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Functional analysis of polar amino-acid residues in membrane associated regions of the NHE1 isoform of the mammalian Na^+/H^+ exchanger

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The NHE1 isoform of the Na^+/H^+ exchanger is a ubiquitous plasma membrane protein that regulates intracellular pH in mammalian cells. Site-specific mutagenesis was used to examine the functional role of conserved, polar amino-acid residues occurring in segments of the protein associated with the membrane. Seventeen mutant proteins were assessed by characterization of intracellular pH changes in stably transfected cells that lacked an endogenous Na^+/H^+ exchanger. All of the mutant proteins were targeted correctly to the plasma membrane and were expressed at similar levels. Amino-acid residues Glu262 and Asp267 were critical to Na^+/H^+ exchanger activity while mutation of Glu391 resulted in only a partial reduction in activity. The Glu262→Gln mutant was expressed partially as a deglycosylated protein with increased sensitivity to trypsin treatment in presence of Na^+. Substitution of mutated Glu262, Asp267 and Glu391 with alternative acidic residues restored Na^+/H^+ exchanger activity. The Glu262→Asp mutant had a decreased affinity for Li^+, but its activity for Na^+ and H^+ ions was unaffected. The results support the hypothesis that side-chain oxygen atoms in a few, critically placed amino acids are important in Na^+/H^+ exchanger activity and the acidic amino-acid residues at positions 262, 267 and 391 are good candidates for being involved in Na^+ coordination by the protein.

Keywords: pH regulation; cation binding; membrane; crown ether; NHE1.

The Na^+/H^+ exchanger is a ubiquitous plasma membrane protein that regulates intracellular pH (pH_i) by exchanging one intracellular proton for an extracellular sodium [1,2]. Six isoforms of the protein have been identified in mammals and are designated NHE1–6. The first isoform discovered was the NHE1 isoform, which is present in all mammalian cells and is sensitive to the inhibitor amiloride and to its derivatives [3]. It is involved in pH regulation [1,2] and control of cell volume [4], and is activated by growth factors [5]. The Na^+/H^+ exchanger family shares a conserved structure, with a membrane-associated N-terminus of approximately 500 amino acids, and a large, regulatory, cytoplasmic domain of approximately 300 amino acids. The primary structure of the membrane-associated domain is more conserved than the cytosolic domain [6].

Surprisingly little is known about the specific amino acids involved in Na^+/H^+ exchange or about their mechanism of operation. It is clear that the membrane domain of the protein transports the cations, as it can function independently of the cytoplasmic domain [1]. A few studies have examined the amino acids and transmembrane segments that play a role in inhibition of the protein by amiloride. One of these studies demonstrated that the sequence VFFLFVLLPPI(164–173), of transmembrane segment 4 of NHE1, was involved in binding amiloride analogs [7,8]. Another study showed that a segment between transmembrane regions 8 and 10 is also involved in amiloride binding [9]. However, it is now thought that the amiloride-binding site is not directly involved in binding and transport of Na^+ [10] and that other regions are important in this role [9,11]. A preliminary report has suggested that amino acid Glu262 is important for activity of the protein, but there was no detailed analysis of the activity and expression of the protein; in addition, the ability of other residues to substitute at this location in the protein was not investigated [12].

A number of studies have shown that acidic and polar residues have important roles within membrane transport proteins, including melibiose permease in *Escherichia coli* [13] bacteriorhodopsin [14,15], mammalian Na^+/Ca^{2+} exchangers [16], ATPases, such as the Ca^{2+}-ATPase of the sarcoplasmic reticulum [17], and Na^+/K^+ ATPase [18–20]. We have recently shown that some conserved acidic residues are important in cation binding and transport by the yeast Na^+/H^+ exchanger sod2 [21]. In the same study we demonstrated that a histidine residue is also important in the activity of sod2 [21]. Nevertheless, in the mammalian Na^+/H^+ exchanger integral membrane His residues are not involved in cation binding and transport [22]. Overall, it is clear that acidic and polar residues are important in cation translocation in many ion transporters, although the exact

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Abbreviations: BCECF-AM, 2′,7-bis(2-carboxyethyl)-5(6) carboxyfluorescein-AM; HA, hemagglutinin; pH_i, intracellular pH; NHE1, Na^+/H^+ exchanger isoform 1; HMA, 5-(*N*,*N*-hexamethylene)-amiloride; TRITC, tetramethyl rhodamine isothiocyanate; RSV, Rous sarcoma virus.

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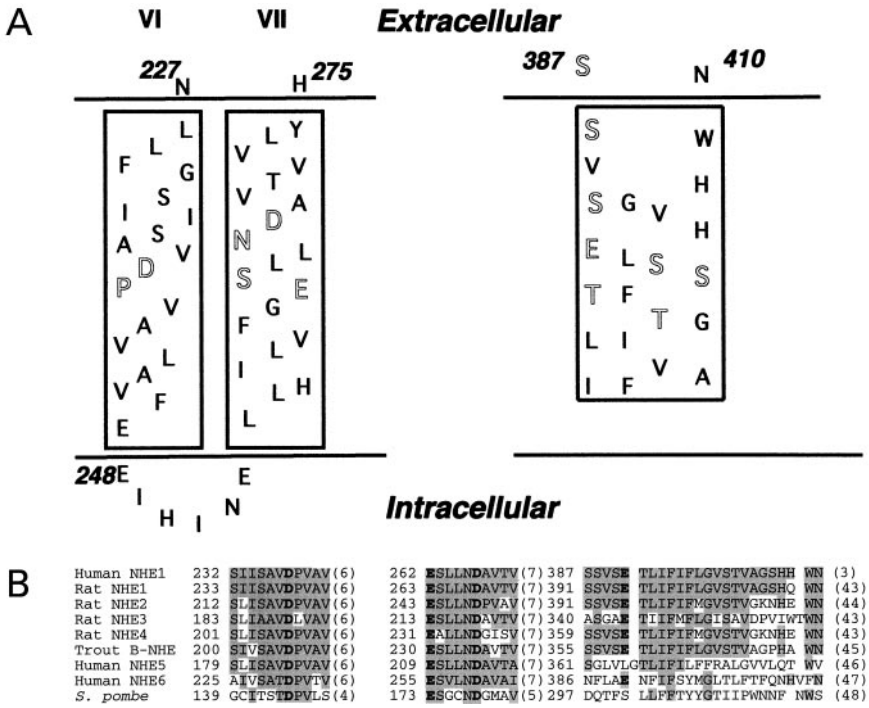


Fig. 1. Topological model of transmembrane segments VI, VII, and of amino acids 387–410 in the membrane- associated segment of the human NHE1 isoform of the Na^+/H^+ exchanger (A), and alignment of membrane associated regions of Na^+/H^+ antiporters (B). In (A), outlined letters indicate the residues mutated in this study. (based on [23]). In (B), numbers preceding sequences indicate the number of the first amino acid. Reference numbers are at the end of each line. Numbers following the amino-acid sequence are the predicted transmembrane segment based on the original papers. Asp238, Glu262, Asp267 and E391 of NHE1 are indicated in bold and corresponding residues on other Na^+/H^+ exchangers are also indicated. Shaded residues indicate conservation with human NHE1.

residues involved vary even between related proteins. In the Na^+/Ca^{2+} exchanger, a surprisingly large number of polar residues appear to be important in transport activity [16].

