Functional characterization of the transmembrane segment VII of the NHE1 isoform of the Na^+/H^+ exchanger[1]

Jie Ding, Raymond W.P. Ng, and Larry Fliegel

Abstract: The Na^+/H^+ exchanger isoform 1 is an integral membrane protein that regulates intracellular pH. It extrudes 1 intracellular H^+ in exchange for 1 extracellular Na^+. It has 2 large domains, an N-terminal membrane domain of 12 transmembrane segments and an intracellular C-terminal regulatory domain. We characterized the cysteine accessibility of amino acids of the critical transmembrane segment TM VII. Residues Leu 255, Leu 258, Glu 262, Leu 265, Asn 266, Asp 267, Val 269, Val 272, and Leu 273 were all mutated to cysteine residues in the cysteineless NHE1 isoform. Mutation of amino acids E262, N266, and D267 caused severe defects in activity and targeting of the intact full length protein. The balance of the active mutants were examined for sensitivity to the sulfhydryl reactive reagents, positively charged MTSET ((2- (trimethylammonium)ethyl)methanethiosulfonate) and negatively charged MTSES ((2-sulfonatoethyl)methanethiosulfonate). Leu 255 and Leu 258 were sensitive to MTSET but not to MTSES. The results suggest that these amino acids are pore-lining residues. We present a model of TM VII that shows that residues Leu 255, Leu 258, Glu 262, Asn 266, and Asp 267 lie near the same face of TM VII, lining the ion transduction pore.

Key words: cation transport, membrane, Na^+/H^+ exchanger, pH regulation.

Résumé : L'isoforme 1 de l'échangeur Na^+/H^+ est une protéine membranaire intégrale qui régule le pH intracellulaire. Elle expulse un H^+ intracellulaire en échange d'un Na^+ extracellulaire. Elle est constituée de 2 grands domaines, un domaine membranaire N-terminal de 12 segments transmembranaires et un domaine régulateur C-terminal intracellulaire. Nous avons caractérisé l'accessibilité à la cystéine des acides aminés du segment transmembranaire crucial TM VII. Les résidus Leu 255, Leu 258, Glu 262, Leu 265, Asn 266, Asp 267, Val 269, Val 272 et Leu 273 ont tous été mutés en résidus cystéine dans l'isoforme NHE1 sans cystéine de l'échangeur Na^+/H^+. La mutation des acides aminés E262, E266 et D267 a entraîné d'importantes altérations dans l'activité et le ciblage de la protéine entière intacte. Le reste des mutants actifs ont été examinés pour leur sensibilité aux réactifs sulfhydryl, MTSET chargé positivement ((2-triméthylammonium)éthyl)méthanethiosulfonate) et MTSES chargé négativement ((2-sulfonatoéthyl)méthanethiosulfonate). Les résidus Leu 255 et Leu 258 ont été sensibles à MTSET mais pas à MTSES. Les résultats donnent à penser que ces acides aminés sont des résidus tapissant le pore. Nous présentons un modèle de TM VII qui montre que les résidus Leu 255, Leu 258, Glu 262, Asn 266 et Asp 267 se situent du même côté du TM VII, longeant le pore de transduction ionique.

Mots-clés : transport de cations, membrane, échangeur Na^+/H^+, régulation du pH.

[Traduit par la Rédaction]

Introduction

The mammalian Na^+/H^+ exchanger isoform 1 (NHE1) is a ubiquitous integral membrane protein that regulates intracellular pH by mediating removal of 1 intracellular proton in exchange for a single extracellular sodium ion (Orlowski and Grinstein 1997). NHE1 has several cellular and physiological functions: it protects cells from intracellular acidification (Grinstein et al. 1989; Pouyssegur et al. 1984), promotes cell growth and differentiation (Grinstein et al. 1989), and regulates sodium fluxes and cell volume after challenge by osmotic shrinkage (Shrode et al. 1996). In addition to these physiological roles, the NHE has been demonstrated to be involved in modulating cell motility and invasiveness of neoplastic breast cancer cells (Paradiso et al. 2004) and is critical to cell motility in some cell types (Denker and Barber 2002). The NHE also plays a critical role in 2 forms of heart disease, heart hypertrophy and the damage that occurs during ischemia and reperfusion. Inhibition of the exchanger protects the myocardium during these disease states (Fliegel 2001; Karmazyn et al. 2003). A new generation of NHE inhibitors is being developed for clinical treatment of heart disease (Lang 2003; Mentzer et al. 2003).

