Functional role of arginine 425 in the mammalian Na\(^+/\)H\(^+\) exchanger\(^1\)
Xiuju Li, Yike Ma, and Larry Fliegel

Abstract: Na\(^+/\)H\(^+\) exchanger isoform 1 (NHE1) is the principal plasma membrane Na\(^+/\)H\(^+\) exchanger of mammalian cells and functions by exchanging one intracellular proton for one extracellular sodium ion. Critical transmembrane segments of Na\(^+/\)H\(^+\) exchangers have discontinuous transmembrane helices, which result in a dipole within the membrane. Amino acid R425 has been suggested to play an important role in neutralizing one such helix dipole. To investigate this hypothesis, R425 was mutated to alanine, glutamine, histidine, or lysine and the mutant NHE1 proteins were expressed and characterized in NHE1-deficient cells. The R425A and R425E mutants exhibited complete loss of expression of mature, fully glycosylated NHE1, reduced expression overall, and greatly reduced cell surface targeting. The cell surface targeting, expression, and activity of the R425H and R425K mutant proteins were also impaired, though residual NHE1 activity remained. When reduced targeting and expression were accounted for, the R425H and R425K mutant proteins had activity similar to that of the wild-type protein. The results suggest that R425 is critical for NHE1 expression, targeting, and activity and that replacement with another basic residue can rescue activity. The findings are consistent with a role for R425 in both neutralizing a helix dipole and maintaining NHE1 structure and function.

Key words: Na\(^+/\)H\(^+\) exchanger, pH regulation, transmembrane segment, helix dipole.

Résumé: L’isoforme 1 de l’échangeur Na\(^+/\)H\(^+\) (NHE1) est le principal échangeur Na\(^+/\)H\(^+\) de la membrane plasmatique des cellules de mammifères et elle fonctionne en échangeant un proton intercellulaire pour un sodium extracellulaire. Des segments transmembranaires cruciaux des échangeurs Na\(^+/\)H\(^+\) comportent des hélices transmembranaires discontinues, ce qui résulte en la formation d’un dipôle à l’intérieur de la membrane. On a suggéré que l’acide aminé R425 joue un rôle important dans la neutralisation d’un tel dipôle de l’hélice. Afin vérifier cette hypothèse, le R425 a été muté en alanine, glutamine, histidine ou lysine, et les mutants de NHE1 ont été exprimés et caractérisés dans des cellules dépourvues de NHE-1. Les mutants R425A et R425E présentaient une perte complète d’expression de NHE1 pleinement glycosylé, une réduction globale réduite et un ciblage à la surface cellulaire grandement réduit. Le ciblage à la surface cellulaire, l’expression et l’activité des protéines mutantes R425H et R425K étaient aussi affectés mais une activité NHE1 résiduelle demeurait. Après correction sur le plan de la réduction du ciblage et de l’expression, les protéines mutantes R425H et R425 K présentaient une activité similaire à celle de la protéine sauvage. Les résultats suggèrent que le R425 est crucial à l’expression, au ciblage et à l’activité de NHE1, et que son remplacement par un autre résidu basique peut préserver l’activité. Ces données sont en cohérence avec le rôle du R425 dans la neutralisation du dipôle de l’hélice et le maintien de la structure et de la fonction de NHE1. [Traduit par la Rédaction]

Mots-clés: échangeur Na\(^+/\)H\(^+\), régulation du pH, segment transmembranaire, dipôle de l’hélice.

Introduction

The Na\(^+/\)H\(^+\) exchanger is a pH regulatory plasma membrane protein present in all mammalian cells. The NHE1 isoform of the protein is an integral membrane protein. It has a membrane domain that transports ions and a cytoplasmic C-terminal domain that regulates activity. NHE1 functions to maintain pH homeostasis in normal and neoplastic cells. It has an important role in promoting cell growth, proliferation, differentiation, and apoptosis. In transport by the Na\(^+/\)H\(^+\) exchanger, one intracellular proton is extruded in exchange for one extracellular sodium ion. The Na\(^+\) gradient drives transport (Kemp et al. 2008; Lee et al. 2013a).

