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The substrate recognition domain in the Na^+/dicarboxylate and Na^+/sulfate cotransporters is located in the carboxy-terminal portion of the protein

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Abstract

The Na^+/dicarboxylate cotransporter, NaDC-1, and the Na^+/sulfate cotransporter, NaSi-1, share 43% sequence identity, but they exhibit no overlap in substrate specificity. A functional chimera, SiDC-4, was prepared from NaDC-1 and NaSi-1 by homologous recombination and expressed in *Xenopus* oocytes. SiDC-4 contains putative transmembrane domains 1–4 of NaSi-1 (amino acids 1–139) and putative transmembrane domains 5–11 of NaDC-1 (amino acids 141–593). SiDC-4 retains the substrate specificity of NaDC-1, which suggests that the substrate recognition domain is found in the carboxy-terminal portion of the protein, past amino acid 141. However, residues that affect substrate affinity and inhibition by furosemide and flufenamate are found in the amino terminal third of the protein. The cation binding properties of SiDC-4, including a stimulation of transport by lithium, differed from both parental transporters, suggesting that cation binding is determined by interactions between the amino- and carboxy-terminal portions of the protein. We conclude that the substrate recognition site of NaDC-1 and NaSi-1 is found in the carboxy-terminal portion of the protein, past amino acid 141, but residues in the amino terminus can affect substrate affinity, inhibitor sensitivity, and cation selectivity. © 1998 Elsevier Science B.V.

Keywords: Sodium; Dicarboxylate; Sulfate; Chimera; Oocyte; (*Xenopus*)

1. Introduction

The transport of dicarboxylates and sulfate in the renal proximal tubule is mediated by distinct sodium-coupled transport proteins [1]. The Na^+/dicarboxylate cotransporter, NaDC-1, absorbs Krebs cycle intermediates such as succinate and citrate from the tubular filtrate [2]. The renal Na^+/sulfate cotransporter, NaSi-1, carries inorganic sulfate [3]. NaDC-1 and NaSi-1 are members of the same gene family and exhibit 43% sequence identity. The hydropathy profiles of NaDC-1 and NaSi-1 resemble one another, and their secondary structures are also likely to be similar. The current model of NaDC-1 secondary structure contains 11 transmembrane domains and a single *N*-glycosylation site located at the C-terminus [4]. Functionally, there is no overlap in substrate specificity between NaDC-1 and NaSi-1,

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but both transporters couple 3 sodium ions to the transport of a divalent anion substrate [2,5].

The structural domains responsible for sodium binding and substrate discrimination in NaDC-1 and NaSi-1 have not yet been determined. Chimeras between structurally-related transporters with distinct functions can help to identify domains that determine these differences in function. In the present study, we report the formation of a functionally active chimera, SiDC-4, which retains the substrate binding characteristics of the parental, NaDC-1. The results suggest that the substrate recognition site in the members of this gene family is found in the carboxy-terminal portion of the protein, distal to amino acid 141.

2. Methods

2.1. Formation of chimeras by homologous recombination

The technique of homologous recombination [6,7] was used to prepare chimeras between the Na^+/dicarboxylate, NaDC-1, and Na^+/sulfate, NaSi-1, cotransporters. Both cDNAs were originally cloned in the *Sal*I and *Not*I sites of the vector pSPORT. A tandem construct, SiDC, was made, which consisted of NaSi-1 cDNA followed by NaDC-1 cDNA in pSPORT separated by two unique restriction sites, *Mun*I and *Sal*I. To prepare the SiDC construct, the *Sal*I site in NaSi-1 plasmid was removed by digestion with *Sal*I, followed by blunting with Klenow, and subsequent relegation. The NaDC-1 cDNA was excised with *Kpn*I and *Not*I and ligated into the *Afl*II and *Not*I sites of NaSi-1, after filling in the *Kpn*I and *Afl*II sites with Klenow. For the homologous recombination procedure, the SiDC tandem construct was linearized between the cDNA inserts using *Mun*I and *Sal*I. DH5α cells were transformed with the linearized construct. Plasmid DNA was purified from carbenecillin-resistant colonies and the size checked on agarose gels. Monomer size plasmids were subjected to restriction digests to determine the approximate location of the junction between the two cDNAs. The precise location of the junction was verified by sequencing. Of the 42 recombinant plasmids tested, most contained complete or almost complete overlaps of the coding region. However, one plasmid contained a chimeric insert, called SiDC-4 (Fig. 1), which consisted of the first four transmembrane domains of NaSi-1 and the last seven transmembrane domains of NaDC-1. Although the reverse tandem construct was made (DCSi), it yielded no circularized plasmids after two separate transformations.

