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INVITED REVIEW

Sodium-coupled dicarboxylate and citrate transporters from the SLC13 family

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Abstract The SLC13 family in humans and other mammals consists of sodium-coupled transporters for anionic substrates: three transporters for dicarboxylates/citrate and two transporters for sulfate. This review will focus on the di- and tricarboxylate transporters: NaDC1 (SLC13A2), NaDC3 (SLC13A3), and NaCT (SLC13A5). The substrates of these transporters are metabolic intermediates of the citric acid cycle, including citrate, succinate, and α-ketoglutarate, which can exert signaling effects through specific receptors or can affect metabolic enzymes directly. The SLC13 transporters are important for regulating plasma, urinary and tissue levels of these metabolites. NaDC1, primarily found on the apical membranes of renal proximal tubule and small intestinal cells, is involved in regulating urinary levels of citrate and plays a role in kidney stone development. NaDC3 has a wider tissue distribution and high substrate affinity compared with NaDC1. NaDC3 participates in drug and xenobiotic excretion through interactions with organic anion transporters. NaCT is primarily a citrate transporter located in the liver and brain, and its activity may regulate metabolic processes. The recent crystal structure of the *Vibrio cholerae* homolog, VcINDY, provides a new framework for understanding the mechanism of transport in this family. This review summarizes current knowledge of the structure, function, and regulation of the di- and tricarboxylate transporters of the SLC13 family.

Keywords Sodium · Citrate · Succinate · Dicarboxylate · Transporter · Indy · DASS · NaDC1 · NaDC3 · NaCT

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Introduction

The SLC13 family contains five members in humans and other mammals: three transporters for di- and tricarboxylates—NaDC1, NaDC3, and NaCT—and two sulfate transporters—NaS1 and NaS2 [62, 73]. All of the mammalian members of the family couple multiple sodium ions with the transport of an anion substrate. The mammalian SLC13 family belongs to the large superfamily of anion transporters called divalent anion sodium symporters (DASS) that includes transporters from other organisms including bacteria [86]. The focus of this review will be the mammalian di- and tricarboxylate transporters of the SLC13 family.

The substrates carried by the SLC13 transporters include the citric acid cycle intermediates citrate, succinate, and α-ketoglutarate. Plasma concentrations of these metabolic intermediates in humans are: citrate ~120 μM [27, 67], succinate ~5 μM [52], α-ketoglutarate ~25 μM [52], and glutarate 0.6–3 μM [5]. These metabolites are important energy sources and biosynthetic precursors, but they have other roles as well. Citrate is a chelator that forms soluble complexes with calcium and prevents the formation of kidney stones [37]. Citrate is also involved in acid–base balance as a base equivalent [65]. Citric acid cycle intermediates have important signaling roles. Citrate concentrations regulate the activity of lipid-metabolizing enzymes, and dysregulation of nutrient sensing in muscle involving malonyl-CoA has been proposed as a mechanism leading to insulin resistance [88]. Citrate sensing by the hypothalamus affects glucose uptake and metabolism by the liver [16]. Other citric acid cycle intermediates act as ligands for G-protein coupled receptors (GPR) [38]. GPR91/SUCNR1, the succinate receptor, has been implicated in blood pressure regulation [20, 89] and may serve as a hypoxia sensor in the liver and eye [19, 90]. GPR99/OXGR1, the α-ketoglutarate receptor, is located in the distal nephron and

collecting duct and is involved in regulating acid–base balance [100].

The connection between SLC13-mediated transport and regulation of metabolic processes was first suggested by studies with the INDY transporter from *Drosophila* [87]. INDY is a homolog of the mammalian SLC13 transporters, with approximately 36–38 % amino acid sequence identity to the human SLC13 transporters. INDY is a sodium-independent, electroneutral anion exchanger that exchanges dicarboxylates across the plasma membrane [49]. Mutations that inactivate the *Indy* gene result in an extension of lifespan [87]. The location of INDY on the basolateral membrane in the fly gut, as well as fat bodies and oenocytes, supports its proposed role in regulating metabolism [49, 50]. Similar results have been seen after inactivation of the *Caenorhabditis elegans* SLC13/DASS transporters, NaC2 and NaC3, although the lifespan extension was more moderate [24, 25]. NaC2 and NaC3 are Na^+/dicarboxylate transporters found in the worm gastrointestinal tract, with properties similar to those of the mammalian NaCT and NaDC3, respectively.