NHE1 is composed of an N-terminal membrane domain of ~500 amino acids and a C-terminal regulatory domain of ~315 amino acids (Fliegel 2001; Orlowski and Grinstein

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Abbreviations: HA, hemagglutinin; PBS, phosphate-buffered saline; MTSES, ((2-sulfonatoethyl)methanethiosulfonate); MTSET, ((2-(trimethylammonium)ethyl)methanethiosulfonate); SDS, sodium dodecyl sulfate; TM, transmembrane (segment).

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Fig. 1. (A) Model of the Na^+/H^+ exchanger (NHE1 isoform). The orientation of transmembrane (TM) segments I-XII of the NHE1 isoform of the Na^+/H^+ exchanger is illustrated. (B) Crude model of amino acids present in TM VII.

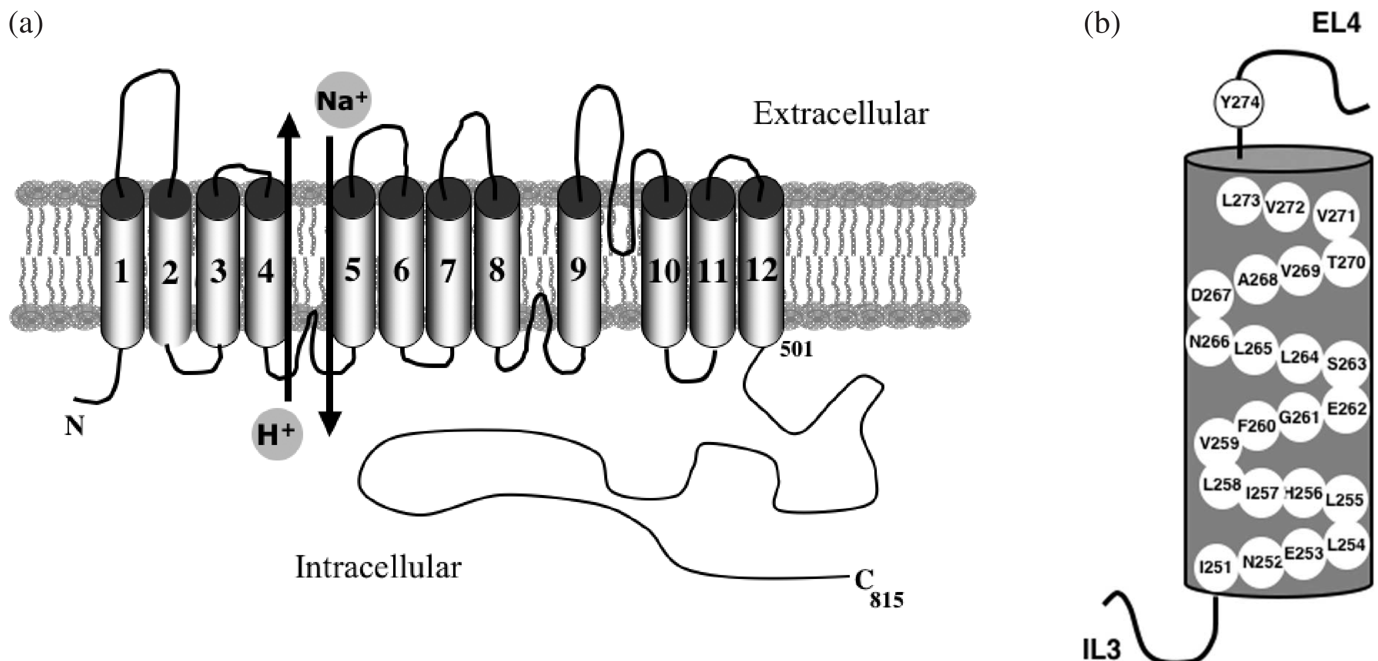

Table 1. Oligonucleotides used for site-directed mutagenesis.

Mutation	Oligonucleotide sequence	Restriction site
L255C	5′-CACATCAATGAGCTctgtCACATCCTTGTTTTTGG-3′	SacI
L258C	5′-CACATCAATGAGCTcCTcCACATCtgTGTTTTTGGGGAGTCC-3′	BseRI
E262C	5′-TCCTTGTTTTTGGctgcagCTTGCTCAATGACGC-3′	BsgI
L265C	5′-GGGGAGTCCTTGtgCAATGACGCCGTCACT-3′	BsrDI
N266C	5′-GTTTTTGGGGAaagCTTGCTCtgCGACGCCGTCACTG-3′	HindIII
D267C	5′-GAGTCCTTGCTCAATtgCGCaGTCACTGTGGTCCTG-3′	FspI
V269C	5′-TTGCTCAATGACGCatgCACTGTGGTCCTGTATCAC-3′	SphI
V272C	5′-GCCGTCACTGTGtgCCTGTATCACCTC TTTGAG-3′	DraIII
L273C	5′-GTCACTGTGGTCtgtTATCAtCTCTTTGAGGAG-3′	(EcoNI)

Note: Mutated nucleotides are in lowercase letters. New restriction sites are underlined. Deleted restriction sites are indicated in parentheses.

