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Topology of the Na^+/dicarboxylate cotransporter: the N-terminus and hydrophilic loop 4 are located intracellularly

Fred F. Zhang, Ana M. Pajor *

Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, TX 77555-0641, USA

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Abstract

The current secondary structure model of the Na^+/dicarboxylate cotransporter, NaDC-1, contains 11 transmembrane domains. The model is based on hydropathy analysis and the extracellular location of the carboxy terminus, which contains an *N*-glycosylation site. In this study, the model was further tested using indirect immunofluorescence of COS-7 cells. The Flag epitope tag (DYKDDDDK) was fused to the amino terminus of NaDC-1 (Flag-NaDC-1), and a monoclonal antibody against the Flag epitope was used to determine the location of the N-terminus. Hydrophilic loop 4 of NaDC-1 was identified using polyclonal antibodies raised against a fusion protein containing amino acids 164–233 of NaDC-1. The expression of NaDC-1 and Flag-NaDC-1 in COS-7 cells was confirmed by functional assays of succinate transport and by Western blots of cell surface biotinylated proteins. Immunofluorescent labeling of cells expressing both NaDC-1 and Flag-NaDC-1 required permeabilization of the plasma membranes with digitonin whereas no immunofluorescence was visible in intact cells. The results of this study show that both the N-terminus and hydrophilic loop 4 of NaDC-1 are located intracellularly, which supports the current model of NaDC-1 structure.

Keywords: Sodium; Dicarboxylate; Flag epitope tag; Succinate transporter; Immunofluorescent staining; COS-7 cell

1. Introduction

Metabolic intermediates of the citric acid cycle, including succinate and citrate, are reabsorbed in the renal proximal tubule by a sodium-coupled transporter called NaDC-1 (Na^+/dicarboxylate cotransporter 1) [1,2]. NaDC-1 has a broad substrate selectivity for a range of di- and tricarboxylates that are carried as divalent anions. The primary function of NaDC-1 in the kidney is to filter citric acid cycle intermediates, which are then metabolized within the proximal tubule cells or are used for exchange with organic anions during organic anion secretion [1]. An important role of NaDC-1 is the regulation of the concentration of urinary citrate, which acts as a calcium chelator. Hypocitraturia has been implicated as a risk factor in the development of kidney stones [3].

NaDC-1 belongs to a distinct superfamily of sodium-coupled anion transporters, called SLC13 in the human gene nomenclature [1]. The family contains several orthologues of NaDC-1 from rabbit, human, rat and mouse [4–7]. NaDC-2, from *Xenopus* intestine, is approx. 62% identical to rabbit NaDC-1 (rbNaDC-1) and it transports both sodium and lith-

* Corresponding author. Fax: +1-409-772-3381;
E-mail: ampajor@utmb.edu

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ium [8]. The high affinity Na^+/dicarboxylate cotransporters, NaDC-3, from rat, human, and winter flounder are approx. 43% identical to rbNaDC-1 [9–11]. The SLC13 family also contains the Na^+/sulfate cotransporters, NaSi-1 and SUT-1, which are approx. 40% identical in sequence to rbNaDC-1 [12–14].

The secondary structure model of NaDC-1 contains 11 transmembrane domains and an extracellular carboxy terminus [15]. All of the members of the SLC13 family contain a conserved *N*-glycosylation consensus sequence at the carboxy terminus. This site is glycosylated in NaDC-1, which places the C-terminus on the outside of the cell [15]. However, several alternate secondary structure models, containing eight, 12 or 13 transmembrane domains, have been proposed for the transporters related to NaDC-1 [9,13,16]. In this study, the secondary structure model of NaDC-1 is tested further using immunofluorescence of COS-7 cells expressing NaDC-1 and a construct of NaDC-1 containing an epitope tag at the N-terminus, called Flag-NaDC-1. The results show that both the N-terminus and hydrophilic loop 4 are located intracellularly, which supports the current model of NaDC-1 topology.

