Structural and Functional Characteristics of Two Sodium-coupled Dicarboxylate Transporters (ceNaDC1 and ceNaDC2) from Caenorhabditis elegans and Their Relevance to Life Span*

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We have cloned and functionally characterized two Na⁺-coupled dicarboxylate transporters, namely ceNaDC1 and ceNaDC2, from Caenorhabditis elegans. These two transporters show significant sequence homology with the product of the Indy gene identified in Drosophila melanogaster and with the Na⁺-coupled dicarboxylate transporters NaDC1 and NaDC3 identified in mammals. In a mammalian cell heterologous expression system, the cloned ceNaDC1 and ceNaDC2 mediate Na⁺-coupled transport of various dicarboxylates. With succinate as the substrate, ceNaDC1 exhibits much lower affinity compared with ceNaDC2. Thus, ceNaDC1 and ceNaDC2 correspond at the functional level to the mammalian NaDC1 and NaDC3, respectively. The nade1 and nade2 genes are not expressed at the embryonic stage, but the expression is detectable all through the early larva stage to the adult stage. Tissue-specific expression pattern studies using a reporter gene fusion approach in transgenic C. elegans show that both genes are coexpressed in the intestinal tract, an organ responsible for not only the digestion and absorption of nutrients but also for the storage of energy in this organism. Independent knockdown of the function of these two transporters in C. elegans using the strategy of RNA interference suggests that NaDC1 is not associated with the regulation of average life span in this organism, whereas the knockdown of NaDC2 function leads to a significant increase in the average life span. Disruption of the function of the high affinity Na⁺-coupled dicarboxylate transporter NaDC2 in C. elegans may lead to decreased availability of dicarboxylates for cellular production of metabolic energy, thus creating a biological state similar to that of caloric restriction, and consequently leading to life span extension.

Two different Na⁺-coupled dicarboxylate transporters (NaDC)³ have been identified in mammalian tissues (1–7).

These are NaDC1 and NaDC3. NaDC1 is Na⁺-coupled, electrogenic, and exhibits low affinity for its dicarboxylate substrates. The 𝐾ₑ value (Michaelis-Menten constant) is in the range of 0.1–4.0 mM (1–4). This isoform is expressed primarily in the brush border membrane of intestinal and renal epithelial cells. The physiological function of NaDC1 is to absorb the intermediates of the citric acid cycle, such as citrate, succinate, α-ketoglutarate, fumarate, and malate, in the intestine and kidney. NaDC3 is also a Na⁺-coupled and electrogenic dicarboxylate transporter, but it exhibits relatively higher affinity for its substrates compared with NaDC1 (5–7). The 𝐾ₑ value is in micromolar range. The NaDC3 is expressed primarily in the basolateral membrane of intestinal and renal epithelial cells. However, it is also found in tissues such as liver, placenta, and brain. NaDC3 in the kidney is involved in generating the driving force for the organic anion transporter OAT1 to facilitate the active entry of organic anions into the tubular cells across the basolateral membrane (8). In the brain, NaDC3 mediates the cellular uptake of N-acetylaspartate, a process closely linked to myelination (9). Therefore, the physiological functions of the NaDCs may extend beyond the mediation of cellular entry of citric acid cycle intermediates. Recently, we have reported on the molecular identification of the third member of this family in mammals (10, 11). This transporter, known as Na⁺-coupled citrate transporter (NaCT), mediates the cellular uptake of citrate in a Na⁺-coupled manner.

In a recent study by Rogina et al. (12), a NaDC-like transporter, coded by the Indy (for I am not dead, yet) gene, has been implicated in the regulation of life span in Drosophila. The investigators of this study suggested that defects in one copy of the Indy gene (heterozygosity) can lead to less efficient production of cellular energy and that, as a consequence, the metabolic profile of the fruit fly changes resulting in life span extension. The eating behavior of the organism is not altered, however. The decreased generation of cellular energy due to the heterozygous mutation in the Indy gene creates a biological situation resembling that of caloric restriction, which in other animal models leads to an extension of life span (13). Recently, we have identified (14) the transport function of Drosophila INDY. This transporter mediates the cellular uptake of a broad spectrum of citric acid cycle intermediates in a Na⁺-independent manner. These characteristics of drINDY have now been confirmed independently by Knauf et al. (15).