Function and tissue distribution

NaDC1/SLC13A2

The Na^+/dicarboxylate cotransporter 1, NaDC1, is found on the apical membranes of renal proximal tubule and small intestinal cells (Fig. 1). NaDC1 is encoded by the SLC13A2 gene on chromosome 17. The rat ortholog has also been called SDCT1 [17]. NaDC1 protein has been immunolocalized to the apical membrane in renal proximal tubule of rat and mouse [2, 92], and jejunal brush border membranes from human and mouse [22, 79]. Northern blots and in situ hybridization experiments have identified NaDC1 message predominantly in kidney cortex and small intestine, and also to a lesser extent in liver, large intestine (rat), bronchiole, trachea, and epididymis [17, 71, 92]. In the kidney, NaDC1 functions to reabsorb citric acid cycle intermediates, such as citrate and α-ketoglutarate, from the tubular lumen. These metabolites, particularly citrate, provide an important source of energy for the kidney [37]. In addition, NaDC1 regulates urinary concentrations of citrate, which is an important calcium chelator and implicated in the development of kidney stones [66]. NaDC1 transport activity also functions to regulate levels of succinate in the tubular lumen. The SUCNR1 succinate receptor on the apical membrane of macula densa cells has been implicated in blood pressure regulation [102]. However, the role of NaDC1 and tubular succinate concentrations in regulating blood pressure has not yet been examined. The NaDC1 transporter in the small intestine mediates the first step of absorption of citric acid cycle intermediates (Fig. 1). There is evidence for transepithelial transport of citric acid cycle intermediates in the intestine [12], but the transporter responsible for release of these metabolites across the basolateral membrane has not been identified. The basolateral transporter appears to be an anion exchanger [108], probably from a different gene family than SLC13 but with a similar transport mechanism as the *Drosophila* Indy transporter that is also found on the basolateral membrane of insect midgut [50].

A detailed analysis of substrate specificity of the Na^+/succinate transporter in renal brush border membrane vesicles (most likely NaDC1) showed a broad substrate specificity with a preference for four carbon dicarboxylates, such as succinate, malate, oxaloacetate, as well as the tricarboxylate, citrate [109] (Table 1). Citrate is transported by NaDC1 in protonated form as a divalent anion [17, 64, 71, 92]. NaDC1 accepts substrates with substitutions at the C2 carbon, such as 2,2-dimethylsuccinate, but not substitutions at the C3 carbon [109]. There are some species differences in substrate selectivity, but overall the substrate profiles are very similar. The K_m for succinate in human NaDC1 is around 600–800 μM, and the K_m for citrate is 6,800 μM [80, 83] (Table 1). However, given that citrate is transported predominantly as $citrate^{2-}$ and the pKa is 5.6 [37], the actual K_m would be less than 100 μM at pH 7.5. In addition to endogenous substrates, NaDC1 appears to mediate nephrotoxicity of diethylene glycol by transporting one of its metabolites, diglycolic acid [54].

There are no specific high affinity inhibitors of NaDC1 to date. NaDC1 and the other SLC13/DASS family members, including the bacterial homologs, are inhibited by anthranilic acids, such as N-(p-amylcinnamoyl) anthranilic acid (ACA) and flufenamate [78, 84]. The human NaDC1 has an IC_{50}/Ki for ACA around 15 μM. ACA behaves as a slow onset, reversible inhibitor that decreases V_{max} without affecting K_m [78]. Therefore, it is likely that ACA binds to an allosteric site on the transporter.

NaDC1 transport is driven by the inwardly directed sodium gradient across the plasma membrane. NaDC1 is an electrogenic transporter that couples the movement of three sodium ions to the transport of each divalent anion substrate [18, 64, 92]. Lithium can substitute for sodium in NaDC1 although the K_m for succinate in lithium is tenfold higher than in sodium [75, 110]. In the presence of sodium, lithium can inhibit transport by binding with high affinity to one of the three cation binding sites [75]. There are some species differences in lithium sensitivity, and the human NaDC1 appears to have a lower affinity for lithium. The human NaDC1 does not accept lithium in place of sodium, although lithium can inhibit transport in the presence of sodium [80].

NaDC3/SLC13A3

The high affinity Na^+/dicarboxylate cotransporter, NaDC3, has the widest tissue distribution of the SLC13 dicarboxylate transporters (Fig. 1). NaDC3 is also referred to as NaC3 and