2.2. Formation of chimeras by PCR

Three additional chimeras were prepared by Overlap Extension PCR [8]. Chimera DS1 consisted of amino acids 1–402 of NaSi-1 followed by amino acids 390–593 of NaDC-1 (note that there are gaps and inserts in the alignment of the two sequences, [2]) (Fig. 1). Chimera DS2 contained amino acids 141–389 of NaDC-1 flanked by NaSi-1 at the N- and C-termini (Fig. 1). Chimera DS3 contained amino

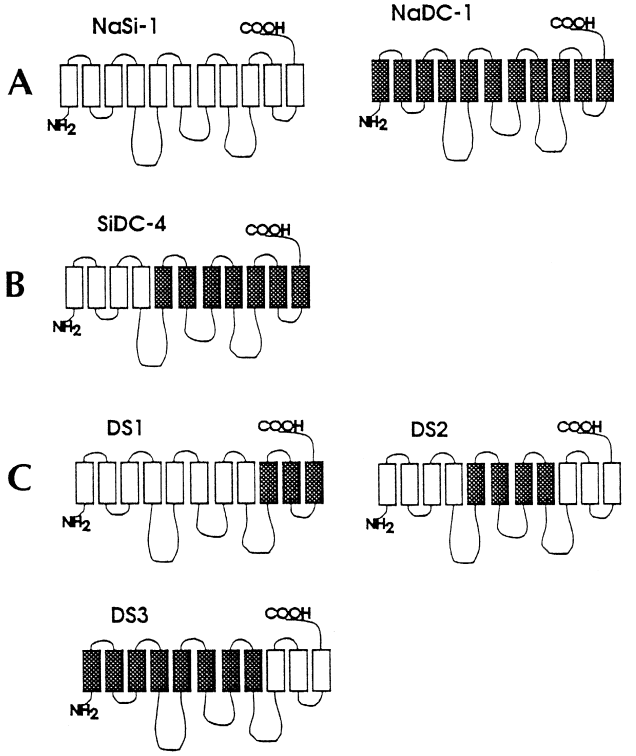

Fig. 1. Wild-type and chimeric transporters. (A) Secondary structure models of the wild-type Na^+/sulfate cotransporter, NaSi-1 (white), and Na^+/dicarboxylate cotransporter, NaDC-1 (grey). (B) Functional chimera SiDC-4, consisting of the first four transmembrane domains (TMD) of NaSi-1 and the last seven TMD of NaDC-1. (C) Non-functional chimeras, DS1, DS2, and DS3, each containing a junction just past TMD 8.

acids 1–389 of NaDC-1 followed by amino acids 403–595 of NaSi-1 (Fig. 1). The primers used for chimera formation were: DS-1: 5′-TTTCTAATTC-CAGCCAAGAAGATCCCAGGACTGACC-3′ and 5′-GGTCAGTCCTGGGATCTTCTTGGCTGGAAT-TAGAAA-3′, DS-2: 5′-TTGTTCATGGTGCCCTCC-AAACTGACAAAAATGACA-3′, and 5′-TGTCAT-TTTTGTCAGTTTGGAGGGCACCATGAACAA-3′. Chimera DS3 was made by subcloning a fragment of NaDC-1 into the DS2 chimera. The ''outside'' primers used in this procedure were vector specific: the T7 and SP6 promoter primers. The PCR amplification reactions were performed using AmpliTaq polymerase (Perkin Elmer–Cetus) with the following parameters: 94°C for 3 min, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, 3 min at 72°C, and a final extension at 72°C for 7 min. The chimeric cDNAs were subcloned into pSPORT1, and verified by sequencing.

2.3. In vitro cRNA transcription

The parental and chimeric cDNAs were used as templates for cRNA synthesis. Plasmids were linearized with *Not*I and in vitro cRNA transcription was done using the T7 mMessage mMachine Kit (Ambion). The cRNAs were resuspended in water to a final concentration of 0.5 to 1 $\mu g/\mu l$.

2.4. Xenopus oocytes

Stage V and VI oocytes from *Xenopus laevis* were dissected and collagenase treated as described previously [2]. Oocytes were cultured at 18°C in Barth's medium supplemented with 5% heat-inactivated horse serum, 2.5 mM sodium pyruvate and 50 mg/l gentamicin. Culture dishes and medium were changed daily.

2.5. Transport experiments

Transport of [^{14}C]-succinate (DuPont-NEN), [^{14}C]-citrate (Moravek), [^{14}C]-glutarate (American Radiolabelled Chemical), and [^{35}S]-sulfate (DuPont-NEN) was measured five to seven days after oocyte injections, also as described [2]. Sodium and choline buffers (in mM) were as follows: 100 mM NaCl or Choline, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes–Tris, pH 7.5. The oocytes were rinsed briefly with choline buffer to remove sodium and serum. Transport was initiated by replacement of the choline rinse with 0.4 or 0.75 ml (kinetic experiments) of the appropriate transport buffer as described in the figure legends. Transport was stopped after the appropriate time by 4×4 ml washes with ice-cold choline buffer. Individual oocytes were transferred to scintillation vials and dissolved in 0.5 ml 10% SDS. Scintillation cocktail was added and radioactivity was counted. Counts in control uninjected oocytes were subtracted from the counts in cRNA-injected oocytes. Data are presented as means $\pm$ SE. Calculations of kinetic constants were done by nonlinear regression to the Michaelis–Menten equation, using the SigmaPlot program (Jandel Scientific). Statistical analysis was done using the SigmaStat Program (Jandel Scientific).

