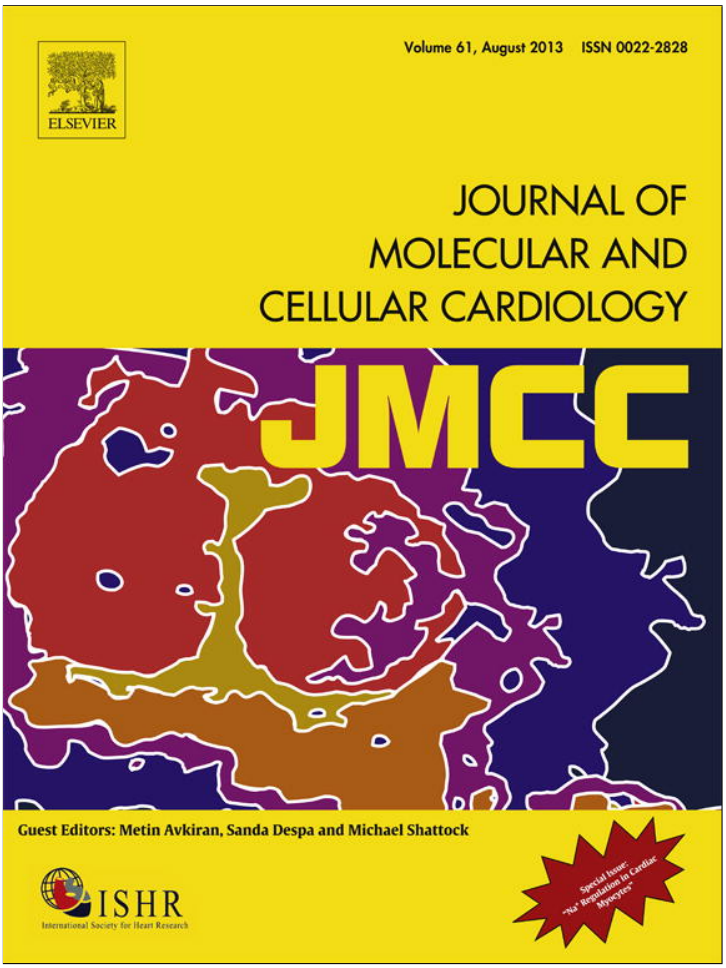

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Original article

Structural changes in the C-terminal regulatory region of the Na^+/H^+ exchanger mediate phosphorylation induced regulation ☆

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ABSTRACT

The Na^+/H^+ exchanger isoform 1 (NHE1) is a plasma membrane pH regulatory protein that removes one intracellular H^+ in exchange for an extracellular Na^+. NHE1 is regulated by phosphorylation of the cytoplasmic regulatory region and amino acids Ser^{770} and Ser^{771} of the regulatory domain are necessary for activation by sustained intracellular acidosis. The phosphomimetic mutations (S770D/S771D) resulted in an activated form of the protein. Immunoprecipitation of full length NHE1 protein showed that the phosphomimetic mutant had increased sensitivity to digestion with trypsin indicating a conformational change. Tryptic digestion of purified C-terminal regulatory region showed that the S770D/S771D mutation altered sensitivity to trypsin digestion. Wild type and phosphomimetic purified C-terminal region (577–815) of human NHE1 were compared and tryptophan fluorescence indicated that there were pH-dependent differences in the conformation of the proteins. Native polyacrylamide gel electrophoresis demonstrated that the phosphomimetic mutant had a more compact structure. Bottom-up hydrogen/deuterium exchange mass spectrometry demonstrated that a peptide fragment containing the phosphomimetic mutations became strongly stabilized relative to the wild type protein. Overall, the results suggested that phosphorylation of S770/S771 changes the conformation of the C-terminal regulatory region in a pH-dependent manner, resulting in a more compact region that affects NHE1 activity. This article is part of a Special Issue entitled "Na^+ Regulation in Cardiac Myocytes".