Studies of life span extension are difficult, if not impossible, to conduct in mammals, particularly in humans. But it is relatively a simple task to monitor the mean and maximum life span in other animal models such as Caenorhabditis elegans. A number of features make C. elegans especially suitable for studies of life span extension. This organism has a short life span with a mean life span of ~15 days. In addition, there are
techniques available to silence genes in this organism as a means of assessing the role of specific genes in the maintenance of life span. Therefore, with an aim to investigate the potential role of NaDC family in life span, we set out to clone the *C. elegans* counterparts of mammalian NaDCs and subsequently to monitor the influence of these transporters on life span by using the RNAi technique to down-regulate their function. Several different genes have successfully been identified and their functional identification of two different Na\(^+\)-coupled dicarboxylate transporters (ceNaDC1 and ceNaDC2) analogous to the mammalian NaDC1 and NaDC3. In addition, studies of the influence of these two transporters on life span have shown that disruption of the function of the high affinity transporter CeNaDC2, but not that of the low affinity transporter CeNaDC1, leads to a significant extension of the average life span in *C. elegans*.

**EXPERIMENTAL PROCEDURES**

Nematode Culture—A wild type nematode strain, *C. elegans* N2 (Bristol-Myers Squibb Co.), was obtained from the Caenorhabditis Genetics Center (St. Paul, MN). Nematode culture was carried out using a standard procedure with a large scale liquid cultivation protocol (16–19 h). The nematodes were cleaned by sedimentation through 15% (w/v) Ficoll 400 in 0.1 M NaCl. The pellet was then used for total RNA preparation.

Extraction and Purification of Poly(A\(^+\)) RNA—Total RNA was isolated using the TRIzol reagent (Invitrogen). Poly(A\(^+\)) mRNA was purified by affinity chromatography using oligo(dT)-cellulose.

Reverse Transcription (RT)-PCR and Hybridization Probe Preparation—A pair of PCR primers specific for the putative *C. elegans nadc1* gene was designed based on the sequence of the cosmid F31F6.6 (GenBank accession number Z69884). The primer contains an incoverted BamHI restriction site at its 5' end. The reverse primer, 5'-GCG TTC AAC CAA AAT GTC TC3-3' (forward primer) and 5'-CTA ACG ACA ATC CTC C3-3' (reverse primer). A second pair of PCR primers specific for putative *C. elegans nadc2* gene was designed based on the cosmid K08E5.2 (GenBank accession number Z30974), 5'-TCA TTC TCC CAA CAC CAT CC3-3' (forward primer) and 5'-ACC ATT CCA CTT CCA AAC AC-3-3' (reverse primer). Poly(A\(^+\)) RNA (−0.5 μg) isolated from mixed stage *C. elegans* was taken as template to perform RT-PCR using an RT-PCR kit from PerkinElmer Life Sciences. A single RT-PCR product was obtained with an estimated size of ~1.0 and ~0.9 kb for the *cenadc1* and the *cenadc2* genes, respectively, as predicted by the distance between the two primers in each pair. The RT-PCR products were gel-purified and subcloned into pGEM-T Easy Vector (Promega, Madison, WI). The molecular identity of the inserts was established by sequencing. These cDNA fragments were used as probes to screen a *C. elegans* cDNA library.

Construction of a Directional *C. elegans* cDNA Library—The SuperScript Plasmid System from Invitrogen was used to establish the cDNA library using the poly(A\(^+\)) RNA from *C. elegans*. The transformation of the ligated cDNA into *Escherichia coli* was performed by electroporation using ElectroMAX DH10B competent cells. The bacteria plating, the filter lifting, the DNA fragment labeling, and the hybridization methods followed the routine procedure (20). The DNA sequencing of the full-length *cenadc1* cDNA and *cenadc2* cDNA clones was performed using an automated PerkinElmer Life Sciences Applied Biosystems 377 Prism DNA sequencer and the Toy DyeDeoxy terminator cycle sequencing protocol.