2.6. Western blots of purified of plasma membranes

Plasma membranes were purified from *Xenopus* oocytes by the method of Geering and co-workers [9], and were blotted onto nitrocellulose as described [4]. The nitrocellulose membranes were blocked for a minimum of 1 h in PBS-TM (phosphate-buffered saline containing 0.05% Tween 20 and 0.5% Carnation instant dried milk). The Western blots were probed with an antibody directed against a fusion protein of NaDC-1 [4]. The primary antibody was diluted 1:10 000 in PBS-TM, and applied for 1 h, after which the secondary antibody, horseradish peroxidase-linked anti-rabbit Ig (Amersham), was diluted 1:5000 in PBS-TM and applied for 1 h. All incubations and washes were done at room temperature. Antibody binding was visualized by enhanced chemiluminescence using the Supersignal CL-HRP substrate system (Pierce). Molecular weights were estimated by comparison with pre-stained protein standards (BioRad). The Western blots were scanned with a Microtek Scanmaker E3 scanner, and peak mass and intensity were calculated using SigmaGel software (Jandel Scientific).

2.7. Deglycosylation

Plasma membranes (4 μg) from *Xenopus* oocytes injected with NaDC-1 cRNA were denatured in 0.5%

SDS, 1% β-mercaptoethanol at 100°C for 10 min. The deglycosylation reaction was carried out in 50 mM sodium phosphate, pH 7.5, 1% NP-40 and between 500–750 units Peptidyl *N*-Glycanase F (PNGase F) (New England Biolabs) at 37°C for 1 h. Control reactions were run in the absence of PNGase F.

3. Results

3.1. Chimera formation and characterization

The method of homologous recombination was used to generate chimeras between NaDC-1 and NaSi-1, which share 43% sequence identity (Fig. 1). Most of the chimeras produced by this method contained complete or almost complete overlaps of the coding region. However, one chimera was formed, SiDC-4, that contained amino acids 1–139 of NaSi-1 followed by amino acids 141–593 of NaDC-1. (Note that the N-terminus of NaSi-1 is shorter than that of NaDC-1, and amino acids 139–141 in NaSi-1 are identical to amino acids 140–142 of NaDC-1.) Chimera SiDC-4 corresponds approximately to the first four transmembrane domains of NaSi-1 followed by the last seven transmembrane domains of NaDC-1. Three additional chimeras, called DS1, DS2 and DS3, were generated using a PCR-based method (Fig. 1).

As shown in previous studies [2,3], the parental transporters, NaDC-1 and NaSi-1, carry completely different substrates. NaDC-1 transports succinate but does not transport sulfate (Fig. 2(A)). Furthermore, the transport of succinate by NaDC-1 is not inhibited by up to 10 mM sulfate [2]. Similarly, NaSi-1 carries only sulfate, and does not interact with succinate (Fig. 2(A)). The chimeric transporter, SiDC-4, retains the transport characteristics of the parental NaDC-1 since it carries succinate but not sulfate (Fig. 2(B)). SiDC-4 also transports citrate and glutarate (Fig. 2(B)), similar to the substrate specificity of NaDC-1 [10]. In three separate experiments, we were unable to measure any succinate, citrate, glutarate or sulfate transport in oocytes expressing chimeras DS1, DS2 or DS3 (not shown).

3.2. Substrate specificity of SiDC-4

The substrate specificity of SiDC-4 was further tested by measuring the transport of succinate in the presence of potential inhibitors (Fig. 3). The substrate specificity of SiDC-4 appears to be very similar to that of the parental NaDC-1. The greatest inhibition of SiDC-4 transport was seen with di- and tricarboxylates including succinate, fumarate, citrate, α-ketoglutarate, and malate. However, SiDC-4 appears to be more sensitive to these inhibitors than NaDC-1 [2],

