Second-Site Revertants of a Low-Sodium-Affinity Mutant of the Na^+/H^+ Exchanger Reveal the Participation of TM4 into a Highly Constrained Sodium-Binding Site†

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ABSTRACT: On the basis of intracellular acidifications in the presence of 30 μM cariporide, we selected a fibroblast cell line termed CR5, expressing a mutated Na^+/H^+ exchanger NHE-1 with a low affinity for cariporide (87 ± 11.6 μM) and extracellular sodium (248 ± 63.7 mM). This mutated exchanger displays a Phe162Ser substitution in its fourth transmembrane segment. Using intracellular acidifications in the presence of 3 mM external sodium on the CR5 fibroblasts, we isolated two revertants which exhibited a complete recovery for sodium affinity but were still resistant to cariporide. Sequencing the cDNAs encoding these revertants revealed the presence of two mutations situated at a distant location from Phe162 in the same fourth transmembrane segment (Ile169Ser and Ile170Thr). Interestingly, introducing these two mutations in the wild-type cDNA did not result in a significant increase in affinity for sodium. Furthermore, all the mutants characterized in this study display an unchanged affinity for lithium, another transported cation. These data suggest that the mutation resulting in the low sodium affinity and the two mutations responsible for the reversion of this phenotype affect the binding of sodium itself instead of the conformational changes triggering substrate translocation. Taken together, these results allow us to propose that optimal sodium binding by the Na^+/H^+ exchangers requires the geometrical integrity of a highly constrained sodium coordination site.

Mammalian Na^+/H^+ exchangers are secondary transporters which use the inwardly directed gradient of sodium to catalyze the electroneutral exchange of one extracellular sodium against one intracellular proton. To date, the cDNAs encoding six isoforms of these transporters (NHEs[1]) have been cloned (*1*−*7*). Basically, while the NHE-1 isoform is ubiquitously expressed and exerts the housekeeping role of protecting the cells against intracellular acidifications and maintaining cell volume, the other isoforms are expressed in a tissue-specific manner and have more specialized physiological roles (for reviews, see refs *8*−*10*). NHE-2 and -3 are expressed on the apical side of epithelial cells in intestine and kidney, where they play a crucial role in the maintenance of sodium and acid−base balance in the organism (*2, 5, 11*). NHE-4, which is expressed predominantly in the stomach, the distal tubule of the kidney, and the brain, is cooperatively activated by high sodium concentrations and has a protective effect against osmotic shocks (*2, 12*). NHE-5 is specifically found in the brain, where it might constitute an additional effector for pH regulation (*13*−*15*), and NHE-6 is mitochondrial (*7*). Thus, these transporters, which derive from a common ancestral gene, are crucial for homeostasis at both the cellular and organism levels. All these proteins are composed of a large conserved transmembrane (TM) domain consisting of 12 transmembrane segments and a cytosolic tail, which significantly differs between the various isoforms (*1*). While the transmembrane region is necessary and sufficient for the ion-transport process, the cytosolic loop is involved in the regulation of the isoform's activities by mitogens and hormones (*16*). The contribution of the Na^+/H^+ exchangers in numerous pathophysiological situations has been hypothesized and documented, for example, in hypertension (*17, 18*), post-ischemic myocardial arrhythmia (*19*−*21*), and regulation of the aqueous humor secretion associated with glaucoma (*22*). Due to their properties, the Na^+/H^+ exchangers are promising targets for structure function studies.

In this article, we focused on the NHE-1 isoform involved in pH regulation. Basically, this antiporter allows cell survival upon acute acidification, and under these conditions, the blockade of NHE-1 using potent inhibitors leads to massive cell death. As reported previously, this observation led to the design of selective tests allowing the selection of fibroblasts which express mutated exchangers with modified pharmacological characteristics (*23*). In this study, we have mutagenized fibroblasts stably expressing the human NHE-1 isoform (pECE expression vector, SV40 promoter) with ethyl methanesulfonate. Then, using repeated intracellular acidifications in the presence of 30 μM of the antiarrhythmic drug cariporide, we selected clones expressing a NHE-1 isoform possessing a decreased affinity for this compound. Among them, the CR5 clone was strongly impaired in its ability to interact with extracellular sodium. These cells, which were able to compensate for intracellular acidifications in the presence of physiological concentrations of extracellular

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[1] Abbreviations: NHE, Na^+/H^+ exchanger; EMS, ethyl methanesulfonate; CR, cariporide-resistant mutant; CRN, cariporide-resistant/Na^+ revertant; TM, transmembrane domain.