**Tissue-specific Expression Pattern of cenadc1 and cenadc2**—To study the tissue-specific expression pattern of the *cenadc1* and *cenadc2* genes in *C. elegans*, transgenic lines expressing these transgenes were developed. The expression pattern of the *cenadc1* and *cenadc2* genes was investigated in live transgenic animals based on the expression pattern of the GFP reporter. A pair of primers for construction of a transcriptional *cenadc1:gfp* fusion gene was designed to amplify the 1.2-kb DNA fragment of the *cenadc1* promoter. A 5'-GCC GTC GAC GCT TAC ATC ATT CCT GTC TTT TTC-3', corresponds to the nucleotide positions 28,630–28,659 of the cosmid F31F6 (GenBank accession number Z69884). The primer contains an incorporated SaI restriction site at its 5' end. The reverse primer, 5'-ATA GGA TTC ATG ATT GGA GCC TCT GTA ATT CAT CTA-3', corresponds to the nucleotide positions 29,797–29,772 in the same cosmid. A BamHI site was incorporated in this primer at the 5' end. The SaI and BamHI sites were introduced into these primers for subsequent cloning into a GFP expression vector. An ~1.2-kb DNA fragment of the *cenadc1* promoter was amplified using the cosmid F31F6 DNA as template. Similarly, a pair of primers for construction of a transcriptional *cenadc2:gfp* fusion gene was designed to amplify the 1.2-kb DNA fragment of the *cenadc2* promoter. The reverse primer, 5'-GCC GTC GAC AAAATA TGT ATT AGC CAT AAC CCC-3', corresponds to the nucleotide positions 13,966–13,998 of the cosmid K08E5 (GenBank accession number Z30974). The reverse primer, 5'-GCG TTT TCC TTC ACG ACA ATC GGT TAT ATC-3', corresponds to the nucleotide positions 15,461–15,432 in the same cosmid. A SaI site and an original SaI site were incorporated into these primers for subsequent cloning purposes. A ~1.5-kb DNA fragment of the *cenadc2* promoter was amplified using the cosmid K08E5 as template. The PCR products were digested with SaI and BamHI and inserted into a GFP expression vector pPD117.01 (a generous gift from Dr. A. Fire, Carnegie Institution, Baltimore, MD) at a multiplicity of 3.99. The resulting PCR products were cloned into the expression vector pPD117.01 (a generous gift from Dr. A. Fire, Carnegie Institution, Baltimore, MD) at a multiplicity of 3.99. The resulting PCR products were cloned into the expression vector pPD117.01.
Sodium-coupled Dicarboxylate Transporters in *C. elegans*

Established using a standard germ line transformation protocol (17, 18). Synctial germ line injection was carried out according to a standard procedure (18). For microinjection, a computerized injection system, Transject 52546 and Micromanipulator 5171 from Eppendorf (Hamburg, Germany), and a Nikon Eclipse TE 300 inverted microscope with Nomarski differential interference contrast optics were used. A cloned mutant collagen gene containing the rol-6 (plasmid pRP4, kindly provided by Dr. M. Koelle, Yale University School of Medicine, New Haven, CT) was used as a dominant genetic marker for DNA transformation. Conjection of this dominant marker with the GFP fusion constructs allowed progeny selection of the transformed animals by their “roller” phenotype. The F1 rollers were picked up according to their characteristic rolling behavior and cultured individually to establish transformed lines. The extrachromosomal arrays were selected by fluorescence microscopy to determine the GFP expression pattern. Stable transgenic lines were established by the y-irradiation method from the F2 rollers, and the background was cleaned up by several times of outcross (17, 21).

**Double Labeling Fluorescent Protein Expression System**—Two modified versions of the *Aequorea victoria* green fluorescent protein (GFP), designated as CFP and YFP with cyan-shifted and yellow-shifted spectra (22), respectively, were used to simultaneously follow the expression patterns of ceNaDC1 and ceNaDC2 in *C. elegans*. For the construction of the *cenadc1* promoter-driven CFP and the *cenadc2* promoter-driven YFP expression vectors, the GFP coding region in the GFP-expression vectors pPD111.17 (pcDNA3.1) and pPD129.36 (pPD111.17) were substituted by the CFP and YFP coding regions, respectively. The CFP and YFP coding regions (∼950 bp) were obtained by an EcoRI/KpnI digestion of the vectors L4666 (pPD133.58) and L4664 (pPD133.51), respectively (kindly provided by Dr. A. Fire, Carnegie Institution, Baltimore, MD). The *cenadc1::cfp* and *cenadc2::yfp* expression vectors were linearized by SacI digestion and connected into the distal arms of the *C. elegans* syncytial gonads as described earlier. The extrachromosomal arrays were used for fluorescence microscopy to compare the expression pattern of CFP and YFP in the same transgenic animal. Epi-fluorescence microscopic analysis of the expression of CFP and YFP was performed using Axiohot microscope (Carl Zeiss, Thornwood, NY). Excitation and emission filter settings are as follows: for CFP examination, excitation at 436 ± 20 nm, dichroic 455 nm LP, and emission at 480 ± 40 nm; for YFP examination, excitation at 500 ± 20 nm, dichroic 515 nm LP, and emission at 535 ± 30 nm LP (22). The filter sets were purchased from Chroma Technology (Brattleboro, VT). The SPOT-cooled CCD color digital camera (Diagnostic Instruments Inc., St. Sterling Heights, MI) and its associated data acquisition software were used to record the fluorescence images.