1. Introduction

The Na^+/H^+ exchanger (NHE) is a ubiquitously expressed plasma membrane protein that regulates intracellular pH (pHi). In mammalian cells it extrudes one intracellular H^+ in exchange for one extracellular Na^+ [1,2]. The Na^+/H^+ exchanger has two major domains. An N-terminal membrane domain of approximately 500 amino acids is followed by a 315 amino acid, hydrophilic, carboxyl-terminal cytosolic domain or tail. The cytosolic tail regulates the membrane domain.

NHE1 is regulated such that it is active at acidic pH values but inactive at neutral physiological pH [3]. Hormonal stimulation of NHE1 activity changes the set point through phosphorylation so that the protein is more active at more alkaline pH values [3,4]. This activation of NHE1 is important in cell growth, and differentiation [5–7]. It is also important in activation of the protein that occurs in cardiovascular disease [2,4,8].

Phosphorylation occurs in the distal 178 amino acids of the regulatory tail [9,10]. Several sites of phosphorylation have been identified on the C-terminal regulatory region. In both CHO cells and in isolated cardiomyocytes, we showed that amino acids Ser^{770} and Ser^{771} are critical to NHE1 activation by sustained acidosis through an ERK1/2 dependent pathway [11,12]. In other reports we showed that amino acids Thr^{717}, Ser^{722}, Ser^{725} and Ser^{728} are phosphorylated in vitro by a p38 mitogen-activated protein kinase dependent pathway [13] and that Ser^{726} and Ser^{729} mediate activation of NHE1 by apoptosis in CHO cells [14]. Others have shown that in vascular smooth muscle cells serum activates Erk1/2, which activates $p90^{rsk}$ and phosphorylates NHE1 at Ser^{703} [15,16].

While some of the mechanisms by which NHE1 is phosphorylated are becoming clearer, how phosphorylation activates the membrane domain is unexplored. The C-terminal regulatory region of the NHE1 protein has been shown to be intrinsically disordered, but contains conserved regions of transient structure [17]. In this study, we examined changes in conformation of the C-terminal regulatory region that occur with phosphorylation, in an effort to determine the mechanism by which phosphorylation alters the activity of the NHE1 protein. We demonstrate that phosphorylation induces a conformational change in the tail of the Na^+/H^+ exchanger, which may be responsible for changes in NHE1 activity.

☆ The abbreviations used are: BCECF-AM,2′,7′-bis(2-carboxyethyl)-5(6) carboxyfluorescein-acetoxymethyl ester; CHO, Chinese Hamster Ovary; HA, hemagglutinin; NHE1, Na^+/H^+ exchanger type 1 isoform; pHi, intracellular pH; TM, transmembrane segment; WT, wild type.

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2. Materials and methods

2.1. Materials

Geneticin was purchased from American Bioanalytical (Natick MA, USA). Cell culture MEM alpha modification medium was from Thermo Scientific Hyclone (Logan UT, USA). Lipofectamine™ 2000 was purchased from Invitrogen Life Technologies (Carlsbad CA, USA). 2′,7′-Bis(2-carboxyethyl)-5(6) carboxyfluorescein-acetoxymethyl ester was purchased from Molecular probes, Inc. (Eugene, OR, USA). PWO DNA polymerase was purchased from Roche Applied Science (Roche Molecular Biochemicals, Mannheim, Germany). Restriction enzymes were obtained from New England Biolabs, Inc. (Mississauga, ON, Canada). Sulpho-NHS-SS-Biotin was from Pierce Chemical Company (Rockford, IL, USA). Immobilized streptavidin resin, nigericin and trypsin and maltose (phenylalanyl chloromethyl ketone–trypsin) and U0126 were from Sigma-Aldrich (St. Louis, MO, USA). Amylose was purchased from New England BioLabs (Pickering, ON, Canada). Ni-NTA resin was obtained from QIAGEN (Toronto, ON, Canada).