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sodium (120−140 mM), could not survive the same acidification when the extracellular sodium concentration was lowered below 10 mM. We took advantage of this observation to design a selection procedure for revertants of this low sodium affinity based on repeated acidifications of fibroblasts expressing the CR5 mutant in the presence of 3 mM NaCl. Following this procedure, we obtained two clones, which had regained a close to wild-type affinity for sodium but still had a very poor ability to interact with cariporide. These results demonstrate for the first time that it is possible to select complete restoration-of-function mutants for a human transporter using a genetic approach. Surprisingly, the mutations resulting in the reversion of the low-affinity-sodium phenotype are clustered in another part of the same segment, instead of being located in other domains of the protein. Taken together with the recent topological characterization of NHE-1 (*24*), the detailed pharmacological characterization of the mutants obtained in this study provides clues to the constraints shaping the substrate-binding site of a cation transporter.

EXPERIMENTAL PROCEDURES

Cell Culture. Mutant Chinese hamster fibroblasts lacking Na^+/H^+ exchange activity (PS120 cell line) were grown in Dulbecco's modified Eagle's medium supplemented with 50 μg/mL streptomycin, 50 units/mL penicillin and 7.5% fetal calf serum at 37 °C, in a humidified atmosphere of 5% CO_2 and 95% air.

Selection of the Cariporide-Resistant Clones. PS120 cells (*25*) stably transfected with the pECE vector expressing the wild-type NHE-1 isoform (about 5×10^6 cells/plate, 5 plates) were randomly mutagenized with 2 μM EMS (Sigma) overnight. This protocol results in about 30−50% cell lethality. The mutagenized cells were then expanded in 100-mm Petri dishes and the selection started on 10 dishes containing approximately 10^7 cells each. The H^+ killing selection consisted in 1-h-long 50 mM NH_4^+ loading, followed by a rapid rinse and 1-h recovery in a medium containing 120 mM NaCl and 30 μM cariporide. This selection procedure was applied twice a week for 3 weeks and the surviving clones were collected individually in 12 well plates for counterselection. The cells were Li^+-loaded for 1 h with a 120 mM LiCl-containing medium and incubated 1 h in a pH 5.5 sodium-free medium. This selection procedure was designed to eliminate the clones in which resistance to the inhibitor was due to overexpression of the wild-type NHE-1 molecule. The clones surviving this treatment were then expanded and aliquots were frozen in liquid nitrogen. To test for the clones exhibiting the most pronounced resistance phenotype, samples of each clone seeded on 6-well plates were subjected to the same acidification procedure in the presence of concentrations of cariporide ranging from 30 μM to 1 mM. The CR5 clone, which was able to resist the highest concentration, was chosen for further analysis and cDNA cloning.

Cloning and Sequencing of the Mutated cDNAs. Total RNA was extracted from the cariporide-resistant fibroblasts using a guanidinium isothiocyanate/pH 4.5 phenol solution (Eurogentec) and ethanol precipitated according to the manufacturer's instructions. Aliquots of 4 μg of total RNA were reverse transcribed (Appligene M-MuLV reverse transcriptase) using random hexaoligonucleotides and oligo-dT as primers. One-tenth aliquots of the reverse transcriptase incubation mix were PCR-amplified using Taq polymerase (Appligene), using the 5′-NHE-1 5′-primer and the 3′-*Age*I 3′-primer. Single-stranded PCR product was then directly ligated into the pGEM vector, using the T/A cloning kit (Promega, pGEM-T Easy Vector Systems). After transfection in XL1-blue strain, white colonies were tested for the presence of the insert. The positive inserts were then sequenced using the T7 DNA polymerase kit (Amersham) according to the method of Sanger et al. (*26*).

Pharmacological Characterization of the Mutated NHE-1 Isoforms. The clones expressing mutated NHE-1 isoforms were seeded on 24-well plates and after 24−48 h were incubated in the NH_4^+ loading medium for 1 h. Following two rapid rinses with choline chloride buffer, the initial rates of $^{22}Na^+$ uptake were determined by incubating the cells for 3 min or less in the $^{22}Na^+$ uptake media containing 1 μCi/mL carrier-free $^{22}Na^+$ (Amersham), 120 mM choline chloride, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 15 mM Hepes pH 7, 1 mM ouabain, and various concentrations of the NHE-1 inhibitors, sodium, lithium or external H^+. Under these conditions, the basal level of $^{22}Na^+$ uptake represents less than 1% of the exchanger's maximal activity. When the effect of various concentrations of NaCl, LiCl or guanidinium was tested in competition experiments, the concentration of choline chloride was adjusted to maintain osmolarity, and the uptake time used was 30 s in order to ensure initial rate conditions. The influx was then stopped by rinsing the cells four times with ice-cold phosphate-buffered saline (150 mM NaCl, 5 mM sodium phosphate pH 7.4), the cells were then solubilized in 0.1 N NaOH, and the radioactivity was determined by γ-counting. When necessary, the protein contents in the wells were determined on 50-μL samples of solubilized cells using the BCA assay (Pierce).