1997). The N-terminal membrane domain mediates ion movement and has 3 membrane associated segments and 12 integral transmembrane segments (Fig. 1A) (Wakabayashi et al. 2000). To understand the mechanism of Na^+/H^+ exchange, we have recently analyzed the transmembrane segment TM IV of NHE1. Prolines 167 and 168 were shown to be critical to NHE1 function (Slepkov et al. 2004). Using cysteine scanning mutagenesis Phe 161 was also shown to be a pore lining residue critical to transport and the structure of TM IV. TM IV was also shown to be composed of 1 region of β-turns, an extended middle region including P167-P168, plus a region helical in character (Slepkov et al. 2005).

TM VII is believed to extend from amino acids Ile 251 to Leu 273 (Fig. 1B) (Wakabayashi et al. 2000). We have shown that TM VII is critical for the function of the NHE1 isoform of the NHE (Murtazina et al. 2001). We recently elucidated the structure of TM VII and showed that it is an interrupted helix. Alanine scanning mutagenesis showed that mutation of several amino acids results in severely reduced activity (Ding et al. 2006). In this manuscript, we use cysteine-scanning mutagenesis to characterize the amino acids that are important in function and are likely pore-lining residues of TM VII. Our study further defines the amino acids that are important to NHE1 function and identifies, for the first time, amino acids of TM VII that may line the pore of the NHE1 protein.

Materials and methods

Materials

PWO DNA polymerase was obtained from Roche Applied Science and Lipofectamine™ 2000 reagent was from Invitrogen (Carlsbad, Calif.). MTSET and MTSES were from Toronto Research Chemicals (Toronto, Ont.).

Site-directed mutagenesis

Mutations were made to an expression plasmid containing a human NHE1 isoform of the NHE. The plasmid pYN4+ contains the cDNA of the entire coding region of the NHE

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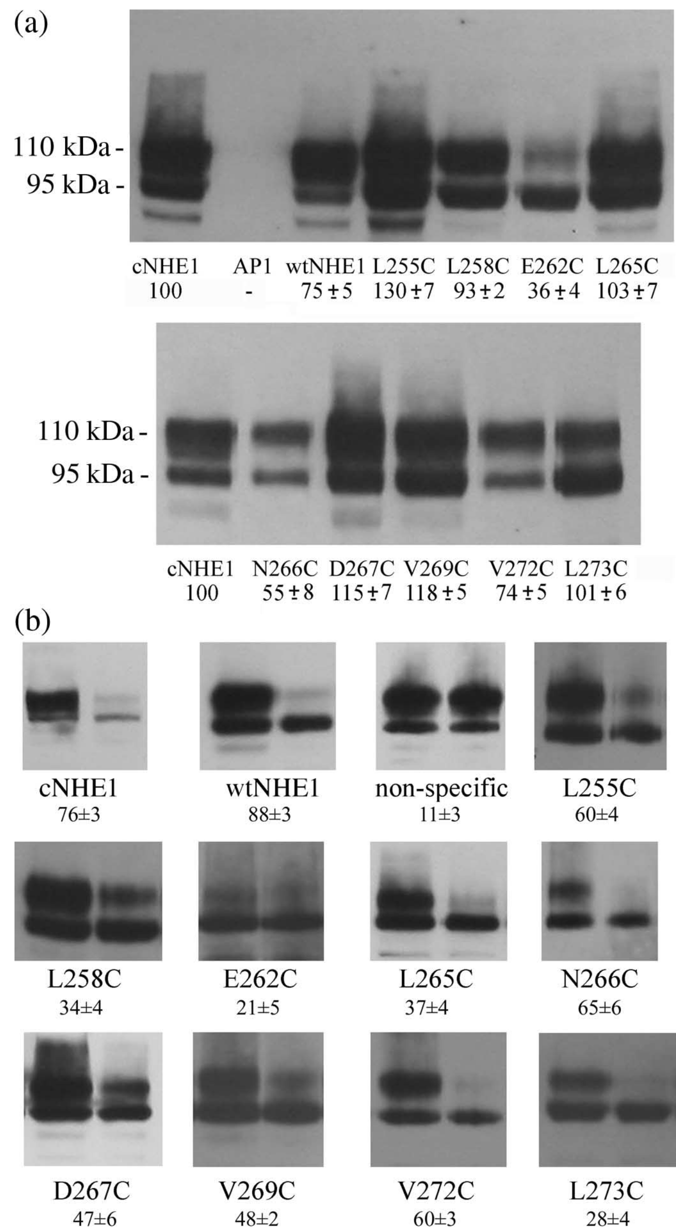