The most recent model of the mammalian Na^+/H^+ exchanger has suggested that this antiporter has 12 transmembrane segments, and one membrane-associated segment that encompasses amino acids 387–406 [23]. Several regions of this protein are of particular interest, for example, the membrane-associated segment that does not traverse the membrane is reminiscent of the selectivity filter of potassium channels [24]. These amino acids (387–406) are within a region that is important for amiloride binding [9]. We have noted that this segment contains several polar amino acids, including a serine at amino acids 387 and 388, and Ser390, Glu391 and Thr392. The double-serine motif at amino acids 387 and 388 is conserved in NHE1 and in some of the other NHE isoforms (Fig. 1B). The transmembrane segments 227–247 and 253–273, predicted to be membrane regions 6 and 7, respectively, are also interesting [23]. Examination of the amino-acid sequences (Fig. 1B) shows that there are several conserved, acidic residues, Asp238, Glu262, and Asp267, within these relatively hydrophobic regions.

In this study, we have examined the functional role of polar amino acids located in transmembrane regions 6 and 7 and in the membrane-associated segment (from amino acids 387–406) of NHE1. We have characterized the effects of these mutations on the activity of the protein, localization, glycosylation and ion selectivity. Our results demonstrate that two of the polar amino acids of transmembrane region 7 are critical for activity of the mammalian Na^+/H^+ exchanger and that one amino acid in the membrane-associated segment from 382 to 404 is also important in activity. The results represent the first systematic investigation of polar amino acids of membrane associated segments involved in cation binding and transport of a mammalian Na^+/H^+ exchanger.

EXPERIMENTAL PROCEDURES

Materials

Restriction endonucleases and DNA modifying enzymes were obtained from New England Biolabs, Inc. (Mississauga, ON, Canada) and Life Technologies Inc. Plasmid kits for DNA purification were obtained from Qiagen (Qiagen Inc., CA, USA). The site-directed mutagenesis kit (Transformer^{TM} Site-Directed Mutagenesis Kit) was from Clontech Inc. (Palo Alto, CA, USA) or from Stratagene (La Jolla, CA, USA) (Quikchange^{TM} site directed mutagenesis kit). Mouse anti-HA Ig was purchased from Boehringer Mannheim (Laval, PQ, Canada) or Berkeley Antibody Co. (Richmond, CA, USA). Tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-(mouse IgG) Ig was from Jackson Immuno Research, Inc. (West Grove, PA, USA). The acetoxymethyl ester of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein was obtained from Molecular Probes Inc. (Eugene, OR). The AP-1 cell line was a generous gift of S. Grinstein

Table 1. Primers used for construction of NHE1 mutants. All primers start at the 5′ end. Lowercase residues indicate mutations, underlined residues indicate restriction sites used to distinguish positive clones.

Mutation or removed*	Mutagenic Primer	Restriction site created
D238N	CCGCCACGGGGTtaACGGCCGAGATG	*Hpa*I
P239A	GCATCATCTCGGCCGTcGACgccGTGGCGGTTCTGGCTGTC	*Sal*I
E262Q	CGGCGTCATTGAGCAAGcttTgCCCAAAAACAAGGATG	*Hin*dIII
E262D	CACATCCTTGTTTTTGGGGAtTCCTTGCTCAATGACGCCGTCACTG	N/A
S263A	CATCCTTGTTTTTGGGGAGgCCTTGCTCAATGACGCCGTCAC	*Stu*I
N266A	TTGGGGAGTCCTTGCTagcTGACGCCGTCACTGTGGTCC	*NHE*I
D267N	GGACCACAGTGACtGCGTtATTGAGCAAGGAC	*Alw*N1
D267E	TTGGGGAGTCCTTGCTCAATGAaGCaGTCACTGTGGTCCTGTATCAC	*Alw*N1
S359A	CATAGGGGCGCATCACCACTCCaGctGCTATGAGCGCCATGATGC	*Pvu*II
S387A/S388A	AAGATGAGGGTCTCGCTGACtgcagcCCACATCTTCAGGAAGTATTTG	*Pst* I
S390A	AAGATGAGGGTCTCGgcGACGgaGCTCCACATCTTCAG	*Sac*I
E391Q	GGAAGATGAAGATcAGGGTCTgGCTGACGCTGCTC	*Alw*NI
E391D	GGAGCAGCGTCAGCGAcACCCTCATCTTCATCTTCCTCG	*Bsa*I*
T392V	CCTGAAGATGTGGAGCtcCGTCAGCGAGgtaCTCATCTTCATCTTCCTCG	*Sac*I
S401A	GCCGGCCACCGTGGcGACGCCGAGGAAG	*Bgl* I
T402V	CCAGTGGTGGGAtCCGGCCACCacGGAGACGCCGA	*Bam*HI
S406A	CAGTTCCAGTGGTGagctCCGGCCACCGTGG	*Sac*I

(Hospital for Sick Children, Toronto, ON, Canada). α-Minimal essential medium and all the tissue culture reagents were purchased from Life Technologies Inc. All other chemicals not listed were of analytical or molecular biology grade and were purchased from Fisher Scientific (Ottawa, ON, Canada), Sigma (St Louis, MO, USA) or BDH (Toronto, ON, Canada).

Plasmid and site-directed mutagenesis

The plasmid pYN4$^+$ incorporated cDNA coding for the Na^+/H^+ exchanger (NHE1 human isoform) with a hemagglutinin (HA) tag on the C-terminus of the protein [25]. It was used for construction of NHE1 mutants and for expression in AP-1 cells. This plasmid contains the Rous sarcoma virus (RSV)-LTR promoter, thymidine kinase poly(A) signal and neomycin resistance gene (aminoglycoside 3′-phosphotransferase).

Site-directed mutagenesis of residues D238N, P239A, E262Q, E262D, S263A, N266A, D267N, D267E, S359A, S387A/S388A, S390A, E391Q, E391D, T392V, S401A, T402V and S406A was performed, with most mutations designed to create a restriction enzyme site that could easily be detected in subsequent analysis. The oligonucleotides used to produce the desired mutations are described in Table 1. The template for mutagenesis was the plasmid pYN4$^+$ [25]. Transformants were screened by digestion with enzymes whose sites were created by the mutagenic primers. Mutants were sequenced to confirm the mutations and fidelity of amplification. We routinely cloned a minimal subfragment containing the mutation of interest back into pYN4$^+$ that had not been used for the mutagenesis procedure.