Selection and Characterization of the Revertants for the Low-Sodium-Affinity Phenotype. The fibroblasts expressing the CR5 clone were mutagenized using EMS as described above. Then, 10 100-mm plates containing about 10^7 cells each were submitted to the same acid load selection as described above, with no cariporide in the recovery medium which contained 3 mM NaCl instead of 120 mM. Following 3 weeks of selection, two clones termed CRN5 and -6 were isolated. These clones were expanded and further analyzed using initial rates of $^{22}Na^+$, and the cDNAs encoding the revertant NHE-1 isoforms were cloned and sequenced as described above.

Site-Directed Mutagenesis and Mutant Analysis. The pECE NHE-1 expression vector containing the wild-type cDNA was used as template for double-strand site-directed mutagenesis using the Stratagene Quickchange kit (Stratagene), according to the manufacturer's instructions. 15 ng of template DNA was used with respectively 200 ng each of 5′- and 3′-homologous primers containing the desired mutation. The codon changes used to create the various NHE-1 mutants are presented in Table 1. The presence of the mutations was verified by sequencing, and for each mutant, two independent plasmids were transfected into antiporter-deficient PS120 cells using DAC-30 (Eurogentech). Stable transfectants were selected for their ability to survive repeated acid loads for 3 weeks, and their kinetic and pharmacological

Table 1: Compilation of All the Amino Acid Substitutions Engineered in the NHE-1 Mutants Presented in This Work[a]

mutation	codon change	oligonucleotides used for site-directed mutagenesis	
Phe162Ser	TTC → TCC	sense	GAC GTC TTC **TCC** CTC TTC CTG
		antisense	CTG CAG AAG **AGG** GAG AAG GAC
Ile169Ser	ATC → AGC	sense	CTG CCG CCC **ATC** ATC CTG GAT
		antisense	GAC GGC GGG **TAG** TAG GAC CTA
Ile170Thr	ATC → ACC	sense	CCG CCC ATC **ATC** CTG GAT GCG
		antisense	GGC GGG TAG **TAG** GAC CTA CGC

[a] The nucleotide changes resulting in the codon changes are underlined.

properties were determined by measuring initial rates of $^{22}Na^+$ uptake in the presence of various extracellular Na^+, Li^+, H^+ and inhibitor concentrations.

Data Treatment and Statistical Analysis. All the data represent the average of at least three experiments, each performed in triplicate. All the numerical results were compiled using Excel software (Microsoft) and analyzed using SigmaPlot. Statistical analysis was performed using SigmaStat version 1.0.

RESULTS

Isolation of Cariporide-Resistant Clones. To obtain cariporide-resistant clones, possessing only one allele encoding the Na^+/H^+ exchanger, 10^8 mutagenized antiporter-deficient fibroblasts (PS120 cell line) stably transfected with NHE-1 (pECE vector) were submitted to repeated intracellular acidifications (see Experimental Procedures) in the presence of 30 μM of this compound. At this inhibitor concentration, the Na^+/H^+ exchanger is totally blocked, and this protocol results in the death of all the cells which express a wild-type Na^+/H^+ exchanger. After 3 weeks of selection with two acid loads a week, several cariporide-resistant clones were obtained. The clones overexpressing a Na^+/H^+ exchanger were counterselected using the Li^+ loading procedure described under Experimental Procedures. Then, following a rapid characterization of the cariporide resistance levels of the surviving clones, we decided to focus our attention on the clone presenting the highest resistance to the inhibitor, which was termed CR5. This mutant fibroblast cell line was recloned and expanded for pharmacological analysis and cDNA cloning.

Pharmacological Characterization of the CR5 Mutant. The CR5 mutant exhibited a dramatic decrease in affinity for cariporide, with a K_i of 87 ± 11.6 μM compared to 0.056 ± 0.004 μM for the wild-type NHE-1 (Figure 1). Different concentrations of amiloride were also tested, resulting in a K_i of about 450 μM for CR5 as compared to 3 μM for the wild-type NHE-1.