**Bacteria-mediated RNA Interference (RNAi)**—A fragment of the coding region of ceNaDC1 cdNA was generated by PCR and subcloned into a pGEM-T easy vector (Promega, Madison, WI). The DNA fragment was released by EcoRI digestion and inserted into a “double T7” plasmid (pPD129.36, pcDNA3.1), digested with EcoRI and KpnI (E. coli DH5α/pET28b, a generous gift from Dr. A. Fire, Carnegie Institution, Baltimore, MD) at an EcoRI site within the multiple cloning site. A host strain DH5α was used for the first transformation. Competent host bacteria HT115 (DE3) (kindly provided by the Caenorhabditis Genetics Center, St. Paul, MN) expressing T7-RNA polymerase from an inducible promoter was prepared using a standard CaCl2 method (20). The double T7 promoter-containing plasmid with the *cenadc1* gene-specific DNA fragment inserted between the two T7 promoter regions was transformed into the competent HT115 (DE3) cells and plated onto standard LB + tetracycline (12.5 μg/mL) + ampicillin (100 μg/mL) plates. HT115 cells harboring the double T7 plasmid were cultured and induced to express dsRNA using 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. The extrachromosomal arrays were transferred onto isopropyl-β-D-thiogalactopyranoside-containing nematode growth medium plates with the induced bacteria HT115 lawn for bacteria feeding experiments. The empty vector pPD129 was processed similarly for use as a negative control, and the bacteria harboring this plasmid were used to feed the control worms. Sufficient quantities of bacteria were used to seed the testing plates for the testing period for the experiments to consume to prevent the worms from starving and to ensure that dsRNA was always present in the testing plates during the entire experimental period for the experimental worms. A similar experimental strategy was used for ceNaDC2. To serve as a positive control for the bacteria-mediated RNAi in the assessment of the influence of ceNaDC1 and ceNaDC2, we monitored the life span of DAF-2 on the surface of DAF-2 bodies by feeding the bacteria using an identical experimental approach. Homozygous *daf-2(e1737)* knockout in *C. elegans* is known to enhance the life span dramatically (~2-fold) (23, 24). Therefore, if the knockdown of DAF-2 function by bacteria-mediated RNAi in *C. elegans* doubles the life span, this can be taken as a positive control for the validity of the experimental approach to assessing the role of ceNaDC1 and ceNaDC2 in the life span. For this purpose, we obtained an ~0.8-kb DNA fragment specific for *C. elegans* DAF-2 by RT-PCR using the following primer pairs: 5′-CGAACAAAAACACTACAGAC-3′ (forward primer) and 5′-TTCCATCATTTCCATACAAACC-3′ (reverse primer) using the nematode total RNA as the template. This fragment was then subcloned into pGEM-T easy vector. The fragment was silenced from the vector by EcoRI digestion, and the released insert was cloned into the double T7 plasmid pPD129.36 at the EcoRI site at the multiple cloning region. Shuttling of this plasmid into HT115 (DE3) bacteria, induction of double-stranded RNA, and feeding of the worms with the bacteria were carried out as described earlier.

**Life Span Measurement**—Life span of age-synchronous nematodes was determined at 20°C. Eggs obtained from gravid hermaphrodites using an alkaline hypochlorite treatment procedure (18) were dispensed on nematode growth medium Petri dishes with bacteria lawn and allowed to hatch. Worms were inspected every day until death and were scored as dead when they were no longer able to move even in response to prodning with a platinum-wire pick. Each day, dead worms were removed from plates and the deaths were recorded. Experiments were started with 60 worms for each RNAi treatment (10 per plate). The worms were transferred to a new plate every day during the reproductive period and every 3 days afterward to avoid contamination by their offspring. Worms that died from matricidal hatching (the bag-of-worms phenotype) and the worms that crawled off the plates or burrowed into the agar were scored and counted. A backup reservoir plate of ~30 spare worms was started at the same time as the experimental worms and was identically treated for this purpose. To avoid the influence of any potential subjective judgment of the experimenter in identifying the dead worms on the experimental outcome, the life span measurement studies were repeated with the experimenter blinded with regard to the identity of the individual experimental groups. Statistical analysis was performed using the Microsoft Excel 2000 analysis ToolPak. Mean life spans from different groups were compared using the Student’s *t* test assuming unequal population variances.

**RESULTS**

**Molecular Cloning and Structural Characterization of ceNaDC1 and ceNaDC2**—The cloned ceNaDC1 cdNA is 1,989 bp long and contains a poly(A) tail (GenBank™ accession number AY90484). The 5′- and 3′-untranslated regions of this cdNA are 12 bp and 150 bp long, respectively. The ceNaDC1 protein, deduced from the coding region of the cdNA, contains 582 amino acids (Fig. 1) with a predicted molecular mass of 64 kDa and an isoelectric point of 6.64. The ceNaDC2 cdNA is 2,250 bp long and contains a poly(A) tail (GenBank™ accession number AY90485). The 5′- and 3′-untranslated regions of this cdNA are 50 and 500 bp long, respectively. The ceNaDC2 protein, deduced from the coding region of the cdNA, contains 566 amino acids (Fig. 1) with a predicted molecular mass of 62 kDa and an isoelectric point of 7.69. According to the Kyte-Doolittle plot with a 21-amino acid window size, ceNaDC1 as well as ceNaDC2 possess 12 putative transmembrane domains.

