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# Mutational analysis of potential pore-lining amino acids in TM IV of the $Na^+/H^+$ exchanger

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**Abstract**

The $Na^+/H^+$ exchanger isoform 1 (NHE1) is an integral membrane protein that regulates intracellular pH by extruding an intracellular $H^+$ in exchange for one extracellular $Na^+$. In this study we examined the effect of site-specific mutagenesis on the pore-lining amino acid Phe161 and effects of mutagenesis on the charged amino acids Asp159 and Asp172. There was no absolute requirement for a carboxyl side chain at amino acid Asp159 or Asp172. Mutation of Asp159 to Asn or Gln maintained or increased the activity of the protein. Similarly, for Asp172, substitution with a Gln residue maintained activity of the protein, even though substitution with an Asn residue was inhibitory. The Asp172Glu mutant possessed normal activity after correction for its aberrant expression and surface targeting. Replacement of Phe161 with a Leu demonstrated that it was not irreplaceable in NHE1 function. However, the mutation Phe161lys inhibited NHE1 function, while the Phe161Ala mutation caused altered NHE1 targeting and expression levels. Our results show that these three amino acids, while being important in NHE1 function, are not irreplaceable. This study demonstrates that multiple substitutions at a single amino acid residue may be necessary to get a clearer picture membrane protein function.
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*Keywords:* Cation transport; Membrane; $Na^+/H^+$ exchanger; pH regulation

## 1. Introduction

The $Na^+/H^+$ exchanger isoform 1 (NHE1) is a plasma membrane glycoprotein that is ubiquitously expressed. It extrudes a single intracellular proton in exchange for one extracellular sodium ion. NHE1 thereby functions to remove excess intracellular acid while facilitating $Na^+$ entry into the cytosol [1]. The nine known isoforms of the $Na^+/H^+$ exchanger are designated NHE1–NHE9, named in order of their discovery [2]. NHE2–9 have more restricted tissue distributions than NHE1 and some have predominantly intracellular localization. Mammalian NHE1 plays a key role in regulation of cell pH, cell volume, cell proliferation and in metastasis of some kinds of tumor cells [1,3]. NHE1 in the myocardium is critical in mediating the damage that occurs during ischemia/reperfusion of the heart [4–6] and is also an important mediator of myocardial hypertrophy [7]. Various NHE1 inhibitors are being developed for the treatment of heart disease [8]. Of the nine isoforms, NHE1 is the most sensitive to NHE inhibitors and it is thought that the NHE1 inhibitor binding site may be overlapping with the sodium binding site [9].

Although the exact mechanisms of transport and inhibitor binding by NHE1 are not known, specific residues within several regions of the membrane domain of NHE1 have been implicated as being important for ion binding and transport (reviewed in [2]). Clearly one of the most important transmembrane segments is TM IV. Numerous residues of TM IV have been implicated in NHE1 function. A Phe165Tyr mutation in hamster TM IV (corresponding to human Phe161) causes both an increase in resistance to inhibitors and a decrease in $V_{max}$ for $Na^+$ [10]. A Leu167Phe mutation (corresponding to human Leu163) causes increased inhibitor resistance with no effect on $Na^+$ transport while a Gly174Ser mutation in TM IV causes a modest increase in resistance to amiloride with no effect on $Na^+$ transport [11]. Also, a Phe162Ser mutation in TM IV was found to cause a dramatic decrease in affinity for cariporide and a 10-fold decrease in $Na^+$ affinity [12]. We found that both Pro167 and Pro168 of TM IV are required for normal NHE1 activity, while Pro178, which is outside the TM region, is not [13]. In

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addition, we examined the sensitivity of active single-cysteine mutants of TM IV residues to sulfhydryl-reactive reagents and found that the mutant Phe161Cys was significantly inhibited by this treatment [14], demonstrating that Phe161 lines the ion transport pore of NHE1.

We recently published the first high-resolution structure of a transmembrane segment of the human $Na^+/H^+$ exchanger [14]. A TM IV peptide was expressed and purified, and its structure was determined in a membrane mimetic environment. TM IV is composed of three sections of four to nine residues that converge structurally and only one region is alpha helical: Asp159-Leu163 form a series of β-turns; Leu165-Pro168 has an extended structure; and Ile169-Pro176 forms an α-helix. Based on our knowledge that Phe161 lines the ion transport pore, and because the structure showed that Asp159 faces the same way as Phe161, we decided to further investigate the importance of these pore facing residues. We also introduced new mutations at Asp172 [14] based on the fact that inactivity of an Asp172Cys mutant indicated this amino acid was clearly important in activity, and on the hypothesis that negatively charged amino acids may be critical in coordination of transported positively charged cations [15]. Our study provides further insights into the mechanism of transport and further information about the structural and functional importance of this pore-lining transmembrane segment of NHE1.