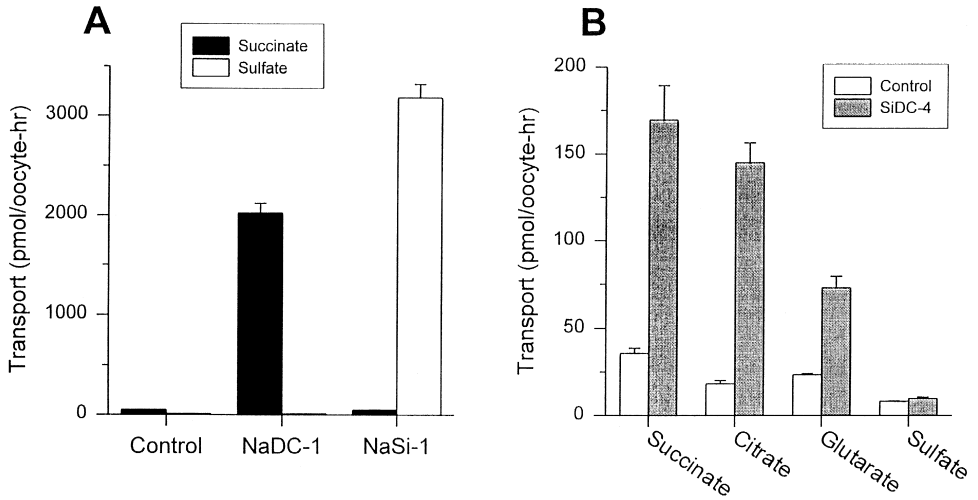

Fig. 2. (A) Transport of succinate and sulfate in control, uninjected oocytes and oocytes expressing the parental transporters, NaDC-1 and NaSi-1. The transport of 100 μM succinate or sulfate was measured over a 15 min time period in sodium containing buffer. Values shown are means ± SE, $n = 5$ oocytes. (B) Transport in SiDC-4-injected oocytes. Transport of succinate, citrate, glutarate and sulfate was compared in control, uninjected oocytes and oocytes expressing SiDC-4. Transport conditions were the same as in A.

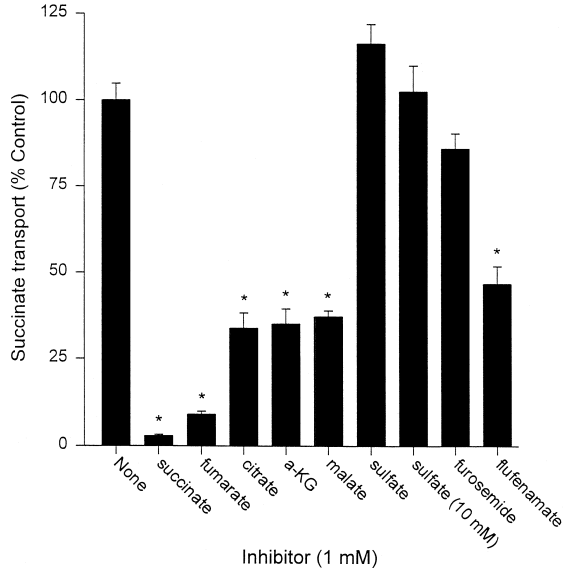

Fig. 3. Substrate specificity of the SiDC-4 chimera. Transport of 10 μM succinate was measured in the presence or absence of 1 mM concentrations of test inhibitors. Sulfate was tested at 1 mM and 10 mM concentrations. Fifteen minute uptakes were measured in sodium containing buffers. Data shown are means ± SE, $n = 5$ oocytes. (* denotes significant difference from control, $P < 0.05$).

suggesting an increased substrate affinity in the chimera compared with the parental transporter. There was no inhibition of succinate transport by up to 10 mM sulfate. Succinate transport by SiDC-4 was inhibited by flufenamate, although the sensitivity of SiDC-4 to flufenamate was lower than seen in the wild-type NaDC-1 [4]. SiDC-4 was not significantly inhibited by 1 mM furosemide, which inhibits NaDC-1 by approximately 50% [4].

3.3. SiDC-4 kinetics

The concentration dependence of succinate transport by SiDC-4 is shown in Fig. 4. Because of the low expression of SiDC-4 relative to control oocytes, the kinetic data should be considered as estimates only. The apparent K_m for succinate was 59 ± 24 μM and the apparent V_{max} was 391 ± 23 pmol/oocyte/h. In a second experiment, the kinetics of succinate transport by SiDC-4 and NaDC-1 were compared in the same batch of oocytes. The apparent K_m for succinate by SiDC-4 was 108 ± 82 μM and the V_{max} was 408 ± 62 pmol/oocyte/h ($n = 5$ oocytes). Oocytes injected with NaDC-1 cRNA had a K_m for succinate of 243 ± 49 μM and a V_{max} of 11 830 ± 696 pmol/oocyte/h ($n = 5$ oocytes). In previous studies, the K_m for succinate in NaDC-1 was 0.5 mM [2,10]. It should be noted that the K_m for succinate in NaDC-1 varies with oocyte batches, probably due to variations in membrane potential [11].

3.4. Cation selectivity in SiDC-4

The SiDC-4 chimera had altered cation specificity compared with NaDC-1 and NaSi-1. The parental Na^+/dicarboxylate cotransporter, NaDC-1, exhibits very low transport of succinate when sodium is replaced by choline (transport of 0.1%) (Fig. 5 and [2]). There is slightly higher transport of succinate in NaDC-1 in 100 mM lithium, approximately 5% of the transport seen in 100 mM sodium. Similarly, the Na^+/sulfate transporter, NaSi-1, has very low sulfate transport and low sulfate-dependent currents when sodium is replaced by choline [3,5]. In contrast, SiDC-4 had a relatively high rate of succinate transport in the presence of choline or lithium, approximately 30% of that seen in sodium (Fig. 5).