When tested for initial rates of $^{22}Na^+$ uptake, the CR5 mutant systematically exhibited much lower rates than the wild-type NHE-1, which was used as a control in the same series of experiments. To understand the reasons for this apparent change in activity, we tested the expression levels of this mutant using semiquantitative Western blot analysis and found that the amounts of expressed antiporter were similar for the wild-type NHE-1 and the CR5 mutant (data not shown). In contrast, testing the initial rates of the CR5 exchanger with different sodium concentrations showed that the mutated antiporter was far from saturated at physiological Na^+ concentrations (Figure 2). Nonlinear analysis of the initial rates of sodium transport showed that the K_m for

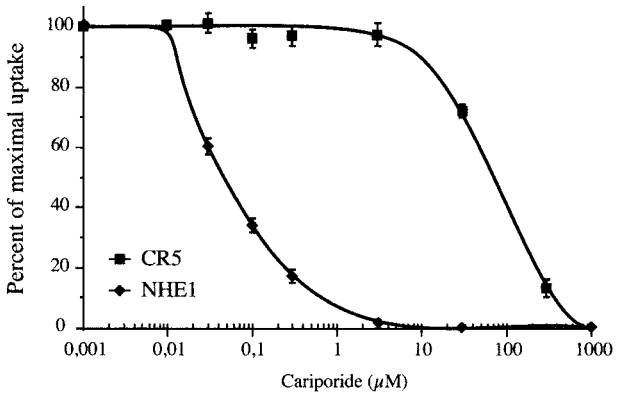

FIGURE 1: Dose−response curves for inhibition of NHE-1 and CR5 mutant by cariporide. Cells were grown to confluence in 24-well plates and then loaded with H^+ using the NH_4Cl prepulse technique. As described in Experimental Procedures, initial rates of $^{22}Na^+$ uptake were determined in the presence of various concentrations of cariporide for the human NHE-1 isoform (◆) and the CR5 mutant (■) containing the Phe162Ser substitution.

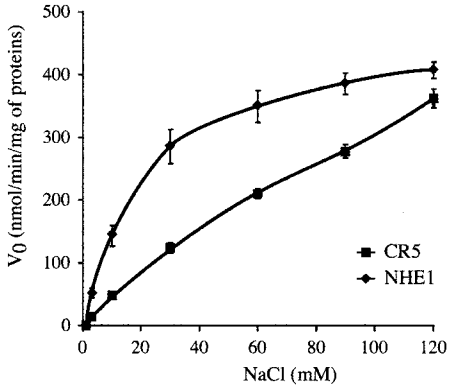

FIGURE 2: Transport activity of the human NHE-1 and CR5 mutant as a function of extracellular Na^+ concentration. Cells were H^+-loaded by the NH_4Cl prepulse technique, and the experimental data were obtained by measuring initial rates of $^{22}Na^+$ uptake as a function of increasing extracellular Na^+ concentration. NHE activity was determined for the wild-type NHE-1 isoform (◆) and the CR5 mutant (■). Note that the CR5 mutant is not saturated at physiological concentrations of extracellular Na^+.

sodium of the CR5 mutant was about 10 times higher than that of the wild-type NHE-1 (Table 2).

As H^+ and Li^+ ions can also interact with the extracellular sodium-binding site and are transported by the Na^+/H^+ exchangers, we investigated whether the CR5 mutation also had modified apparent affinities for these cations. As shown in Table 2, the mutation affecting sodium affinity does not affect the K_m values for extracellular protons and lithium (0.15 ± 0.023 versus 0.17 ± 0.019 μM for protons, and 13 ± 0.41 versus 11± 0.32 mM for lithium).

Protonated guanidinium, the moiety that is absolutely required for the biological activity of the Na^+/H^+ exchanger

Table 2: Comparison of the Different Affinities for the Inhibitor Compound and Monovalent Cations between All the NHE-1 Mutants Constructed and Characterized in This Work[a]

NHE-1 mutants	half-inhibition constant	apparent affinity constants			
	cariporide (μM)	Na^+ (mM)	guanidinium (mM)	Li^+ (mM)	H^+ (μM)
NHE-1	0.056 ± 0.004	23 ± 6.07	25 ± 0.88	11 ± 0.32	0.17 ± 0.019
CR5 Phe162Ser	87 ± 11.6	248 ± 63.7	22 ± 3.2	13 ± 0.41	0.15 ± 0.023
CRN5 Phe162Ser/Ile169Ser	143.7 ± 17.3	21.14 ± 2	15 ± 2.7	9 ± 0.63	0.18 ± 0.016
CRN6 Phe162Ser/Ile170Thr	260 ± 36.53	30.5 ± 1.77	20 ± 1.23	5 ± 0.48	0.16 ± 0.004
CRN6 Ile169Ser	0.093 ± 0.0055	18.22 ± 5.3	28 ± 4.2	9 ± 0.51	0.19 ± 0.020
CRN6 Ile170Thr	0.27 ± 0.029	12.74 ± 1.98	25 ± 2.9	6 ± 0.79	0.18 ± 0.005
CRN6 Ile169Ser/Ile170Thr	0.59 ± 0.089	75 ± 4.96	33 ± 3.1	8 ± 0.408	0.19 ± 0.010
CRN6 Phe162Ser/Ile169Ser/Ile170Thr	194.25 ± 13.9	15.23 ± 3.16	19.5 ± 0.8	2 ± 0.204	0.15 ± 0.011