Following a multiple protein sequence alignment of the two ceNaDCs, the three members of the human NaDC family, and the drINDY using the PILEUP and in combination with the MOTIFS program in the GCG package, a sodium symporter family signature motif was identified within these transporter proteins (Fig. 1). A consensus pattern established for the signature sequence is as follows: (S)SX(2)(FX(2)P)V(GX(3)X)NX(1V), where the X denotes the flexible amino acid residues preceding the number in parentheses, and the numerical value indicates the permitted number of the flexible amino acid residues in the consensus. This sodium symporter family is a group of integral membrane proteins that mediate the cellular uptake of a wide variety of molecules including di- or tricarboxylates and sulfate by a transport mechanism involving sodium cotransport (sodium symporters). They are grouped into a single gene family on the basis of sequence and functional similarities. This group consists of the following proteins: the sodium/sulfate cotransporters and sodium/dicarboxylate co-transporters identified in yeast, *C. elegans*, *Drosophila*, and...
mammals; the putative sulfur deprivation response regulator (SAC1) from Chlamydomonas reinhardtii; and the hypothetical protein YfbS from E. coli (25). These transporter proteins usually consist of 430–620 amino acids. They are highly hydrophobic and contain 11 or 12 putative transmembrane regions. The highly conserved sodium symporter signature motif is located in or near the penultimate transmembrane domain.

The molecular identity of mammalian or C. elegans functional counterpart of drINDY is not known at present. Therefore, we compared the primary structure of ceNaDC1 and ceNaDC2 with that of drINDY and mammalian NaDC1, NaDC3, and NaCT. With a pairwise comparison analysis, ceNaDC1 is more closely related to drINDY (51% similarity and 37% identity) than ceNaDC2 (46% similarity and 35% identity). Similarly, hNaDC1 and hNaCT are more closely related to drINDY (52% similarity and 40% identity) than hNaDC3 (50% similarity and 37% identity). However, the differences are small, and it is difficult to conclude whether ceNaDC1 or ceNaDC2 is the C. elegans functional counterpart of drINDY based on the structural comparison. Similarly, this structural analysis does not allow definitive conclusion with regard to the question of whether NaDC1 or NaDC3 is the mammalian functional counterpart of drINDY. Structural comparison reveals that both ceNaDCs have similar sequence homology with hNaDC1, hNaDC3, and hNaCT. Thus, the sequence data have failed to provide any useful hint with respect to the functional identity of the two ceNaDCs.

The cenadc1 and cenadc2 genes are located on chromosomes X and III, respectively. Both genes, excluding the unidentified promoter region in respective genes, are ~3.5 kbp in size (C. elegans data base, ACeDB, data version WS57). The presence of 10 exons in the cenadc1 gene and 8 exons in cenadc2 gene was deduced by a comparison between the sequences of the cloned cDNAs and the respective genes in the GenBank™ data base (F31F6.6 and K08E5.2) from the nematode genome sequence project. The structural organization of the cenadc1 and cenadc2 genes is shown in Fig. 2.

Functional Characterization of ceNaDC1 and ceNaDC2 Using a Heterologous Expression System—The functional analysis of the cloned ceNaDCs was carried out by heterologous expression of the cDNAs in HRPE cells using the vaccinia virus expression system (5, 7, 9). Cells transfected with vector alone served as the control. The transport function was monitored by the uptake of [3H]succinate. Initial studies on the time course of uptake indicated that the uptake was linear at least up to 5 min. All subsequent studies were therefore carried out with a 2-min incubation. With a succinate concentration of 10 μM and in the presence of Na⁺, the uptake of succinate increased 12-fold in cells expressing ceNaDC1 compared with control cells (Fig. 3A). Under similar conditions, the increase in succinate uptake was 24-fold in the case of ceNaDC2. Thus, both
ceNaDC1 and ceNaDC2 mediate the uptake of succinate in the presence of Na\(^+\). The uptake via these two transporters was, however, obligatorily dependent on the presence of Na\(^+\) because substitution of Na\(^+\) with Li\(^+\), K\(^+\), or NMDG abolished completely the cDNA-induced increase in succinate uptake. There was no involvement of anions in the uptake process as indicated by comparable uptake activities for both transporters in the presence of NaCl or sodium gluconate (data not shown).