2. Methods

2.1. Construction of Flag-NaDC-1

The N-terminus of the rabbit NaDC-1 was fused with the peptide DYKDDDDK, containing the epitope of the Flag monoclonal antibody, to form the Flag-NaDC-1 fusion protein. The epitope tag allowed immunological localization of the N-terminus of NaDC-1. The fusion protein was constructed using polymerase chain reaction (PCR), with a sense primer similar to that used for the Na^+/I^- symporter [17]. The sequence of the sense oligonucleotide used in the PCR was 5′-G GAA TTC ATG GAC TAC AAG GAC GAC GAT GAC AAA GCC ACC TGC TGG CAG GGC CTG-3′, which contains an *Eco*RI restriction site, a start codon, the nucleotides coding for the eight amino acid Flag peptide, followed by 21 nucleotides of the 5′ end of NaDC-1 coding for amino acids 2–8. The antisense primer was complimentary to nucleotides 841–862 of NaDC-1. The template for the PCR amplification was the plasmid pSV-201 containing the coding region of the rabbit NaDC-1 in the pSV-SPORT1 expression vector [18]. The PCR reaction was run using the Failsafe PCR kit (Epicenter Technologies) with the following cycles: a denaturation step of 1 min at 95°C, followed by 25 two-step reaction cycles (95°C×1 min, 72°C×2 min), and a final 15 min elongation step at 72°C. The resulting PCR fragment was cloned into the pCR2.1 vector (InVitrogen) and a 575 nucleotide *Eco*RI-*Sac*I fragment of the insert was subsequently subcloned into the *Eco*RI and *Sac*I sites of plasmid pSV-201. The final construct was called pSV-201-Flag. The identity of the amplified cDNA was verified by sequencing.

2.2. Transient transfections of COS-7 cells

COS-7 cells (CRL 1651) were used for expression of NaDC-1 and Flag-NaDC-1 in this study. Cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) containing 4.5 g/l glucose, 25 mM HEPES, 10% heat-inactivated fetal calf serum and 1% antibiotic-antimycotic (Gibco/BRL), in 10% CO_2 at 37°C as described in our previous study [18]. One day prior to transfection, cells were seeded at a density of 5×10^5 cells per well of a six-well plastic dish (immunofluorescence, Western blots) or 2×10^5 cells per well of a 12-well dish (transport assays). For immunofluorescence experiments, the cells were plated onto 22 mm^2 glass coverslips placed into each well. The cells were transfected using the GenePORTER protocol (GTS) following the manufacturer's directions. Briefly, the transfection mixture contained 4 μg of plasmid DNA and 15 μl of GenePORTER transfection reagent per ml D-MEM medium (without serum or antibiotics). The six-well plates were transfected with 1 ml of the transfection mixture per well and the 12-well plates were transfected with 0.5 ml of the transfection mixture per well. Experimental groups were transfected with pSV-201 or pSV-201-Flag whereas control groups were transfected with pSV-SPORT1 vector or with the transfection reagent only. Duplicate or triplicate wells were transfected for each group. All of the experiments were carried out at 48 h after transfection, since our previous study showed peak expression of NaDC-1 at that time [18].

2.3. Succinate transport assay

The transport of [^{3}H]succinate was measured in COS-7 cells transfected with pSV-201, pSV-201-Flag or the control plasmid, pSV-SPORT1, using a modification of our previous protocol [18]. The transport buffers and washing solutions were added to each well of a plate simultaneously using specially constructed manifolds made from culture plate tops into which were glued 1 ml or 5 ml plastic test tubes, as described [19,20]. Each well containing attached cells was washed with 2 ml choline buffer (140 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES-Tris, pH 7.4) to remove medium and serum, then the transport of 100 μM [^{3}H]succinate was measured in sodium buffer (same as choline buffer but with 140 mM sodium in place of choline) for 15 min. Succinate transport is linear through 15 min in COS-7 cells expressing pSV-201 [18]. The dishes were placed on a rocker during the incubation period after which the transport solution was poured off. The uptakes were stopped with three 2 ml washes of ice-cold choline buffer and the remaining buffer was aspirated off. The cells were then lysed in 250 μl of 0.1% sodium dodecyl sulfate (SDS). Half of each lysed cell sample (125 μl) was used for scintillation counting and the rest was used for measurement of protein concentration with the Detergent Compatible (DC) Protein Assay kit (Bio-Rad).

2.4. Cell surface and total cell biotinylation of COS-7 cells

The cell surface and total cell expression of NaDC-1 and Flag-NaDC-1 was tested by biotinylation with a membrane impermeant reagent, Sulfo-NHS-LC-Biotin (Pierce), followed by streptavidin precipitation and immunoblotting, a method similar to that described in our previous study with *Xenopus* oocytes [15]. The transfected COS-7 cells grown in six-well plates were detached from the culture dish with 1 ml 0.4% trypsin for 5 min at 37°C, followed by the addition of 1 ml culture medium (D-MEM containing serum and antibiotics). Three wells were combined for each group. The suspended cells were rinsed three times with 1 ml ice-cold PBS buffer, pH 8, and surface biotinylated by incubating cells with 0.5 mg/ml Sulfo-NHS-LC-Biotin in 0.5 ml PBS buffer, pH 8, for 30 min at room temperature. The biotinylation reagent was removed with two washes with ice-cold PBS, pH 8. The cells were then dissolved in 50 μl lysis buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.6, 1% (v/v) Triton X-100) for 30 min on ice. For measurement of total cell biotinylation the cells were lysed before incubation with biotinylation reagent. After biotinylation and solubilization, the cells were centrifuged for 15 min at $14\,000 \times g$ and the supernatants were transferred to new tubes. The biotinylated proteins were incubated with 50 μl Immunopure Immobilized Streptavidin (Pierce) on a rocker for 1 h at 4°C. The biotin-streptavidin-agarose bead complexes were then washed four times with lysis buffer and the final pellets were resuspended in SDS-PAGE sample buffer [21].