Cell culture and stable transfection

A Chinese hamster ovary cell line (AP-1 cells) that was previously selected to lack endogenous NHE activity [26] was routinely grown in a humidified atmosphere of 5% CO_2 and 95% air in α-MEM medium supplemented with 10% (v/v) fetal bovine serum, 25 mM Hepes, penicillin (100 U·mL^{-1}) and streptomycin (100 μg·mL^{-1}), pH 7.4 at 37 °C. The transfection and selection of clones was essentially as described previously [25] with minor modifications. Briefly, 1.3×10^6 cells were seeded in 100 mm Petri dish, in 8 mL of growth media. Cells were grown until 70% confluent and transfected with 20 μg of wild-type or mutagenized plasmid constructs by the calcium phosphate coprecipitation technique. After 12–16 h of incubation with DNA, the cells were washed with fresh media. After 24 h of incubation the 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^{-1} geneticin (G418) that was used to maintain selection pressure without acute acid load selection. Only AP-1 cells transfected with wild-type cDNA encoding NHE1 were further selected for survival following an acute acid load, essentially as described previously [25]. Cultures were regularly re-established from frozen stocks, and cells from passage numbers in the range 3–15 were used for experiments. For analysis of enzyme activity, at least three independently isolated clones of each Na^+/H^+ exchanger mutant were tested.

Na^+/H^+ exchange activity

NHE activity was estimated as the initial rate of Na^+-induced recovery of cytosolic pH (pH_i) after an acute acid load caused by preloading with NH_4Cl and pH_i was measured fluorometrically using 2′,7-bis(2-carboxyethyl)-5(6) carboxyfluorescein-AM (BCECF-AM) essentially as described previously [25,27]. Stably transfected and untransfected cells were seeded on glass coverslips (2×10^5 cells per coverslip) and grown until they reached 70–80% 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^{-1} BCECF-AM and incubated in 'Normal' buffer containing 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, and 10 mM Hepes, pH 7.4 at 37 °C. Normal buffer is nominally bicarbonate free and under these conditions the contribution of and bicarbonate-based pH regulatory systems is minimal. Intracellular acidosis was induced by NH_3/NH_4^+ prepulse/withdrawal (5 min in Normal buffer containing 25 mM NH_4Cl, pH 7.4), followed by withdrawal for 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. Fluorescence was measured in a Shimadzu RF-5000 spectrofluorophotometer as described previously [27]. The initial rate of rise of pH_i was calculated from the first 20 s of recovery. Figures show the entire recovery period for illustration purposes only. Measurements of each type of stably transfected cell were repeated at least eight times. Results are shown as means ± SE of at least 8–10 experiments. Statistical significance was determined with a Mann–Whitney *U*-test. A calibration curve for intracellular BCECF in AP-1 cells was carried out using the K^+/nigericin method [28,29]. There was no difference in the calibration curve between control and transfected cells. Hydrogen ion efflux rates (mM $H^+ \cdot min^{-1}$), equivalent to the rate of Na^+/H^+ exchange were determined as described previously [29]. Intracellular buffering capacity for different mutant AP-1 cell lines and for AP-1/NHE1 was determined using the methods described previously [29]. Where results are given as $\Delta pH \cdot min^{-1}$, cells were acidified to the same levels and differences in buffering capacity were insignificant from one cell line to another. The determination of kinetic parameters of the Na^+/H^+ exchanger was essentially as described previously [29]. Na^+ and Li^+ concentrations were varied while maintaining the osmolarity with *N*-methyl-D-glucamine. For these experiments cells were acidified to the same level with 40 mM NH_4Cl. To determine the proton affinity, intracellular pH was varied by acidifying to various degrees with different amounts of NH_4Cl as described previously [29].

SDS/PAGE and immunoblotting

We used Western blotting to determine the presence and relative levels of expression of wild-type and mutant NHE1 protein expressed in transfected AP-1 cells. For immunoblot analysis total membrane (microsomal) fractions (10–30 μg) were isolated from transfected and untransfected AP-1 cells. The cells were recovered from plates manually (in the absence of trypsin) and centrifuged at 5000 ***g*** for 3 min in a clinical centrifuge. Pelleted cells were suspended in 5 mL of lysis buffer consisting of 10 mM Tris, pH 8.0, 25 mM KCl, 2 mM MgCl2, 2 mM EGTA, 2 mM EDTA and incubated on ice for 10–15 min. A protease inhibitor cocktail [30] was added and the sample was homogenized with a tight fitting Dounce homogenizer. After 40 strokes, 7.5 mL of lysis buffer with 250 mM sucrose and 6 mM 2-mercaptoethanol was added and the sample was homogenized for a further 20 strokes. The sample was centrifuged at 16 000 ***g*** for 15 min and the supernatant was spun at 137 000 ***g*** for 75 min. The final pellet was suspended in 10 mM Tris/HCl, 1 mM EDTA, pH 7.4. Samples were resolved on 9.5% or 10% SDS/polyacrylamide gels. The gel was transferred onto nitrocellulose membranes, immunostained with anti-HA Ig (1 : 2000) [25] and examined using the Amersham enhanced chemiluminescence Western blotting and detection system as described by the manufacturer. X-ray films were scanned, and densitometric analysis was using IMAGE GAUGE V.3 software.

Immunocytochemistry

To determine the intracellular localization of wild-type and mutant NHE1 protein *in vivo*, transfected cells were grown on glass coverslips to 60–70% confluence. The coverslips were washed three times with $NaCl/P_i$, pH 7.4 and cells were fixed with 4% formaldehyde in $NaCl/P_i$. Fixation was terminated with 100 mM glycine in $NaCl/P_i$ for 15 min. Cells were then permeabilized with 0.1% Triton-X-100 and 0.1% BSA in $NaCl/P_i$ for 15 min. The coverslips were thoroughly washed with $Tris/NaCl/P_i$ (50 mM Tris, 200 mM NaCl), pH 7.4 followed by blocking in 10% goat antiserum in $Tris/NaCl/P_i$ for 1 h at room temperature. After five washes with $Tris/NaCl/P_i$, coverslips were incubated with mouse monoclonal anti-HA Ig (1 : 100) for 1–3 h at room temperature. The cells were washed again with $Tris/NaCl/P_i$ and reacted with TRITC-conjugated goat anti-(mouse IgG) Ig (1 : 50) for 1 h. Coverslips were mounted in 50% glycerol containing 1% propyl gallate, an autofluorescence photobleacher and visualized using an Olympus fluorescent microscope. Some cells were treated with 4′,6-diamidino-2-phenyl indole to show localization of DNA.

Trypsin treatment of microsomal membranes

Total membrane (microsomal) fractions were diluted to a protein concentration of 2 mg·mL^{-1} in 1 mM EDTA, adjusted to pH 7.4 with Tris/HCl (no protease inhibitors). Microsomal suspension (10 μL) was added to 10 μL of Normal buffer. After preincubation for 3 min at 37 °C, *N*-α-tosyl-L-phenylalanylchloromethane (Sigma) was added to give a desired trypsin/protein ratio (1 : 20 or 1 : 30). The reaction was terminated by adding 2 μL (7 mg·mL^{-1}) of trypsin inhibitor (Sigma) followed by addition of sample buffer for SDS/PAGE. The samples were resolved on 10% SDS/polyacrylamide gels followed by transfer onto nitrocellulose membranes. Western blotting was performed as described above. It should be noted that using this type of technique, we have been able to detect changes in protein conformation caused by site-specific mutagenesis of other amino acids of the Na^+/H^+ exchanger [25] (H. Wang, D. N. Singh & L. Fliegel, unpublished observations).