## 2. Materials and methods

### 2.1. Materials

Anti-HA antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). BCECF-AM was from Molecular Probes, Inc. (Eugene, OR). PWO DNA polymerase was from Roche Molecular Biochemicals, Mannheim, Germany. LIPOFECTAMINE™ 2000 Reagent was from Invitrogen Life Technologies, Carlsbad, CA, USA. All other chemicals were of analytical grade and were purchased from Fisher Scientific (Ottawa, ON), Sigma or BDH (Toronto, ON). EMD87580 was a generous gift of Merck, Darmstadt, Germany.

### 2.2. Site-directed mutagenesis

The plasmid pYN4+ contains the cDNA of the entire coding region of human NHE1 with a C-terminal hemagglutinin (HA) tag that we have previously shown does not affect activity [14]. Site-directed mutagenesis was performed using amplification with PWO DNA polymerase followed by use of the Stratagene (La Jolla, CA, USA) QuikChange™ site-directed mutagenesis kit as recommended by the manufacturer. Mutations were designed to create a new restriction enzyme site for use in screening transformants (Table 1). Construction and initial characterization of the basic activity of the Phe161Lys mutant was described earlier and is included here for comparative purposes and because of the novel data found in resistance to NHE1 inhibition. DNA sequencing to confirm the accuracy of the mutations and fidelity of the product was performed by the DNA Services laboratory of the Department of Biochemistry.

### 2.3. Cell culture and stable transfection

AP-1 cells are a Chinese hamster ovary cell line that lacks an endogenous $Na^+/H^+$ exchanger. They were used to examine NHE1 activity and were routinely grown in a humidified atmosphere of 5% $CO_2$ and 95% air in α-MEM supplemented with 10% (v/v) bovine growth serum, 25 mM HEPES, penicillin (100 U/ml) and streptomycin (100 μg/ml), pH 7.4 at 37 °C. The transfection and selection of stable cell lines were carried out as described previously and transfection was done with LIPOFECTAMINE™ 2000 Reagent [14]. Briefly, $1.3\times10^6$ cells were seeded in 100-mm Petri dish, in 8 ml of growth media. Cells were grown until 90% confluent and transfected with 20 μg of wild-type or mutagenized plasmids. Post-transfection cells were trypsinized, diluted 5 or 10 times with α-MEM medium and plated in 100-mm dishes in α-MEM media containing 800 μg/ml geneticin (G418) that was used to maintain selection pressure without acute acid load selection. After the initial selection single clones of trasnsfected cells were obtained and stable cell lines were maintained in 400 μg/ml G418 and were regularly re-established from frozen stocks at passage numbers between 5 and 15. Results shown are from at least two independently obtained clones for each mutant cell line.

### 2.4. SDS-PAGE and immunoblotting

For Western blot analysis equal amounts of each sample were resolved on a 9% SDS-PAGE gel and the gel was transferred onto a nitrocellulose membrane. Immunoblot analysis with anti-HA antibody confirmed NHE1 expression in samples from total cell lysates of transfected AP-1 cells made as described earlier [13,16]. The Amersham enhanced chemiluminescence Western blotting and detection system was used to detect immunoreactive proteins. Densitometric analysis of X-ray films was carried out using NIH Image software (National Institutes of Health, Bethesda, MD, USA).

### 2.5. Cell surface expression

Cell surface expression was measured as described earlier [14]. Cells were labeled with Sulpho-NHS-SS-Biotin (Pierce Chemical Company, Rockford, IL, USA) and immobilized streptavidin resin was used to remove surface labeled $Na^+/H^+$ exchanger. Equal amounts of the total and unbound proteins were analyzed by SDS-PAGE and Western blotting against the HA tag. Relative amounts of NHE1 on the cell surface were calculated by comparing

Table 1
Oligonucleotide primers for site-directed mutagenesis

| Mutation | Primer | RE site |
|---|---|---|
| Asp159Asn | CCCCCCTTCCTGCA**a**TCC**a**ACGTCTTCTTCCTC | −*Pst*I |
| Asp159Gln | CACCCCCCTTCCTGCA**a**TCC**c**A**g**GTCTTCTTCCTCTTC | −*Pst*I |
| Asp159Glu | CCCCCCTTCCTGCA**a**TCCGA**g**GTCTTCTTCCTCTTC | −*Pst*I |
| F161Ala | CCTGCAGTCCGACGT**ggc**CTTCCTCTTCCTGCTG | −*Aat*II |
| F161Leu | CCTGCAGTCCGACGT**gc**TCTTCCTCTTCCTGC | −*Aat*II |
| F161Lys | CTGCAGTCCGACGT**gaaa**TTCCTCTTCCTGCTG | −*Aat*II |
| Asp172Asn | CCGCCCATCATCCTG**a**ATGC**c**GGCTACTTCCTGCC | +*Nae*I |
| Asp172Gln | CCGCCCATCATCCTG**c**A**g**GC**c**GGCTACTTCCTGCCAC | +*Nae*I |
| Asp172Glu | GCCCATCATCCTGGA**g**GC**c**GGCTACTTCCTGCC | +*Nae*I |

Mutated nucleotides are in lower case letters and bold. Mutated amino acid residues are indicated using three-letter notation and new (+) or removed (−) restriction endonuclease sites are underlined. In each case the forward direction of the primer pair is illustrated.