2.5. Western blots

The NaDC-1 and Flag-NaDC-1 transporters were detected in the pool of biotinylated proteins using Western blotting, as described previously [21]. The polyclonal anti-NaDC-1 antibodies used in this study were raised in rabbits and directed against a fusion protein containing amino acids 164–233, from hydrophilic loop 4 of NaDC-1 [15,22]. The primary antibody was used at 1/5000 dilution and the secondary antibody, horseradish peroxidase linked anti-rabbit Ig (Amersham), was used at 1/2000 dilution. The mouse monoclonal M5 anti-Flag antibody (Sigma) was tested at 1/500 dilution, but did not produce any signal in Western blots (results not shown). Antibody binding was visualized by enhanced chemiluminescence using the Supersignal CL-HRP substrate system (Pierce).

2.6. Permeabilization of cells grown on glass coverslips

For immunofluorescence studies, some of the COS-7 cells grown on glass coverslips were permeabilized using digitonin [23,24]. The cell monolayers were washed three times with ice-cold KHM buffer (110 mM K-acetate, 2.5 mM Mg-acetate, 20 mM HEPES, pH 7.2), followed by 4 min incubation with ice-cold 20 μg/ml digitonin (Sigma) dissolved in KHM buffer. The digitonin was removed by wash-

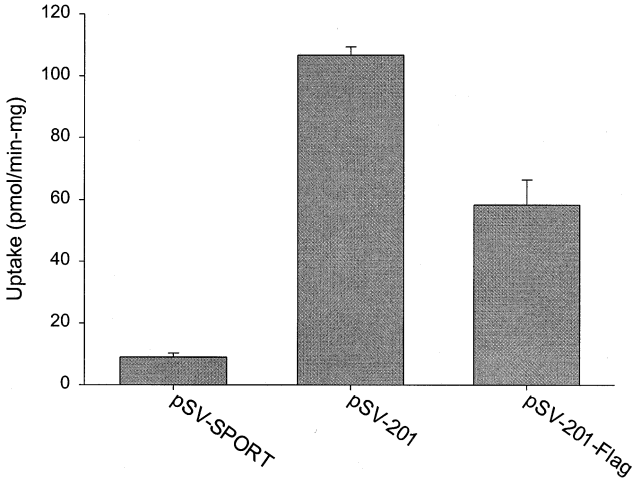

Fig. 1. Transport of succinate in COS-7 cells transfected with plasmid pSV-SPORT1 (control), pSV-201 (coding for NaDC-1) or pSV-201-Flag (coding for Flag-NaDC-1). The uptake of 100 μM succinate was measured for 15 min in sodium-containing buffer. The data shown represent means ± ranges of duplicate wells.

ing the cells twice with 5 ml ice-cold PBS buffer (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4). The optimal concentration of digitonin was determined by incubating the COS-7 cells with digitonin concentrations ranging from 5 μg/ml to 100 μg/ml and examining the percentage of cells staining with 4% trypan blue. The cells were considered to be completely permeabilized when more than 90% were stained with trypan blue.

2.7. Immunofluorescent staining

The permeabilized and non-permeabilized COS-7 cells on glass coverslips were used for immunofluorescent staining with the anti-NaDC-1 and M5 anti-Flag antibodies. Prior to staining, cells were incubated for 1 h with blocking buffer consisting of D-MEM cell culture medium with 1% goat serum. The cell monolayers were then incubated for 2 h with primary antibody (1/500 dilution of anti-NaDC-1 or M5 anti-Flag antibody in blocking buffer) and 1 h with secondary antibody (1/1000 dilution of FITC-conjugated anti-rabbit IgG antibody or anti-mouse IgG antibody (Molecular Probes)). The cell monolayers were washed three times with 5 ml PBS buffer after each antibody incubation. Control experiments examining trypan blue exclusion showed that the cells were not permeabilized by the washes and antibody incubations (results not shown). Finally, the cells were fixed with 1% formaldehyde in PBS buffer for 1 h and mounted with FluorSave reagent (Calbiochem).

