RESEARCH COMMUNICATION

Functional identity of *Drosophila melanogaster* Indy as a cation-independent, electroneutral transporter for tricarboxylic acid-cycle intermediates

Katsuhisa INOUE*, You-Jun FEI*, Wei HUANG*, Lina ZHUANG*, Zhong CHEN* and Vadivel GANAPATHY*^{†1}

*Department of Biochemistry and Molecular Biology, Medical College of Georgia, 1120 15th Street, CB2208, Augusta, GA 30912, U.S.A., and [†]Department of Obstetrics and Gynecology, Medical College of Georgia, Augusta, GA 30912, U.S.A.

Indy is a gene in *Drosophila melanogaster* which, when made dysfunctional, leads to an extension of the average adult life span of the organism. The present study was undertaken to clone the *Indy* gene-product and to establish its functional identity. We isolated a full-length *Indy* cDNA from a *D. melanogaster* cDNA library. The cDNA codes for a protein of 572 amino acids [(*Drosophila* Indy (drIndy)). In its amino acid sequence, drIndy exhibits comparable similarity to the two known Na⁺-coupled dicarboxylate transporters in mammals; namely, NaDC1 (35% identity) and NaDC3 (34% identity). We elucidated the functional characteristics of drIndy in two different heterologous expression systems by using mammalian cells and *Xenopus laevis* oocytes. These studies show that drIndy is a cation-independent electroneutral transporter for a variety of tricarboxylic acid-cycle

intermediates, with preference for citrate compared with succinate. These characteristics of drIndy differ markedly from those of NaDC1 and NaDC3, indicating that neither of these latter transporters is the mammalian functional counterpart of drIndy. Since drIndy is a transporter for tricarboxylic acid-cycle intermediates, dysfunction of the *Indy* gene may lead to decreased production of metabolic energy in cells, analogous to caloric restriction. This might provide the molecular basis for the observation that disruption of the *Indy* gene function in *Drosophila* leads to extension of the average adult life span of the organism.

Key words: caloric restriction, citrate transport, dicarboxylate transporter, life span.

INTRODUCTION

In a recent study, Rogina et al. [1] have identified a gene, which when made dysfunctional by P-element insertional mutations, doubled the average adult life span in *Drosophila melanogaster*. They named the gene *Indy* (an acronym from 'I'm not dead yet'). The protein product of the *Indy* gene is most closely related in amino acid sequence to mammalian Na⁺-coupled dicarboxylate transporters, known as NaDCs. NaDCs are secondary active transporters for dicarboxylate intermediates of the tricarboxylic acid cycle [2]. On the basis of this known function of mammalian NaDCs, Rogina et al. [1] hypothesized that mutations in the *Indy* gene may reduce the availability of these key metabolic intermediates to the cell, thus reducing the generation of metabolic energy. These mutations may thus create a metabolic state resembling caloric restriction. It has been documented in rodents and in non-human primates that caloric restriction can extend the maximal life span of the animal [3,4].

Two different NaDCs have been identified so far in mammalian tissues [2]. These are NaDC1 and NaDC3 (a unique NaDC identified in *Xenopus laevis* is currently referred to as NaDC2). Therefore the question arises as to which one of the two NaDCs is the mammalian counterpart of *Drosophila* Indy (drIndy) in terms of biological function. NaDC1 is Na⁺-coupled, electrogenic and exhibits a low affinity for its dicarboxylate substrates. The Michaelis–Menten constant for the prototypical substrate succinate is in the range of 0.1–4.0 mM [5–7]. This isoform is expressed primarily in the brush-border membrane of intestinal and renal epithelial cells. NaDC3 is also Na⁺-coupled and electrogenic, but exhibits relatively higher affinity for its

dicarboxylate substrates compared with NaDC1. The Michaelis–Menten constant for succinate is in the range of 2–50 μ M [8–11]. This isoform is expressed primarily in the basolateral membrane of intestinal and renal epithelial cells, sinusoidal membrane of hepatocytes, brush-border membrane of placental trophoblasts and in the plasma membrane of neurons and glial cells. Since NaDC1 and NaDC3 differ significantly in transport characteristics and tissue-expression pattern, it is important to identify the isoform of NaDC that is the mammalian functional counterpart of drIndy. This cannot be achieved without information on the functional nature of drIndy. Rogina et al. [1] derived the amino acid sequence of the putative Indy protein on the basis of the genomic sequence and expressed sequence tags ('ESTs'). Neither has the full-length *Indy* cDNA been cloned nor has the transport function of Indy been established. We do not even know whether drIndy is actually a Na⁺-coupled transporter for dicarboxylate anions, as predicted by Rogina et al. [1]. Therefore the present studies were undertaken to clone the full-length drIndy cDNA and identify its transport function.