[a] Data were fitted using SigmaPlot ($p < 0.001$). Half-inhibition constants and apparent affinity constants deduced from the fits represent the mean ± SD of at least three independent experiments, each point being determined in triplicate.

inhibitors, possesses a structure reminiscent to that of hydrated sodium. As this compound has been shown to exhibit a competitive inhibition pattern for sodium, with a K_i close to the Na^+ apparent K_m for NHE-1 (*27*), we tested the dose response of the CR5 mutant for inhibition by guanidinium at sodium tracer concentrations. In these experimental conditions, both wild-type and mutant exchangers are far from being saturated by their substrate. Thus, the half-inhibitory concentrations of guanidinium are equal to the real K_i values. Surprisingly, the K_i of guanidinium for the CR5 mutant was found to be identical to the K_i measured for wild-type NHE-1.

Identification of the Mutation Responsible for the Cariporide Resistance Phenotype. The cDNA encoding the transmembrane region of the CR5 mutant was cloned by RT-PCR and subcloned in the pGEM-T vector (Promega Kit, pGEM-T Easy Vector Systems). Two independently obtained clones were completely sequenced, and their nucleotide sequence was compared to the wild-type human NHE-1 cDNA sequence. A thymidine to cytosine substitution was identified at position 486 in the mutated cDNA, resulting in substitution of Phe162 to a serine residue in the putative TM4 segment of the antiporter.

This substitution was reintroduced into the wild-type NHE-1 cDNA using double-stranded DNA site-directed mutagenesis. After sequence verification, two independently obtained clones were transfected into PS120 cells, and after acid load selection, stable transfectants were pharmacologically characterized. Exactly the same pharmacological profiles as the CR5 mutant were obtained for cariporide, sodium, guanidinium, and lithium, demonstrating that the Phe162Ser substitution is necessary and sufficient to produce the CR5 phenotype.

Revertant Selection. The CR5 mutant exhibits a decreased affinity for extracellular sodium. We used this property as a basis for selecting second-site revertants of this mutation. For this purpose, 10^8 cells expressing the CR5 cDNA were mutagenized using the same EMS treatment as described above and submitted to repeated acidifications at an extracellular sodium concentration of 3 mM, a treatment which is lethal for cells expressing the CR5 mutant but allows the cells expressing an antiporter possessing a normal affinity for sodium to survive. Following 3 weeks of selection, eight clones were obtained, recloned twice, expanded, and tested for their apparent affinity for Na^+; pharmacological profiles were obtained.

Pharmacological Characterization of the Revertants. Among the different clones, which were tested for their initial rates in different sodium concentrations, five exhibited biphasic patterns for Na^+ affinity and were left for further analysis. By contrast the CRN (for cariporide-resistant/Na^+ revertant) -3, -5, and -6 clones exhibited a purely Michaelian behavior with respect to extracellular sodium (Figure 3a). The CRN3 clone is totally identical to the wild-type, while the CRN5 and -6 clones possessed apparent sodium affinities of 21.14 ± 2 and 30.5 ± 1.77 mM, respectively (Table 2). Furthermore, the CRN3 clone exhibited the same K_i for