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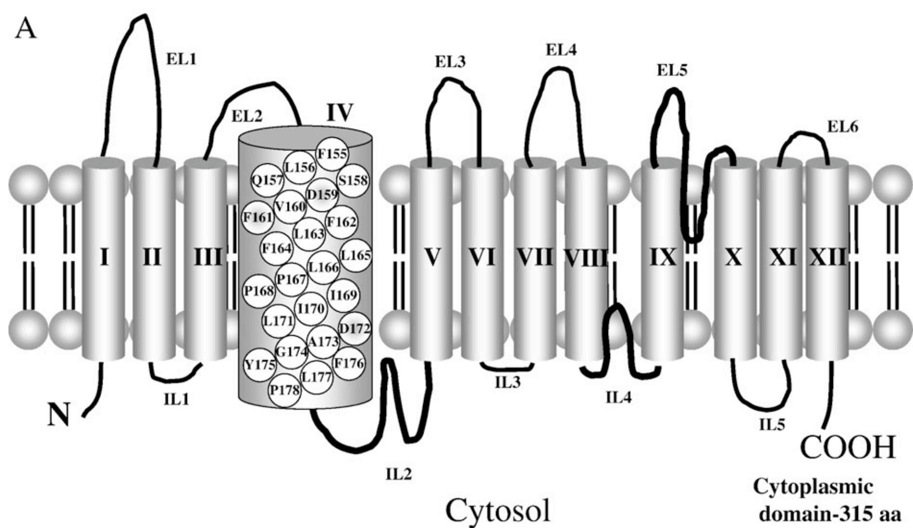
both the 110-kDa and the 95-kDa forms of NHE1 in the total and unbound fractions.

### 2.6. $Na^+/H^+$ exchange activity

NHE1 activity was measured using a PTI Deltascan spectrofluorometer as described earlier [17]. Stably transfected cells were seeded on glass coverslips ($2 \times 10^5$ cells per coverslip) and grown until they reached 80–90% confluency. The coverslip was then transferred to a cuvette holder with constant stirring at 37 °C. The cells were loaded with 0.15 μg/ml 2′,7-*bis*(2-carboxyethyl)-5 (6) carboxyfluorescein-AM (BCECF-AM) and incubated in “Normal buffer” containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.5 mM glucose, and 10 mM HEPES, pH 7.4 at 37 °C. Normal buffer is nominally bicarbonate free and under these conditions so the contribution of bicarbonate-



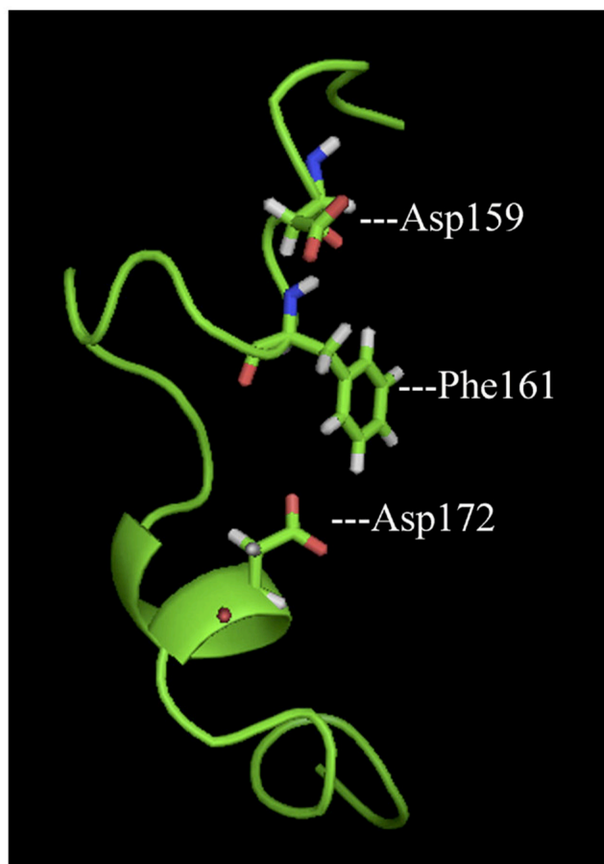




Fig. 1. Models of the membrane domain of the NHE1 isoform of the $Na^+/H^+$ exchanger. (A) Topology of the entire membrane domain [24]. TM IV is enlarged to illustrate its amino acid sequence. Asp159, Phe161, and Asp172, examined in the present study, are shaded. EL, extracellular loop, IL, intracellular loop. (B) Deduced structure of TM IV [14]. The side chains of Asp159, Phe161 and Asp172 are illustrated.