Glycosidase treatment of microsomal membranes

Microsomal membrane fractions prepared as described above, were washed twice with 5 mL of glycosidase buffer (50 mM KCl, 20 mM NaH_2PO_4, pH 7.2) and resuspended to 1 mg·mL^{-1} in the same buffer also containing 50 mM EDTA and proteinase inhibitors [30]. Membrane samples (50 μg) were incubated in the presence of *N*-glycosidase F (Boehringer Mannheim; (4 mU·μg^{-1} of protein) or with neuraminidase (Boehringer Mannheim; 0.5 mU·μg^{-1} of protein) in glycosidase buffer plus 1% 2-mercaptoethanol.

The samples were incubated at 37 °C for 16 h. SDS/PAGE and Western blotting analysis were performed as described above.

RESULTS

Expression of mutant NHE1 proteins in AP-1 cells

Fig. 1A illustrates the membrane-associated segments of NHE1 that we examined and Fig. 1B shows an alignment of parts of these segments from different isoforms of the Na^+/H^+ exchanger. Note the conserved acidic residues, D238, E262, and D267 within the hydrophobic regions of transmembrane segments VI and VII. Amino acid D238 is part of a characteristic DPV sequence conserved across several of the isoforms including the yeast Na^+/H^+ exchanger sod2. The amino acids E262 and D267 are also highly conserved among all the isoforms. Note also the presence of a considerable number of hydrophilic amino acids in the membrane-associated segment (amino acids 387–410). In addition residue E391 is conserved among most of the isoforms except NHE5 and the yeast exchanger sod2. On the basis of these sequence observations we constructed a series of specific Na^+/H^+ exchanger mutants.

In an initial series of experiments we simply neutralized charged residues, using the mutations D to N, E to Q, S to A and T to V (Table 1). Western blot analysis was performed to confirm that the mutant proteins were expressed and that their level of expression was similar to the wild-type Na^+/H^+ exchanger. We detected the Na^+/H^+ exchanger with an anti-HA Ig that reacted with the HA tag on the C-terminus of the protein [25]. Figure 2 shows that all the mutants were expressed in AP-1 cells. In all cases, the mutant and wild-type exchanger show the same pattern of immunoreactive bands, the largest of which is approximately 105-kDa and probably represents the glycosylated form of the mature NHE1 protein. Untransfected cells showed no immunoreactivity with anti-HA Ig (data not shown). Some smaller immunoreactive bands were usually seen on the blots. The electrophoretic mobility of these reactive bands suggests they may be immature, unglycosylated forms of the Na^+/H^+ exchanger [31]. Interestingly, the E391Q mutant appeared to have a slightly larger proportion of the band migrating at 90-kDa and the E262Q mutant migrated principally as the 90-kDa band. In

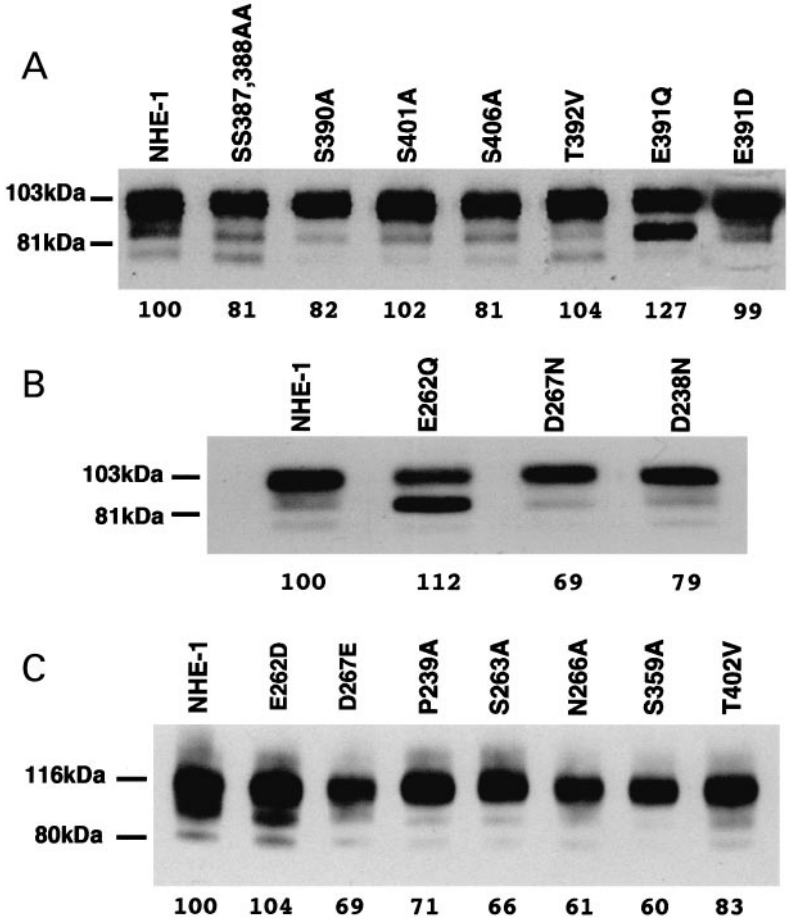


Fig. 2. Western blot analysis of microsomes from AP-1 cells transfected with control and mutated NHE1 cDNA including a HA tag. Cells were transfected and prepared for SDS/PAGE and Western blotting with anti-HA antibody as described in Experimental procedures. Western blots with 10 μg of wild-type NHE1 protein and NHE1 proteins with the indicated mutations. Numbers underneath the lanes indicate the values obtained from densitometric scans relative to wild-type NHE1. Results are mean of three measurements and densitometry values included proteins from the 80-kDa to 110-kDa range. (A) Western blot of S401A, S406A, T392V, E391Q and E391D mutants. (B) Western blot of E262Q, D267N and D238N mutants. (C) Western blot of E262D, D267E, P239A, S263A, N266A, S359A and T402V.