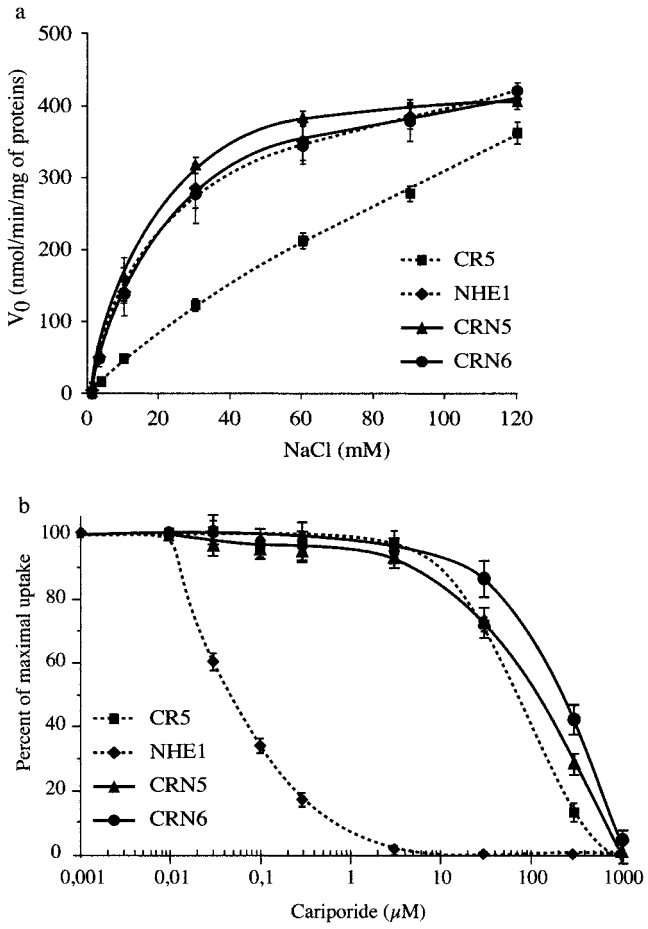

FIGURE 3: Pharmacological profiles and kinetic properties of CRN revertants compared to NHE-1 and CR5 isoforms. Cells were grown to confluence in 24-well plates and then loaded with H^+ using the NH_4Cl prepulse technique. As described in Experimental Procedures, initial rates of $^{22}Na^+$ uptake were determined in the presence of various concentrations of cariporide (a) or as a function of increasing extracellular Na^+ concentration (b). NHE activity was determined for the human NHE-1 isoform (◆), the CR5 mutant (■), and the two CR5 revertants, CRN5 (▲) and CRN6 (●).

cariporide as the wild-type, while CRN5 and -6 had retained the parental resistance phenotype (Figure 3b).

Identification of the Mutations Responsible for the Revertant Phenotype. The cDNAs encoding the transmembrane region of the CRN3, -5 and -6 exchangers were cloned using RT-PCR, subcloned into the pGEM vector, and completely sequenced. CRN3 appeared to have regained a phenylalanine at position 162 and is therefore a true revertant of the CR5 mutation. This observation explains why its phenotype was exactly the same as NHE-1. By contrast, CRN5 and -6 had conserved the same Phe162Ser mutation, but further sequencing revealed that secondary mutations occurred in TM4, at position 169 or 170, respectively. These two positions correspond to conserved isoleucines that have been both changed into a serine and a threonine, respectively.

Site-Directed Mutagenesis on CR5 and NHE-1. To verify that these two mutations indeed confer the reversion phenotype, the corresponding changes were introduced by site-directed mutagenesis in the CR5 mutant encoding cDNA. Similarly, the same mutations were introduced into the wild-type NHE-1 cDNA, to determine the ability of each of these restitution-of-function mutations to increase the apparent affinity of the exchanger for extracellular sodium. The double mutation Ile169Ser/Ile170Thr was also introduced into the NHE-1 and CR5 cDNAs.

Two independently mutated cDNA clones were transfected for each combination of mutations, and following the selection of the stably transfected cell population, the different mutants were tested using $^{22}Na^+$ uptakes (Table 2). The mutations mimicking the substitutions detected in the Phe162Ser/Ile169Ser and Phe162Ser/Ile170Thr mutants resulted in exactly the same pharmacological profiles as observed for the original revertants (data not shown). These results confirm that these mutations are indeed responsible for the gain of affinity for sodium of the CRN mutants when compared to the parental CR5 sodium-resistant isoform.

Surprisingly, when introduced in the wild-type NHE-1 cDNA, the Ile169Ser and Ile170Thr mutations do not result in any statistically significant increases in the exchanger affinity for extracellular sodium (K_m of 18.22 ± 5.3 mM for Ile169Ser and 12.74 ± 1.98 mM for Ile170Thr). Furthermore, when both mutations were introduced in the NHE-1 cDNA, a 3-fold loss in apparent affinity for the mutated antiporter was observed.

Effect of the Various Mutations on Lithium Transport and Guanidinium Inhibition. To further understand the effects of the different mutations discovered in this study on the Na^+/H^+ exchanger's transport characteristics, we determined the K_m for lithium and the K_i values for the guanidinium ion. As summarized in Table 2, none of the mutants were significantly affected in their ability to interact with guanidinium, and only the Phe162Ser/Ile169Ser/Ile170Thr triple mutant presented a 4−5-fold increase in its K_m for lithium, thus possessing a true gain-of-function for the interaction with this substrate.