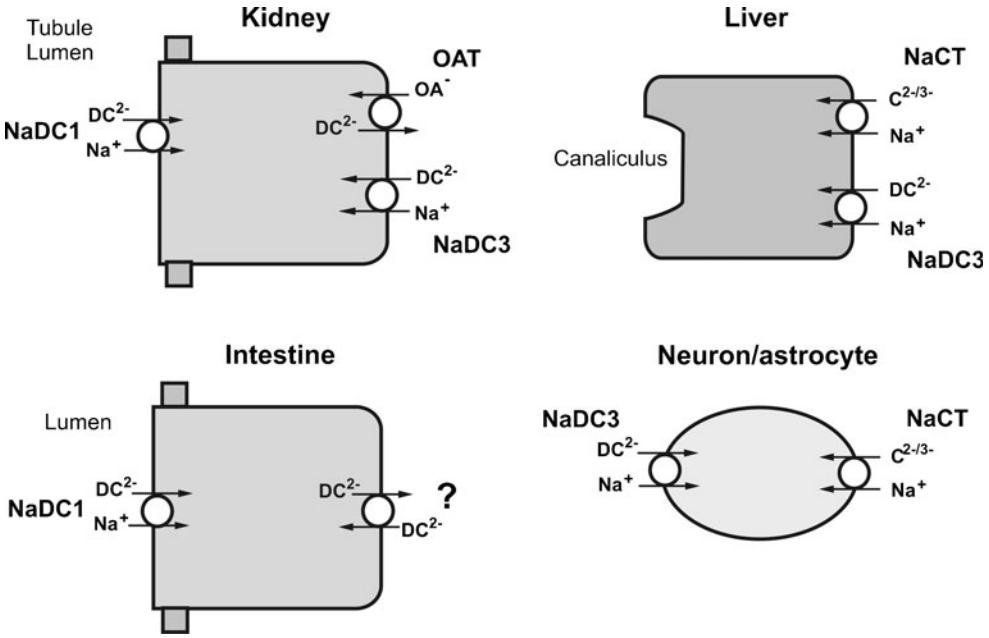

Fig. 1 Tissue distribution of SLC13 dicarboxylate/citrate transporters. For details, please see text. NaDC1 is found on the apical membrane in kidney proximal tubule and small intestinal cells. NaDC3 is located on the basolateral membrane in the kidney and liver; NaDC3 is also found in neurons and astrocytes. NaDC3 is also abundant on the brush border membrane of the placental syncitiotrophoblast (not shown). NaCT is found on the basolateral (sinusoidal) membrane of hepatocytes and on the plasma membrane of neurons and astrocytes. Dicarboxylate transport across the basolateral membrane of enterocytes is mediated by an unknown anion exchanger. Abbreviations: DC^{2-} dicarboxylate, OA^{-} organic anion, $C^{2-/3-}$ di or tricarboxylate

SDCT2 (rat) in some papers. Sodium-dependent succinate transport has been characterized in renal basolateral and placental brush border membrane vesicles [29, 111], and the cDNAs for NaDC3 were isolated from kidney, placenta, and brain libraries [18, 48, 74, 104]. NaDC3 message also has been found in hepatocytes [18], choroid plexus [74], astrocytes and neurons [53], and eye [30]. NaDC3 protein has been immunolocalized to the basolateral membrane of kidney cells [3].

NaDC3 has a high affinity for substrates and will accept a broad range of dicarboxylates (Table 1). NaDC3 transports four carbon dicarboxylates, similar to NaDC1, including succinate, malate, and fumarate. However, NaDC3 will also accept longer chain dicarboxylates containing five and six

Table 1 Transport properties of the human SLC13 dicarboxylate transporters

Protein name	Gene	Substrates	K_m succinate (μM)	K_m citrate (μM)	References
NaDC1	SLC13A2	Succinate, citrate^{2-}, fumarate, malate, 2-methylsuccinate, α-ketoglutarate, 2,2-dimethylsuccinate, tricarballylate	590–800	6,800	[80, 83, 112]
NaDC3, NaC3	SLC13A3	Similar to NaDC1 plus 2,3-dimethylsuccinate, glutarate, oxaloacetate, adipate, 2,3-dimercaptosuccinate, 3-OH-glutarate, *N*-acetyl-aspartate	2–25	220 (rat)	[13, 14, 17, 40, 47, 104]
NaCT, NaC2	SLC13A5	Citrate^{3-}, succinate, malate	1,900 (IC_{50})	600	[42]

carbons, such as glutarate and adipate [34]. Dicarboxylates with substitutions at the C3 carbon are also substrates of NaDC3, including 2,3-dimethylsuccinate and meso-2,3-dimercaptosuccinate (succimer) [13, 14]. Citrate is transported by NaDC3 but the affinity is lower than for succinate, with K_m values of 220 μM for citrate compared with 15 μM for succinate, and lower maximal currents with citrate than succinate [18, 104]. The most important physiological substrates for NaDC3 are glutarate and α-ketoglutarate. NaDC3 contributes these substrates to the organic anion transporters and thus plays an important role in the excretion of drugs and xenobiotics [93] (Fig. 1). The brain metabolite, *N*-acetyl aspartate (NAA), is also a substrate of NaDC3, and this is particularly important in mediating toxicity in Canavan disease in which levels of NAA accumulate in brain and eye [30, 40]. NaDC3 transports glutarate derivatives, including 3-hydroxyglutarate (3-OH-glutarate) that accumulate in the brain in glutaric aciduria type 1 [34, 95]. One treatment for this disease is lithium, which inhibits the activity of NaDC3 and the accumulation of toxic glutarate derivatives. Finally, there has been a suggestion that NaDC3 may mediate the sodium-dependent transport of glutathione in the eye and kidney [55, 56]. However, the transport of glutathione by NaDC3 has not been demonstrated directly yet.

The $K_{0.5}$ for succinate in the human NaDC3 is 25 μM, and for glutarate and α-ketoglutarate the $K_{0.5}$ is around 40 μM [14]. However, when the $I_{max}/K_{0.5}$ ratios are compared, succinate and glutarate are equally good substrates, while α-ketoglutarate is transported slightly less efficiently [14]. The amino acid, *N*-acetylaspartate, is transported by hNaDC3 with a $K_{0.5}$ ~300 μM [40]. As with NaDC1, transport by NaDC3 is sodium-dependent and electrogenic, with three Na^+ ions coupled to the transport of each divalent anion substrate [18, 104]. The $K_{0.5}$ for sodium is 46 mM in human NaDC3 [47]. Lithium does not support succinate transport in rat or human NaDC3, but the presence of Li^+ inhibits Na^+-dependent transport suggesting that lithium binds to one or more of the sodium binding sites and prevents the optimal conformation for substrate binding [18, 104].