The parental NaDC-1 is sensitive to inhibition by millimolar concentrations of lithium [2,10], with an apparent K_i for lithium of around 2 mM [10]. Similar

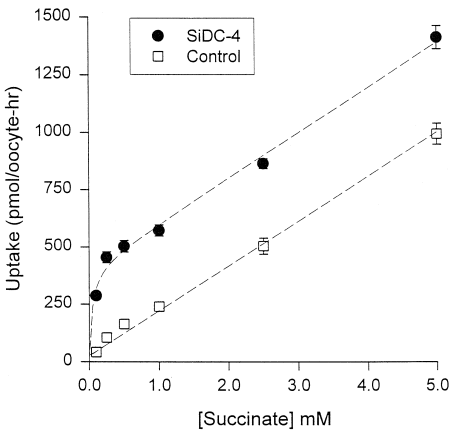

Fig. 4. Kinetics of succinate transport in control uninjected oocytes and oocytes expressing the SiDC-4 chimera. Concentrations of succinate ranged from 50 μM to 7 mM, and 10 min uptakes were measured. Data shown are means ± SE ($n = 5$). The K_m for succinate in SiDC-4 is 59 ± 24 μM and the V_{max} is 391 ± 23 pmol/oocyte/h ($n = 5$), after correction for counts in uninjected oocytes.

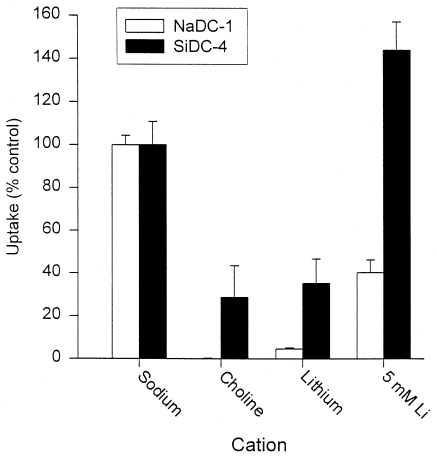

Fig. 5. Cation specificity of SiDC-4 compared with the parental transporter, NaDC-1. The transport of 100 μM succinate was measured in transport buffers containing 100 mM sodium, choline or lithium. Succinate transport was also measured in a combination of 95 mM sodium with 5 mM lithium. Fifteen minute uptakes were measured. Data shown are means ± SE ($n = 5$), expressed as a percentage of control uptakes measured in sodium buffer. Control uptakes were 2856 ± 126 pmol/oocyte/h (NaDC-1) and 116 ± 13 pmol/oocyte/h (SiDC-4). Uptakes were corrected for counts in uninjected oocytes.

to our previous results, succinate transport by NaDC-1 was inhibited by 60% in the presence of 5 mM lithium (Fig. 5). NaSi-1 is relatively insensitive to inhibition by lithium. Transport of 100 μM sulfate by NaSi-1 decreased only 20% in 5 mM lithium (results not shown). Unlike either of the parental transporters, however, the chimera SiDC-4 showed a 1.7-fold increase in the transport of succinate in the presence of 5 mM lithium (Fig. 5).

3.5. Characterization of anti-NaDC-1 antibodies

The anti-NaDC-1 antibody used in this study was prepared by injecting the same fusion protein as in our previous study [4] into rabbits rather than chickens. In our previous study, the antifusion protein antibodies raised in chickens recognized several proteins in the *Xenopus* oocyte plasma membranes, which often made it difficult to identify NaDC-1 expressed in oocytes [4]. The rabbit antibodies prepared in this study recognized the same 63 kDa protein in rabbit renal brush border membrane vesicles (not shown) as the chicken antibodies [4].

Plasma membranes prepared from oocytes injected with NaDC-1 contain a major immunoreactive protein at 56.7 kDa and a less abundant, broad band of protein centered at 74 kDa (Fig. 6). The 66 kDa protein recognized by the chicken antibodies in our previous study [4] appears to correspond to a component of the broad 74 kDa band recognized by the rabbit antibodies. Enzymatic deglycosylation of NaDC-1 in oocyte membranes using PNGase F resulted in the disappearance of both the 56.7 kDa and the 74 kDa proteins, and the appearance of an immunoreactive signal at 55.5 kDa. Plasma membranes prepared from oocytes injected with a mutant NaDC-1, called GM2, which lacks *N*-glycosylation sites [4], had only a single immunoreactive protein at 55.5 kDa. Therefore, the 55.5 kDa protein probably represents the unglycosylated form of NaDC-1, and the larger proteins (56.7 and 74 kDa) represent heterogeneously glycosylated forms of the transporter. Interestingly, the GM2 mutant expresses only ~55% of wild-type transport ([4] and not shown), and it had a ~65% reduction in the amount of protein at the plasma membrane relative to oocytes expressing NaDC-1 (the lane containing the control NaDC-1 membranes