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based pH regulatory systems is minimal. Intracellular acidosis was induced by $NH_3/NH_4^+$ prepulse/withdrawal (3 min in "Normal buffer" containing 50 mM $NH_4Cl$, pH 7.4), followed by withdrawal for 1–1.5 min in "$Na^+$-free buffer" (135 mM *N*-methyl-D-glucamine, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, and 10 mM HEPES, pH 7.4). Intracellular pH recovery was obtained by transferring cells to "Normal buffer" allowing the cells to recover for at least 3 min. There were no differences in buffering capacities of stable cell lines (not shown). Following the $pH_i$ recovery, a three-point pH calibration curve was made using the $K^+$/nigericin method with $Na^+$-free calibration buffers (135 mM *N*-methyl glucamine, 135 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, 10 mM HEPES, at pH 6, 7, and 8) and 10 μM nigericin [18]. The NHE1 protein activity was determined by measuring the slope of the first 20 s of the recovery period, which was linear and activity was expressed as ΔpH/second. The determination of kinetic parameters of the $Na^+/H^+$ exchanger was essentially as described earlier [18,19]. $Na^+$ and $Li^+$ concentrations were varied while maintaining osmolarity with *N*-methyl-D-glucamine. To investigate the inhibitor sensitivity of some mutants, cells were treated with EMD87580 of varying concentrations. EMD87580 is a highly selective NHE1 inhibitor [20]. EMD87580 was dissolved in water and the inhibitory effect of EMD87580 was documented using a double-pulse acidification assay. In this assay, cells were treated with ammonium chloride two times as described above and allowed to recover in NaCl containing medium following each pulse. The first pulse and recovery was in the absence of EMD87580 while the second pulse recovery was in the presence of inhibitor. The rate of recovery from acid load was compared ±inhibitor. Results are shown as mean±SE and statistical significance was determined using a Mann–Whitney *U* test.

## 3. Results

Based on our knowledge of TM IV of the NHE1 isoform of the $Na^+/H^+$ exchanger, we further characterized amino acids Asp159, Phe161 and Asp172 of the transmembrane domain. Fig. 1A illustrates a general model of the topology of TM IV. Fig. 1B illustrates a more detailed model of the deduced structure of a TM IV peptide [14]. In this structure, amino acids Asp159 and Phe161 were within one well-defined region of the peptide and have side chains that project on a similar face. However, because the peptide TM IV is not constrained by protein–protein interactions as it would be in the full-length NHE1, there were points of rotation in the peptide at amino acids 164, 168 and 169. Thus, the relative position of amino acid 172 to either 159 or 161 is uncertain.

To further study these potentially pore-lining residues in TM IV, we made a series of mutations to amino acids Asp159, Phe161 and Asp172 (Table 1). Both Asp159 and Asp172 were