MATERIALS AND METHODS

Materials

[³H]Succinate (specific radioactivity, 40 Ci/mmol), [¹⁴C]citrate (specific radioactivity, 55 mCi/mmol), and [¹⁴C]pyruvate (specific radioactivity, 15 mCi/mmol) were purchased from Moravsek Biochemicals (Brea, CA, U.S.A.). The human retinal pigment epithelial (HRPE) cell line, used in functional expression studies, was routinely maintained in Dulbecco's modified Eagle's

Abbreviations used: drIndy, *Drosophila* Indy; HRPE, human retinal pigment epithelial; (h)NaDC, (human) Na⁺-coupled dicarboxylate transporter; poly(A)⁺, polyadenylated; RT, reverse transcription.

¹ To whom correspondence should be addressed (e-mail vganapat@mail.mcg.edu).

The nucleotide sequence data for drIndy cDNA will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AF509505.

medium/F-12 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Frogs (*Xenopus* species) were purchased from Nasco (Fort Atkinson, WI, U.S.A.).

Cloning of the drIndy cDNA

The nucleotide sequence of the putative mRNA coding for the Indy protein was first deduced from the *Drosophila* gene sequence (GenBank® accession no. AE003519; reverse complement). This sequence was used to design primers for reverse transcriptase (RT)-PCR to obtain a cDNA probe specific for Indy. The forward primer was 5'-CTCCAACCTTCTTCGCTAACC-3' and the reverse primer was 5'-CTAGTGCGTCTTGTTCCTCC-3'. The predicted size of the RT-PCR product was 1675 bp. This primer pair was used to obtain a fragment of Indy cDNA by using the commercially available polyadenylated [poly(A)⁺] RNA from adult *D. melanogaster* (ClonTech, Palo Alto, CA, U.S.A.). This yielded a RT-PCR product of expected size. The product was subcloned in pGEM-T vector and sequenced to establish its molecular identity. A unidirectional *Drosophila* cDNA library was then established using the commercially available poly(A)⁺ RNA. The SuperScript™ plasmid system (Life Technologies, Gaithersburg, MD, U.S.A.) was employed for this purpose. The Indy-specific cDNA probe derived from RT-PCR was labelled with [α -³²P]dCTP and used to screen the *Drosophila* cDNA library under high-stringency conditions [12,13].

DNA sequencing

Both the sense and antisense strands of the cDNA were sequenced by primer walking. Sequencing was done by Taq DyeDeoxy terminator cycle sequencing using an automated PerkinElmer Applied Biosystems 377 Prism DNA sequencer. The sequence was analysed using the National Center for Biotechnology Information server (<http://www.ncbi.nlm.nih.gov/>).

Functional expression of drIndy cDNA in mammalian cells

The functional expression of drIndy cDNA was accomplished in HRPE cells using the vaccinia virus expression system [14–16]. Subconfluent HRPE cells grown on 24-well plates were first infected with a recombinant (VTF₇₋₃) vaccinia virus encoding T₇ RNA polymerase and then transfected with the plasmid carrying the full-length drIndy cDNA. After 12–15 h post-transfection, uptake measurements were made at 37 °C with radiolabelled succinate, citrate or pyruvate. The uptake medium was 25 mM Hepes/Tris, pH 7.5, containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄ and 5 mM glucose. The time of incubation was 15 min, a time period representing initial-transport rates as determined from time course studies. Endogenous transport was always determined in parallel using cells transfected with pSPORT1 vector alone. The transport activity in cDNA-transfected cells was adjusted for the endogenous activity to calculate the cDNA-specific transport activity. Experiments were performed in triplicate, and each experiment was repeated at least three times. Results are expressed as the means $\pm$ S.E.M. Since infection with vaccinia virus 'shuts off' host-cell proliferation, the cell number of HRPE cells determined immediately prior to infection with the virus was used for calculation of transport activity.

