

Mouse and rat NaCTs were expressed functionally in *X. laevis* oocytes by injection of respective cRNAs. Citrate-induced currents were monitored in these oocytes using the two-microelectrode voltage-clamp technique. The membrane potential was clamped at -50 mV. The perfusion buffer contained *N*-methyl-D-glucamine chloride ($-Na^+$), NaCl or sodium gluconate ($-Cl^-$).

This raises three different possibilities in terms of transport mechanism. First, both citrate and succinate are transported as bivalent anions and the Na^+ /substrate stoichiometry is 3:1. In this case, the charge-to-substrate ratio would be 1 for both substrates. Secondly, citrate is transported as a trivalent anion and succinate is transported as a bivalent anion, but the Na^+ /substrate stoichiometry changes from 4:1 for citrate to 3:1 for succinate. In this case also, the charge-to-substrate ratio would remain as 1. Thirdly, citrate is transported as a trivalent anion and succinate is transported as a bivalent anion, and the Na^+ /substrate stoichiometry remains as 4:1 for both substrates. In this case, the charge-to-substrate ratio would vary depending on the substrate. This ratio would be 1 for citrate but 2 for succinate. These three possibilities can be differentiated by an experimental method in which the NaCT-expressing oocytes are perfused with radiolabelled citrate or succinate for a given time period in which the substrate-induced inward currents are monitored. At the end of the perfusion period, the amount of citrate or succinate transported into the oocytes is measured. The amount of charge transferred into the same oocyte can be calculated from the inward currents. This would allow us to determine the charge-to-substrate ratio. However, this requires a robust electrogenicity of the transport process so that the radiolabelled substrate can be mixed with adequate amounts of unlabelled substrate to allow measurable inward currents for charge-transfer calculations and, at the same time, to allow measurable transfer of radiolabel into the oocyte so that the amount of substrate transferred can be measured. From our experience [14,20], the substrate-induced currents via mouse NaCT are not sufficient to determine the charge-to-substrate ratio using our electrophysiological set-up. Therefore we used rat NaCT to see if this transporter is associated with a higher magnitude of substrate-induced currents compared with mouse NaCT. This indeed turned out to be the case. Perfusion of the oocytes, which expressed rat NaCT, to 0.5 mM citrate was found to induce 3-fold higher currents compared with mouse NaCT (Figure 6). We also compared the ability of rat NaCT to transport other intermediates of the tricarboxylic acid cycle by comparing the magnitude of inward currents induced by these compounds (Figure 7). When the concentration of the substrate was kept constant at 0.5 mM, citrate induced the maximum current compared with various putative substrates tested. Fumarate, succinate, malate and 2-oxo-glutarate, the dicarboxylate intermediates of the tricarboxylic acid cycle, also induced appreciable currents, but the magnitude of the currents was significantly less when compared with that

Figure 7 Relative ability of rat NaCT to transport various tricarboxylic acid cycle intermediates and other related compounds

Rat NaCT was expressed functionally in *X. laevis* oocytes by injection of cRNA. Oocytes were perfused with various monocarboxylates, dicarboxylates and tricarboxylates (0.5 mM) and the substrate-induced inward currents were monitored using the two-microelectrode voltage-clamp technique. The currents induced by various substrates are given as percentage of the current induced by citrate. The data are from three different oocytes and the citrate-induced current in each oocyte was normalized by taking this value as 100%. The value for citrate-induced current in three different oocytes was 87 ± 8 nA.

induced by citrate (20–60% of citrate-induced currents). *cis*-Aconitate induced currents that amounted to approx. 10% of citrate-induced currents. Isocitrate, pyruvate and lactate induced no or little current. Since rat NaCT induced marked currents with citrate as well as succinate, we analysed the charge-to-substrate ratio for the transport process using rat NaCT rather than mouse NaCT. The results of charge transfer for the transport of citrate and succinate via rat NaCT are presented in Figure 8. Even though the transport of both substrates via the transporter is electrogenic, the quantity of the charge transferred into the oocytes for a given amount of the substrate transfer is relatively higher for succinate when compared with that for citrate. The charge-to-substrate transfer ratio for succinate is 2, whereas the corresponding value for citrate is 1. This suggests that the Na^+ /substrate stoichiometry is 4:1 irrespective of whether transported substrate is citrate or succinate.