2.2. Site-directed mutagenesis

Silent mutation restriction enzyme sites were introduced during site directed mutagenesis as described earlier [18]. The plasmid pYN4+ was used as a template. It encodes for the entire human NHE1 cDNA with a hemagglutinin (HA) tag at the C-terminal of the protein [18]. HA-tagged NHE1 functions the same as wild type NHE1 [19]. The oligomers for site directed mutagenesis are indicated in Table 1. DNA sequencing was used to confirm mutations and fidelity of amplification and was performed at the University of Alberta, Department of Medicine, Applied Genomics Centre.

2.3. Cell culture and transfections

Mutant forms of NHE1 were stably expressed in the AP1 cell using the plasmidpYN4+ [18,19]. AP1 cells do not express endogenous active NHE1. Stably transfected cells were established using LIPOFECTAMINE™ 2000 Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). pYN4+ plasmid, which expresses hemagglutinin (HA) tagged NHE1,encodes a neomycin resistance gene, which allows the selection of transfected cells using geneticin (G418) antibiotics. Stable cell lines for experiments were used at passage numbers between 5 and 11.

2.4. SDS-PAGE and immunoblotting

To examine NHE1 levels in cultured cells, cells were grown in 60 or 35 mm dishes until 80–90% confluent, and then were harvested as described earlier [20]. Growth medium was removed by aspiration and cell monolayers were washed with cold (4 °C) phosphate-buffered saline. RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% Triton X-100, 5 mM EGTA, 0.1 mM PMSF, 0.1 mM benzamidine, and a protease inhibitor cocktail [21]) was added for 1–3 min. Cell debris was removed by centrifugation at 14,000 rpm for 5 min at 4 °C. The supernatants were either frozen for storage, or prepared for subsequent trypsin treatment and SDS-PAGE. Cell lysates containing NHE1 were examined using 10% SDS/polyacrylamide gels which were subsequently transferred onto nitrocellulose membranes. Immunoblotting followed and was with anti-HA monoclonal antibody as described earlier [18]. The secondary antibody was peroxidase-conjugated goat anti-mouse antibody. NHE1 was visualized by enhanced chemiluminescence, and X-ray films (Fuji medical X-ray film) were processed by Kodak X-OMAT 2000 M35 processor. To quantify band intensities, ImageJ 1.35 software (National Institutes of Health, Bethesda, MD, USA) was used where indicated.

2.5. Cell surface expression

Cell surface expression was measured essentially as described earlier [19]. Briefly, the surface proteins of intact cells were labeled with sulfo-NHS-SS-biotin (Pierce) and immobilized streptavidin resin was used to remove solubilized plasma membrane Na^+/H^+ exchanger. Equal amounts of total and unbound proteins were separated by SDS-PAGE and were analyzed by Western blotting followed by densitometry to measure immunoreactive (HA-tagged) Na^+/H^+ exchanger protein. It was not possible to efficiently and reproducibly elute proteins bound to immobilized streptavidin resin. The relative amount of NHE1 on the cell surface was calculated by comparing both the 110- and 95-kDa HA-immunoreactive species in Western blots of the total and unbound fractions.

2.6. Measurement of NHE activity

NHE1 activity was determined using a PTI Deltascan spectrofluorometer essentially as described earlier [7,19]. Experiments were performed at 37 °C and all solutions and relevant equipment was pre-warmed to 37 °C. NHE1 mutants were grown on coverslips (THOMAS® Red Label® MICRO COVER GLASSES) in 35 mm dishes until approximately 80–90% confluent. Cells were then incubated in 400 μl serum free α-MEM medium containing 1.875 μg/ml 2′,7′-bis(2-carboxyethyl)-5(6) carboxyfluorescein-acetoxymethyl ester (BCECF-AM; Molecular Probes Inc., Eugene, OR, USA) at 37 °C for 20 min to allow BCECF-AM to penetrate into the cell. Intracellular pH was measured in normal buffer pH 7.3 (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, 10 mM HEPES), 50 mM ammonium chloride was added to the cells to induce an acidification after its withdrawal 3 min later. After this time the solution was changed to sodium free buffer pH 7.3 (135 mM N-methyl glucamine, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, 10 mM HEPES) for 30 s. Cells were then returned to normal sodium containing buffer, allowing pH recovery via

Table 1
Oligonucleotides used for site-directed mutagenesis (A) and cloning (B). A, Nucleotides mutated are indicated in lower case, and restriction sites introduced are underlined.