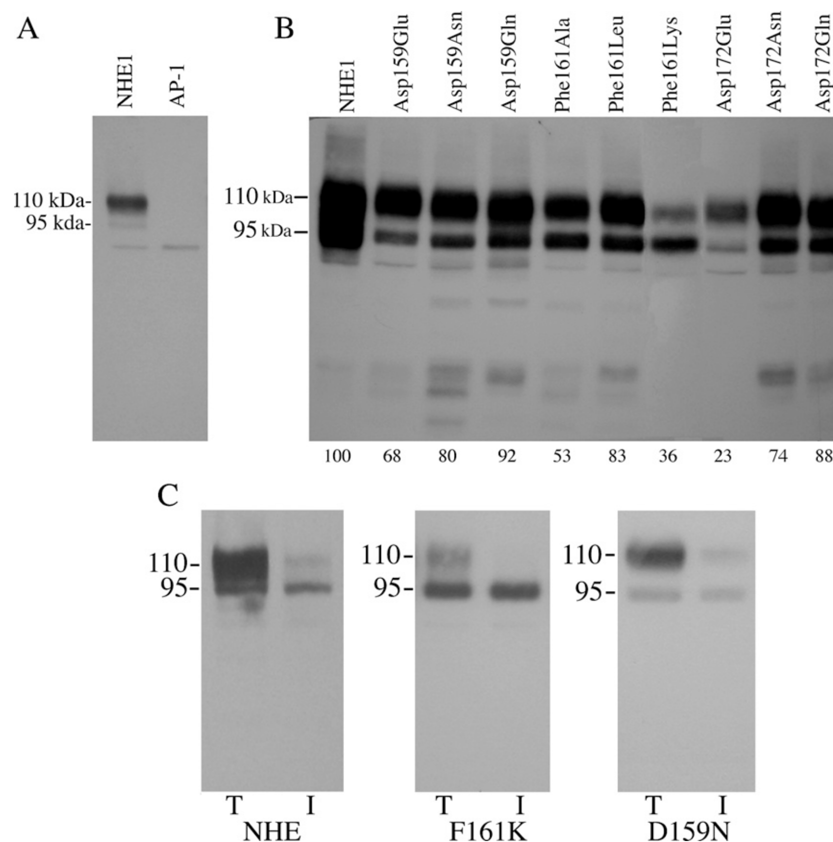


Fig. 2. Western blot analysis of stable cell lines expressing Asp159, Phe161 and Asp172 mutants of NHE1. (A) Western blot of cell extracts from AP-1 cells transfected with HA-tagged wild-type NHE1 (NHE1) or transfected with plasmid not expressing NHE1 (AP-1). (B) Representative Western blot showing NHE1 expression in AP-1 cells transfected with HA-tagged wild-type NHE1 (NHE1) and the NHE1 mutants Asp159Glu, Asp159Asn, Asp159Gln, Phe161Ala, Phe161Leu, Phe161Lys, Asp172Glu, Asp172Asn, and Asp172Gln. In each lane 100 μg of total protein was loaded. Numbers underneath the lanes indicate the mean values obtained from densitometric scans of the 110-kDa band relative to wild-type NHE1 for at least three trials. (C) Subcellular localization of control NHE and TM IV mutants Phe161Lys and Asp159Asn. Sulfo-NHS-SS-biotin-treated cells were lysed and their proteins were solubilized and subsequently treated with streptavidin agarose to bind labeled protein as described in the Materials and methods. Equal samples of total lysates (T) and unbound (representing intracellular) lysates (I) were run on SDS-PAGE and blotted with anti-HA antibody to identify NHE1 protein.

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Table 2
Plasma membrane localization of Asp159, Phe161, and Asp172 NHE1 mutants

| Cell line | Plasma membrane (percent of total) |
|---|---|
| NHE1 | 76.9±5.2 |
| Asp159Glu | 71.1±6.9 |
| Asp159Asn | 78.8±3.5 |
| Asp159Gln | 67.4±1.8 |
| Phe161Ala | 42.8±7.8* |
| Phe161Leu | 86.3±3.0 |
| Phe161Lys | 46.6±6.4* |
| Asp172Glu | 87.0±4.0 |
| Asp172Asn | 53.2±3.3* |
| Asp172Gln | 62.7±1.8 |

Plasma membrane targeting of the $Na^+/H^+$ exchanger in AP-1 cells transfected with wild-type NHE1 (NHE1) and with the mutants Asp159Glu, Asp159Asn, Asp159Gln, Phe161Ala, Phe161Leu, Phe161Lys, Asp172Glu, Asp172Asn, and Asp172Gln was determined as described in Materials and methods. The percent of the total NHE1 protein localized to the plasma membrane is indicated. The results are mean±standard error for at least 4 determinations.

* Indicates significantly reduced plasma membrane targeting in comparison to NHE1 (Mann–Whitney *U*-test, $P<0.05$).

changed to the similarly charged amino acid Glu, and also to the uncharged amino acids Gln and Asn, while Phe161 was changed to Ala, Leu and Lys. Western blotting using anti tag (HA) antibodies was used to characterize the proteins expression (Fig. 2). Fig. 2A shows that transfection with plasmid containing the HA-tagged wild-type NHE1 resulted in the presence of an immunoreactive species of 110 kDa, plus a smaller band at 95 kDa that represents an immature form of the exchanger that is not fully glycosylated [14]. Untransfected cells or cells transfected with plasmid with no insert show no such immunoreactivity. A smaller, weak cross band of approximately 80 kDa in size was often present, and its identity is not known. Fig. 2B examined the expression levels of wild-type NHE1 and the mutant NHE1 proteins used in this study. The amount of mature 110-kDa NHE1 relative to the wild-type is quantified below each lane. All of the mutants were expressed as mostly mature NHE1 with the exception of the Phe161Lys. The Asp172Glu mutant had substantially decreased expression of mature NHE1. For the Phe161Lys mutant there was a substantial increase in the relative amount of the 95-kDa protein.

We also studied the plasma membrane targeting of the mutant NHE1 proteins. Table 2 summarizes the results and Fig. 2C illustrates examples of the surface targeting experiments for wild-type NHE1 and for two types of the mutant $Na^+/H^+$ exchanger proteins. The majority of the wild-type NHE1 protein was present on the plasma membrane and most of the mutant $Na^+/H^+$ exchanger proteins did not have significantly less plasma membrane localization in comparison with the wild-type protein. However, three NHE1 mutants, Phe161Ala, Phe161Lys and Asp172Asn did have significantly reduced plasma membrane targeting compared to the wild-type NHE1 protein (Table 2 and Fig. 2C).