NaCT/SLC13A5

The SLC13A5 gene codes for the Na^+/citrate transporter, NaCT, that is predominantly located in the liver and brain [42, 43] (Fig. 1). Other names that have been used for NaCT include NaC2 and mINDY [8, 103]. In situ hybridization studies show a wide distribution of NaCT message in the brain: cerebral cortex, cerebellum, hippocampus, and olfactory bulb [43]. However, there also appear to be some species differences in brain cellular distribution: in rat, NaCT is found in neurons and NaDC3 is found in astrocytes [113], whereas in mouse, the distribution of NaCT overlaps with that of NaDC3 in both neurons and astrocytes [53]. NaCT protein has been immunolocalized to the sinusoidal membrane in rat and human liver [31]. NaCT message has also been reported in rat (but not human) testis [42, 43]; mouse and rat kidney [8, 103, 113]; mouse adipose tissue, skeletal muscle, and pancreas [8]; and human salivary glands [98].

The NaCT transporter appears to handle a narrower range of substrates than NaDC1 and NaDC3, although there are some species differences. The human NaCT appears to be predominantly a citrate transporter (Table 1). In two-electrode voltage clamp studies of the human NaCT, citrate induces inward currents in the presence of sodium, but there are no currents with α-ketoglutarate, glutarate, or 3-OH-glutarate [10]. In mammalian cells expressing hNaCT, the K_m for citrate is 600 μM, whereas the IC_{50} for inhibition of citrate transport by succinate is 1.9 mM, and for inhibition by malate, 3 mM [42]. Succinate kinetics by hNaCT have not been reported. By comparison, the rat and mouse NaCT have broader substrate selectivities and appear to transport succinate and citrate equally well. There is inhibition of ^{14}C-citrate transport by citrate, succinate, fumarate, malate, α-ketoglutarate, *cis*-aconitate, and isocitrate in mouse NaCT, and the rat NaCT exhibits inward currents in the presence of α-ketoglutarate and *cis*-aconitate [41]. However, there appears to be a discrepancy in reported results of succinate and citrate transport kinetics in mouse NaCT. In one study, the V_{max}/K_m ratio is 4.8 for citrate and 2.3 for succinate, indicating that the efficiency of transporting citrate is only about twofold higher than for succinate [41]. However, in a separate study, the V_{max}/K_m ratios for citrate and succinate are ~18-fold different, making the transporter much more efficient with citrate than succinate, and more like the human NaCT [8]. This point would be important to resolve because it affects the interpretation of results from the knockout mouse model.

NaCT is a sodium coupled transporter but, unlike NaDC1 and NaDC3, NaCT appears to couple four sodium ions to the transport of trivalent citrate or divalent succinate [41]. Transport is electrogenic and substrate-dependent inward currents have been measured in mouse, rat, and human NaCT [10, 41]. Lithium does not fully substitute for sodium but can interact with at least one of the sodium binding sites in NaCT in the presence of sodium. There appear to be species differences in cation handling among the NaCT transporters. The rat NaCT is inhibited by lithium [44], similar to NaDC1 and NaDC3. In contrast, the kinetics of lithium interaction with hNaCT are complex and produce decreases in both K_m and V_{max} when measured in the presence of sodium [44]. As a consequence, at low citrate concentrations relative to the K_m value of 600 μM, lithium stimulates transport by hNaCT in the presence of sodium, whereas at high citrate concentrations, lithium behaves as an inhibitor [44]. At typical plasma citrate concentrations of ~100 μM, lithium would stimulate transport in hNaCT. The binding order of cations and substrate in NaCT would be useful to establish and would help in interpreting the mechanism of the effect of lithium in hNaCT.

Protein structure

VcINDY crystal structure

The first high-resolution crystal structure was recently solved of a bacterial homolog, named VcINDY (*Vibrio cholerae*), from the SLC13/DASS family [61]. The structure of VcINDY, at 3.2 Å resolution, is in an inward-facing conformation together with a bound citrate molecule and one sodium ion [61] (Fig. 2a). Interestingly, citrate is not an effective inhibitor of succinate transport by VcINDY [61], nor is citrate a substrate of the other bacterial members of the SLC13/DASS family [36, 96, 114]. Because the bacterial members of the family typically couple two sodium ions to the transport of a dicarboxylate substrate, it is likely that the crystal structure represents a conformational state after the release of one of the two sodium ions [61].

The structure of VcINDY contains 11 transmembrane helices (TM) and two opposing hairpin structures: HP_{in} and HP_{out}. The first half of the protein is related in sequence to the second half, but they are in opposite orientations in the membrane, known as inverse twofold symmetry [61] (Fig. 2b). The substrate and cation binding sites of VcINDY are composed of amino acids from both halves of the protein, particularly the tips of the two opposing hairpin loops and unwound portions of TM5 and TM10 [61]. As shown in Fig. 2b, the N-terminal half of the protein contains TM2-3 (shown as a narrow rectangle A in the figure) connected with the larger bundle of TM4-6 (rectangle B). Similarly the C-terminal repeat has a smaller bundle of TM7-8 (rectangle C) connected with the larger bundle formed by TM9-11 (rectangle D). Transport by VcINDY is proposed to occur by an alternating access mechanism by changes in conformation of the connectors between the helix bundles as well as tilting of helix bundles (shown in Fig. 2b). Although VcINDY has a unique protein fold that is unrelated to those of the large neurotransmitter (LeuT fold) or glutamate (GltPh fold) transporter families, there is a similar topology pattern of two halves related by inverse symmetry [51]. This inverted symmetrical structure allows the helices to tilt and reorient, also termed a "rocking-bundle" [26, 51], and is a very common feature of transporters with an alternating access mechanism of transport.