Mutation	Oligonucleotide sequence	Site
A		
S703D	5′-GGGCCCGCATCGGCgacGACCCgCTaGCCTATGAGCCG-3′	Nhe I
S726/729D	5′-CGGCTTCCCCGCAGgatCCgGAGgaTGTGGACCTGGTG-3′	BspEI
S770/771D	5′-CGGAGCAAGGAGACTgatgaCCCgGGAACCGACGATGTC-3′	Sma I
S770/771A	5′-GCATCATGATGCGGGCCAAGGAGACTTCGGCCC-3′	Bsa JI
B		
FP1	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACCATGTACCACAAGATGGAGATGAAGCAGGCC	
RP1	5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACTATTAGTGATGGTGATGGTGATGCTGCCCCTTGGGGAAGAACGGTTCTCC	
FP2	5′-ACACACAAGATCTGGCGGTGGCGGTGGCGGTATGTACCACAAGATGGAGATGAAG	
RP2	5′-TAATGCGAATTCCTACTATTAGTGATGGTGATGGTG	

the activity of NHE1. The first 20 s of recovery was used to calculate the NHE1 activity. Each measurement of NHE1 activity was followed by a 3 point pHi calibration at pH values of 6, 7, and 8 with 10 μM nigericin in calibration buffer (5 mM N-methyl glucamine, 135 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, 10 mM HEPES).

We used a double pulse assay to examine NHE1 activity in AP1 cells stable transfected with wild type and mutant NHE1 essentially as described earlier [11,12]. Cells were treated with ammonium chloride two times, and allowed to recover in NaCl containing medium after each treatment. After the first treatment, cells were rinsed with Na^+-free medium for 10–20 s allowing the pHi to stabilize. NaCl was then added to allow for intracellular pH recovery. The second pulse was similar except cells were maintained in Na^+-free medium for 3 min to cause sustained acidosis prior to addition of 135 mM NaCl. The value obtained for the second pulse in the presence of sustained intracellular acidosis, was compared to that obtained for a second pulse in the absence of sustained acidosis. In some experiments the MEK inhibitor U0126 (10 μM) was present in the second pulse of the assay.