Once we confirmed that the Phe161, Asp159, and Asp172 mutants were at least partially expressed and targeted to the plasma membrane, we examined the activity of the mutant

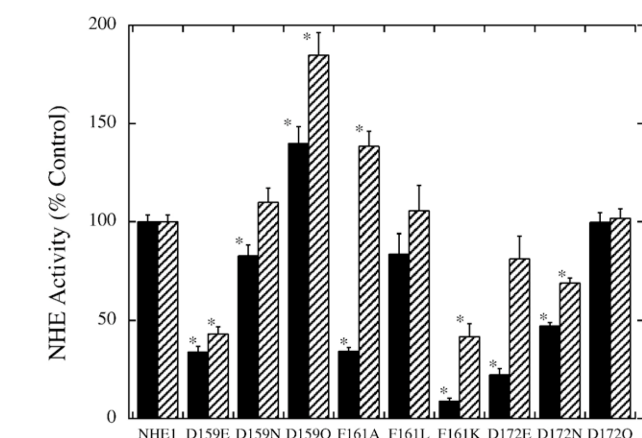


Fig. 3. $Na^+/H^+$ exchanger activity of cell lines expressing control and NHE1 mutants. NHE activity was measured as described in the Materials and methods in stable cell lines expressing NHE1. NHE1, wild-type NHE1. Mutations are indicated below the black and hatched bars. Hatched values indicate the activity after correction for the level of expression and surface targeting. Black bars are uncorrected. The results are the mean±the S.E. of at least 14 determinations from two independently made cell lines. *Indicates significantly different from the wild type at $P<0.01$.

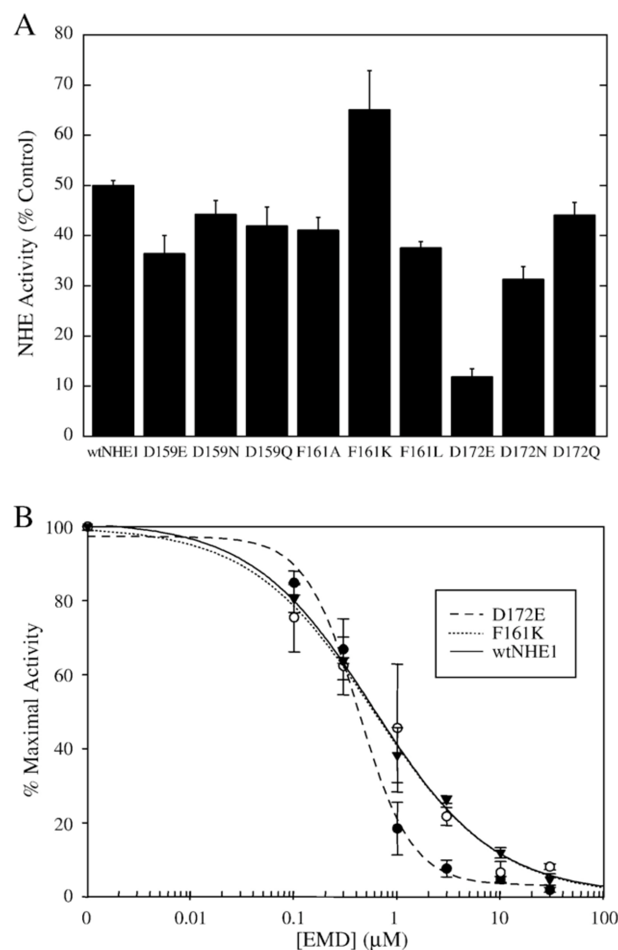


Fig. 4. Effect of EMD87580 on activity of control and NHE1 mutant proteins. NHE1 activity was determined as described in the Materials and methods. (A) Effect of 0.7 μM EMD87580 on activity of all mutants. (B) Dose dependence of effects of EMD87580 on wild-type NHE1 and on the Asp172Glu and Phe161Lys mutants.

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exchangers. Fig. 3 shows both the uncorrected activity (black bars) and the activity corrected for expression and surface processing (striped bars) relative to wild-type NHE1 for the Phe161, Asp159, and Asp172 mutants. The mutants Asp159Glu, Asp159Asn, Phe161Ala, Phe161Lys, Asp172Glu, and Asp172Asn had significantly decreased activity relative to wild-type NHE1. However, after correction for expression and plasma membrane targeting the Asp159Asn, Phe161Ala and Asp 172Glu mutants were not significantly reduced from controls. Surprisingly, the Asp159Gln mutant resulted in increased exchanger activity, and this increase in activity was not due to increased expression and plasma membrane targeting of the exchanger. After correction for surface targeting and expression levels the Phe161Ala mutant was also slightly elevated in comparison to control.

Alterations in sensitivity to inhibition have been reported earlier upon mutation of amino acids in transmembrane segments of NHE1 [2]. EMD87580 is a potent and selective NHE1 inhibitor [20]. To determine whether alterations in NHE1 inhibitor sensitivity had occurred with the amino acids mutated in this study we initially tested the effect of 0.7 μM EMD87580 on the mutants. We have earlier determined that this concentration of EMD87580 inhibits approximately 50% of the activity of the