Functional expression of drIndy cRNA in *X. laevis* oocytes

Capped cRNA from the cloned drIndy cDNA was synthesized using the MEGAscript kit (Ambion, Austin, TX, U.S.A.). Mature oocytes from *X. laevis* were isolated by treatment with

collagenase A (1.6 mg/ml), manually defolliculated and maintained at 18 °C in modified Barth's medium supplemented with 10 mg/l gentamicin [17]. On the following day, oocytes were injected with 50 ng of cRNA in 50 nl of water. Oocytes injected with 50 nl of water served as a control. The oocytes were used for electrophysiological studies 6 days after cRNA injection. Electrophysiological studies were performed by the conventional two-micro-electrode voltage-clamp method [9,11,16]. Oocytes were superfused with a NaCl-containing transport buffer (100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 3 mM Hepes, 3 mM Mes and 3 mM Tris, pH 7.5) followed by the same buffer containing 2 mM succinate. The membrane potential was clamped at -50 mV. The dependence of succinate-induced currents on Na⁺ was assessed by comparing the succinate-induced currents in the Na⁺-containing transport buffer with those in a Na⁺-free transport buffer (NaCl in the transport buffer was replaced iso-osmotically by choline chloride). Oocytes injected with human NaDC3 (hNaDC3) cRNA [9] were used as a positive control for succinate-induced currents.

Uptake of succinate in water-injected and cRNA-injected oocytes was measured as described previously [18,19]. At 6 days after injection with water or cRNA, oocytes were incubated with [³H]succinate (7.5 µCi/ml; succinate concentration, 0.1 µM) in a NaCl-containing transport buffer at room temperature for 1 h. After the incubation, oocytes were washed with fresh transport buffer in the absence of radiolabelled succinate four times, and then each oocyte was transferred individually into scintillation vials for determination of radioactivity.

RESULTS

Structural features of drIndy

The cloned drIndy cDNA (GenBank® accession no. AF509505) is 2602 bp long with an open reading frame (258–1976 bp) coding for a protein of 572 amino acids. When compared with the amino acid sequences of hNaDC1 (592 amino acids) and hNaDC3 (602 amino acids), there is significant similarity among the three proteins. The sequence identity between drIndy and hNaDC1 is 35%, and that between drIndy and hNaDC3 is 34%.

Functional features of drIndy

To establish the functional identity of drIndy, we expressed the cloned cDNA heterologously in mammalian cells and assessed the ability of the clone to transport succinate. When measured in the presence of Na⁺, the uptake of succinate (40 nM) in HRPE cells transfected with drIndy cDNA was 20-fold higher than in cells transfected with vector alone (Figure 1A). This shows that drIndy indeed possesses the ability to transport the dicarboxylate succinate. However, surprisingly, drIndy was able to transport succinate not only in the presence of Na⁺, but also in the absence of Na⁺. When measured in the absence of Na⁺, the uptake of succinate in cells transfected with drIndy cDNA was still 15-fold higher than in cells transfected with vector alone. These results are in contrast with those obtained with hNaDC3 under identical conditions (Figure 1B). The uptake of succinate (40 nM), when measured in the presence of Na⁺, increased 200-fold in HRPE cells as a result of transfection with hNaDC3 cDNA. This cDNA-induced uptake was, however, completely abolished when Na⁺ was omitted in the uptake medium. Similar is the case with NaDC3s from other animal species [8,10]. Studies by other groups [5–7] have shown that the uptake of succinate mediated by NaDC1 from different animal species is also obligatorily dependent on the presence of Na⁺. Thus,

Figure 1 Comparison of succinate uptake by drIndy (A) and hNaDC3 (B) in the presence or absence of Na⁺

HRPE cells were transfected with vector alone, drIndy cDNA or hNaDC3 cDNA. Uptake of succinate (40 nM) was measured in the presence of either NaCl (+Na⁺) or *N*-methyl-D-glucamine chloride (−Na⁺). Data (means ± S.E.M.) are from nine independent measurements.

Table 1 Ion-dependence of drIndy-mediated succinate transport

Uptake of succinate (40 nM) was measured in HRPE cells transfected with either vector alone or drIndy cDNA. Uptake buffer (25 mM Hepes/Tris), pH 7.5, contained one of the indicated salts (140 mM) or mannitol (280 mM). In addition, all buffers contained 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄ and 5 mM glucose. Data (means ± S.E.M.) are from six independent measurements. NMDG, *N*-methyl-D-glucamine.