Human NHE1 is 815 amino acids long. The N-terminal 500 amino acids form the membrane domain responsible for ion transport. The C-terminal 315 amino acids form a cytosolic regulatory domain (Lee et al. 2013a). Although the complete structure of human NHE1 is not known, insights into its structure and function have been gained from studies of the protein, its fragments, and related Na\(^+/\)H\(^+\) exchangers (reviewed in Lee et al. 2013a). Early studies using the cysteine scanning accessibility method predicted a topology of 12 transmembrane segments (Wakabayashi et al. 2000). NHE1 is distantly related to the bacterial Na\(^+/\)H\(^+\) exchanger NhaA, and the crystal structure of NhaA has been solved (Hunte et al. 2005). A key feature of NhaA is that 2 of the 12 transmembrane segments, TM 4 and TM 11, contain extended regions in their center and cross each other, generating a novel fold. Key residues of this novel fold include negatively charged D133 on TM 4 and positively charged K300 on nearby TM 10. These charged residues are believed to compensate for the helix dipole partial charges introduced by TM 4 and TM 11 in the center of the membrane (Hunte et al. 2005; Kemp et al. 2008; Lee et al. 2013a). Lee et al. (2013b) recently deduced the crystal structure of the Na\(^+/\)H\(^+\) exchanger NapA from Thermus thermophilus. The NapA and NhaA structures are very similar, with NapA having residue K305.
which is equivalent to K300 of NhaA and functions similarly to neutralize the dipole of a discontinuous helix. In their analysis, they also suggested that residue R425 of human NHE1 plays an equivalent role to these 2 residues. Nygaard et al. (2010) also came to a similar conclusion, aligning R425 with K300 of *E. coli* NhaA.

In this study, we examined the hypothesis that R425 is a key residue of the NHE1 protein and that the positive charge is important for NHE1 function. We found that this residue is critical for NHE1 function but that some substitutions, such as to the basic residue lysine, are partially tolerated. This study, which analyzed multiple R425 mutants and characterized their activity and targeting in detail, is the first complete examination of this critical residue.

**Materials and methods**

**Materials**

The plasmid pYN4+ (Slepkov et al. 2004) contains cDNA for the human NHE1 protein and was used for transient and stable expression. It has a HA (hemagglutinin) tag that allows detection of NHE1 but does not affect NHE1 activity (Li et al. 2004). 2',7'-Bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was purchased from Molecular Probes, Inc. (Eugene, Ore., USA). Sulfo-NHS-SS-biotin was purchased from Pierce. Synthetic DNA was purchased from Integrated DNA Technologies (Coralville, Iowa, USA). Pwo DNA polymerase for site-directed mutagenesis was from Roche. All other chemicals used were of high-quality analytical grade and were from Sigma (St. Louis, Mo., USA), BDH (Toronto, Ont., Canada), or Fisher Scientific (Ottawa, Ont., Canada).

**Cell culture and stable transfection**

To express and characterize wild-type versus mutant Na+/H+ exchangers, we used AP-1 cells. AP-1, a mutant cell line derived from Chinese hamster ovary cells, lacks its own NHE1 protein (Li et al. 2006). Stable or transient transfections were performed with LIPOFECTAMINE® 2000 Transfection Reagent (Invitrogen Life Technologies, Carlsbad, Calif., USA) (Slepkov et al. 2006). Stably transfected cells were selected with G418. The plasmid, pYN4+, contains a neomycin resistance gene (Ding et al. 2006). Cell lines were regularly reestablished from frozen stocks at passage numbers between 5 and 11. Results are typical of at least 2 stable cell lines and of transient transfections.

**Site-specific mutagenesis**

Mutagenesis of NHE1 was carried out using the plasmid pYN4+ as described earlier (Slepkov et al. 2005). Table 1 lists the synthetic oligonucleotides used for mutagenesis. Mutations created a restriction enzyme site for use in screening for mutants. DNA sequencing confirmed the fidelity of DNA amplification and the presence of the mutation.

**Cell surface expression**

To determine targeting of the NHE1 protein to the cell surface, cell surface proteins were labeled with sulfo-NHS-SS-biotin and cells were solubilized (Slepkov et al. 2004). Cell surface proteins (including cell surface NHE1) were bound to immobilized streptavidin resin. Equivalent amounts of unbound and total proteins were separated by SDS-PAGE. Western blotting was used to check for immunoreactive (HA-tagged) Na+/H+ exchanger (NHE1). It was not possible to efficiently and reproducibly elute NHE1 bound to the immobilized streptavidin resin because of the high affinity of streptavidin for biotin. Both the upper and lower HA-immunoreactive species of NHE1 were used for plasma membrane targeting estimations.