The substrate selectivity of the uptake process mediated by ceNaDCs was then studied by competition analysis by monitoring the ability of various monocarboxylates and dicarboxylates (5 mM) to compete with succinate for the uptake process (Fig. 3B). Uptake measurements were made in parallel in vector-transfected cells and in cDNA-transfected cells, and then the cDNA-specific uptake was calculated by subtracting the uptake in vector-transfected cells from the uptake in cDNA-transfected cells. Only the cDNA-specific uptake was used in the analysis. Among the various dicarboxylates tested, the ceNaDC1-mediated succinate uptake was inhibited markedly by fumarate, malate, \(\alpha\)-ketoglutarate, dimethylsuccinate, and \(N\)-acetylglutamate. In contrast to fumarate, its stereoisomer maleate failed to compete with succinate for transport via ceNaDC1. Malonate, a structural homolog of succinate, not only failed to inhibit the uptake of succinate but actually caused a significant stimulation of succinate uptake. The monocarboxylates pyruvate, lactate, and \(\beta\)-hydroxybutyrate caused only a minimal inhibition of succinate uptake.

The substrate selectivity of ceNaDC2 was more or less similar to that of ceNaDC1. The uptake of succinate mediated by ceNaDC2 was inhibited significantly by fumarate, malate, \(\alpha\)-ketoglutarate, dimethylsuccinate, and \(N\)-acetylglutamate, whereas the monocarboxylates had only a minimal effect. However, there were some notable differences between ceNaDC1 and ceNaDC2. Maleate was able to inhibit ceNaDC2-mediated succinate uptake, whereas ceNaDC1-mediated succinate uptake was not affected. Malonate, which caused a significant stimulation of succinate uptake via ceNaDC1, had minimal effect on succinate uptake via ceNaDC2. Another notable feature was that fumarate and maleate were much more potent in inhibiting ceNaDC2-mediated succinate uptake than in inhibiting ceNaDC1-mediated succinate uptake, suggesting that there may be significant differences in substrate affinities between the two transporters. But, interestingly the trend in the inhibitory potency was opposite for dimethylsuccinate and \(N\)-methylglutamate. These two dicarboxylate derivatives were more potent in inhibiting ceNaDC1-mediated succinate uptake than in inhibiting ceNaDC2-mediated succinate uptake.

The cDNA-specific succinate uptake was saturable for ceNaDC1 as well as for ceNaDC2, and the data conformed to the Michaelis-Menten equation describing a single saturable system (data not shown). The Michaelis-Menten constant (\(K_m\)) was 0.73 ± 0.05 mM for ceNaDC1 and 60 ± 9 \(\mu\)M for ceNaDC2. Thus, with succinate as the substrate, ceNaDC2 exhibits a 10-fold greater affinity than ceNDY1. The competition studies suggest that the same may be true for other dicarboxylate substrates such as fumarate and maleate. These data show that...
ceNaDC1 is a low affinity Na\(^+\)/sucinate cotransporter, and ceNaDC2 is a high affinity Na\(^+\)/sucinate cotransporter. Therefore, these two C. elegans dicarboxylate transporters correspond at the functional level to mammalian NaDC1 and NaDC3, respectively. NaCT shows very little ability to transport succinate and thus is not related to either ceNaDC1 or ceNaDC2 in terms of transport function.

Drosophila INDY does have the ability to transport various dicarboxylate intermediates of citric acid cycle (14, 15). But the transport function is not Na\(^+\)/H\(^+\)-dependent. Furthermore, Drosophila INDY has a much higher affinity for citrate, a tricarboxylate, than for dicarboxylates (14).

The effect of Na\(^+\) on the uptake of succinate was then investigated by measuring the uptake in the presence of varying concentrations of extracellular Na\(^+\) in cells transfected with either ceNaDC1 cDNA or ceNaDC2 cDNA. Again, the uptake values were adjusted for the endogenous uptake activity measured under identical conditions in cells transfected with vector alone. The concentration of Na\(^+\) in the uptake medium was varied from 0 to 140 mM. The osmolality of the medium was maintained by adding appropriate concentrations of NMDG chloride as a substitute for NaCl. The relationship between the cDNA-specific uptake and Na\(^+\) concentration was sigmoidal for both ceNaDC1 and ceNaDC2, suggesting the involvement of multiple Na\(^+\) ions per succinate molecule transported. The uptake rates failed to reach saturation within the concentration range of Na\(^+\) employed in these studies.