The crystal structure of the VcINDY protein is a homodimer, with contacts between one protomer at TM3, TM4a, TM9b interacting with TM4b, TM8, and TM9a of the opposing protomer (see also Fig. 3) [61]. Size exclusion chromatography experiments also showed that the transporter forms dimers in detergent [61]. At present, there is no information yet whether the functional unit of the transporter is a monomer or dimer, although some of the bacterial SLC13/DASS homologs exhibit sigmoid succinate transport kinetics [84].

SLC13 transporter topology

The experimental findings of the mammalian SLC13 transporter topology are in general agreement with the VcINDY crystral structure. Figure 3 shows the secondary structure model of NaDC1 based on the sequence alignment with vcINDY [61]. The other mammalian NaDC3 and NaCT transporters have similar secondary structure models, with slight differences in the length of loops between TM helices (see alignments in Supplemental section [61]). Experimental evidence from both NaDC1 and NaDC3 shows that the N-terminus is intracellular and the C-terminus, containing the conserved N-glycosylation site, is extracellular [4, 115]. Furthermore, the loop before helix 5a containing the epitope for

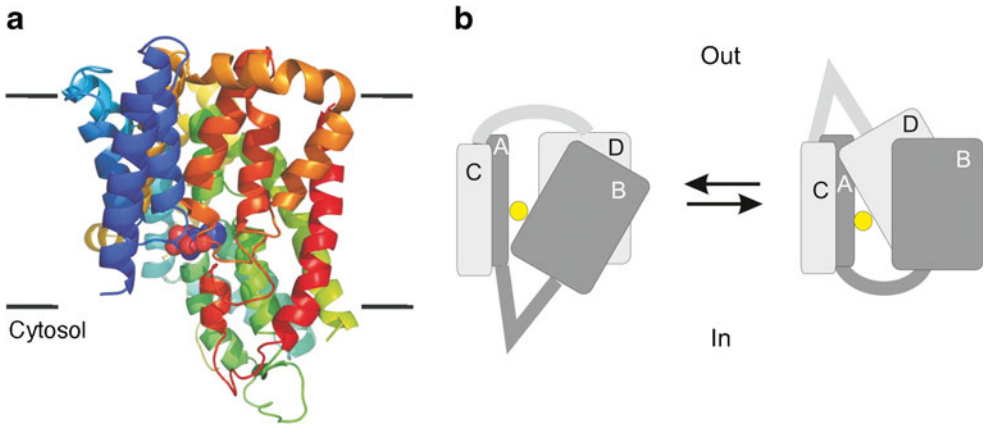

Fig. 2 **a** Model of bacterial homolog, VcINDY, based on the crystal structure. The image shows a single protomer, but the structure contains two protomers, each with a citrate and sodium binding site. The inside of the cell is at the *bottom* of the figure. Figure from [61], reprinted by permission of MacMillan Publishers Ltd: Nature, copyright 2012. **b** Cartoon showing conformational changes in a single protomer of the SLC13 transporters, based on figure of dimer from [61]. Helix bundles are represented by *rectangles*: *A* TM2-3; *B* TM4-6; *C* TM7-8; *D* TM9-11. Note that the orientation of helix bundles A and B is inverted relative to bundles C and D. The *filled circle* represents a substrate molecule

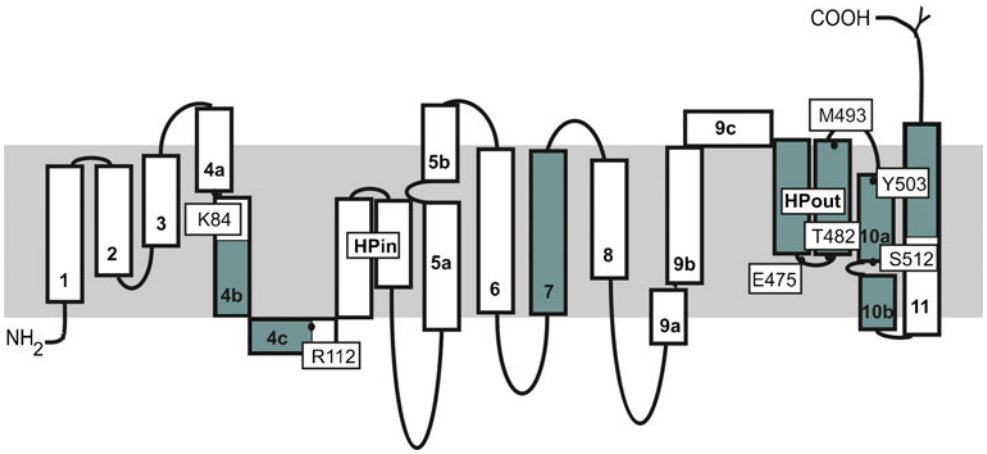

Fig. 3 Secondary structure model of NaDC1, based on sequence alignment with VcIndy [61]. Transmembrane helices are shown by *numbered rectangles*. Functionally important residues are shown in *boxes* and are discussed further in the text. The *light gray shading* represents the plasma membrane and the *darker gray shading* represents the region of the protein that has been analyzed in cysteine scanning mutagenesis experiments [72, 77, 82, 85, 106]. HP_{in} and HP_{out} represent the two hairpin helices that are important for substrate and cation binding