DISCUSSION

The amino acid sequence of mouse NaCT cloned from the brain is exactly the same as that predicted from the mouse gene sequence by computational analysis except for a stretch of 76 amino acids as predicted by the GenomeScan but not found in the cloned NaCT. This additional sequence arises from the use of alternative splice junctions predicted by the GenomeScan in intron 1. The nucleotide sequence of the cloned NaCT cDNA shows that exon 1 is 102 bp long (starting from the translational-initiation codon ATG) and that exon 2 is 129 bp long. However, according to the GenomeScan prediction, exon 1 contains an additional 194 bp sequence at its 3'-end and exon 2 contains an additional 34 bp sequence at its 5'-end. This stretch of 228 bp, predicted to be a part of the coding region, gives rise to the extra 76 amino acids. This sequence is, however, not found in the cDNA cloned from mouse brain. Whether these alternative splice junctions are used in tissues other than the brain is not known. NaCT has been cloned from rat brain and from a human liver cell line [1,2], but there

Figure 8 Determination of charge-to-substrate ratio for rat NaCT with citrate and succinate as substrates

Rat NaCT was expressed functionally in *X. laevis* oocytes by injection of cRNA. The oocytes were perfused with 50 μ M citrate or succinate (radiolabelled plus unlabelled substrates) for 10 min and the substrate-induced currents were monitored in these oocytes using the two-microelectrode voltage-clamp technique. The membrane potential was clamped at -50 mV. At the end of the experiment, the oocytes were washed with the perfusion buffer and the radioactivity associated with the oocytes was determined. The quantity of charge transferred into the oocytes during perfusion with the substrates was determined from the integration of the area covered by the time versus inward current curves and the quantity of the substrates transferred into the oocytes was determined from the radioactivity associated with the oocytes. (A) Relationship between substrate uptake and charge transfer for citrate (6 oocytes) and succinate (5 oocytes). (B) Charge-to-substrate ratio for citrate and succinate.

is no evidence for the presence of alternative splice variants in these tissues. Therefore it seems very unlikely that the stretch of the additional 76 amino acids predicted by the GenomeScan is actually found in NaCT expressed in any tissue.

Analysis of the amino acid sequence of the cloned mouse NaCT using the TMpred program predicts two different topology models, one with 13 transmembrane domains with its N-terminus placed on the cytoplasmic side of the membrane and C-terminus placed on the external surface of the membrane and the other with 12 transmembrane domains with its N- and C-termini placed on the external surface. This protein also possesses three putative N-glycosylation sites, of which Asn-566 is conserved in rat and human NaCTs. We have obtained evidence that the corresponding asparagine residue in human NaCT (Asn-562) is most probably N-glycosylated because treatment of HepG2 cells with tunicamycin, an inhibitor of N-linked glycosylation, leads to a significant inhibition of NaCT activity (E. Gopal, L. Zhuang, K. Inoue and V. Ganapathy, unpublished work). These results suggest that Asn-566 in mouse NaCT is also probably N-glycosylated. Both topology models with either 12 or 13 transmembrane domains are in agreement with the potential N-glycosylation of Asn-566 because in both models this residue is located on the exoplasmic side of the membrane. Interestingly, analysis of the amino acid sequences of rat and human NaCTs using the same

TMpred program indicates that these proteins probably possess 12 transmembrane domains instead of 13. However, the program predicts that both the N- and the C-terminus are most probably located on the external surface of the membrane. This model places the conserved N-glycosylation site in the C-terminal tail in rat and human NaCTs on the external side of the membrane. The proposed models for the topology of mammalian NaCTs are only speculative at the time of writing this paper and the actual topology of these transporters remains to be established with epitope-specific antibodies and by other experimental approaches.