Fig. 2. Characterization of Na^+/H^+ exchanger isoform 1 (NHE1) expression in AP-1 cells. (A) Western blot analysis of cell extracts from control AP-1 cells and stably transfected AP-1 cells with cysteine-scanning mutations in TM VII. Cell extracts were prepared from control and stably transfected cell lines. One hundred micrograms of total protein was loaded in each lane. Numbers below the lanes indicate the values obtained from densitometric scans of both the 110 and 95 kDa bands relative to wild-type NHE. Results are mean ± SE of at least 3 measurements. (B) Subcellular localization of control NHE1 and TM VII cysteine scanning mutants in AP-1 cells. Sulfo-NHS-SS-biotin-treated cells were lysed and the solubilized proteins were treated with streptavidin agarose beads to bind labeled proteins. Equal samples of total cell lysate (left side of panel) and unbound lysate (right side of panel) were run on SDS–PAGE and blotted using anti-HA antibody directed toward the tag on the NHE1 protein. Non-Sp refers to an experiment in which nonspecific binding to streptavidin-agarose beads was carried out following the standard procedure but without labeling cells with biotin. cNHE1 is cysteineless NHE1 and wtNHE1 is the wild-type NHE1. Other mutants are indicated. The percent of the total NHE1 protein that was found within the plasma membrane is indicated for each mutant. For Non-Sp, the numbers indicate the amount of nonspecific binding to streptavidin-agarose beads. The results are the mean ± SE of at least 6 determinations.

with a hemagglutinin (HA) tag (Slepkov et al. 2004). The mutations made (Table 1) changed the indicated amino acids to cysteine, using the background of the functional cyteineless NHE1 protein that we have described earlier (Table 1) (Slepkov et al. 2005). Site-directed mutagenesis was using amplification with PWO DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) followed by using the Stratagene (La Jolla, Calif.) QuikChange™ site-directed mutagenesis kit. Mutations created a new restriction enzyme site or deleted a restriction site for use in screening transformants. DNA sequencing confirmed the mutations and fidelity of PCR.

Cell culture and transfections

To examine NHE expression and activity, AP-1 cells, Chinese hamster ovary cells that lack an endogenous NHE (Murtazina et al. 2001), were used. Transfection with LIPOFECTAMINE™ 2000 Reagent (Invitrogen Life Technologies, Carlsbad, Calif.) was used to make stable cell lines of all mutants as described earlier (Slepkov et al. 2004). Transfected cells were selected using 800 µg/mL geneticin (G418), and stable cell lines for experiments were regularly reestablished from frozen stocks at passage numbers between 5–9.

SDS–PAGE and immunoblotting

To confirm NHE1 expression, cell lysates were made from AP-1 (Slepkov et al. 2004). Western blot analysis was on equal amounts of up to 100 µg of each sample resolved on a 10% SDS–polyacrylamide gel. Nitrocellulose transfers were immunostained using a primary antibody of anti-HA monoclonal antibody (Boehringer Mannheim, Laval, Que.) and a secondary antibody of peroxidase-conjugated goat anti-mouse antibody (Bio/Can, Mississauga, Ont.). An enhanced chemiluminescence blotting and detection system (Amersham) was used to visualize immunoreactive proteins. Densitometric analysis of X-ray films was via NIH Image 1.63 software (National Institutes of Health, Bethesda, Md.,).

Cell surface expression

Cell surface expression was measured with sulpho-NHS-SS-biotin (Pierce Chemical Company, Rockford, Ill.) (Slepkov et al. 2004). Sulfo-NHS-SS-biotin labeled cell surface proteins and immobilized streptavidin resin was used to remove plasma membrane NHE. Equivalent amounts of the total and unbound proteins were analyzed by Western blotting and densitometry as described above. The relative amount of NHE1 on the cell surface was calculated by comparing both the 110 kDa and 95 kDa HA-immunoreactive species in Western blots of the total and unbound fractions. It was not possible to efficiently elute proteins bound to immobilized streptavidin resin.