Fluorescent staining of the cell monolayers was visualized with a Nikon epifluorescence microscope (Model Eclipse E800, Japan). Fluorescence signals were obtained using band pass filters at 480 nm and 535 nm for the excitation and emission paths, respectively. Images were taken through a digital video camera (Dage CCD 300T, Dage MTI) and recorded and analyzed with MetaMorph imaging software (MetaMorph v. 3.5, Universal Imaging, PA). Both phase-contrast and fluorescent images were taken for every given field of cells, which contained at least 5–10 cells.

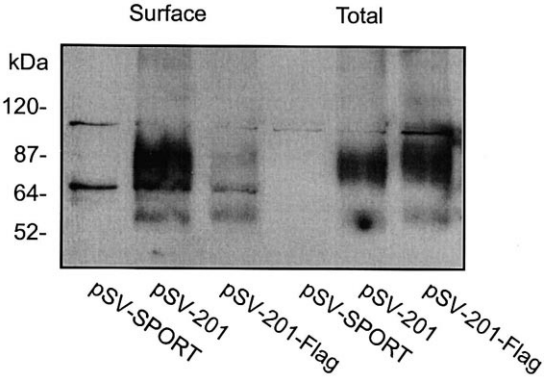

Fig. 2. Immunoblot analysis of NaDC-1 and Flag-NaDC-1 expressed in COS-7 cells. COS-7 cells transfected with pSV-SPORT1, pSV-201 or pSV-201-Flag were biotinylated with a membrane impermeant reagent, Sulfo-NHS-LC-Biotin, before cell lysis (cell surface biotinylation) or after cell lysis (total cell biotinylation). The biotinylated proteins were precipitated with streptavidin-agarose beads and separated using SDS-PAGE. The separated proteins were transferred to nitrocellulose, probed with the anti-NaDC-1 antibody and visualized with enhanced chemiluminescence. The position of size standards (Benchmark Prestained size standards, Gibco/BRL) is shown at left.

3. Results

3.1. Expression of NaDC-1 and Flag-NaDC-1 in COS-7 cells

The cell surface expression of NaDC-1 and Flag-NaDC-1 was monitored by measurement of succinate transport activity. As shown in Fig. 1, COS-7 cells transiently transfected with plasmids pSV-201 (coding for NaDC-1) and pSV-201-Flag (coding for Flag-NaDC-1) exhibited transport of succinate that was higher than the transport in control cells transfected with pSV-SPORT1 vector only. The uptake rate of cells expressing NaDC-1 is similar to that seen in our previous study, approx. 100 pmol/mg protein per min, and the background transport in control cells is somewhat lower [18]. The rate of succinate transport in cells expressing Flag-NaDC-1 was approx. 50% of that in cells expressing the wild-type NaDC-1 (Fig. 1).

Western blots of NaDC-1 expressed in COS-7 cells show two predominant protein bands at approx. 58 and 75 kDa, which probably represent differently glycosylated forms of the transporter (Fig. 2), similar to what is seen in *Xenopus* oocytes [21]. The control COS-7 cells transfected with the control plasmid pSV-SPORT have two sharp protein bands that react with the antibodies, suggesting some cross-reactivity of the antibodies with proteins in the COS-7 cells. Similar to the results of transport assays, the cell surface abundance of Flag-NaDC-1 protein was lower than that of wild-type NaDC-1 (Fig. 2). Although the transport activity of Flag-NaDC-1 (Fig. 1) appears to be higher than the relative protein abundance, it should be noted that the uptakes are normalized for protein content, which decreased in the Flag-NaDC-1 groups, whereas the biotinylations compare wells of cells. Possibly the expression of Flag-NaDC-1 protein is more toxic to the cells. When the wells of cells are compared, the transport