Even though we have already reported on the cloning and functional characteristics of rat and human NaCTs, the present study provides important information, for the first time, on the transport mechanism of NaCT with respect to its Na^+ /substrate stoichiometry and electrogenicity. Mouse NaCT, when expressed in mammalian cells, transports not only citrate but also succinate. This is similar to rat NaCT [1]. In contrast, human NaCT exhibits very little ability to transport succinate [2]. pH dependence of the transport process mediated by mouse NaCT shows that the transport of succinate remains almost unaffected over the pH range 7.0–8.5, whereas the transport of citrate increases markedly when the pH is changed from 8.5 to 7.0. These results can be taken as evidence that citrate is recognized by NaCT in its protonated bivalent anionic form (H-citrate^{2-}) because the fraction of the bivalent form of citrate increases as the pH is changed from 8.5 to 7.0. The bivalent form of succinate (succinate^{2-}) does not change significantly over the pH range 7.0–8.5. Similar conclusions have been drawn for NaDC1 and NaDC3 [10,11]. Na^+ -activation kinetics of mouse NaCT shows that multiple Na^+ ions are involved in the transport process. The Na^+ /substrate stoichiometry is 3:1 for citrate and 2:1 for succinate. These results, together with the results from the pH-dependence studies, would suggest that citrate transport is electrogenic whereas succinate transport is electroneutral. However, this is not the case as is evident from the oocyte expression system. In contrast with the studies with the mammalian cell expression system, electrophysiological studies with the oocyte expression system provide evidence for a completely different transport mechanism for mouse NaCT. The transport of citrate as well as succinate occurs via an electrogenic process. Exposure of NaCT-expressing oocytes to citrate or succinate is associated with inward currents in the presence of Na^+ , indicating that, for citrate as well as succinate, the transport process results in the transfer of net positive charge into the oocytes. But, the amount of positive charge transferred into the oocytes varies depending on whether the transported substrate is citrate or succinate. With citrate, the charge-to-substrate ratio is 1, indicating that citrate transport is associated with the transfer of one positive charge per citrate molecule. Therefore the Na^+ /citrate stoichiometry should be 3:1 if citrate is recognized by the transporter as a bivalent anion (H-citrate^{2-}) or 4:1 if the trivalent anionic form (citrate^{3-}) is recognized instead. In contrast with citrate, the charge-to-substrate ratio is 2 for succinate which indicates that succinate transport is associated with the transfer of two positive charges per succinate molecule. Since succinate exists predominantly as a bivalent anion (succinate^{2-}) under the experimental conditions, the Na^+ /succinate stoichiometry should be 4:1. Taken collectively, the most probable conclusion that can be drawn from these results is that mouse NaCT transports succinate as well as citrate by an electrogenic process with an Na^+ /substrate stoichiometry of 4:1. Furthermore, the charge-to-substrate ratio of 2 for succinate and 1 for citrate strongly suggests that NaCT is capable of transporting citrate as a trivalent anion and succinate as a bivalent anion. Thus NaCT is an Na^+ -coupled transporter for dicarboxylates as well as tricarboxylates. This characteristic is distinct from that of NaDC1 and NaDC3,

which recognize their substrates only in their bivalent anionic form.

The electrophysiological data from the oocyte expression studies demonstrate that the number of Na^+ involved in the transport process is 4 irrespective of whether the transported substrate is citrate or succinate. In contrast, the Na^+ -activation kinetics in mammalian cell-expression studies indicated that the number of Na^+ ions involved in the transport process is 3 if the transported substrate is citrate and that the number changes to 2 if the transported substrate is succinate. However, since studies on oocyte expression demonstrate unequivocally the electrogenic nature of the transport process for succinate, which exists almost entirely as a bivalent anion under the experimental conditions, the Na^+ /succinate stoichiometry of 2 is probably incorrect. Obviously, the value obtained for the number of Na^+ ions involved in the transport of succinate in the mammalian cell expression system is an underestimate. The value of 3 for the Na^+ /citrate stoichiometry can be reconciled with the electrogenic nature of the transport process if one hypothesizes that citrate is transported as a bivalent anion. However, the charge-to-substrate transfer ratios, calculated from the oocyte expression studies, suggest that citrate is recognized as a trivalent anion whereas succinate is recognized as a bivalent anion. Therefore the value obtained for the number of Na^+ ions involved in the transport of citrate in the mammalian cell expression system is also probably an underestimate. There is precedence for such discrepancies between the Na^+ /substrate coupling ratio determined from Na^+ -activation kinetics (i.e. the Hill coefficient) and the actual coupling ratio [12,21]. For mouse NaDC1 and NaDC3, the Hill coefficient calculated from Na^+ -activation kinetics is 2 with succinate as the substrate although the transport process is undoubtedly electrogenic. The electrogenicity of the transport process mandates that the Na^+ -to-succinate coupling ratio should be at least 3. This apparent discrepancy arises because the Hill coefficient does not always represent the absolute value. The Hill coefficient represents the minimal number of Na^+ -binding sites involved in the activation process and the value for the Hill coefficient determined from Na^+ -activation kinetics is influenced significantly by the strength of co-operativity among the binding sites. Another significant difference observed in the mammalian cell expression system is in the nature of Na^+ -activation kinetics between the transport of succinate and citrate. The transport of citrate saturates within the concentration range of Na^+ tested whereas the transport of succinate does not saturate under identical conditions. Apparently, the co-operativity among the different Na^+ -binding sites differs depending on whether the transported substrate is succinate or citrate. Whether this is due to the difference in the ionic nature (i.e. bivalent nature of succinate and the trivalent nature of citrate) or the bulkiness of these two substrates is not known.

The substrate specificity of mammalian NaCTs merits discussion. Human NaCT appears to be rather specific for citrate as its interaction with succinate and other dicarboxylates occurs with a very low affinity. In contrast, both rat and mouse NaCTs interact with citrate as well as succinate with comparable affinity. In this respect, the rodent NaCTs are similar to *Drosophila* NaCT (Indy). This functional difference can be used as a diagnostic criterion for the identification of the protein domains involved in substrate binding using human-mouse or human-rat chimaeric NaCTs. We have used a similar approach to obtain valuable information on the substrate-binding site of other transporters

such as NaDC3 [14] and OCTN2 (novel organic cation transporter 2) [22].

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