**SDS-PAGE and immunoblotting**

NHE1 expression in AP-1 cells was confirmed by immunoblotting using antibodies against the HA tag on the C-terminus of the NHE1 protein. Cell lysates were run on 10% SDS-PAGE gels and then transferred to nitrocellulose membranes. Anti-HA monoclone antibody was the primary antibody used to identify HA-tagged NHE1. The secondary antibody was peroxidase-conjugated goat anti-mouse antibody (Bio/Can, Mississauga, Ont., Canada). Reactive protein was visualized on X-ray film using the Amersham enhanced chemiluminescence western blotting and detection system. Quantification of NHE1 on western blots was done using Image J 1.35 software (National Institutes of Health, Bethesda, Md., USA).

**Intracellular pH measurement**

BCECF was used to measure Na+/H+ exchanger activity and intracellular pH (pHi) after an acute acid load that was induced as described earlier (Slepkov et al. 2005). Cells were grown to approximately 80%-90% confluence on coverslips and loaded with BCECF, and fluorescence was measured using a FTI DeltaScan spectrofluorometer. Acute acidosis was induced by adding 50 mmol/L NH₄Cl for 3 min. After removal of NH₄Cl, recovery (ΔpH/s) in medium containing NaCl was quantified for 20 s. A calibration curve of pH vs. fluorescence was constructed for each sample (Murtazina et al. 2001; Silva et al. 1997; Slepkov et al. 2005). Results are the mean ± S.E. of at least 6 experiments.

**Results**

To characterize amino acid R425 of NHE1, we made several different kinds of mutations and examined the activity, targeting, and expression of the mutant proteins relative to the wild-type protein. Initially, we examined the expression of the wild-type protein and the R425 mutants in AP-1 cells using western blot analysis. Figure 1 illustrates the results of blotting for the HA tag present on the C-terminus of NHE1. As shown earlier, wild-type NHE1 appears as 2 bands. One band represents a protein of approximately 110–120 kDa. The second, smaller band represents

**Table 1. Oligonucleotides used for site-directed mutagenesis of plasmid pYN4+ containing cDNA for NHE1. Mutated nucleotides are indicated in lowercase. Changed amino acids have new codons indicated in boldface. Restriction sites that were introduced are underlined. The forward oligonucleotide of the pair used for mutagenesis is shown.**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Oligonucleotide</th>
<th>Restriction site</th>
</tr>
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<tbody>
<tr>
<td>R425A</td>
<td>CTGCCTCATCGCCgcCTGGTGCGGGGTGCTG</td>
<td>PstI</td>
</tr>
<tr>
<td>R425E</td>
<td>CTGCCTCATCGCCgcCTGGTGCGGGGTGCTG</td>
<td>Ar II</td>
</tr>
<tr>
<td>R425H</td>
<td>CTGCCTCATCGCCCaCTGGTGCGGGGTGCTG</td>
<td>Pml I</td>
</tr>
<tr>
<td>R425K</td>
<td>CTGCCTCATCGCCaaCTGGTGCGGGGTGCTG</td>
<td>Sca I</td>
</tr>
<tr>
<td>R425W</td>
<td>CTGCCTCATCGCCggCTGGTGCGGGGTGCTG</td>
<td>Bgl II</td>
</tr>
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partially or completely deglycosylated NHE1 (Slepkov et al. 2005). When we examined the expression levels of the various R425 NHE1 mutants, we found that the expression was reduced in all cases to 40%–18% of the wild-type NHE1 expression level. The R425A and R425E mutants were expressed almost exclusively as lower molecular weight, deglycosylated protein. Small amounts of the R425H and R425K mutant proteins were fully glycosylated.

We have previously mutated the NHE1 protein to study its structure and function (Ding et al. 2006; Slepkov et al. 2005). Some mutations result in mistargeting of the NHE1 protein, leading to elevated intracellular levels of NHE1. To determine whether the R425 mutations resulted in aberrant targeting of NHE1, we quantified cell surface targeting of the mutant proteins in comparison with wild-type NHE1. The results are shown in Fig. 2. Approximately 73% of wild-type NHE1 was targeted to the cell surface. In contrast, cell surface targeting of all of the mutant proteins was reduced, from a minimal level of 10% for the R425A mutant to approximately 50% for the R425K mutant.