antibodies raised against NaDC1 and NaDC3 is accessible only from the inside [4, 115], consistent with the crystal structure (Fig. 3). Interestingly, the L1 antibody directed against amino acids 35–53 of NaDC3, which corresponds to TM2, was able to label the protein only after membrane permeabilization suggesting that TM2 is accessible from the inside of the cell, possibly to a water-filled vestibule [4].

Direct labeling of substituted cysteines [46] is another experimental approach that has been used to study membrane topology in the SLC13 transporters. MTSEA-biotin labeling shows that the K84C mutant of NaDC1 is accessible from the outside of the cell [106]. Lys84 is predicted to be in the hinge region connecting TM4a and TM 4b (Fig. 3) and it is a functionally important residue. Labeling of the K84C mutant with the membrane-impermeant reagent, MTSET, inhibits transport. The K84C mutant has different extracellular accessibility at different conformational states and exhibits substrate protection of alkylation [106], which likely reflects changes in conformational state of the transporter through the transport cycle. We have also shown that Lys84 is important for distinguishing between succinate and citrate in the transition state [106], although it is not predicted to be in the substrate binding domain. The equivalent residue, Asn108, in the bacterial homolog SdcS is accessible from both sides of the membrane in the presence of sodium [45]. In the human NaDC3 transporter, the V81C mutant predicted to be in helix 4a (very close to the location of Lys84) was only accessible after membrane permeabilization, but there is no information yet about changes in accessibility with conformational state [4]. Furthermore, surface helix 4c, predicted to be at the inner membrane surface, is accessible to membrane-impermeant reagents in both NaDC1 and NaDC3: R112C of NaDC1 can be labeled by MTSEA–biotin and A113C of NaDC3 can be labeled by fluorescein-5′-maleimide [4, 115]. This result provides evidence for a large water-filled cavity in the SLC13 transporters.

Protein topology has also been examined by direct labeling of cysteine-substituted mutants of NaDC1 with MTSEA–biotin. This reagent labels T482C at the tip of HP_{out}, predicted to be near the substrate/cation binding site, and M493C and L499C at the extracellular loop before TM10. There is no labeling of residues predicted to be in TM10a (Y503C, M505C, and A511C), indicating that those positions are buried from the extracellular solvent, at least in the conformational state seen with sodium [77] (Fig. 3). In similar experiments with cysteine-substituted mutants of hNaDC3, direct labeling with fluorescein-5′-maleimide showed that G454C (surface helix 9c) and A499C (at the outer edge of HP_{out}) are both accessible to the extracellular medium, whereas A438C (helix 9b) and the endogenous Cys517 (near unwound portion of TM10a) are not accessible [4].

Structure–function studies

The VcIndy crystal structure identified two key regions of the transporter for substrate and cation binding. At the N-terminus, the tip of the HP_{in} hairpin loop interacts with the unwound portion of TM5, and at the C-terminus, the HP_{out} hairpin loop interacts with the unwound portion of TM10 (Fig. 3) [61]. In the three-dimensional structure of the protein, the two opposing hairpin structures are located close to one another [61] (Fig. 2a). The tips of the hairpins contain conserved substrate and cation binding motifs, including SNT in HP_{in} and SNT/A/V/P in HP_{out}. Thr379 in HP_{out} of VcINDY is important for substrate recognition, and the T379P mutant of VcINDY was able to interact with both succinate and sulfate [61]. The dicarboxylate transporters have Thr/Ala/Val at this position whereas the sulfate transporters have Pro [61].

The amino acids around the SNA motif in HP_{out} from NaDC1 (Fig. 3) have been examined in mutagenesis studies and the results are consistent with the VcINDY crystal structure. The corresponding HP_{out} sequence in rbNaDC1 between amino acids 475–483 is ^{475}ECTSNAATT^{483}. The first in this sequence, Glu475, is important for both substrate and cation binding affinity in NaDC1, as well as affecting chloride conductance [32]. The adjacent cysteine at position 476 is the inhibitory binding site for the membrane-impermeant, cysteine-selective reagent, pCMBS [76]. Asn479 in the SNA motif at the tip of HP_{out} is a key residue. The N479C mutant was expressed on the plasma membrane but had no transport activity [72]. Cysteine-substituted mutants at positions Ser478, Ala480, Ala481, Thr482, and Thr483 are sensitive to inhibition by membrane-impermeant methanethiosulfonate (MTS) reagents [72, 77]. These cysteine mutants showed substrate protection and cation dependence of inhibition [72, 77], providing evidence for conformational movements of HP_{out} during the transport cycle. Also within this loop region, the naturally occurring polymorphism in the human NaDC1, V477M (aligns with Ala480 of rbNaDC1), had a large decrease in substrate and cation affinity and altered handling of Li^+ [83]. Finally, although not predicted to interact with substrate, Met493 at the outer portion of HP_{out} exhibits substrate protection from labeling and changes in accessibility to the outside during the transport cycle [77].