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NHE activity

NHE activity was measured using a PTI Deltascan spectrofluorometer as described previously (Slepkov et al. 2005). After ammonium chloride induced acute acid load, the initial rate of Na^+-induced recovery of cytosolic pH (pH_i) was measured using 2′,7-bis(2-carboxyethyl)-5(6) carboxyfluorescein-AM (BCECF-AM; Molecular Probes Inc., Eugene, Ore.). Recovery was in the presence of 135 mmol/L NaCl. There was no difference in the buffering capacities of stable cell lines. Where indicated, the activity of the NHE mutants was corrected for the level of protein expression and for the targeting of the protein to the cell surface. Results are shown as mean ± SE and statistical significance was determined using the Mann–Whitney *U* test.

To test the effect of MTSET and MTSES on activity of the NHE1 mutants, we used the standard NHE assay with ammonium chloride-induced acidification of the cells. In this case, cells were acidified 2 times as described above. After a first control acidification and recovery, either MTSET or MTSES was added to a final concentration of 10 mmol/L for 10 min in Na^+-free buffer. The cells were subsequently washed 3 times in Na^+-free buffer prior to the second ammonium chloride-induced acidification and recovery. To calculate residual activity, the following formula was used:

$$\% \text{ residual activity} = \frac{\text{pH change after (Reagent)}}{\text{pH change without (Reagent)}} \times 100\%$$

Results

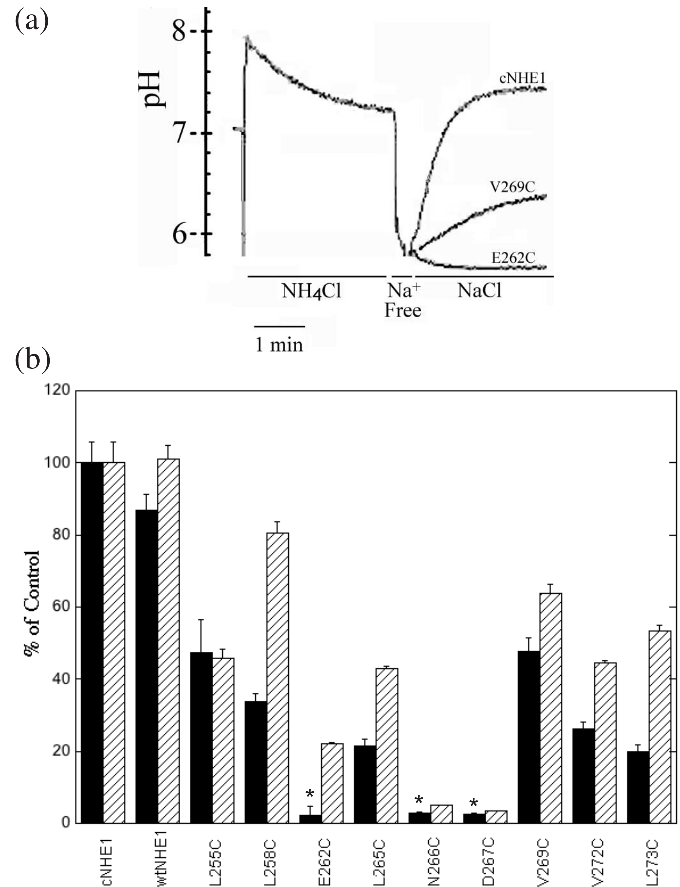
Activity and expression of NHE1 mutants

Figure 1 illustrates a general model of the NHE (Fig. 1A) (Wakabayashi et al. 2000) and a schematic model illustrating TM VII (Fig. 1B). To examine which amino acids of TM VII are critical to activity of the NHE and which amino acids are pore-lining, we used the cysteineless NHE1 (cNHE1), which we previously determined functions normally (Slepkov et al. 2005). Nine amino acids of TM VII were mutated to cysteine residues in the background of the cysteineless NHE1 protein. These amino acids were chosen because they represent a cross section through the transmembrane segment and were previously shown to be important in NHE1 function (Murtazina et al. 2001; Slepkov et al. 2005).

Experiments examined whether these mutant forms of the NHE expressed and targeted properly and had activity. Figure 2A shows a representative Western blot of the expression of the cysteineless NHE1, the wild-type, and the cysteine-scanning mutants expressed in AP-1 cells. The expression levels of the E262C and the N266C mutants were reduced greatly compared with that of cysteineless NHE1. There was also a slight decrease in expression of the V272C mutant. It was notable that most of the E262C mutant was present as a lower molecular mass-deglycosylated or partially glycosylated form.