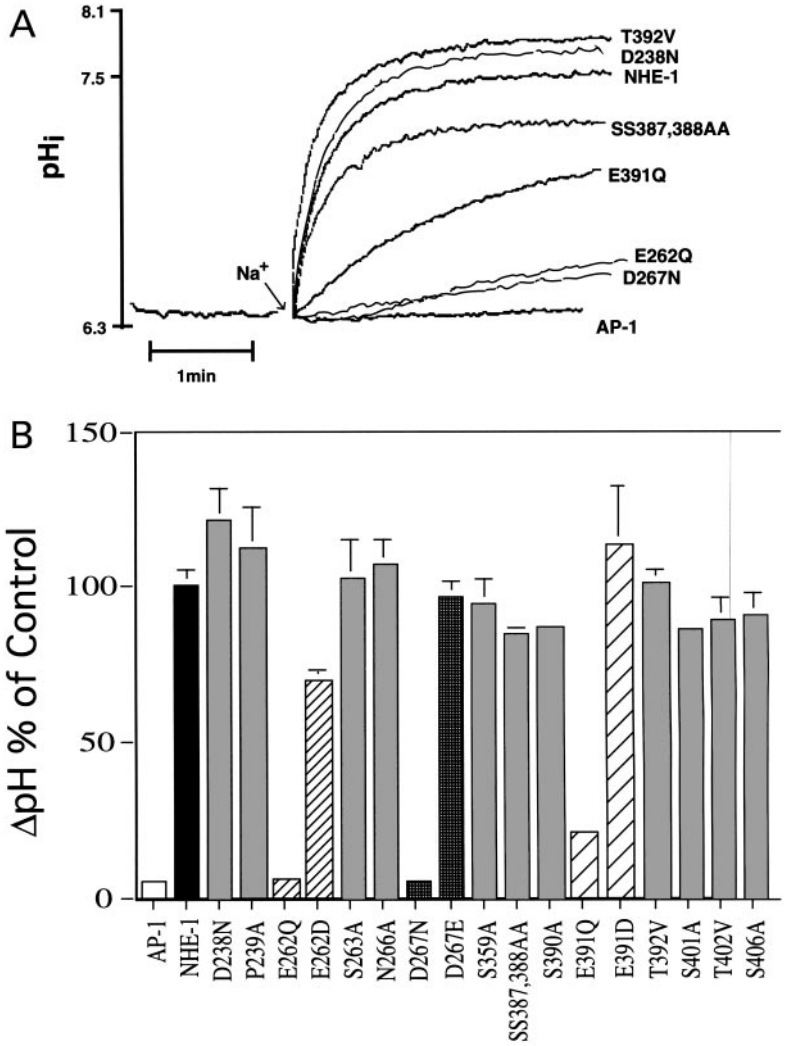
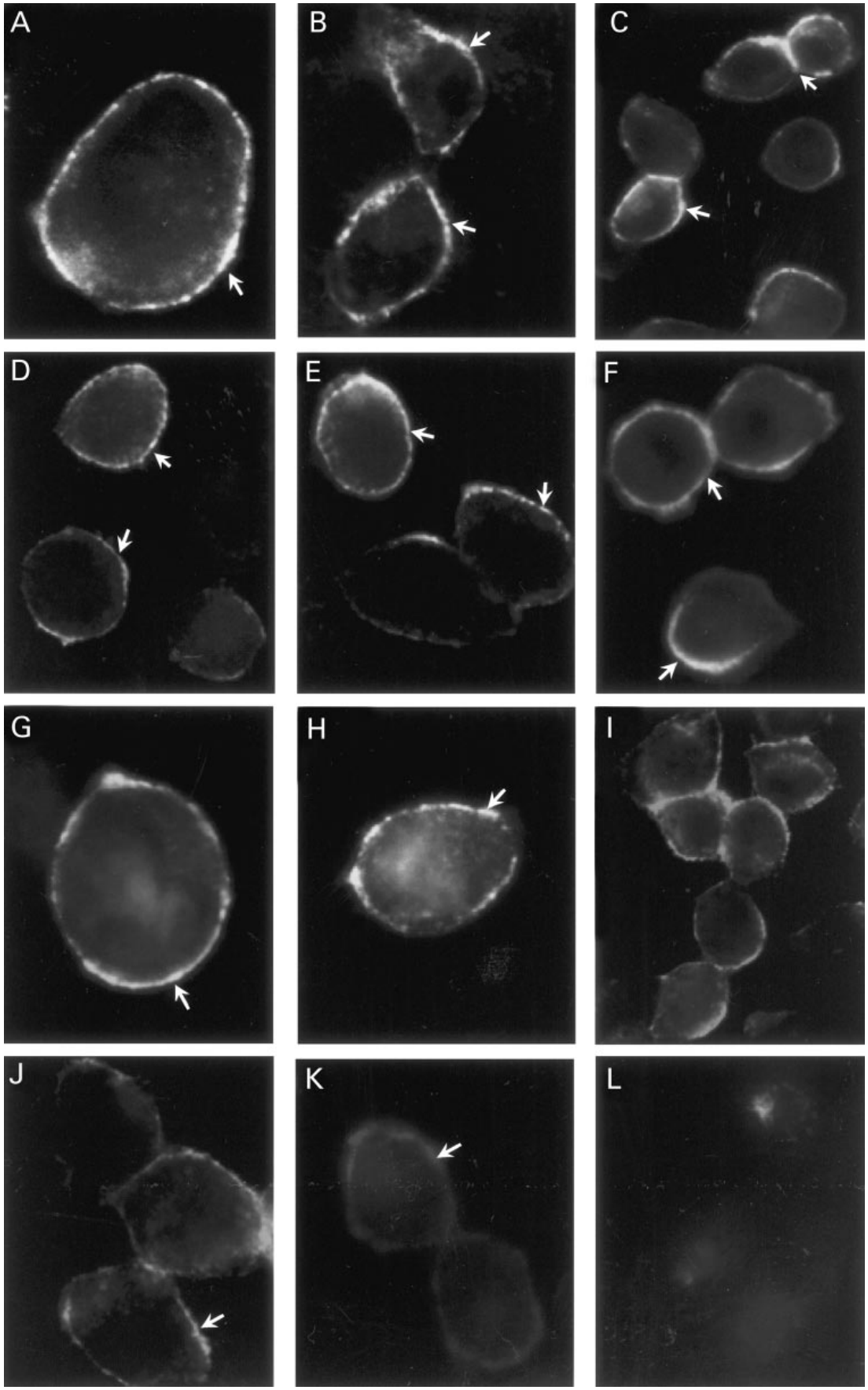


Fig. 3. Recovery from an acute acid, load by AP-1 cells stably transfected with wild-type Na^+/H^+ exchanger (NHE1) and mutated Na^+/H^+ exchangers. Cells were prepared and pH_i was measured after acute acid load as described in Experimental procedures. (A), Representative traces illustrate typical rates of pH_i recovery of AP-1 cells transfected with wild-type NHE1 and mutated Na^+/H^+ exchangers and mock-transfected (AP-1) cells. Arrow indicates the beginning of pH_i recovery after the cells were re-exposed to Na^+ containing Normal buffer. The initial pH_i was typically 6.3 for all cell types. Traces are representative of eight experiments for each mutant and wild-type NHE1 transfectant. Representative traces for some cell lines were not show as they overlapped greatly with the control, the summary of these values are in B. (B) Bar graph summarizing the effects of mutations on initial rates of pH_i recovery. Results are mean ± SE for at least eight measurements. Where not shown the SE was too small to be displayed.

contrast, the E262D mutant did not show an increased amount of 90-kDa band nor did the E391D mutant. Densitometric analysis of the levels of expression of mutant and wild-type NHE1 proteins are beneath the Western in units relative to the controls. The analysis included the larger 105-kDa and the smaller 85 to 95-kDa species. The mutant Na^+/H^+ exchanger proteins were all expressed at similar, though not identical, levels.