Developmental Stage-specific Expression Pattern of ceNaDC1 and ceNaDC2

To monitor the relative expression levels of ceNaDC1 mRNA and ceNaDC2 mRNA during different stages of C. elegans development, synchronized cultures were obtained, and total RNA was isolated at each of the following four stages of development: embryo, early larva (larva stages 1 and 2), late larva (larva stages 3 and 4), and adult. The steady state levels of mRNAs for ceNaDC1 and ceNaDC2 were then determined by semi-quantitative RT-PCR with 18 S rRNA as an internal control for variations in RNA input into RT-PCRs. The levels of ceNaDC1 mRNA and ceNaDC2 mRNA were compared at different developmental stages based on relative intensities of ceNaDC-specific RT-PCR products compared with that of 18 S rRNA-specific RT-PCR product (Fig. 4). ceNaDC1 mRNA expression was not detectable at the embryo stage. Abundant expression of this mRNA was evident, however, at the early larva stage. There was a transient decrease in ceNaDC1 mRNA levels at the late larva stage, but the levels increased again during subsequent development into the adult stage. The relative mRNA levels for ceNaDC1 at the four stages (embryo, early larva, late larva, and adult) were 0, 0.87, 0.24, and 0.77.

Tissue-specific Expression Pattern of cenadc1 and cenadc2 Genes

We first studied the tissue expression pattern of cenadc1 and cenadc2 genes in C. elegans using the transgenic GFP fusion technique in which the transgene consisted of the cen-
adc1 promoter fused with GFP cDNA or the cenadc2 promoter fused with GFP cDNA. In both cases, the expression of GFP is controlled by the respective promoter. Thus, the expression pattern of GFP would match the expression pattern of the respective gene promoters in stably transformed transgenic C. elegans. The insets A1 and B1 are the bright field images (low magnification) of the animals for A and B, respectively. C, (YFP expression driven by the cenadc2 promoter) and D (CFP expression driven by the cenadc1 promoter) show the expression pattern of YFP and CFP in the same animal. The inset C1/D1 is the bright field image of the same worm for both C and D.

We have described in this paper the cloning and functional characterization of two transporters in C. elegans that mediate the transport of several intermediates of the citric acid cycle. Both transporters are Na+-coupled and exhibit broad substrate specificity for dicarboxylates. They do not interact with monocarboxylates. At the functional level, these two transporters, named ceNaDC1 and ceNaDC2, resemble the mammalian Na+-coupled dicarboxylate transporters NaDC1 and NaDC3, respectively.

Even though ceNaDC1 and ceNaDC2 generally resemble NaDC1 and NaDC3, respectively, in terms of functional characteristics, there is one important difference. This difference relates to the interaction of these transporters with certain derivatives of succinate such as dimethylsuccinate and N-acetylasparginate. Dimethylsuccinate is considered to be a specific substrate for mammalian high affinity transporter NaDC3 (26, 27). The low affinity transporter NaDC1 does not tolerate substitutions in the carbon backbone of succinate. Thus, dimethylsuccinate and dimercaptosuccinate are recognized preferentially by NaDC3. In contrast to the mammalian counterparts, it is ceNaDC1, the low affinity transporter in C. elegans, that interacts with dimethylsuccinate with much higher affinity compared with ceNaDC2. Interaction with N-acetylasparg-
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The knockdown of ceNaDC1 and ceNaDC2 shows high affinity for this succinate analog (9). In contrast, it is not ceNaDC1, not ceNaDC2, that shows high affinity for this compound.

Na⁺-activation kinetics of succinate uptake mediated by ceNaDC1 and ceNaDC2 shows that multiple Na⁺ ions are involved in the transport mechanism. Succinate exists as a divalent anion under the experimental conditions (i.e. pH 7.5), and therefore the number of Na⁺ ions involved per transport cycle will determine whether or not the transport process is influenced by membrane potential. However, the exact number of Na⁺ ions transported with succinate per transport cycle could not be determined in the present studies because the activation of succinate uptake by Na⁺ did not saturate within the range of Na⁺ concentrations employed in the study. We tried to express ceNaDC1 and ceNaDC2 in Xenopus laevis oocytes to evaluate the electrogenic nature of these two transporters by using the two-microelectrode voltage clamp method, but the transporters were not functionally expressed in this heterologous system. We do not know the reasons for the lack of expression. We are currently trying different expression vectors for this purpose. Successful expression of these transporters in X. laevis oocytes may become essential to demonstrate unequivocally whether or not ceNaDCs are electrogenic.