We examined intracellular targeting of the NHE1 expressing cell lines within AP-1 cells since we have shown that

Fig. 3. Na^+/H^+ exchanger (NHE) activity of AP-1 cells stably transfected with cysteineless NHE1 (cNHE1), and Na^+/H^+ exchanger mutants (L255 to L273) individually changed to Cys. NHE activity was measured after transient induction with an acid load. (A) Examples of measurement of NHE activity of cysteineless NHE1 (cNHE1), NHE1 with a V269C mutation, and NHE1 with an inactive mutant, E262C. Bars indicate the presence of ammonium chloride, Na^+-free, and NaCl-containing solutions. Traces are shown for the entire ammonium chloride treatment and recovery of cNHE1. For clarity, only the recovery from acidosis is shown for V269C and E262C. Lines are slightly offset for easier viewing. (B) Summary of activity of cNHE1 stably transfected with NHE1 activity set to 100%. All mutations to cysteine were done in the background of cNHE1 and activities are percent of those of cNHE1. All results are mean ± SE of at least 6 determinations from 2 independently made stable cell lines. Results are shown for mean activity both uncorrected (solid bars) and normalized for surface processing and expression (hatched). *Indicates mutants with unnormalized activity that is <20% of cNHE1.



mutation of transmembrane amino acids can affect surface targeting of the NHE (Slepkov et al. 2005). After cells were treated with sulfo-NHS-SS-biotin, labeled proteins of lysates were bound to streptavidin-agarose beads. Western blotting with anti-HA antibody examined equal amounts of total cell lysates and unbound lysates to identify the relative amounts of tagged NHE1 protein. Figure 2B illustrates examples of the results and a summary of quantification of at least 6 experiments. Both the 110 and 95 kDa bands were included in the analysis. The results showed that in several cases mutation of the amino acids to cysteine caused a decrease in the

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Fig. 4. Effect of MTSET and MTSES on activity of cysteineless Na^+/H^+ exchanger (cNHE1) and cysteine mutant NHE1-containing cell lines. NHE activity was measured after acid load. Cells were then treated with 10 mmol/L reagent before another transient acidification. Example of effect of MTSET on NHE activity of (A) cNHE1 and (B) L255C NHE1 proteins. Traces are shown for the entire ammonium chloride treatment and recovery of cNHE1 and L255C in the absence of MTSET. For clarity, only the recovery from acidosis is shown in the presence of MTSET. (C) Summary of results which represent the % of activity after the second acid load, in comparison with the first acid load. $*p < 0.05$ and $^+p < 0.01$ second recovery from acid load vs. cNHE1. Filled bars represent MTSES treatments and open bars MTSET treatments.