DISCUSSION

In the absence of atomic resolution molecular models, the identification of functional domains in membrane transporters represents a crucial task for the understanding of the structure−function relationships of such proteins. Numerous studies on other membrane proteins have involved the extensive site-directed mutagenesis of various residues and domains within such proteins. However, the interpretation of the obtained results is often made difficult by the fact that many amino acid substitutions can unpredictably affect the protein structure, resulting in inactive or poorly active mutants. To circumvent this difficulty we have previously developed somatic cell genetics techniques that enabled us to obtain fully functional mutants possessing decreased affinity for the NHE-1 inhibitors. The aim of the present study was to expand this approach in order to identify residues involved in the binding of sodium by the exchanger. In a first attempt, we tried to select cells able to resist acute intracellular acidifications in the presence of a low extracellular NaCl concentration (3 mM), expecting to isolate mutants possessing an increased affinity for extracellular sodium. This protocol, which was assayed on more than 600 million cells, failed to provide us with any gain of function mutant, although the procedures used for the isolation of mutants of the inhibitor binding site had proven to be reliable and successful. However, inhibitor binding and ion transport are largely different, since ion transport consists of binding and translocation, a mechanism having multiple steps. Thus, direct selection of gain of function mutants may be impossible, if simultaneous mutations are needed to increase the global efficiency of the transport mechanism. An alternate hypothesis is that a strong interaction with sodium may impair the cation translocation, thus making the antiporter much less efficient to compensate for the intracellular acidification used in the H^+ killing selections. In parallel to these experiments, we used about 100 million of the same mutagenized pool of cells to select cariporide-resistant clones. Among these clones, the CR5 mutant appeared to be the most interesting, because it possessed both a 1500-fold decrease in affinity for cariporide and at least a 10-fold decrease in apparent affinity extracellular sodium. On the basis of the low sodium affinity of this new mutant, we developed a strategy enabling us to isolate restitution of function mutants for sodium affinity. Two clones, CRN5 and -6, were successfully isolated. They possessed an apparent affinity for sodium close to that of the wild-type NHE-1 (Table 2), with a Michaelian behavior for sodium binding (Figure 3a), and were still highly resistant to cariporide (Table 2, Figure 3b). The comparison of their sequences with the wild-type NHE-1 cDNA revealed the substitution Phe162Ser in the CR5 mutant and two additional substitutions: Ile169Ser and Ile170Thr in the CRN5 and -6 mutants, respectively. These results demonstrate for the first time that alterations of substrate interaction in a eukaryotic transporter can be totally reverted by introducing secondary mutations at a distant location in the same transmembrane segment (Figure 4).

Two hypotheses can account for this result: the apparent affinity constant characterizing the interaction of a transporter with its substrate is a combination of the equilibrium constants of ion binding and of the kinetic constants of ion translocation. It might therefore be possible that the observed mutations do not modify the sodium binding directly but affect the kinetics of the conformational changes responsible for translocation. This would result in a modified K_m value.

An alternate hypothesis is that the sodium-binding site is a highly geometrically constrained region of the antiporter, in which an optimal conformation for the coordination of

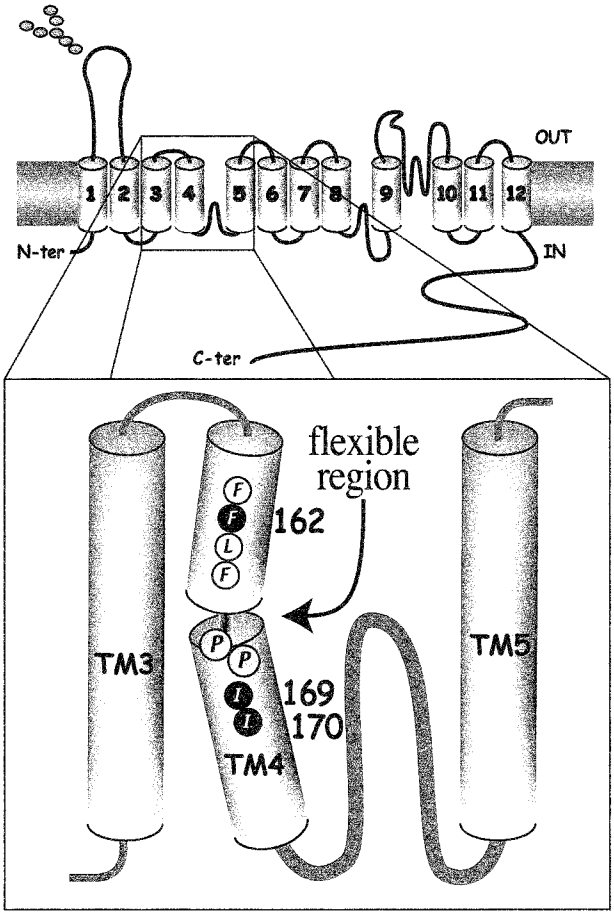

FIGURE 4: Schematic representation of NHE topology and TM4. (Top) Schematic representation of the NHE-1 topology based on the work of Wakabayashi and colleagues (*24*). (Bottom) Enlargement of the TM4 domain, showing (in black) the mutations discovered in this study and (in gray) the doublet of prolines producing a flexible region one helix turn above.

the transported cation must occur. A first mutation in this region could disrupt this geometry coordination and result in a decreased affinity, while the secondary mutations observed in the revertants could, by a distance effect, restore the proper geometry of the coordination site.