In mammals, the expression of NaDC1 is restricted primarily to the intestine and kidney, whereas the expression of NaDC3 is evident not only in the intestine and kidney but also in the liver, brain, and placenta (26). Furthermore, NaDC1 and NaDC3 exhibit differential distribution in the apical versus basolateral membrane of the polarized cells in the intestine, kidney, liver, and placenta. NaDC1 is localized to the apical membrane of the intestinal and renal tubular cells. In contrast, NaDC3 is localized to the basolateral membrane of the renal tubular cells, sinusoidal membrane of the hepatocytes, and the brush border membrane of the placental syncytiotrophoblast (26). The physiological function of NaDC1 in the intestine and kidney is to facilitate the absorption of exogenous dicarboxylates in the intestine and the reabsorption of endogenous dicarboxylates in the kidney. In the liver and placenta, NaDC3 may play a role in the cellular entry of circulating dicarboxylates for subsequent metabolic utilization. Because these dicarboxylates are present in the circulation only in micromolar concentrations, the high affinity transporter NaDC3 has obvious advantages over the low affinity transporter NaDC1 to perform this function. In C. elegans, the low affinity transporter NaDC1 as well as the high affinity transporter NaDC2 are expressed predominantly in the intestinal tract. The C. elegans intestinal tract is a tubular structure made up of a single layer of 20 donut-shaped cells (28). Unlike in mammals, the intestinal tract in C. elegans performs a variety of functions in addition to the digestion and absorption of dietary nutrients. It is a primary site of synthesis and storage of fat as the energy source, a function similar to that of liver and adipose tissue in mammals. The cells of the intestinal tract in C. elegans are polarized, with numerous microvilli on the luminal surface analogous to the apical membrane of the enterocytes in mammals. The basolateral membrane of the intestinal cells is in contact with the pseudocoelomic space that is filled with fluid that supplies nutrients to the rest of the cells in the body. In this respect, there is a lot of similarity between the intestinal tract in C. elegans and the liver and adipose tissue in mammals. We have provided evidence in this paper in support of coexpression of NaDC1 and NaDC2 in the cells of the intestinal tract in C. elegans. It is not known, however, whether these two transporters are distributed differentially in the apical versus basolateral membrane of the intestinal cells.

The physiological functions of NaDC1 and NaDC2 in C. elegans intestinal tract are not known. We used the RNAi technique to silence the function of these two transporters to evaluate their influence on life span in this organism. This technique is very effective in silencing the function of any specific protein as evidenced by the doubling of the average life span by silencing the function of DAF-2. Homozygous mutations in daf-2 gene lead to doubling of life span in C. elegans (23, 24). Since RNAi-mediated targeting of daf-2 also doubles the life span, we conclude that this technique is very effective in silencing the function of any targeted gene. RNAi-mediated interference of NaDC1 function does not have any noticeable effect on the average life span as well as on the maximal life span, whereas targeting NaDC2 by this approach results in a significant increase in the average and maximal life span. We speculate that NaDC2 is localized to the basolateral membrane of the intestinal cells where it functions in the cellular entry of endogenous dicarboxylates for subsequent metabolic utilization and energy production. Interference with this function leads to a metabolic state analogous to that of caloric restriction, thus resulting in life span extension. It has been well established in C. elegans that caloric restriction (29) or suppression of metabolic energy production within the mitochondria (30) is associated with a significant increase in life span.

**Fig. 6. Influence of the knockdown of ceNaDC1 and ceNaDC2 on life span in C. elegans.** The knockdown of ceNaDC1 and ceNaDC2 was carried out by feeding the worms with bacteria producing ceNaDC1- or ceNaDC2-specific dsRNA. The knockdown of DAF-2 was used as a positive control. The survival curves were plotted according to the Kaplan-Meier algorithm using Sigma Plot (version 6.0, SPSS Inc., Chicago). These curves show the survival probability of the wild type animals at a given day after hatching under the influence of the gene-specific dsRNAs. Each group was from a total of four experiments. The total number of worms in each group at the beginning of the life span experiment was 240.
It is interesting to note that *Indy* gene is expressed in *Drosophila* in tissues such as the fat body, midgut, and oenocytes (12, 15). The fat body in this organism is involved in the metabolism and storage of major energy sources (fat, glycogen, and protein). The metabolic functions of this organ are similar to those of liver in mammals. The same is true with the intestinal tract in *C. elegans* where NaDC2 expression is seen. However, the transport characteristics of ceNaDC2 are very different from those of *Drosophila* INDY even though disruption of NaDC2 function enhances life span in *C. elegans* as disruption of INDY does in *Drosophila*. In addition to NaDC1 and NaDC2 reported in this paper, a recent search of the *C. elegans* data base has revealed that there are three other genes coding for putative transporters with structural similarity to *Drosophila* INDY. Cloning and functional characterization of these putative transporters will be required to establish the molecular identity of the gene that is the *C. elegans* functional counterpart of *Drosophila* INDY. The present studies have clearly shown that NaDC2 is involved in the regulation of life span in *C. elegans*, but it is likely that additional transporters with NaDC2-like transport function may exist in this organism and function in the regulation of life span.

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