The effect of mutations on Na^+/H^+ exchanger activity

We next tested the effect of the various mutations on activity of the Na^+/H^+ exchanger. Mutants were expressed under the control of the RSV-LTR promoter, and several independent colonies of each mutant cell line were isolated. Na^+/H^+ exchanger activity was evaluated by monitoring initial rates of recovery in response to acid-loading with NH_4Cl. At least three independent clones of each mutant cell line were examined. Figure 3A illustrates some examples of the effects of the various mutations on the rate of recovery from acid load, compared with recovery in the wild-type NHE1 and untransfected AP-1 cells. The rate of recovery after ammonium chloride prepulse is shown upon Na^+ addition. Cells transfected with the wild-type Na^+/H^+ exchanger are referred to as NHE1. The results show that control cells (NHE1), T392V and S387A/S388A initiate a rapid and immediate recovery from an acid load. The traces for P239A, S263A, N266A, D267E, S359A, S387A/S388A, S390A, E391D, T392V, S401A, T402V and S406A were almost identical in initial rate and steady state pH_i. Their traces are not shown because of the large degree of overlap. In all cases the activity of



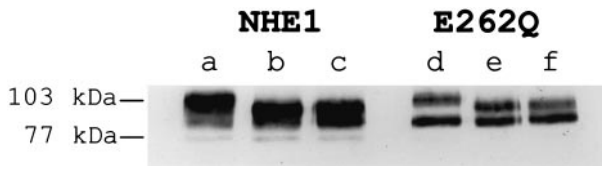


Fig. 5. Carbohydrate analysis of the wild-type and E262Q Na^+/H^+ exchangers transfected into AP-1 cells. Samples containing 50 μg of protein were incubated with *N*-glycosidase F (4 $U \cdot mg^{-1}$) in glycosidase buffer (lanes b and e); with neuraminidase (0.5 $U \cdot mg^{-1}$) for 16 h at 37 °C (lanes c and f) or in the absence of enzyme (lanes a and d). The samples (15 μg) were then analyzed by SDS/polyacrylamide gel electrophoresis and Western blotting with anti-HA Ig (as described in Fig. 2). The relative positions of Bio-Rad prestained markers are indicated in the margin.

the Na^+/H^+ exchanger was abolished by amiloride analogue 5-(*N*,*N*-hexamethylene)-amiloride (HMA) (20 μM) confirming that we were dealing with the NHE1 isoform of Na^+/H^+ exchanger (not shown).

Figure 3B illustrates our quantitative analysis of effects of the various amino-acid substitutions on initial rate of recovery from an acid load. There were no significant differences in the degree of acidification or in the buffering capacity of mutant or wild-type transformants. The mutants for D238N, P239A, S263A, N266A, D267E, S359A, S387A/S388A, S390A, E391D, T392V, S401A, T402V and S406A conferred essentially the same rate of recovery as the wild-type Na^+/H^+ exchanger. In contrast, the initial rate of recovery conferred by the E262Q and the D267N mutants was essentially the same as that seen in untransfected cells. The transfected cells showed a very small upward drift in their pH_i after acidification but this was small and was not inhibited by HMA. Some untransfected AP-1 cells also displayed this characteristic. The E391Q mutant showed greatly reduced, but not abolished, Na^+/H^+ exchanger activity. Mutant E262D showed activity that was less than 25% of that of the activity of the wild-type exchanger.

Subcellular localization of the mutant and wild-type NHE1 proteins

Analysis of the expression of the mutant Na^+/H^+ exchanger proteins, by Western blotting, showed that all were targeted to a microsome fraction. As we analyzed a crude total microsomal fraction from the AP-1 cells, it might contain both plasma membranes and intracellular membranes. Therefore, we examined the subcellular localization of the mutant proteins in whole cells, to determine if they were correctly targeted. To do this we used immunofluorescence microscopy with an antibody against the HA-tag included at the C-terminus of the protein (Fig. 4). Figure 4A shows that when AP-1 cells are transfected with wild-type NHE1, localization of the protein is predominantly in the plasma membrane. Untransfected cells (Fig. 4L) show no staining with the anti-HA antibody and only a weak background signal from within the cell. Cells transfected with mutants T392V, S387A/S388A, S390A, S401A, T402V, S406A, E391Q, E262Q, D267N and E262D (B-K, respectively) also show predominant plasma membrane localization of the protein. Other mutants such as E391D also behaved similarly (not shown). Clearly, the point mutations introduced into the Na^+/H^+ exchanger protein did not prevent its targeting to the plasma membrane.

Table 2. Kinetic characterization of Na^+/H^+ exchanger mutant proteins with conservative amino-acid substitutions. The activity of the various Na^+/H^+ exchanger mutants was estimated in the presence of varying concentrations of Na^+ and Li^+ as described in Experimental procedures.

	Na^+		Li^+	
	K_m (mM)	V_{max} ($\Delta pH \cdot min^{-1}$)	K_m (mM)	V_{max} ($\Delta pH \cdot min^{-1}$)
NHE1	41.5	1.8	9.8	0.60
E262D	38.3	1.6	17.1*	0.70
D267E	41.6	1.9	10.4	0.74
E391D	33.4	1.8	9.1	1.03

* Iindicates significantly different from unmutated Na^+/H^+ exchanger (NHE1) at $P < 0.05$.

Carbohydrate analysis

To characterize the lower molecular mass form of the E262Q mutant we carried out carbohydrate analysis using *N*-glycosidase and neuraminidase. Treatment of wild-type NHE1 protein with either *N*-glycosidase or neuraminidase resulted in the appearance of immunoreactive bands smaller than the 105-kDa protein and resulted in the disappearance of the 105-kDa protein (Fig. 5, lanes b and c). Treatment of the E262Q mutant with *N*-glycosidase F (lane e) and neuraminidase (lane f) resulted in disappearance of 105-kDa band and appearance of a smaller band. At the same time, treatment of the E262Q mutant with *N*-glycosidase F (lane e) and neuraminidase (lane f) did not affect the size of the 90-kDa immunoreactive band.

Trypsinolysis of Na^+/H^+ exchanger proteins

To determine whether the mutant Na^+/H^+ exchanger proteins were properly folded we carried out limited trypsinolysis. This method examines the accessibility of Arg and Lys residues to proteolytic attack, and has been used previously to examine the structure of membrane proteins such as the H^+-ATPase of yeast [32]. We initially

Fig. 4. Immunocytochemical localization of the Na^+/H^+ exchanger protein in transfected AP-1 cells. Cells were transfected and collected and prepared for immunocytochemical analysis with anti-HA Ig as described in Experimental procedures. A–K: immunofluorescent images of AP-1 cells transfected with (A), NHE1 (wild-type); (B), T392V; (C), S387A/S388A; (D), S390A; (E), S401A; (F), T402V; (G), S406A; (H), E391Q; (I), E262Q; (J) D267N; (K), E262D. Arrows indicate plasma membrane labeling. L: immunofluorescent image of nontransfected AP-1 cells prepared with anti-HA Ig as with A–H. G and H were treated with DAPI to show nuclear localization.