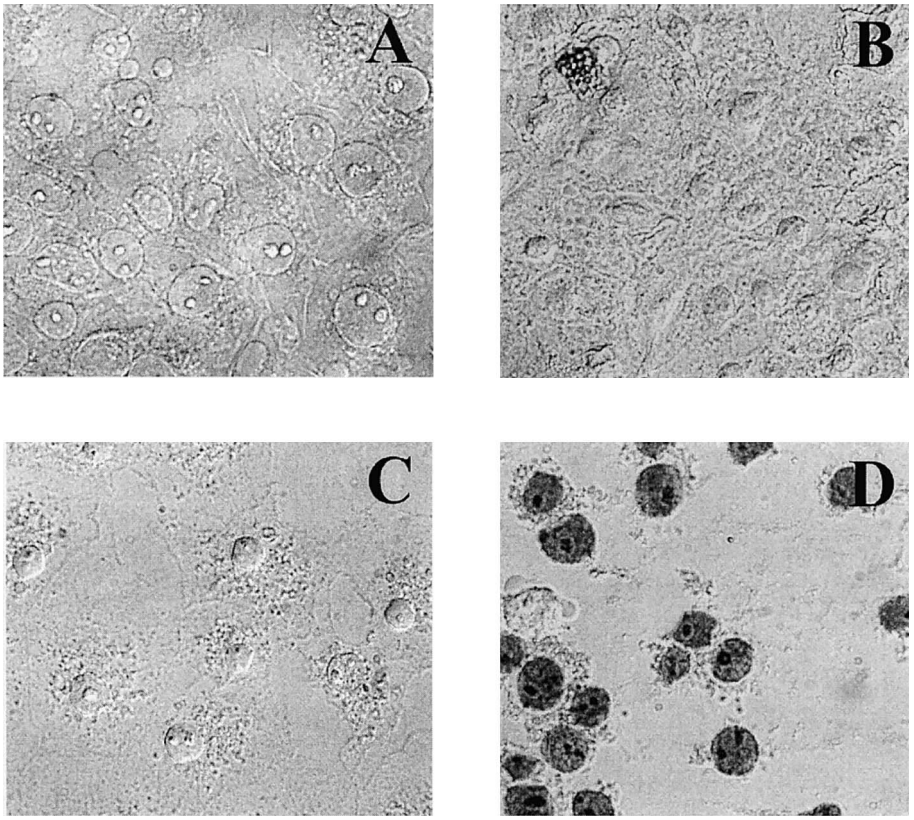

Fig. 3. Phase-contrast images of COS-7 cells grown on glass coverslips. (A) Non-permeabilized COS-7 cells; (B) non-permeabilized COS-7 cells treated with trypan blue dye; only one cell has taken up the dye; (C) COS-7 cells permeabilized with 20 μg/ml digitonin; (D) permeabilized COS-7 cells treated with trypan blue; most of the cell nuclei have taken up the dye.