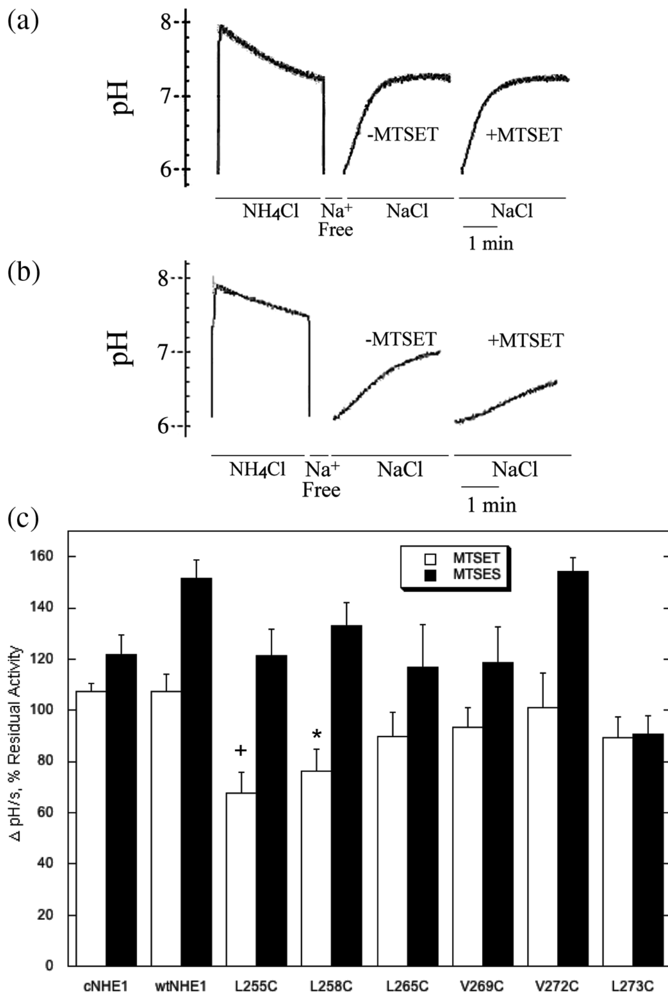
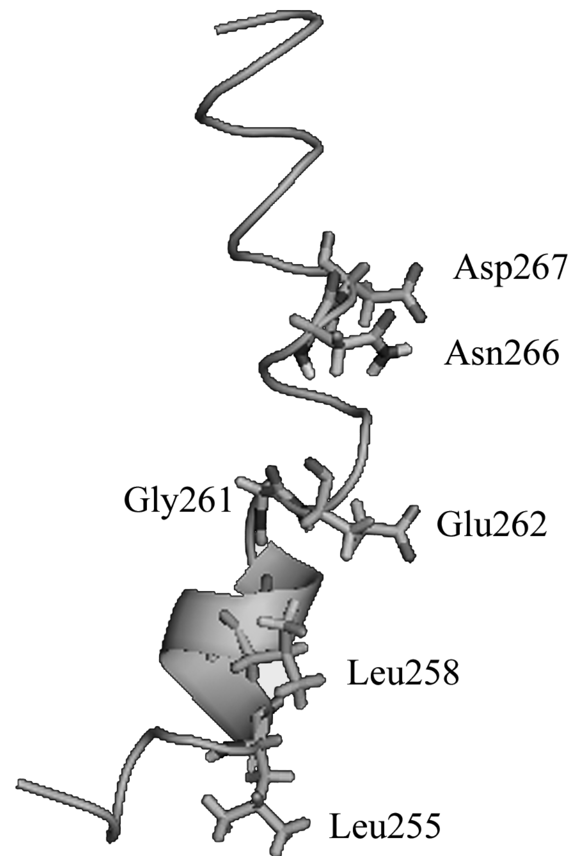


Fig. 5. Representative structure of TM VII of the Na^+/H^+ exchanger isoform 1 (NHE1). The structure was from 2HTG of protein data bank (Ding et al. 2006). Leu 255, Leu 258, Glu 262, Asn 266, and Asp 267 are shown facing 1 side of a possible ion pore of the protein. Gly 261 is shown as a reference point.



targeting of the protein to the cell surface. Most notable was the E262C mutant, which was now mostly intracellular. The L265C and the L273C mutant proteins were now also targeted much less efficiently to the cell surface. All of the other mutant proteins exhibited a decrease in surface targeting relative to cysteineless NHE1; however, the decreases were not as large in magnitude as that of Glu 262, Leu 265, and Leu 273. Nonspecific binding of NHE1 protein to streptavidin agarose beads was ~11%, so the values shown overestimate the level of surface protein.

As part of our analysis of the effect of the mutations, we determined how the mutations of the protein affected the activity. The rate of recovery from an acute acid load induced by ammonium chloride was determined as described earlier (Li et al. 2004). Figure 3 illustrates the rates of recovery from stable cell lines transfected with either wild-type NHE or mutants of TM VII. Figure 3A shows examples of the cysteineless NHE1 with full activity, of V269C with partial activity, and of the near inactive E262C. Figure 3B shows a summary of the results. The rate of recovery is also shown when corrected for both the level of expression and surface targeting so that we could determine whether the mutations affect the activity of the protein directly or indirectly. Three of the mutants were severely affected by mutation to cysteine: Glu 262, Asn 266, and Asp 267. Correction for surface targeting and expression improved the relative activity of all these mutants, particularly in the case of Glu 262. However, all 3 had less than 20% (uncorrected) activity of the cysteineless NHE1 protein. Because of their low NHE1 activity, these mutants were not useful for any further experiments.

We examined the sensitivity to MTSET or MTSES of the mutant NHE that had greater than 20% residual activity of the cysteineless NHE1 (Fig. 4). Figure 4A and 4B show representative results of mutants that were either unaffected or affected by MTSET, respectively. Figure 4C shows a summary of the results. MTSES caused slight stimulations of activity of both the wild-type NHE1, the cysteineless NHE1, and some of the other mutant proteins. The positively

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charged MTSET significantly inhibited the mutant NHE L255C and L258C, whereas the negatively charged reagent MTSES did not result in significant reductions in their activity. For the L273C mutant, MTSET and MTSES resulted in some slight reductions in activity but these were not significantly different from cNHE1.