We then characterized the effect of the mutations on NHE1 activity in stable cell lines. Figure 3A shows examples of NHE1 activity in various cell types. For ease of viewing, only the recovery from acidosis is shown for the mutant NHE1 proteins. NH₄Cl, treatment with ammonium chloride. After NH₄Cl treatment, there was a brief “Na Free” treatment to induce acidosis. NaCl, recovery from acidosis in buffer containing NaCl. (B) Summary of activity of WT and R425 mutant NHE1 proteins in stably transfected AP-1 cells. Results are the change in intracellular pH per second. Black bars indicate uncorrected activity (relative to control). Light gray stippled bars indicate activity corrected for differences in surface processing. Medium gray stippled bars indicate activity corrected for differences in expression. Dark stippled bars indicate activity corrected for both expression and surface processing relative to the WT protein. Mean wild-type NHE1 activity was 0.0208 ΔpH/s. n = at least 6 determinations. AP-1, mock-transfected AP-1 cells. Mutant cell lines are indicated.

Fig. 4. Analysis of NHE1 activity of transiently transfected AP-1 cells expressing wild-type (WT) or R425 mutant NHE1 proteins. Plasmids containing cDNA for WT NHE1 protein or the indicated mutants were transiently transfected into AP-1 cells, and cells were assayed for Na⁺/H⁺ exchanger activity. AP-1, mock-transfected AP-1 cells. n = at least 6 determinations.

### Figure 2
Surface localization of NHE1 in AP-1 cells expressing wild-type (WT) or R425 mutant NHE1 proteins. Equivalent amounts of the total cell lysate (T) and the unbound intracellular lysate (I) were examined by western blotting with anti-HA-tag antibody to identify the tagged NHE1 protein. Lysates from cell lines expressing WT or mutant NHE1 proteins are shown. Numbers indicate the mean ± S.E. n = at least 3 determinations. The arrow indicates the position of full-length glycosylated NHE1 protein.

### Figure 3
Analysis of NHE1 (Na⁺/H⁺ exchanger) activity of stable cell lines expressing wild-type (WT) or R425 mutant NHE1 proteins. NHE1 activity was assayed in AP-1 cells stably expressing the indicated mutants, as described in the Materials and methods section. (A) Examples of NHE1 activity in various cell types. For ease of viewing, only the recovery from acidosis is shown for the mutant NHE1 proteins. NH₄Cl, treatment with ammonium chloride. After NH₄Cl treatment, there was a brief “Na Free” treatment to induce acidosis. NaCl, recovery from acidosis in buffer containing NaCl. (B) Summary of activity of WT and R425 mutant NHE1 proteins in stably transfected AP-1 cells. Results are the change in intracellular pH per second. Black bars indicate uncorrected activity (relative to control). Light gray stippled bars indicate activity corrected for differences in surface processing. Medium gray stippled bars indicate activity corrected for differences in expression. Dark stippled bars indicate activity corrected for both expression and surface processing relative to the WT protein. Mean wild-type NHE1 activity was 0.0208 ΔpH/s. n = at least 6 determinations. AP-1, mock-transfected AP-1 cells. Mutant cell lines are indicated.

### Figure 4
Analysis of NHE1 activity of transiently transfected AP-1 cells expressing wild-type (WT) or R425 mutant NHE1 proteins. Plasmids containing cDNA for WT NHE1 protein or the indicated mutants were transiently transfected into AP-1 cells, and cells were assayed for Na⁺/H⁺ exchanger activity. AP-1, mock-transfected AP-1 cells. n = at least 6 determinations.
of R425A and R425E. The R425H and R425K mutants possessed some activity but the uncorrected activity was less than 15% of the wild-type protein. The activity of all of the mutant proteins was too low to perform detailed analysis of the kinetics of NHE1 activation.

Since we had observed that the mutants had reduced targeting and expression levels, we corrected the mutants’ activity to determine whether the measured activity was due to these factors or to a defective protein. After correction of the activity of mutants R425A and R425E for targeting, expression, or both, the activities were still severely impaired (Fig. 3B). For the R425H and R425K mutants, correction of the activity indicated that the proteins had intrinsic NHE1 activity comparable to that of the wild-type protein.