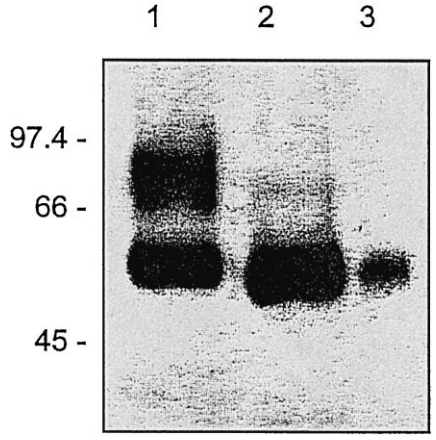

Fig. 6. Western blot of plasma membranes prepared from *Xenopus* oocytes. Two μg of protein were loaded into each well of a 10% acrylamide gel. The sample volumes were 10 μl for lane 3 and 30 μl in lanes 1, 2. The blot was probed with a 1:10 000 dilution of primary antibody directed against NaDC-1, followed by a 1:5000 dilution of anti-rabbit IgG. The position of size standards (kDa) is shown at left. Lane 1: NaDC-1-injected oocytes, membranes incubated with buffers for deglycosylation reaction (same as lane 2) but without enzyme; 2: NaDC-1-injected oocyte membranes treated with PNGase F; 3: oocytes expressing GM2 mutant (NaDC-1 lacking *N*-glycosylation sites).

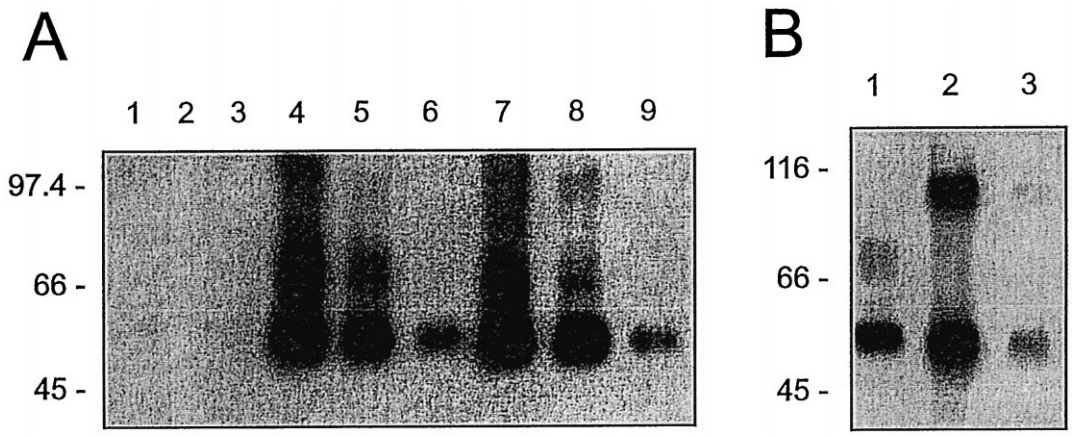

Fig. 7. (A) Western blot of plasma membranes prepared from control uninjected oocytes (lanes 1,2,3), and oocytes expressing NaDC-1 (lanes 4,5,6) and SiDC-4 (lanes 7,8,9). Three different protein concentrations were loaded onto the gel for each sample: 5 μg (lanes 1,4,7), 2.5 μg (lanes 2,5,8) and 0.5 μg (lanes 3,6,9). Immunoblot conditions were the same as in Fig. 6. Size standards (kDa) are indicated at left. (B) Western blot of plasma membranes prepared from oocytes expressing NaDC-1 (lane 1) and the mutant chimeras DS2 (lane 2) and DS3 (lane 3). Two μg protein were loaded in each lane. Other conditions were the same as described in Fig. 6.

is not shown) (Fig. 6), suggesting that the removal of glycosylation sites impairs protein trafficking or stability.