Discussion

TM VII (residues Ile 251–Leu 273) of the NHE1 isoform has been implicated in ion transport and inhibitor binding properties of NHE (Ding et al. 2006). Using alanine-scanning mutagenesis, we showed that a number of amino acid side chains are sensitive to substitution in the intact protein. Substitution of amino acids Leu 255, Leu 258, Glu 262, Asn 266, and Asp 267 with alanine resulted in significant decreases in activity of the NHE1 protein (Ding et al. 2006). Leu 273, Val 272, Leu 255, and Leu 265 also showed significant changes in sensitivity to inhibition by EMD87580, indicating they may be important in protein function (Ding et al. 2006). We have also shown that conservation of the charge at amino acids Glu 262 and Asp 267 is critical for NHE activity. For this reason, and because of a helical wheel analysis of TM VII that suggested that these residues might be pore lining (not shown), we chose these amino acids and Val 269 for analysis by cysteine-scanning mutagenesis in combination with reaction with sulfhydryl reagents. Cysteine-scanning mutagenesis uses the fact that the sulfhydryl moiety is the most reactive functional group in a protein (Mattson et al. 1993). It can determine pore-lining residues in membrane proteins of various types (Akabas et al. 1992; Doering et al. 1998; Dunten et al. 1993; Slotboom et al. 2001; Yan and Maloney 1995). Two sulfhydral reactive reagents that are typically used in these studies are MTSET and MTSES (Stauffer and Karlin 1994), which are membrane impermeant (Holmgren et al. 1996; Liu and Siegelbaum 2000). They react with pore lining residues surrounded by water and cannot reach with residues within the hydrophobic bilayer. MTSET is positively charged and MTSES is negatively charged.

Initially, we tested to determine whether mutation to cysteine affected activity and targeting of the mutated amino acids. Mutation of amino acids Glu 262, Asn 266, and Asp 267 to cysteine eliminated most of the activity of the protein. Whereas this negated the possibility of doing further experiments with these mutants, it further confirmed the importance of these specific amino acids at these locations. When the remaining mutants were examined for reactivity with MTSET and MTSES, Leu 255 and Leu 258 were significantly inhibited with the positively charged MTSET but not with negatively charged MTSES. Reactivity with MTSET supports the hypothesis that these amino acids are facing towards the pore of the protein. The fact that the negatively charged MTSES did not inhibit NHE1 function is in keeping with the idea that these amino acids face towards, or nearly towards, the pore. A positively charged reagent might be expected to inhibit transport of a cation by repelling it, whereas a negatively charged reagent might not have this effect. In fact, negatively charged amino acids have been suggested to be important in ion coordination of NHE proteins (Dibrov and Fliegel 1998) and negative charges on the outer vestibule of K^+ channels are thought to increase the flow of positively charged ions, facilitating transport (Haug et al. 2004).

We earlier elucidated the structure of TM VII (Ding et al. 2006). This transmembrane segment was predominantly alpha helical with a break in the helix at the functionally critical residues of Gly 261 and Glu 262. Since the structure was determined as a peptide that rotates at the break at amino acid 261 and 262, it was not possible to orient the side chains above and below the break to each other. Figure 5 shows the representative structure of TM VII. The functionally important residues Asp 267, Asn 266, and Glu 262 are indicated. Gly 261 is indicated as a reference point for the previously demonstrated break in structure. Below Gly 261, the residues that were reactive with MTSET, Leu 258 and Leu 255, are indicated. We suggest that they face the pore of the NHE through which Na^+ and H^+'s are transported. The residues Asp 267, Asn 266, and Glu 262 are shown in a similar face of the transmembrane segment. Because of the break in structure at amino acid Gly 261, it is not possible to confirm this from the structural data alone. However, the critical nature of these amino acids suggests that they may lie on the same or similar plane to that of Leu 258 and Leu 255 as illustrated. We propose that these 5 amino acids are therefore on a similar side of this transmembrane segment that faces the ion transducing pore of the NHE1 protein.

Our study gives the information on the pore-lining residues of TM VII. Combined with our earlier structural data on the protein, our data suggests that amino acids Leu 255, Leu 258, Glu 262, Asn 266, and Asp 267 line the pore of the protein. We have earlier shown that Phe 161 of TM IV also faces the pore of theNHE. Future studies will elucidate the balance of pore-lining residues and give us a clearer picture of how this critical protein functions.

Acknowledgements

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