To confirm the effects we observed in the stable cell lines, we used AP-1 cells transiently transfected with plasmids containing cDNA for the wild-type NHE1 protein or the mutants. The results are shown in Fig. 4. The pattern of NHE1 activity was the same as that of the stably transfected cells. The R425A and R425E mutants were barely active. The activity of the R425H and R425K mutants was significantly higher but still greatly reduced in comparison with the wild-type activity. In another series of experiments, using transiently transfected cells, we examined the effect of mutating R425 to tryptophan. We found that this substitution resulted in complete abolition of NHE1 activity (not shown).

Discussion

The NHE1 isoform of the Na+/H+ exchanger is a critical membrane protein that is involved in pH regulation, cell growth, and...
heart hypertrophy (Amith and Fliegel 2013; Fliegel 2005, 2009). It is also critical in metastasis of cancer cells (Amith and Fliegel 2013; Boedtkjer et al. 2012; Reshkin et al. 2014). While the structures of the NhaA and NapA Na+/H+ exchangers have been elucidated, studies of the mammalian NHE1 isofrom are less advanced because of the resistance of the protein to expression and crystallization (Moncoq et al. 2008). Both NapA and NhaA have interrupted helices in locations critical for function (Hunte et al. 2005; Lee et al. 2013b). As noted above, the interrupted helices form a dipole that must be neutralized within the membrane to stabilize the structure of the Na+/H+ exchanger. This is accomplished by amino acids K300 and K305 in NhaA and NapA, respectively (Hunte et al. 2005; Lee et al. 2013b). While it has been hypothesized that amino acid R425 is important in NHE1 in this regard (Lee et al. 2013b; Nygaard et al. 2010), this hypothesis has not been well tested.

R425 resides in the middle of putative transmembrane segment 10 of human NHE1. The residue’s location within the protein makes it a likely candidate for neutralization of a helix dipole. Nygaard et al. (2010) examined the effect of mutating R425 to alanine and found that there was a marked reduction in plasma membrane targeting and ion transport; however, further characterization of the effect of this mutation and mutations to other residues was not done. Here we generated multiple R425 mutations and examined the effects of these mutations to characterize this site. We confirmed that mutation to alanine markedly reduced transport and targeting of NHE1 to the plasma membrane. We also report the novel observations that total expression levels of the R425A protein are greatly reduced and that this mutant is present as a non-glycosylated protein. We further demonstrated that changing the positively charged R425 residue to negatively charged glutamate had similar effects on expression, targeting, and function (as did mutation to a bulky tryptophan residue). Of interest were the intermediate effects of mutation of R425 to histidine or lysine. Both of these mutations had strong effects on activity, targeting, and expression, though mutation to lysine had the least detrimental effects. When the activities of the R425K and R425H proteins were corrected for the strong effects of the mutations on targeting and expression levels, the activities of the proteins were close to wild-type. Therefore, the mutant NHE1 proteins themselves were functional and the low activities were mostly due to the low amounts of protein present on the cell surface. Substitution of lysine for arginine conserves the positive charge, whereas replacement with negatively charged glutamate does not. The situation with histidine is uncertain. This amino acid has a pKₐ of approximately 6.0, but this can vary depending on the local environment, so this residue could also be positively charged. The effect of histidine was not simply due to insertion of a bulkier residue, as substitution with tryptophan was not complimentary to activity. Overall, these experiments are consistent with the hypothesis that a positive charge at this position is required for activity and that this amino acid functions to neutralize a helix dipole.

Figure 5 shows a model of R425, along with transmembrane segments IV, X, and XI, within the lipid bilayer. The structure is from the model of Landau et al. (2007). R425 is placed near the end of a helical segment of transmembrane segment XI near amino acid G455. In this position it could act similarly to K300 and K305 of NhaA and NapA, respectively (Hunte et al. 2005; Lee et al. 2013b). While the model of Landau et al. has not been verified, it provides support for this putative role of R425.

The observation that mutation of R425 to any amino acid resulted in marked effects on targeting and expression is an indication that this amino acid is critical to the structure of NHE1. We have previously shown that mutation of amino acids in critical locations of NHE1 transmembrane helices tends to disrupt the targeting and expression of the protein (Lee et al. 2009; Tzeng et al. 2010, 2011). It is likely that disruption of these critical residues leads to recognition of an aberrant structure by the cellular quality control system, leading to the protein’s retention, as occurs with the cystic fibrosis transmembrane regulator (Amalar 2004; Choi et al. 2005; Sharma et al. 2004). Future studies will examine this phenomenon and potential mutations of NHE1 in the human genome.

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