3.6. Western blots of oocyte membranes

Plasma membranes were prepared from control, uninjected oocytes or oocytes expressing NaDC-1 or the chimera, SiDC-4. The membrane proteins were separated by SDS-PAGE in two gels run in parallel. Each gel contained three different amounts of each protein sample (5, 2.5, and 0.5 μg). One of the gels was used for Coomassie blue staining, to verify the amount of protein loaded into each well, and the second gel was used for Western transfer and blotting. The exposure time of the film was varied to ensure a linear response. The Coomassie Blue stained gel showed that sample loading was approximately the same among the three samples (not shown). As shown in Fig. 7(A), there was no immunoreactive signal in Western blots of membranes from uninjected oocytes. A strong immunoreactive protein band at 56.7 kDa and a less intense signal at 74 kDa were seen in membranes from oocytes injected with NaDC-1 or SiDC-4. The relative intensities of SiDC-4 compared with NaDC-1 were 98% (5 μg protein), 132% (2.5 μg) and 139% (0.5 μg), indicating that the abundance of the two proteins was very similar. Fig. 7(B) shows an immunoblot of membranes prepared from oocytes injected with the DS2 and DS3 chimeras, both of which contain the epitope for the anti-NaDC-1 antibodies. Equal amounts of protein were loaded onto each lane and the film exposure was varied to ensure a linear response. In plasma membranes from oocytes expressing the DS2 and DS3 chimeras there were two immunoreactive proteins. One protein had the same apparent mass as the non-glycosylated form of NaDC-1, 55 kDa. The second protein, at 106.7 kDa, could represent aggregation of the 55 kDa protein. There was no evidence of the usual proteins seen in NaDC-1-injected oocytes at 56.7 and 74 kDa. The abundance of the 55 kDa protein in DS3 plasma membranes was very low, only 34% of NaDC-1, whereas the DS2 chimera appeared to be more abundant than NaDC-1 (212%).

4. Discussion

The Na^+/dicarboxylate and Na^+/sulfate cotransporters are members of the same gene family, sharing 43% sequence identity and similar hydropathy profiles. The two transporters are therefore likely to have very similar protein structures. Functionally, NaDC-1 and NaSi-1 are similar in coupling 3 sodium ions to the transport of a divalent anion substrate. However, the substrate specificities of these transporters are distinct from one another. NaDC-1 carries Krebs

cycle intermediates, such as succinate and citrate, whereas NaSi-1 carries inorganic sulfate and thiosulfate. The chimera SiDC-4 contains the first 4 transmembrane domains (amino acids 1–139) of NaSi-1 and the last 7 transmembrane domains (amino acids 141–593) of NaDC-1, but it retains the substrate selectivity of the NaDC-1 parent. Therefore, the residues involved in discriminating between succinate and sulfate are located distal to amino acid 141.

There is very little information about the structures of the substrate and ion binding domains in ion-coupled transporters. In sugar transporters, the substrate binding site appears to be located in the carboxy-terminal half of the protein. For example, the carboxy-terminal half of lac permease has been expressed in functional form, and it catalyzes downhill lactose transport [12]. The residues that discriminate between sugar substrates are found in transmembrane segment 10 in the yeast glucose (Hxt2) and galactose (Gal2) transporters [13]. Furthermore, the carboxy-terminal half of the low and high affinity Na^+/glucose cotransporters, SGLT1 and SGLT2, contains residues that determine substrate affinity [14]. However, the location of the substrate recognition site in transporters that do not carry sugars is not necessarily at the carboxy-terminus. For example, the H^+-peptide cotransporters appear to have their substrate binding sites located near the amino terminus [15]. The Na^+-coupled dopamine and norepinephrine transporters have domains that determine substrate affinity located in both the amino- and carboxy-terminus, whereas the residues that discriminate between substrates are found near transmembrane domain 2 [7].

The sequences of NaDC-1 and NaSi-1 are not related to the sequences of other transporters. The predicted secondary structure of NaDC-1 (and probably also NaSi-1) is also slightly different than that of other transporters, having 11 transmembrane domains and an extracellular, *N*-glycosylated, carboxy-terminus [4]. However, the location of the substrate recognition domain in NaDC-1 and NaSi-1 appears to be in the carboxy-terminal portion of the protein, past amino acid 141, similar to the sugar transporters. The SiDC-4 chimera has a substrate selectivity that resembles the substrate selectivity of the parental NaDC-1. Just like NaDC-1, SiDC-4 transports succinate, citrate and glutarate but does not interact with sulfate. Succinate transport by SiDC-4 was inhibited by a range of di- and tricarboxylic acids, similar to the substrate specificity of NaDC-1.

The SiDC-4 chimera exhibited an increase in substrate affinity compared with NaDC-1. SiDC-4 had an increased sensitivity to inhibition of succinate transport by test substrates and a lower apparent K_m for succinate. This result suggests that substrate affinity could be affected by residues in the amino-terminus of the protein, similar to the neurotransmitter transporters [7]. However, another possible explanation is that the alteration in cation binding seen in SiDC-4 affects its substrate affinity. The turnover number for succinate appears to be reduced in the SiDC-4 chimera because the V_{max} was much lower than the V_{max} of NaDC-1 although the abundance of the two proteins in the plasma membrane was similar.

Interestingly, the residues involved in binding substrates and those involved in binding the inhibitors, flufenamate and furosemide, are found in different portions of the proteins. The parental rabbit NaDC-1 is sensitive to inhibition by flufenamate and furosemide with IC_{50}'s of 250 μM and 2 mM, respectively [10]. The chimera SiDC-4 was not inhibited by furosemide and was relatively insensitive to inhibition by flufenamate, which resembles the properties of the human isoform, hNaDC-1, more than the parental NaDC-1 [10]. It is possible that amino acids in the amino-terminus that are common between hNaDC-1 and NaSi-1 could determine the sensitivity to these inhibitors. Alternately, the interaction between residues in the amino- and carboxy-terminus of the protein could determine inhibitor sensitivity.