Salt	Succinate uptake (fmol/10 ⁶ cells per min)		Fold increase
	Vector	drIndy	
NaCl	5.9 ± 0.8	118.0 ± 7.8	20.0
KCl	7.0 ± 1.2	111.0 ± 8.5	15.9
LiCl	5.0 ± 0.6	122.3 ± 4.3	24.5
NMDG chloride	4.9 ± 0.7	84.7 ± 10.0	17.3
Sodium gluconate	6.0 ± 1.0	76.6 ± 2.6	12.9
Mannitol	5.3 ± 0.4	83.3 ± 7.1	15.7

although the mammalian NaDC1 and NaDC3 are Na⁺-dependent succinate transporters, drIndy is an Na⁺-independent succinate transporter.

The ability of drIndy to transport succinate remained almost the same even when Na⁺ in the uptake medium was replaced with K⁺, Li⁺, *N*-methyl-D-glucamine or mannitol, suggesting that

Table 2 Substrate specificity of drIndy

Uptake of [³H]succinate (40 nM) was measured in HRPE cells transfected with either vector alone or drIndy cDNA. The uptake buffer was 25 mM Hepes/Tris, pH 7.5, containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄ and 5 mM glucose. Concentration of the inhibitors was 2.5 mM. Uptake in vector-transfected cells was subtracted from uptake in cDNA-transfected cells to determine drIndy cDNA-specific uptake. Data (means ± S.E.M.) are from four independent measurements.

Inhibitor	cDNA-specific [³ H]succinate uptake (fmol/10 ⁶ cells per min)	% Control
Control	143.0 ± 10.8	100
Succinate	4.6 ± 0.4	6
2-Oxoglutarate	3.0 ± 1.0	5
Malate	6.8 ± 2.7	8
Fumarate	4.8 ± 0.3	6
Dimethylsuccinate	14.1 ± 0.8	13
<i>N</i> -Acetylaspartate	58.4 ± 1.5	43
Maleate	123.1 ± 7.6	85
Malonate	98.8 ± 7.1	71
Pyruvate	28.1 ± 3.0	21
Lactate	111.2 ± 3.2	77
β-Hydroxybutyrate	134.0 ± 9.0	91
Citrate	18.6 ± 3.0	13

Figure 2 Saturation kinetics of succinate uptake via drIndy measured in the presence of Na⁺

Data (means ± S.E.M.) represent only the cDNA-specific uptake and are from four independent measurements. Inset: Eadie–Hofstee plot [succinate uptake/succinate concentration (v/s) versus succinate uptake (v)].

drIndy is a cation-independent succinate transporter (Table 1). We then tested whether the drIndy-mediated transport process is dependent on an H⁺ gradient by measuring the uptake of succinate at different pH values between pH 5 and 8. The uptake of succinate (40 nM) mediated by drIndy decreased gradually from 127 ± 5 to 52 ± 2 fmol/10⁶ cells per min (means ± S.E.M.) when the pH of the uptake medium was reduced from 8 to 5. These data show that drIndy is not a H⁺-coupled succinate transporter either.

We then studied the substrate specificity of drIndy by assessing the ability of various monocarboxylate, dicarboxylate and tricarboxylate compounds (at a concn. of 2.5 mM) to compete with succinate (40 nM) for the transport process mediated by drIndy (Table 2). The dicarboxylate compounds 2-oxoglutarate, malate, fumarate and dimethylsuccinate were the most potent

Figure 3 Comparison of the ability of drIndy and hNaDC3 to transport citrate and pyruvate

Uptake of citrate (35 μ M) or pyruvate (135 μ M) was measured in the presence of NaCl in HRPE cells transfected with vector alone, drIndy cDNA or hNaDC3 cDNA. Data (means $\pm$ S.E.M.) are from three independent measurements.

inhibitors of succinate transport mediated by drIndy. The dicarboxylate compounds maleate and malonate, and the monocarboxylate compounds lactate and β -hydroxybutyrate, were not effective. Surprisingly, the monocarboxylate and tricarboxylate compounds (pyruvate and citrate respectively) were very potent in competing with succinate for transport via drIndy. The amino acid derivative *N*-acetyl aspartate was moderately effective in inhibiting succinate transport. Kinetic analysis revealed that the transport of succinate via drIndy was saturable (Figure 2). The Michaelis–Menten constant (K_m) for the transport process was 40 ± 4 μ M.