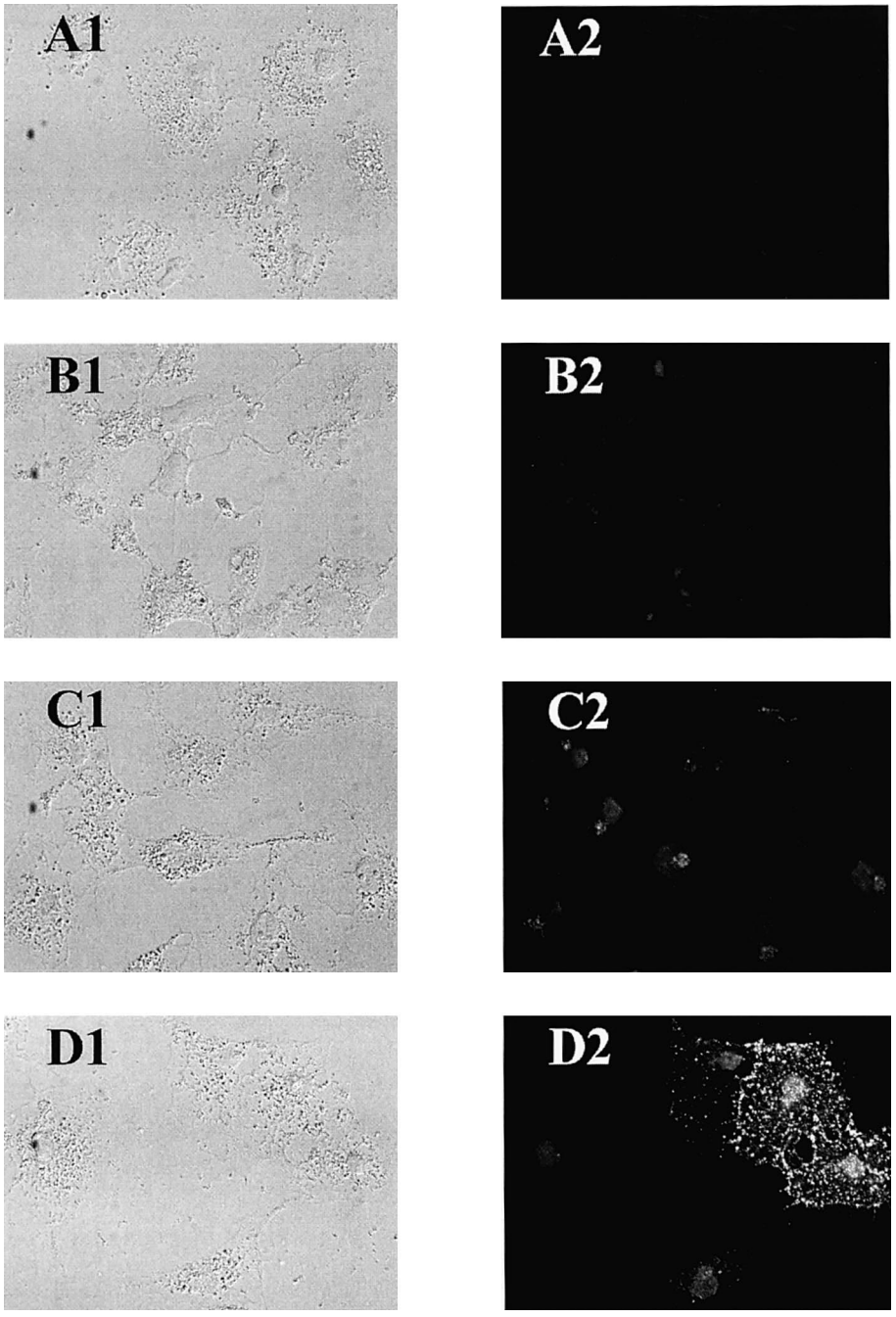

Fig. 4. Immunofluorescence and phase-contrast images of COS-7 cells treated with anti-NaDC-1 antibodies followed by FITC-conjugated secondary antibodies. The left column (A1–D1) shows phase-contrast images and the right column (A2–D2) shows the corresponding fluorescent images of the same microscopic field. Samples: (A) non-permeabilized COS-7 cells transfected with pSV-SPORT1 control vector; (B) non-permeabilized COS-7 cells transfected with pSV-201; (C) digitonin-permeabilized COS-7 cells transfected with pSV-SPORT1 control vector; (D) permeabilized COS-7 cells transfected with pSV-201.

activity of Flag-NaDC-1 is about 35% of the NaDC-1 group. Fig. 2 also shows that the total cell abundance of Flag-NaDC-1 protein is similar to the NaDC-1 protein. Therefore, the results indicate that Flag-NaDC-1 is expressed functionally on the plasma membrane of COS-7 cells but the presence of the Flag epitope at the N-terminus affects the trafficking or cell surface stability of the protein.