The unwound portion of the helix between TM10a and TM10b is predicted in the structure to interact directly with substrate [61]. A key residue within that loop, Ser512 in rbNaDC1, the equivalent Ala504 in mNaDC1 [70], or Thr509 in hNaDC1 [107], was found to determine differences in substrate specificity and cation affinity between transporters. Also in this loop, the F500L mutant of hNaCT was found to have altered citrate affinity and interaction with Li^+ [44]. Phe500 is conserved in hNaDC3 and hNaS1 but both rat NaCT and hNaDC1 have Leu at this position.

The intracellular surface helix, H4c, may have a functional role in addition to being accessible from the outside (Fig. 3). Mutants made at the Lys114 position in fNaDC3 are expressed on the plasma membrane and have normal transport activity, but do not exhibit substrate-dependent currents [35]. This result suggests a role of Lys114 relating to cation coupling or binding. VcINDY transports two sodium ions with each dicarboxylate substrate, but the mammalian members of the family have three or four cation binding sites.

SLC13 transporter regulation

NaDC1 regulation

As mentioned earlier, one of the primary physiological functions of NaDC1 in the renal proximal tubule is the regulation of urinary citrate concentrations. Citrate is an important chelator of calcium, and many patients with kidney stones exhibit hypocitraturia, often related to metabolic acidosis [66]. Acidosis stimulates NaDC1-mediated citrate transport, which would result in a decrease in tubular citrate. Acidic pH stimulates transport by increasing the concentration of divalent citrate, the transported species [71]. In addition, chronic exposure to acidosis increases expression of the SLC13A2 gene and NaDC1 protein abundance [2, 68]. The mechanism of acid activation of the SLC13A2 gene is mediated by the endothelin B receptor as part of a concerted response of the proximal tubule to an acid load [59]. Other studies have shown that Na^+/citrate transport activity in renal brush border membrane vesicles is increased with a high protein diet [1], and NaDC1 protein abundance in the kidney is decreased by ischemia [21]. In the intestine, the Slc13a2 gene is upregulated after 24 h of fasting [101].

A number of factors have been shown to affect protein expression, activity, and targeting of NaDC1. For example, protein kinase C activation results in a decreased abundance of NaDC1 protein on the plasma membrane [81]. Other kinases have been shown to increase the V_{max} of NaDC1 including SGK1 and SGK 3, protein kinase B, and NHERF2 [9]. Cyclophilin B appears to act as a chaperone that mediates increased expression of renal NaDC1 [7]. Protein/protein interactions with other transporters are also important for modulating NaDC1 function. A recent study has shown that direct interaction with the oxalate transporter, SLC26A6, regulates transport activity of NaDC1 [69]. SLC26A6 coexpression does not affect the cell surface abundance of NaDC1, but it reduces the transport activity [69]. The interaction domain was narrowed down to amino acids 45–118 [69], which includes TM3 and TM4 in the new topology of NaDC1 (Fig. 3). Interestingly, this domain was identified in the VcINDY crystal structure as part of the protein/protein interface between two protomers [61]. It will be important to determine whether NaDC1 functions normally as a homodimer.

The role of NaDC1 in reabsorbing citric acid cycle intermediates has been verified using a knockout mouse model, which has greatly increased urinary excretion of citrate, α-ketoglutarate, fumarate, and malate and a modest increase in succinate [39]. Despite the increased excretion, there is no significant change in plasma citrate concentration in the knockout mice [39]. This finding provides evidence of a strong homeostatic mechanism to maintain plasma levels of citric acid cycle intermediates. Rodents reabsorb most of the filtered citrate, with a fractional excretion of citrate around 1 % [99]. Citrate is reabsorbed in the proximal tubule on NaDC1, and there is no tubular secretion of citrate [11]. Therefore, a decrease in plasma citrate in the knockout mouse would be expected unless there was a source of citrate release to the plasma from reservoirs such as bone [37]. The citrate

release transporter responsible has not yet been identified although a sodium-independent citrate transporter has been found in prostate [63]. NaDC1-deficient mice do not appear to have any changes in growth or response to renal injury [39]. It is not clear yet whether the changes in urinary succinate and α-ketoglutarate in the knockout mice have any effects on blood pressure or acid–base regulation mediated by the succinate receptor SUCNR1 in macula densa [102] or the α-ketoglutarate receptor OXGR1 in the distal nephron [100]. Finally, the possible compensation by other transporters in the NaDC1 knockout mice is not known, although NaDC3 was not increased [39]. NaCT may be present in mouse kidney [8] and the mouse diet is very high in citrate [15]. It would be helpful to know if there is upregulation or compensation by other transporters and whether a high citrate diet affects the outcome.