Since the potent inhibition of drIndy-mediated, succinate transport by pyruvate and citrate was a surprise finding, we assessed the ability of drIndy to transport these two compounds directly by using radiolabelled pyruvate and citrate (Figure 3). The ability of hNaDC3 to transport these two compounds was also assessed under identical conditions, for the purposes of comparison. These experiments showed that drIndy possesses a marked ability to transport citrate (Figure 3A). The uptake of citrate (35 μ M) in cells transfected with drIndy cDNA was approx. 30-fold higher than in cells transfected with vector alone. The uptake of pyruvate was also stimulated in these cells as a result of transfection with drIndy cDNA, but the magnitude of stimulation was comparatively much smaller. The increase in pyruvate (135 μ M) uptake as a result of transfection with drIndy cDNA was only 1.5-fold compared with transfection with vector alone, but this increase was statistically significant ($P < 0.05$). hNaDC3 differed markedly from drIndy in terms of transport of these two compounds. hNaDC3 exhibited a much higher ability

Table 3 Ion-dependence of drIndy-mediated citrate transport

Uptake of citrate (35 μ M) was measured in HRPE cells transfected with either vector alone or drIndy cDNA. The composition of the uptake buffers was as described in Table 1. Data (means $\pm$ S.E.M.) are from six independent measurements. NMDG, *N*-methyl *D*-glucamine.

Salt	Citrate uptake (pmol/10 ⁶ cells per min)		
	Vector	drIndy	Fold increase
NaCl	2.2 $\pm$ 0.4	57.4 $\pm$ 3.3	26.1
KCl	1.7 $\pm$ 0.1	33.8 $\pm$ 2.7	19.9
LiCl	1.9 $\pm$ 0.1	52.1 $\pm$ 2.2	27.4
NMDG chloride	1.9 $\pm$ 0.1	25.4 $\pm$ 1.8	13.4
Sodium gluconate	2.2 $\pm$ 0.1	144.2 $\pm$ 1.6	65.6
Mannitol	1.7 $\pm$ 0.1	26.3 $\pm$ 0.3	15.5

to transport pyruvate than to transport citrate (compare Figures 3B and 3A). These studies show a significant difference between drIndy and hNaDC3 in their relative abilities (i.e. the fold increase in transport in cDNA-transfected cells compared with vector-transfected cells) to transport pyruvate, succinate and citrate when measured under identical conditions (for drIndy: citrate > succinate > pyruvate; and for hNaDC3, succinate $\gg$ pyruvate > citrate).

Since drIndy transports succinate in a cation-independent manner, we investigated whether the transport of citrate mediated by the transporter is also cation-independent. The results of these studies show that citrate transport via drIndy is also cation-independent, as is the transport of succinate (Table 3). There was, however, an interesting difference between the transport of these two substrates. While the transport of succinate was not influenced by chloride, the transport of citrate was enhanced markedly when chloride was absent. Thus chloride has differential influence on the transport of succinate and citrate mediated by drIndy.

The intracellular levels of various tricarboxylic acid-cycle intermediates in HRPE cells are not known. Since the influx of succinate in these cells was enhanced by drIndy, we investigated whether this influx was coupled with efflux of tricarboxylic acid-cycle intermediates from the cells. Control cells and drIndy-expressing cells were first incubated in a Na⁺-containing medium for 30 min in the absence or presence of 0.1 mM succinate, fumarate, malate or 2-oxoglutarate. The cells were then washed, and the influx of [³H]succinate was determined. These studies showed that pre-loading of the cells with these compounds did not facilitate the influx of succinate (results not shown), suggesting that drIndy-mediated succinate influx does not involve counter-transport of intracellular tricarboxylic acid-cycle intermediates.

drIndy and hNaDC3 exhibit similar affinities for succinate, the K_m values for the two transporters being 40 ± 4 (the present study) and 20 ± 1 μ M [9] respectively. The preferential ability of drIndy to transport citrate compared with hNaDC3 indicated that there may be a significant difference in the affinities of these two transporters for citrate. We therefore compared the potency of citrate to inhibit the transport of succinate mediated by drIndy and hNaDC3 under identical conditions (Figure 4). Citrate inhibited the drIndy-mediated transport of succinate with a K_i of 105 ± 35 μ M. The corresponding value for hNaDC3 was 2.1 ± 0.3 mM. Thus hNaDC3 exhibits a 20-fold lower affinity than drIndy for citrate.