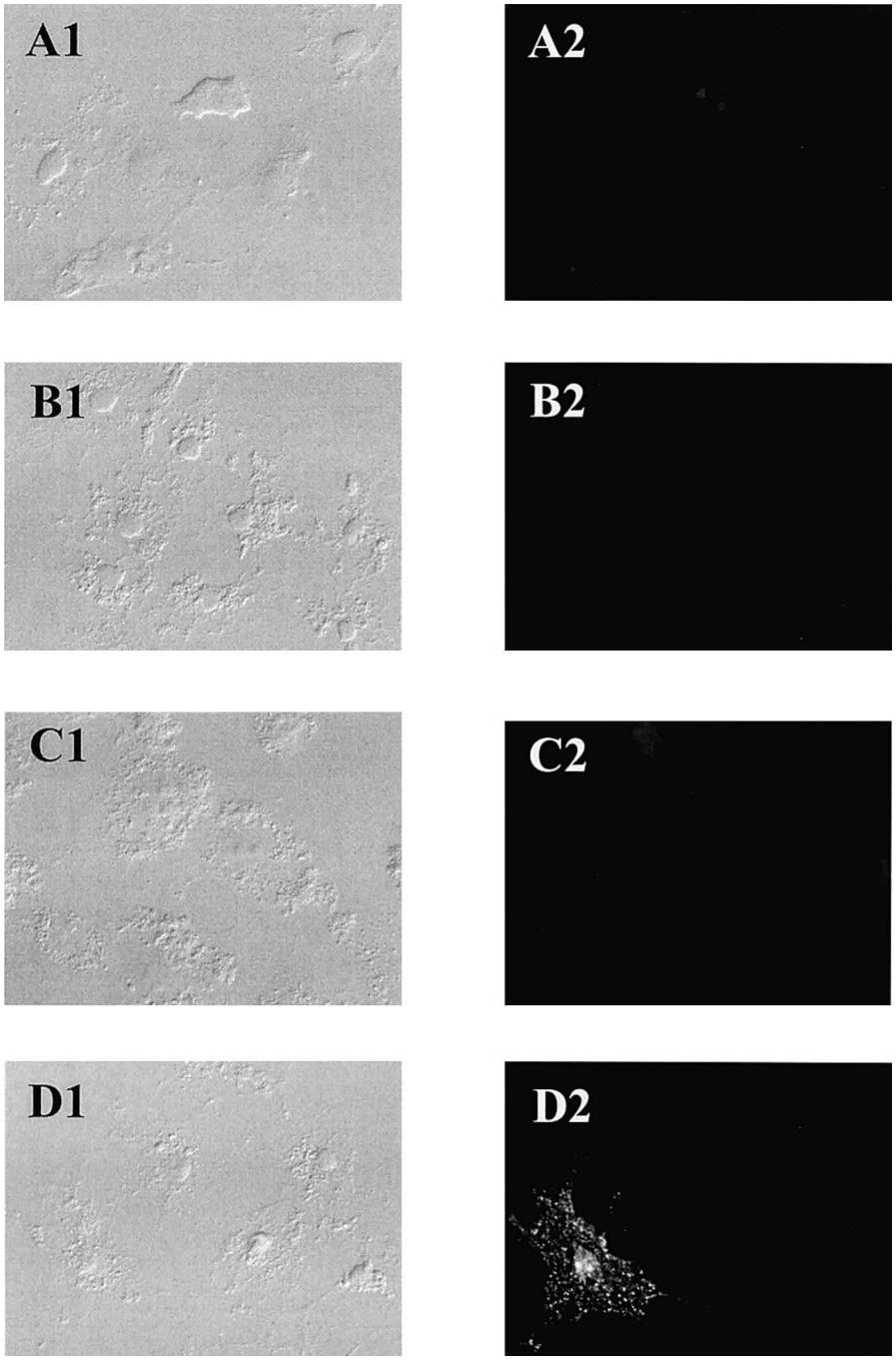

Fig. 5. Immunofluorescence and phase-contrast images of COS-7 cells treated with M5 anti-Flag antibodies followed by FITC-conjugated secondary antibodies. The left column (A1–D1) shows phase-contrast images and the right column (A2–D2) shows the corresponding fluorescent images of the same microscopic field. Samples: (A) non-permeabilized COS-7 cells transfected with pSV-SPORT1 control vector; (B) non-permeabilized COS-7 cells transfected with pSV-201-Flag; (C) digitonin-permeabilized COS-7 cells transfected with pSV-SPORT control vector; (D) permeabilized COS-7 cells transfected with pSV-201-Flag.

3.2. Digitonin permeabilization of COS-7 cells grown on coverslips

The optimal conditions for cell permeabilization were determined by incubating COS-7 cells for 4 min with different concentrations of digitonin ranging from 5 to 100 μg/ml. The lowest concentration of digitonin that produced more than 90% staining with trypan blue was 20 μg/ml, and this concentration was used in subsequent experiments. As shown in Fig. 3, almost all of the cells treated with 20 μg/ml digitonin were stained with trypan blue.

3.3. Localization of amino acids 164–233

Rabbit polyclonal antibodies raised against amino acids 164–233 of NaDC-1 were used to determine the cellular location of hydrophilic loop 4. The phase-contrast and fluorescent images from a single experiment are shown in Fig. 4. The highest intensity immunofluorescent signal was seen in cells expressing NaDC-1, but only when the cell membranes were permeabilized with digitonin (Fig. 4B2 vs. D2). Approx. 40–50% of the permeabilized cells transfected with pSV-201 had fluorescence signals that were higher than control cells, indicating a cell transfection efficiency of at least 40%. Intact cells had little or no immunofluorescence. There was some fluorescence associated with nuclei in some experiments with permeabilized cells transfected with the control plasmid, pSV-SPORT1 (Fig. 4C2), but the fluorescence intensity was much lower than that seen in cells transfected with pSV-201. The immunofluorescence was not seen in cells incubated with secondary antibody only (results not shown). Therefore, the epitope for the anti-NaDC-1 antibodies is located intracellularly.

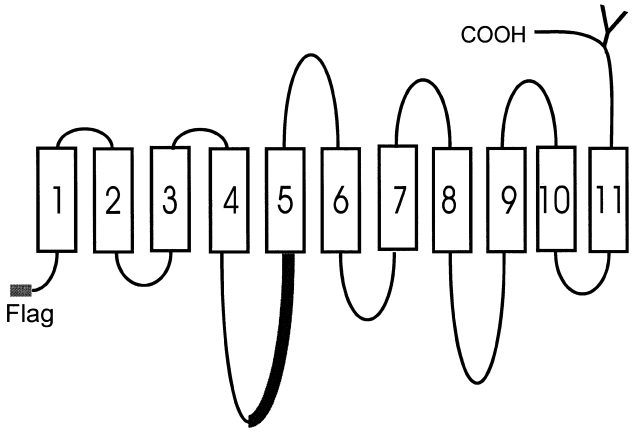

Fig. 6. Secondary structure model of NaDC-1. The open rectangles represent 11 transmembrane α-helices joined by hydrophilic loops. The Y indicates the *N*-glycosylation site at the C-terminus. The heavy line in hydrophilic loop 4, between transmembrane domains 4 and 5, shows the location of amino acids 163–233 used to produce the anti-NaDC-1 antibodies. The gray rectangle represents the Flag peptide fused to the N-terminus of NaDC-1.