We decided to challenge these two hypotheses further, by characterizing the effect of these various mutations when introduced alone or in combination in the wild-type sequence. Examination of the results concerning the K_m values for sodium of our different mutants (Table 2) shows that introducing the Ile169Ser and Ile170Thr substitutions in the wild-type sequence does not result in a spectacular decrease in the sodium K_m, such as was observed in the CR5 mutant. Moreover, introducing these mutations together in the same molecule produces a double mutant possessing a significantly decreased affinity for sodium. By contrast, both these mutations have the ability to increase the affinity of the CR5 mutant for sodium. The inability of these mutations, which occur at some distance away from Phe162, to decrease the K_m of NHE-1 for sodium suggests that they restore the proper geometry of the sodium-binding site of the CR5 mutant but do not modify the transport parameters of the protein. Moreover, it can be observed that none of the mutations introduced strongly affects the K_m for lithium, which is also a substrate of the Na^+/H^+ exchangers. Lithium probably uses the same translocation pathway as sodium but is significantly smaller and might be accommodated differently in the external binding site. On the basis of these observations, we propose that the effects of the substitutions found in the CR5 and CRN5 and -6 mutants only affect the binding site for sodium, with no significant modification of the kinetic parameters for cation translocation.

As the guanidinium ion blocks the exchanger in a competitive manner, with a K_i close to the apparent K_m for sodium, we tested whether the CR5 mutant also displayed a decreased affinity for guanidinium. Surprisingly, guanidinium inhibition of $^{22}Na^+$ uptake initial rates is similar for the wild-type NHE-1, the CR5 mutant, and its revertants (CRN5 and -6). This could be simply explained by the fact that guanidinium may bind to a site which is physically different from the sodium site but close enough to block the sodium access in a seemingly competitive manner. Similarly, the affinities for amiloride and cariporide, which possess a guanidinium moiety, are modified in the CR5 mutant, while the affinity for guanidinium is left untouched. This can be easily explained by the fact that the hydrophobic groups substituted to the guanidinium ion are responsible for the high affinity of these inhibitors, when compared to the guanidinium group itself. It is reasonable to hypothesize that amino acid substitutions affecting the interaction of these groups with the exchanger can drastically modify the affinity constants of amiloride and cariporide, without modifying the binding of guanidinium.

The TM4 segment possesses an unusual doublet of prolines in the middle of its sequence (Figure 4). Therefore the region situated one helix turn N-terminal from this doublet is not stabilized by hydrogen bonding and must be either highly flexible or maintained by a precise set of interactions with other parts of the protein (*28*). This region contains Phe162, which was identified as a crucial residue for sodium binding in this study. Noticeably, this lack of hydrogen bonding due to the prolines also leaves free backbone carbonyls, which may be involved in the coordination of the cations. This situation is reminiscent of the pore geometry of the potassium channel of *Streptomyces lividans* (*29*). In this case, free carbonyls, which precisely coordinate the cations, are maintained in the proper geometry by the pore helices, and any mutation in this region results in dramatic changes in the channel properties. From our data, the most reasonable model is that the Phe161 to Phe164 region of the exchanger is highly constrained by other parts of the transmembrane domain, producing a proper geometry for sodium interaction, which might involve the free carbonyls left at positions 163 and/or 164. The positions in which substitutions produce a reversion of the low-sodium-affinity phenotype are situated just below the proline doublet. They probably restore the proper geometry and optimal sodium coordination of the sodium-binding pocket situated above. This could explain why introducing the Ile169Ser or Ile170Thr mutation does not increase the affinity of the wild-type exchanger for sodium, as its binding site is probably already constrained in the optimal conformation. This hypothesis is confirmed by the observation that introducing these two substitutions together in the NHE-1 sequence is deleterious for sodium interaction. In addition, the presence of a ring of phenylalanine residues may favor the interaction of inhibitors such as amiloride or cariporide.

Taken together, these results suggest the presence of a geometrically constrained domain of the Na^+/H^+ exchangers, which is crucial for cation binding. This domain is probably composed of the TM4 segment, with its particular sequence, and might also contain the loop situated between TM4 and TM5, which has been recently shown to enter deeply into the membrane part of the antiporter (*24*) (Figure 4). These conclusions are in good accordance with the work of Orlowski and Kandasamy on chimeric NHE-1/NHE-3 exchangers (*30*). We are at present using modified protocols of random mutagenesis, which will enable us to conserve the sequence encoding the TM4 segment of the CR5 mutant. This may allow us to identify other domains of the molecule which shape the substrate-binding site.

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