It has been well established that NaDC1 and NaDC3 mediate the Na⁺-coupled transport of succinate by an electrogenic mechanism [5–10]. In contrast, the transport process mediated by

Figure 4 Comparison of affinity for citrate for the transport process mediated by drIndy and hNaDC3

HRPE cells were transfected with vector alone, drIndy cDNA (●) or hNaDC3 cDNA (○). Uptake of succinate (80 nM) was measured in the presence of NaCl with or without increasing concentrations of citrate. Data (means $\pm$ S.E.M.) represent only the cDNA-specific uptake and are from three independent measurements.

drIndy occurs via a Na^+ -independent mechanism. This raises the question as to whether or not the drIndy-mediated transport process is electrogenic. To address this issue, we expressed the cloned drIndy in *X. laevis* oocytes and assessed its transport function by measuring the uptake of radiolabelled succinate, as well as by monitoring the succinate-induced changes in membrane potential by the two-micro-electrode voltage-clamp method. Water-injected oocytes served as the control. For comparison, we also performed these experiments under identical conditions with oocytes expressing hNaDC3. The results are shown in Figure 5. The uptake of radiolabelled succinate in oocytes injected with drIndy cRNA was 12-fold higher than in oocytes injected with water. This drIndy-induced uptake of succinate

was, however, not influenced by the absence of Na^+ (Figure 5A). In contrast, even though the induction of radiolabelled succinate uptake by hNaDC3 in oocytes was similar to the induction caused by drIndy when measured in the presence of Na^+ , the hNaDC3-induced uptake was abolished completely when Na^+ was absent in the uptake medium (Figure 5B). We then monitored the changes in membrane potential in oocytes expressing drIndy or hNaDC3 in response to succinate in the medium. Even though drIndy induced the uptake of radiolabelled succinate in oocytes, there was no detectable change in membrane potential associated with the transport process (Figure 5C). This was the case irrespective of whether Na^+ was present or absent in the medium. In contrast, the presence of succinate in the medium induced marked inward currents in oocytes expressing hNaDC3, and this current was obligatorily dependent on the presence of Na^+ (Figure 5D). There was no detectable current in oocytes expressing hNaDC3 in response to succinate in the medium when Na^+ was absent. These data show that the transport process mediated by drIndy is electroneutral.

DISCUSSION

The drIndy cDNA described in this paper (GenBank® accession no. AF509505) is 2602 bp long with a poly(A)⁺ tail. This cDNA is longer than the drIndy mRNA sequence (1872 bp long) reported by Rogina et al. [1] (GenBank® accession no. NM_079426), who derived the sequence from the *Drosophila* genomic sequence (GenBank® accession no. AE003519). The additional sequence is located in the 5'-untranslated region, as well as in the 3'-untranslated region of the cloned drIndy cDNA. Comparison of the nucleotide sequence of the cloned cDNA with that of the genomic clone reveals that the *Indy* gene consists of nine exons, as deduced by Rogina et al. [1], except that the first exon is 97 bp longer than that reported by these investigators. Rogina et al. [1] predicted this 97 bp sequence to be a part of the first intron, but this portion of the gene is indeed expressed in mRNA, as evidenced from the sequence of the cloned cDNA. The start codon is within exon 2 and the stop codon is within

Figure 5 Transport characteristics of drIndy and hNaDC3 in *X. laevis* oocytes

drIndy and hNaDC3 were expressed functionally in oocytes by injection of the respective cRNA. Water-injected oocytes served as control. (A and B) Uptake of succinate (0.1 μM) was measured in the presence of either NaCl ($+\text{Na}^+$) or choline chloride ($-\text{Na}^+$). Data (means $\pm$ S.E.M.) are from ten oocytes. (C and D) Succinate (2 mM)-induced inward currents were monitored using the two-micro-electrode voltage-clamp method in drIndy- and hNaDC3-expressing oocytes or in water-injected oocytes. The membrane potential was maintained at -50 mV. Perfusion buffers contained either NaCl ($+\text{Na}^+$) or choline chloride ($-\text{Na}^+$).