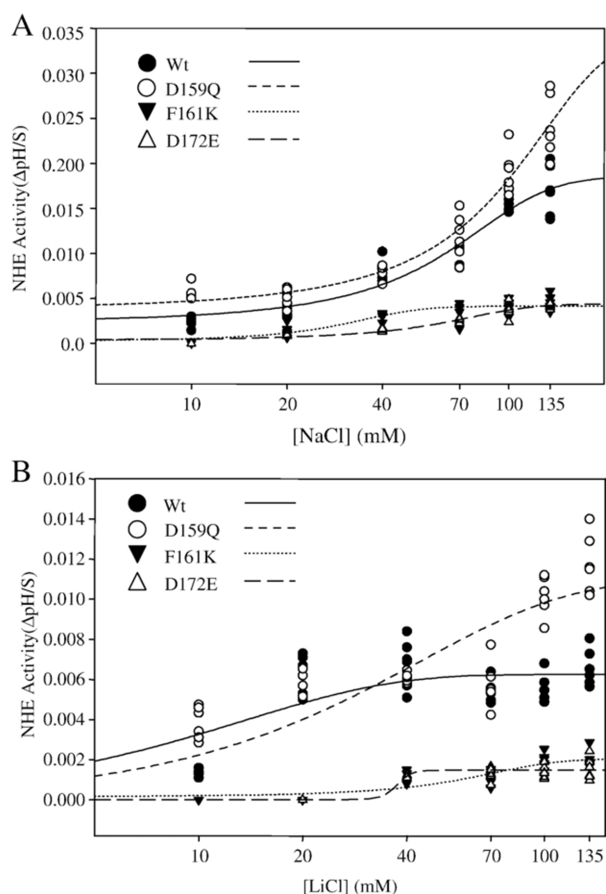


Fig. 5. Effect of varying $Na^+$ and $Li^+$ concentration on activity of control and NHE1 mutant proteins. NHE1 activity was determined as described in the Materials and methods. (A) Effect of varying $Na^+$ concentration on NHE1 activity. (B) Effect of varying $Li^+$ concentration on NHE1 activity.

NHE1 protein [17]. Fig. 4A shows the results. Most of the mutants had no apparent change in the level of inhibition in comparison to the wild type. However the Asp172Glu mutant appeared to be somewhat more sensitive to inhibition. The Phe161Lys mutant showed a slight change in resistance to inhibition, though this was small and variable. To further investigate these results these two mutants were examined in detail along with the control. The results are shown in Fig. 4B. Wild-type NHE1 and the Phe161Lys mutant were affected to the same extent by EMD87580 with IC50s of 0.70 and 0.69 μM respectively. The sensitivity of the Asp172Glu mutant was increased and it had an IC50 of 0.24 μM.

We characterized several of the mutants in more detail to gain insights into the nature of the effects of the mutations. The activity of the wild-type NHE1 protein and of the mutants Asp159Asn, Phe161Lys and Asp172Glu was examined in the presence of varying amounts of $Na^+$ or $Li^+$. Fig. 5A illustrates the effects of altering the $Na^+$ levels on the activity of the protein. The Asp159Gln mutant had greater activity than the wild type, as suggested by the results of Fig. 3. The Phe161Lys and Asp 172Gln mutants had much lower activities than the wild type. The $V_{max}$ values of the wild-type and Asp159Gln, Phe161Lys and Asp172Glu mutants were 0.018, 0.034, 0.004 and 0.004 ΔpH/second respectively. The $K_m$ for these proteins was 58, 95, 29 and 60 mM respectively. It appeared as though the Asp159 Gln mutant was not at maximal activity even in the presence of 135 mM NaCl. Unfortunately, because higher osmotic concentrations are known to activate the NHE1 protein and cause osmotic effects on the cells, we were unable to use higher NaCl concentrations. Similar experiments were done using LiCl instead of NaCl (Fig. 5B). In this case the data did not fit as well to a curve fitting equation (not shown). Nevertheless, the $V_{max}$ for the Asp159Gln mutant was elevated and the calculated $K_m$ was again increased. It was noted that for the wild-type NHE1 protein the LiCl concentrations of 20 mM were saturating and further increases in LiCl did not stimulate NHE1 activity further. It has been previously reported that lower concentrations of LiCl are required for activation of NHE1 in comparison to NaCl concentrations [21].

## 4. Discussion

Transmembrane segment IV of the NHE1 isoform of the $Na^+/H^+$ exchanger has been implicated as being critical in the function of the protein. Through site-specific mutagenesis a variety of the amino acids of TM IV have been shown to be critical to NHE1 function [2]. Mutations of TM IV sometimes alter $Na^+$ affinity and sensitivity to inhibitors [2,11]. We recently deduced the structure of a TM IV peptide and used cysteine scanning mutagenesis to show that amino acid Phe161 lines the ion transport pore of the protein. Based on the structure of TM IV, amino acid Asp159 is a likely candidate for facing the transport pore of NHE1 [14]. We have also earlier suggested that negatively charged amino acids may be critical in coordination of the positive cations that are transported [15]. In the present study we therefore further characterized Phe161 and two acidic residues, Asp159 and Asp172.