compared the wild-type with mutant proteins that had reduced activity. We used both Na^+-containing and Na^+-free buffers as Na^+ might cause a change in conformation of the protein and therefore a change in sensitivity to proteolysis. This kind of change in sensitivity to proteolysis by ions has been seen with vanadate ions and the H^+-ATPase yeast [32]. An example of our typical results is shown in Fig. 6A–C. Figure 6A shows that treatment of wild-type NHE1 with trypsin resulted in a gradual decline in the amount and size of the 105-kDa immunoreactive band in both Na^+-containing and Na^+-free buffer. This was accompanied by a more gradual decrease in the 90-kDa immunoreactive band. A similar pattern was seen for the D267N mutant except that, in the Na^+-containing buffer, the 90-kDa protein was not apparent after 1 min of incubation with trypsin. For the E262Q mutant the same gradual decrease in the 105-kDa immunoreactive band was observed. However, we consistently found a difference between the 90-kDa band of the E262Q mutant and wild-type protein. Specifically, the 90-kDa band in the mutant was more sensitive to trypsin in the presence of Na^+. This effect was very pronounced and reproducible. Figure 6B shows another set of mutants, E391Q, S406A and T402V compared with the wild-type exchanger. We have found no consistent qualitative or quantitative differences in the sensitivity of these proteins to trypsinolysis. It was of note that the 90-kDa band from the E391Q mutant, appeared to be more sensitive to trypsin, especially in the presence of Na^+. Figure 6C shows the E262D and D267E mutants. Again, there were no consistent differences between these mutants and the wild-type protein in their sensitivity to trypsinolysis, in either the presence or absence of Na^+.

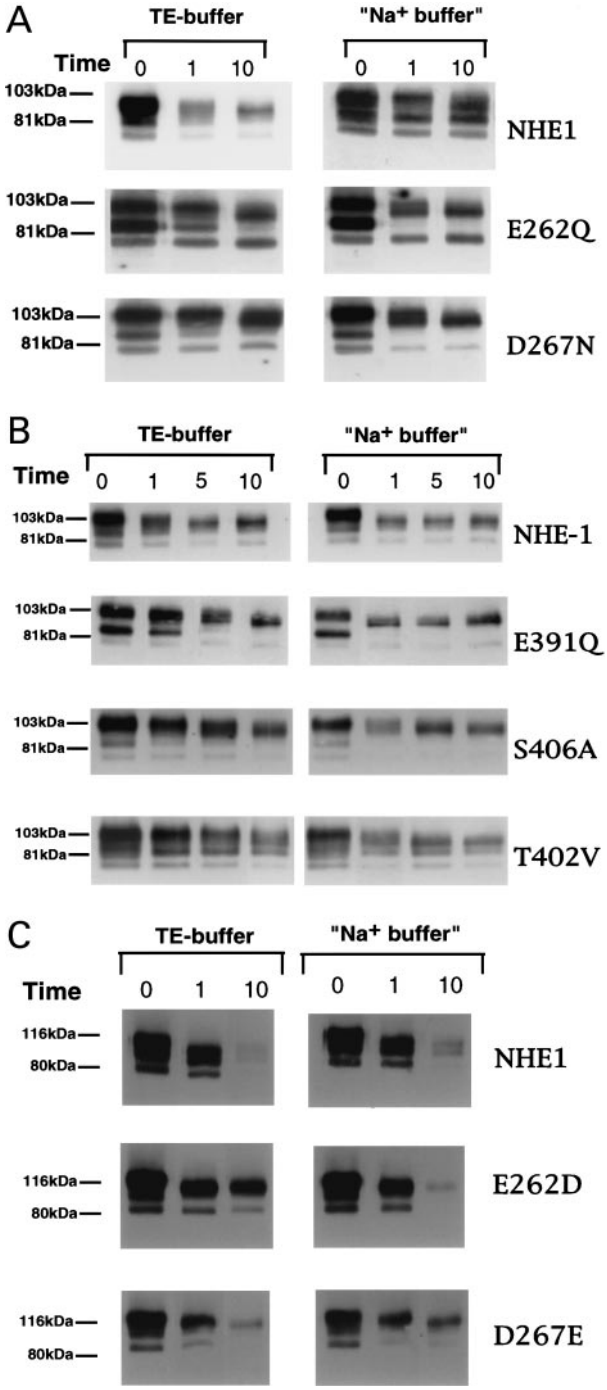


Fig. 6. Time course of trypsinolysis of wild-type (NHE1), E391Q, S406A and T402V Na^+/H^+ exchangers. Total microsomal fractions were incubated with trypsin for 0–10 min at 37 °C as described in Experimental procedures. Incubations were in the presence of Normal buffer containing NaCl (67.5 mM) or Tris/EDTA buffer that was NaCl free. The samples (18 μg of protein in each lane) were then analyzed by SDS/PAGE and Western blotting (as with Fig. 2).

Cation selectivity of Na^+/H^+ exchanger mutants with conservative mutations

We examined the activity of the conservative mutants E262D and D267E, and of the wild-type protein, in the presence of varying concentrations of Li^+ and Na^+, to determine whether the affinity of the exchanger for either cation was altered by the mutation (Table 2). In the presence of Na^+, there were, essentially, no differences in Na^+/H^+ exchanger activity among the proteins except for a decrease in the maximal activity of the E262D mutant compared with the wild-type protein. There were also no differences in the activation and maximum transport activity of the mutant proteins with respect to protons. However, the K_m for Li^+ was significantly increased in the E262D mutant compared with the wild-type exchanger. There was no change in the kinetic properties of transport for protons between the mutants and the controls (not shown).

DISCUSSION

Polar residues are important in binding and coordination of cations in a variety of membrane transporters [13–20]. Therefore we compared the amino-acid sequences of several Na^+/H^+ exchangers looking for conserved polar residues within several membrane segments of the NHE1 isoform. We hypothesized that polar residues within these membrane segments are involved in cation binding, coordination and transport. We initially examined conserved Asp and Glu residues in transmembrane segments VI and VII (Fig. 1) and all polar residues within the membrane-associated segment spanning from amino acid

387–406. In total, we constructed 17 different mutants of the Na^+/H^+ exchanger protein, encompassing 15 amino acids. In all cases, the mutations did not prevent expression of the protein or greatly alter its localization to the cell surface.

To investigate the role of the polar amino acids we made mutations designed to reduce their polarity. The substitutions we selected were typical of those used for this type of study [16]. Our measurements of the activity of these various mutants showed that only few of these polar amino acids contribute to Na^+/H^+ exchange activity. The mutation of amino acids Ser263, Ser387 and Ser388, Thr392, Ser401, Thr402 and Ser406 all had no appreciable effect on exchanger activity. Any minor variations in activity seen in these mutants could be accounted for by minor variations in the levels of protein expression especially in the case of the S387A/S388A and the S390A mutations. These results are surprising given the high degree to which these amino acids are conserved (Fig. 1B). It was particularly surprising to us that mutation of the double serine motif at residues 387, 388 had no effect on activity. This motif is conserved among the NHE1 isoform of the protein and in NHE1, NHE2 and NHE4 (Fig. 1B). In addition, its location is similar to that of the double Asp motif that is important in activity of the yeast and *E. coli* Na^+/H^+ exchangers [21,33,34]. However it is clear that residues 387 and 388 are not critical in cation binding and transport.