NaDC3 regulation

There have been relatively few studies of NaDC3 regulation. There is evidence that protein kinase C activation inhibits NaDC3 [33, 94], whereas there is no effect of activating other protein kinases (protein kinase A, tyrosine kinases, MAPK) [28, 94]. The sequence for basolateral targeting of NaDC3 was identified as AKKVWSARR, located at the amino terminus [3].

NaDC3 is upregulated in the livers of NaCT knockout mice, possibly to compensate for reduced hepatic uptake of citrate [8], but there is no change of NaDC3 in the kidney of NaDC1 knockout mice [39]. Other gene expression profiling studies have suggested that NaDC3 message is upregulated in the kidney during chronic metabolic acidosis and downregulated in the frontal cortex in ovariectomized rats [68, 91]. The SLC13A3 gene may be downregulated in livers of glycerol kinase mice, a model of glycerol kinase deficiency [60]. Furthermore, single nucleotide polymorphisms in two introns of SLC13A3 (NaDC3) have been associated with type 2 diabetes, although the mechanism has not been determined [6]. NaDC3 expression in the kidney increases with age and may be related to cellular senescence [57].

The PITX transcription factor, which is important for eye development and normal eye function, was found to directly regulate SLC13A3 gene expression [97]. This study reported an involvement of NaDC3 in the eye response to oxidative stress, although the mechanism was not determined. It will be important to verify whether NaDC3 transports glutathione or if another substrate, such as *N*-acetylaspartate, is important for eye function [97]. To date, there are no reports of NaDC3 knockout mice. Because of the role of NaDC3 in drug and xenobiotic excretion, we would expect that the NaDC3 knockout mice would have changes in drug pharmacokinetics. However, NaDC1 could potentially compensate for the lack of NaDC3 in the kidney because it can contribute glutarate and α-ketoglutarate to the organic anion transporters [93].

NaCT regulation

The physiological and metabolic changes in the Slc13a5 knockout mouse have been examined in a detailed study [8]. The mNaCT transporter in this study was renamed mINDY, after the *Drosophila* Indy transporter that extends lifespan through a mechanism similar to caloric restriction [87, 105]. Deletion of the mINDY transporter in mice has profound metabolic effects, and the mice exhibit changes in signaling pathways regulating energy balance. The knockout mice are smaller in size, have lower plasma glucose levels, and are resistant to the effects of high fat feeding, such as weight gain and insulin resistance [8]. Plasma levels of citrate and malate were slightly elevated in the knockout mice, but there were no significant changes in succinate or fumarate [8]. This may be related again to strong homeostatic mechanisms regulating plasma levels of these metabolites. NaDC3 was upregulated in livers of NaCT knockout mice fed a normal diet, but not in mice fed a high fat diet. The mechanism by which NaCT inhibition affects metabolism has not yet been determined. It is not clear whether the citrate levels in the liver or brain provide the important signal. Citrate injections within the brain are known to affect metabolism by the liver [16]. It will be interesting to determine whether citrate interacts with specific receptors, analogous to the SUCNR1 and OXGR1 receptors. For example, the SUCNR1 receptor in the liver senses succinate released from hepatocytes during oxidative stress [19]. In any case, the metabolic changes in the mINDY knockout mice suggest that NaCT may be an important therapeutic target for treating human metabolic diseases such as obesity and type 2 diabetes.

Besides the knockout mouse model, there are only a few preliminary studies reporting the regulation of NaCT and the mechanisms have not been determined. For example, microarray expression profiling of cortical bone from Hyp mice, a model of X-linked hypophosphatemic rickets, shows a downregulation of SLC13A5 [58], which also suggests that NaCT may be found in bone. In glioblastoma patients, SLC13A5 was one of the genes found to be underexpressed, possibly as a consequence of a hypermethylated promoter region for the SLC13A5 gene [23]. Glioblastomas have been associated with alterations in citric acid cycle intermediates, particularly glutarate, but there is as yet no information on any potential involvement of NaCT.

Conclusion

In conclusion, there have been many recent advances in the field of SLC13 transporters. The VcINDY crystal structure

has allowed us to examine the effects of site-directed mutagenesis in the context of the structure, which provides a much better understanding of the mechanism of these transporters. However, the crystal structure shows a single conformational state bound with citrate, which is not the preferred substrate, and only one of the two sodium ions bound. Structures showing more conformational states would provide information on the protein movements during the transport cycle. Furthermore, a crystal structure of one of the mammalian SLC13 transporters will be important for verifying the location of substrate binding domains and for identifying the additional cation binding sites; the bacterial transporters couple two sodium ions whereas mammalian transporters couple three or four. The functional unit of the transporter as a monomer or a dimer is still to be determined. Similarly, we are only starting to understand the physiological roles of the SLC13 transporters in regulating citric acid cycle intermediates. The homeostatic mechanism regulating these metabolites is still poorly understood and there is very little information on sodium-independent transporters for release of citric acid cycle intermediates from cells. The mechanisms regulating the activity and expression of the SLC13 transporters are still poorly understood. Future studies are likely to focus on the SLC13 transporters as potential therapeutic targets for treatment of metabolic disesases.

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