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The structure of TM IV suggests that Asp159 points in the same general direction as Phe161, albeit with approximately a 20–30° difference in its orientation. Asp159 was examined by changing it to Glu, Asn and Gln. The Glu mutation maintains the negative charge with an increase in the side chain length. In this case there was a significant decrease in activity of the protein. When the mutation was to the uncharged residue Asn, this mutant retained normal activity. These results indicated that conservation of the charge of this residue was not necessary for activity of the NHE1 protein. Surprisingly, when we mutated Asp159 to Gln, the maximum velocity of the NHE1 protein was increased though the mutation did not alter the resistance to EMD87580. These results suggest that amino acid Asp159 is important in TM IV, but that an absolute conservation of the side chain is not necessary. It is clear that the size of the side chain was important. The larger side chains of Glu and Gln both affected activity dramatically while a change to Asn had little effect. It is possible that the presence of the larger more protruding negative side chain of Glu interacts with a cation to impede its progression along the pore. A change from Asp to the neutral residue Gln might not be so intrusive to the passage of a cation. It is conceivable that the uncharged side chain of Gln, as compared to the carboxyl of Asp, allows a dissociation of a coordinated cation more quickly thereby enhancing activity. However, further experiments are necessary to support this suggestion. We previously showed that mutation to Cys virtually eliminated activity of this mutant [14]. It is therefore clear that the nature of the change in the side chain is critical for NHE1 function. It is also possible that the side chain does not function directly in cation coordination. It could be involved in transmembrane segment packing and helix–helix interactions. Nevertheless, it is clear that this amino acid and side chain are important in NHE1 function.

Mutation of Asp172 had varying effects when changed to Glu, Gln or Asn. Simply removing the charge and changing to Asn had an inhibitory effect. However, mutation to Gln did not inhibit protein activity and the change to Glu also did not affect activity when correcting for expression levels of the protein. Therefore, removal of the charged residue was inhibitory, but replacement with a larger residue either charged or uncharged was not. Therefore, similar to Asp159, this amino acid residue is important in function, but there is not an absolute requirement for this amino acid or this side chain. It was interesting that the Asp172Glu mutant was more sensitive to inhibition by EMD87580. A relationship has been suggested between the sodium binding site and the site of binding of inhibitors of NHE1 although the site of inhibitor binding is not necessarily the same as the $Na^+$ binding site [2,9].

In the case of Phe161 we previously showed that the Phe 161Cys mutant was impaired in activity but was not inactive [14]. A previous study also found that mutation of Phe165Tyr in hamster NHE1 (corresponding to Phe161 in the human sequence) caused a 4-fold decrease in the rate of $Na^+$ transport [22]. Our present results showed that mutation to the smaller Ala residue resulted in a large decrease in activity that was due to a combination of effects on surface targeting and expression levels. Substitution with the hydrophobic but relatively large Leu residue had little effect on activity but substitution with a positively charged lysine had a large inhibitory effect on activity independent of effects on expression and targeting. This effect did not modify the sensitivity to EMD87580. These results support the critical role for this residue in NHE1 function. The inhibitory effect of a positively charged Lys at this position could be due to a side chain providing repulsion to cations in the transport pore. It was interesting that mutation to Leu had little effect, while mutation to Ala affected both targeting and expression levels. It may be that alanine affected targeting and expression because a minimum size is required to maintain stability of the protein. When we corrected for effects of the Ala mutation on targeting and expression, the activity was actually slightly elevated, indicating that the protein itself was functional. Overall, these results confirm that Phe161 is involved in NHE1 function but that similar to Asp159 and Asp172, the Phe side chain itself is not critical for activity and can be substituted for by some amino acids.

It is interesting that the Phe161Lys mutant was largely expressed as an inactive protein that was mostly in a deglycosylated form. We have earlier found this same phenomenon with other mutants when we mutated Asp159, Phe161, Phe162, Pro167, Pro168 and Leu171 in TM IV to cysteine [14]. In addition both Ala and Gln mutations of Glu262 of TM VII caused the same phenomenon [17]. The effect is specific to the type of mutation since mutation of Phe161 to Ala or Leu did not cause this effect. What is the cause of this? At present this is not clear. However, we speculate that some disruption or alteration of the transmembrane structure may be involved. This could be caused by a disruption of direct interactions between transmembrane segments by the altered the side chain. Alternatively, this could occur more indirectly as a result of disruption of an association with the pore-lining residues of the transporter. The propensity for various amino acids to pack in transmembrane segments has been examined. In this regard, it is worth note that Lys has a low packing value in alpha helices compared to Phe [23], which could theoretically account for the effect of the Phe161Lys mutation. However, because residue 161 was shown to be a pore-lining residue, and because this region of TM IV was demonstrated to not be an alpha helix [14], this explanation may not be plausible. It is interesting however that Ala and Leu have higher packing values than Lys, and mutation of Phe161 to either residue did not inhibit NHE1 activity.

Overall, it seems clear that Asp159, Phe161 and Asp172 are critical to NHE1 function, but effects of mutation of these amino acids varied. In all cases, substitutions could be made that retained NHE1 activity. For Asp159 and Asp172, conservation of the charged side chain was not essential. It is therefore clear that either the carboxyl of these amino acids does not directly coordinate a cation, or that there is enough redundancy in the cation coordination so that their mutation does not have a severe detrimental effect.

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