3.4. Localization of the N-terminus

The M5 anti-Flag monoclonal antibody was used to detect the Flag peptide fused to the N-terminus of NaDC-1 (Flag-NaDC-1). Similar to the results with the anti-NaDC-1 antibodies, immunostaining was only positive in COS-7 cells that were both permeabilized and transfected with pSV-201-Flag (Fig. 5D2). Approx. 20% of the permeabilized cells transfected with pSV-201-Flag showed immunofluorescence above controls, which indicates a lower amount of protein expression or lower transfection efficiency with the pSV-201-Flag plasmid. There was no immunostaining in intact cells and only occasional immunostaining of nuclei in permeabilized cells transfected with the control plasmid, pSV-SPORT1. The positive staining was also not due to the FITC-conjugated anti-mouse secondary antibody (not shown). The results of these experiments show that the N-terminus of NaDC-1 is located intracellularly.

4. Discussion

The secondary structure model of NaDC-1 was originally based on an analysis of the amino acid sequence using the Rao-Argos buried helix parameter scale [15]. This model of NaDC-1 contains 11 membrane-spanning α-helices and the N- and C-termini on opposite sides of the membrane. The only experimental verification of this model prior to this study has come from the identification of the *N*-glycosylation site at Asn-578, which places the C-terminus on the extracellular side of the plasma membrane [15]. The results of this study are in agreement with the current model of NaDC-1, with the N-terminus

and hydrophilic loop 4 both located inside the cell (Fig. 6).

The anti-NaDC-1 antibodies used in this study were raised against a fusion protein of amino acids 164–233 of NaDC-1 and glutathione *S*-transferase [15]. The sequence of amino acids 164–233 is very hydrophilic, containing 16 charged amino acids. According to our model of NaDC-1, this peptide is located in hydrophilic loop 4 between transmembrane domains 4 and 5. Since it was possible to identify an immunofluorescent signal in cells expressing NaDC-1, it is likely that the epitope for the anti-NaDC-1 antibodies is exposed rather than being buried in the membrane. Furthermore, the fluorescence signal was dependent on the permeabilization of the cell membrane to allow the antibodies access to the inside of the cell. Therefore, the results support our current secondary structure model.

To detect the location of the N-terminus of NaDC-1, a fusion protein was made containing the epitope of the Flag antibody at the N-terminus of NaDC-1. This fusion protein was detected using the M5 anti-Flag antibody. The addition of the Flag epitope did not appear to affect the function of NaDC-1, although it did result in lower cell surface protein expression. Cells expressing the Flag-NaDC-1 fusion protein showed immunofluorescent staining only after the cell membranes had been permeabilized with digitonin, consistent with the prediction that the N-terminus of NaDC-1 is intracellular. In addition, since both the N-terminus and hydrophilic loop 4 are located on the same side of the membrane, the results indicate that there are an even number of transmembrane domains between them.

It is likely that all of the members of the family related to NaDC-1 have very similar protein structures. The mammalian members of the SLC13 superfamily have approximately the same number of amino acids (594–602 amino acids) and, when analyzed using the same method, the hydropathy plots are virtually superimposable. All of the members of this family contain a conserved *N*-glycosylation site at the C-terminus [1]. However, there is considerable variation in the secondary structural models that have been proposed for the members of this family, probably because the C-terminal half of the protein is very hydrophobic. For example, the flounder high affinity Na^+/dicarboxylate cotransporter, fNaDC-3, is predicted to have eight transmembrane domains [16], the rat NaDC-1 is predicted to have 12 [9], and the mouse Na^+/sulfate cotransporter, mNaSi-1, is thought to have 13 [13]. However, none of these models has been tested experimentally. Since the results of this study show that NaDC-1 has an odd number of transmembrane domains, it is likely that the eight and 12 transmembrane domain models for the other family members are incorrect. Further studies will be required to refine the secondary structure model.

In conclusion, the N-terminus and hydrophilic loop 4 of the Na^+/dicarboxylate cotransporter, NaDC-1, are both located on the cytoplasmic side of the cell membrane. The results of this study support our current secondary structure model of NaDC-1 with 11 transmembrane domains, an intracellular N-terminus and an extracellular C-terminus.

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