exon 9. The drIndy mRNA reported by Rogina et al. [1] provides sequence information only up to the stop codon. The cloned cDNA provides the sequence information on the 3'-untranslated region located in exon 9. The primary structure of the cloned drIndy protein is, however, 100% identical with that predicted by Rogina et al. [1]. The mammalian proteins most similar in amino acid sequence to drIndy are the Na⁺-coupled dicarboxylate ion transporters NaDC1 and NaDC3. The sequence identity between drIndy and NaDC1 or NaDC3 is 34–35%. Thus, on the basis of the primary structure alone, we could not predict which one of these two transporters is the mammalian functional counterpart of drIndy. Therefore we carried out the functional characterization of drIndy. This was done in an attempt to establish the functional identity of drIndy, and also to determine which one of the two mammalian Na⁺-coupled dicarboxylate transporters is similar to drIndy in functional characteristics. These studies have led to an unexpected conclusion. Even though drIndy is indeed a succinate transporter, neither NaDC1 nor NaDC3 is the mammalian functional counterpart of drIndy.

There are three important functional differences between drIndy and the two mammalian Na⁺-coupled dicarboxylate transporters. The first notable difference is in the ion-dependence of succinate transport mediated by the three proteins. NaDC1 and NaDC3 are strictly Na⁺-coupled succinate transporters [5–10]. In the absence of Na⁺, the mammalian transporters do not exhibit any detectable ability to transport succinate. In contrast, Na⁺ is not essential for the transport of succinate via drIndy. The ability of drIndy to mediate the transport of succinate remains the same even when Na⁺ in the medium is replaced isosmotically by other univalent inorganic cations, such as K⁺ or Li⁺, or by the non-ionizable organic solute mannitol. The second notable difference is in substrate selectivity. drIndy transports the tricarboxylate citrate much more efficiently than the dicarboxylate succinate. This is not the case with NaDC1 and NaDC3. These two mammalian proteins transport succinate much more efficiently than citrate. With respect to pyruvate, drIndy possesses a small, but detectable, ability to transport this monocarboxylate. Surprisingly, NaDC3 shows a much higher ability to transport pyruvate. The third important difference is in the electrogenic nature of these transporters. NaDC1 and NaDC3 are electrogenic transporters for which the transport function is associated with a net transfer of positive charge into the cells. In contrast, drIndy is electroneutral. The transport function of drIndy is not associated with membrane depolarization, as evidenced from the absence of substrate-induced inward currents in oocytes functionally expressing drIndy. Thus, whereas the mammalian NaDCs are Na⁺-coupled electrogenic transporters with preference towards dicarboxylate groups, drIndy is a cation-independent electroneutral transporter with preference for the tricarboxylate groups of citrate. The exact mechanism by which drIndy transports succinate and citrate in an electroneutral manner is not known. The process may involve exchange with intracellular anions, but this needs to be determined. However, our studies have already shown that drIndy-mediated influx of succinate is not likely to be associated with exchange of intracellular dicarboxylate groups. Interestingly, there may be a differential role for intracellular chloride in the drIndy-mediated influx of succinate, as compared with citrate. Since removal of chloride from the extracellular medium stimulates citrate influx without affecting succinate influx, it is possible that influx of citrate, but not that of succinate, is coupled with the efflux of chloride. Additional studies are required to investigate this possibility.

The functional identity of drIndy reported in the present study as a transporter for a variety of tricarboxylic acid-cycle inter-

mediates supports the hypothesis of Rogina et al. [1] that disruption of the *Indy* gene in *Drosophila* decreases cellular availability of these important substrates for the generation of metabolic energy. This will lead to a condition equivalent to caloric restriction. Thus the functional identity of drIndy as a transporter for tricarboxylic acid-cycle intermediates provides the molecular basis for the observations made by Rogina et al. [1] that suppression of the *Indy* gene function results in an extension of the average adult life span of the organism. The exact mechanism by which caloric restriction delays the aging process is not known. There is evidence, however, that decreased production of toxic free radicals, due to diminished mitochondrial function [4], and enhanced autophagy, resulting in promotion of renewal, and rejuvenation of cellular components [20] may underlie the positive influence of caloric restriction on life span.

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