A number of other conserved amino acids were tested for their contribution to Na^+/H^+ exchanger activity. Ser359, is present in transmembrane segment IX, and its mutation to Ala had no effect on activity. We also mutated Pro239 and Asn266. Prolines usually function as helix breakers within the membrane [35] and we hypothesized that mutation of Pro239 to Ala might disrupt a particular conformation of the protein. However, this residue proved to be unimportant in function of the protein. Asp238, together with Pro239 comprises a well-conserved motif in almost all the mammalian Na^+/H^+ exchangers and in the yeast Na^+/H^+ exchanger sod2 (Fig. 1B). Again however, it was surprising that this residue was not important in function of the Na^+/H^+ exchanger.

In this study the most dramatic effects on Na^+/H^+ exchanger activity resulted from mutation of the acidic residues in membrane segment VII, Glu262 and Asp267. Mutation to the neutral amino acids Gln and Asn, respectively, effectively eliminated Na^+/H^+ exchanger activity. Substitution of these amino acids with other acidic amino acids restored activity in the presence of Na^+, with the exception of a minor reduction in activity of the E262D mutant (Fig. 3). These experiments demonstrate that an acidic side chain at position 262 and 267 is essential for the activity of the Na^+/H^+ exchanger. When we examined the kinetic characteristics of mutants with acidic substitutions the only major difference was that the affinity of E262D for Li^+ was reduced compared with the wild-type exchanger. The ionic radii of Na^+ and Li^+ are 0.95 and 0.65 Å, respectively [36]. If E262 is involved in coordination of Na^+ and Li^+ cations, the substitution to Asp with its smaller side chain may reduce the ability of the protein to coordinate the smaller lithium ion, while still allowing for coordination of the larger Na^+ ion.

Within the membrane-associated segment that contains amino acids 387–406, we found that only amino acid 391 played a significant role in activity of the Na^+/H^+ exchanger. Indeed, changing this residue to Gln reduced the activity of the Na^+/H^+ exchanger greatly. Because of the low activity of this mutant it was not possible to get accurate readings of the effect of this mutation on the affinity for Na^+ or H^+, however, it was clear that overall activity was greatly reduced. Substitution with the alternative acidic residue Asp, restored activity. It is surprising that this was the only polar amino acid within this membrane-associated segment whose substitution affected activity of the antiporter. It is also somewhat surprising that Glu391 was clearly important in activity while the conserved polar amino acids immediately prior to (Ser390) and following it (Thr392) were not. Our results suggest that there is not a strict correlation between the conservation of a particular residue and its importance to Na^+/H^+ exchanger activity. The latest model for the topology of the Na^+/H^+ exchanger [23] suggests that amino acids 387–406 enter or are associated with the bilayer from the outside and then return to the outside of the membrane (Fig. 1). Such a conformation is somewhat similar to that of the selectivity filter of the potassium channel [24]. It is possible that main chain carbonyl oxygen atoms are important in cation binding and coordination within this segment, as seen in the K^+-channel selectivity filter [37]. Proof of this hypothesis will require a detailed structural analysis of the mammalian Na^+/H^+ exchanger. It is clear, however, that side-chain oxygens from Glu391, Glu262 and Asp267 are important in exchanger activity.

With the E262Q mutation, a significant fraction of the protein existed as a smaller molecular mass form (Fig. 2) which resulted from reduced protein glycosylation (Fig. 5). This form accounted for more than half of the E262Q protein expressed. There was also a slight increase in the smaller form of the mutant E391Q, which was also defective in transport. However, a defect in glycosylation could not account for the absence of activity in the E262Q mutant as the total amount of this mutant protein expressed was greater than that for the wild-type and the larger form of the protein was over one third of the total protein expressed. This suggests that the elimination of activity is not due to a simple defect in glycosylation but probably results from an effect on cation coordination and transport by this amino acid. In keeping with this suggestion, we [38] have previously shown that carbohydrates are not necessary for activity of the Na^+/H^+ exchanger and their removal does not alter activity of the NHE1 isoform of the antiporter.

While it is possible that some of the effects we observed were due to an altered structure of the Na^+/H^+ exchanger rather than effects on cation binding and transport, this seems unlikely for several reasons. Firstly, the effects were specific. In 15 different amino-acid mutations, only three had any effect on activity, and the effects were highly specific according to both location and amino acid. For example, mutation of Glu262 completely eliminated activity while mutation of the adjacent amino acid, Ser263, had no effect. Secondly, the proteins were all properly targeted to the plasma membrane. In previous studies we found that some mutations can affect activity of

the Na^+/H^+ exchanger through aberrant targeting [25] but this was not the case here. Finally, we checked for any effects on conformation of the protein using limited proteolytic digestion. Our results suggest that, at least within the parameters of this assay, there were no effects overall on the structure of the protein.

The only possible change in protein conformation was shown by the smaller molecular mass form of the E262Q mutant that was more sensitive to trypsin in the presence of Na^+. This smaller protein did not account for all of the E262Q protein expressed however, and therefore could not be responsible for all of the effects on activity. A more likely explanation for the differential sensitivity to trypsin of this mutant is that the decreased glycosylation leaves it more susceptible to proteolysis. This phenomenon has been reported for other proteins [39]. As the sensitivity of the deglycosylated E262Q mutant to trypsin was only increased in the presence of Na^+, it is possible that this region of the protein is involved in a Na^+-induced conformational change. It was only possible to detect this sensitivity in the protein with reduced carbohydrates, that normally are protective from proteolysis. A similar observation has been recorded for NhaA, the Na^+/H^+ exchanger of *E. coli*. In this case a pH-induced conformation change in the protein allowed increased accessibility for trypsin to a segment of the protein involved in pH-regulation of activity [40]. As the E262Q mutation causes a Na^+-dependent increase in sensitivity to trypsin, and the E262D mutant has a reduced ability to coordinate Li^+, we suggest that this amino acid is probably involved in cation coordination by the Na^+/H^+ exchanger.

We have earlier hypothesized that Na^+/H^+ exchangers may act by coordination of substrate cations through a crown ether-like cluster of polar amino acids [34]. We have also shown that side-chain groups of another eukaryotic Na^+/H^+ exchanger, sod2, are also important in cation binding and transport [34]. Boyer [41] suggested that cations could be coordinated by various electronegative atomic groups, such as oxygen atoms. The results of the current study support the hypothesis that the oxygen in the side chains of E262, D267 and E391 may, at least partially, serve this role for the mammalian Na^+/H^+ exchanger.

Our study is the first systematic analysis of amino-acid residues important in membrane-associated segments of the mammalian Na^+/H^+ exchanger. It is the first clear demonstration of the importance of the negatively charged amino acids E262, D267 and E391 in activity of the protein. It identifies certain polar residues that appear to be important in cation binding and transport activity of this protein. Current models suggest that these residues are part of important membrane-associated segments of the protein which are transmembrane segments or segments 'associated' with the membrane. Future experiments are necessary to determine the exact conformation of this part of the protein. It is of note that relatively few polar amino acids in these segments appear to be critical to transport activity of NHE1. This contrasts to results obtained with the Na^+/Ca^+ exchanger [16]. Our data support the hypothesis that a few, critically placed, conserved amino acids may be sufficient to carry out Na^+/H^+ exchange, even when they are dispersed throughout the protein [42].

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