The two parental transporters, NaDC-1 and NaSi-1, couple three sodium ions to the transport of each divalent anion substrate. However, they differ in their sensitivity to inhibition by lithium, which is thought to bind to one of the sodium binding sites in NaDC-1 [16]. This functional difference suggests that the structure of at least one of the sodium binding sites is different between NaDC-1 and NaSi-1. Surprisingly, the SiDC-4 chimera had cation binding properties that were unlike those of either parental transporter. SiDC-4 exhibited a relatively low cation selectivity, with transport seen in the presence of both choline and lithium. Furthermore, rather than being inhibited by lithium, the transport of succinate by SiDC-4 was stimulated by 5 mM lithium. This result suggests that

the lithium can substitute for sodium with a high affinity in SiDC-4. Therefore, the cation binding sites in NaDC-1 and SiDC-4 may be composed of interacting residues from both the amino- and carboxy-terminal portions of the protein.

Further testing of the domains responsible for substrate discrimination in these transporters was not possible in our study. The formation of chimeras, although a useful approach in structure-function studies, may have limitations when the parental proteins are not very similar in sequence. NaDC-1 and NaSi-1 are only 43% identical in sequence. The homologous recombination approach, which relies on sequence similarities, only yielded one viable chimera. The PCR-based approach, although more directed, did not produce any functional chimeras with the substrates tested, succinate, citrate, glutarate and sulfate. It is likely, based on the Western blots of DS2 and DS3, that these chimeras were misfolded or improperly targeted to the plasma membrane. Others have reported similar problems in forming chimeras between proteins with such different sequences. For example, attempts to produce chimeras between the norepinephrine and serotonin transporters (47% identical) yielded no functional chimeras containing switch points past the amino-terminal tail [6].

In conclusion, the SiDC-4 chimera retains the substrate specificity of the parental Na^+/dicarboxylate cotransporter, NaDC-1, suggesting that the residues determining substrate selectivity are found in the 7 transmembrane domains near the carboxy-terminus of the protein. However, the SiDC-4 chimera has an increased substrate affinity, suggesting that residues in the first four transmembrane domains may also influence substrate binding. The binding site for the inhibitors furosemide and flufenamate appears to reside in the amino terminal portion of the protein. Finally, although the parental transporters have similar cation binding properties, the SiDC-4 chimera had altered cation selectivity, and exhibited a stimulation of transport in the presence of lithium.

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References

[1] H. Murer, M. Manganel, F. Roch-Ramel, in: E.E. Windhager (Ed.), Handbook of Physiology-Renal Physiology, Am. Physiol. Soc., New York, 1992, pp. 2165–2188.
[2] A.M. Pajor, J. Biol. Chem. 270 (1995) 5779–5785.
[3] D. Markovich, J. Forgo, G. Stange, J. Biber, H. Murer, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 8073–8077.
[4] A.M. Pajor, N. Sun, Am. J. Physiol. 271 (1996) C1808–C1816.
[5] A.E. Busch, S. Waldegger, T. Herzer, J. Biber, D. Markovich, H. Murer, F. Lang, J. Biol. Chem. 269 (1994) 12407–12409.
[6] K.R. Moore, R.D. Blakely, Biotechniques 17 (1994) 130–136.
[7] K.J. Buck, S.G. Amara, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 12584–12588.
[8] A.N. Vallejo, R.J. Pogulis, L.R. Pease, in: C.W. Dieffenbach, G.S. Dveksler (Eds.), PCR Primer: a laboratory manual, Cold Spring Harbor Laboratory Press, 1995, pp. 603–612.
[9] K. Geering, I. Theulaz, F. Verrey, M.T. Hauptle, B.C. Rossier, Am. J. Physiol. 257 (1989) C851–C858.
[10] A.M. Pajor, N. Sun, Am. J. Physiol. 271 (1996) F1093–F1099.
[11] S.H. Wright, B. Hirayama, J.D. Kaunitz, I. Kippen, E.M. Wright, J. Biol. Chem. 258 (1983) 5456–5462.
[12] J. Wu, J. Sun, H.R. Kaback, Biochemistry 35 (1996) 5213–5219.
[13] M. Kasahara, E. Shimoda, M. Maeda, FEBS Lett. 389 (1996) 174–178.
[14] M. Panayotova-Heiermann, D.D.F. Loo, C.T. Kong, J.E. Lever, E.M. Wright, J. Biol. Chem. 271 (1996) 10029–10034.
[15] F. Doring, D. Dorn, U. Bachfisher, S. Amasheh, M. Herget, H. Daniel, J. Physiol. 497 (3) (1996) 773–779.
[16] E.M. Wright, S.H. Wright, B.A. Hirayama, I. Kippen, Proc. Natl. Acad. Sci. U.S.A. 79 (1982) 7514–7517.