2.7. Limited trypsin digestion

Limited trypsin digestion of the NHE1 protein was used to demonstrate changes in conformation essentially as described earlier [22]. Cell lysates were prepared as described above using RIPA lysis buffer without proteinase inhibitors. Trypsin (phenylalanyl chloromethyl ketone–trypsin, Sigma, St., Louis, MO) was prepared and dissolved in TE buffer. Equal amounts of proteins (80 μg) from cell lysates were treated with indicated amounts of trypsin and incubated at 30 °C for 10 min. The reaction was terminated by addition of SDS-PAGE loading buffer followed by boiling at 100 °C for 3 min. Samples were resolved by SDS-PAGE and NHE1 was visualized by western blot analysis using an antibody against the HA tag of against the NHE1 protein itself as indicated. For some experiments a crude membrane fraction was made. In this case the cell lysate was sonicated for 15 s on ice, the fraction was centrifuged at 5000 rpm for 20 min at 4 °C. The supernatant was then centrifuged at 35,000 rpm for 1 h at 4 °C and the pellet was resuspended and examined for trypsin sensitivity as described above. For protein expressed in *Escherichia coli*, 20 μg of wild or mutant proteins was treated with the indicated amount of trypsin and incubated at 30 °C for 10 min. The reaction was stopped by addition of SDS-PAGE loading buffer followed by boiling at 100 °C for 3 min. Samples were then resolved by SDS-PAGE and visualized by coomassie staining. For some experiments limited trypsin digestion was done as above for 15 min. The reactions were then analyzed using an Orbitrap mass spectrometer (Thermo Scientific). All samples were diluted in 50% v/v water/ACN and 0.2% v/v formic acid and were resolved and ionized by using nanoflow HPLC (Easy-nLC II, Thermo Scientific) coupled to the LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Scientific). Nanoflow chromatography and electrospray ionization were accomplished by using a PicoFrit fused silica capillary column (ProteoPepII, C18) with 100 μm inner diameter (300 Å, 5 μm, New Objective). Samples were injected onto the column at a flow rate of 3000 nl/min and resolved at 500 nl/min using 85 min linear ACN gradients from 0 to 75% v/v aqueous ACN in 0.2% v/v formic acid. The mass spectrometer was operated recording high-accuracy and high-resolution survey Orbitrap spectra using external mass calibration, with a resolution of 60 000 and m/z range of 400–2000. The wild type and the mutant peptides were identified using the ExPASy bioinformatics Resource Portal.

2.8. Expression and purification of the NHE1 C-terminal regulatory region

To produce the C-terminal regulatory region of the human NHE1 protein we expressed this region using the Gateway™ cloning system (Life Technologies, Inc., Rockville, MD) for *E. coli* and purified it using an added Histidine tag. The cDNA coding for the C-terminal 239 (WT) amino acids, 577–815 were amplified with the forward primer 1 (FP1, Table 1) and reverse primer 1 (RP1, Table 1). The primers had cloning sites, attB1 and attB2 designed for use in the Gateway™ cloning system and a C-terminal six histidine tag was added to the sequence. The PCR product was cloned into the expression vector pDest14. The S770771D mutant plasmid was obtained by site-directed mutagenesis as described earlier [18]. Protein purification was via immobilized metal (nickel) affinity chromatography essentially as described earlier [23].

To express the C-terminal regulatory region of NHE1 protein for mass spectrometry analysis, the cDNA coding for the C-terminal 238 amino acids (WT) and S770/771D mutant with a six histidine tag at the C-terminal were amplified by using the plasmids constructed above

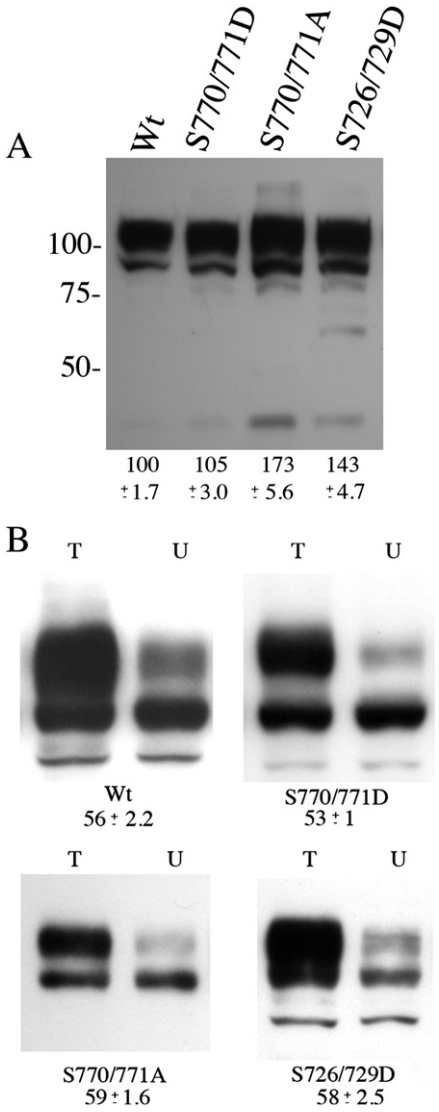
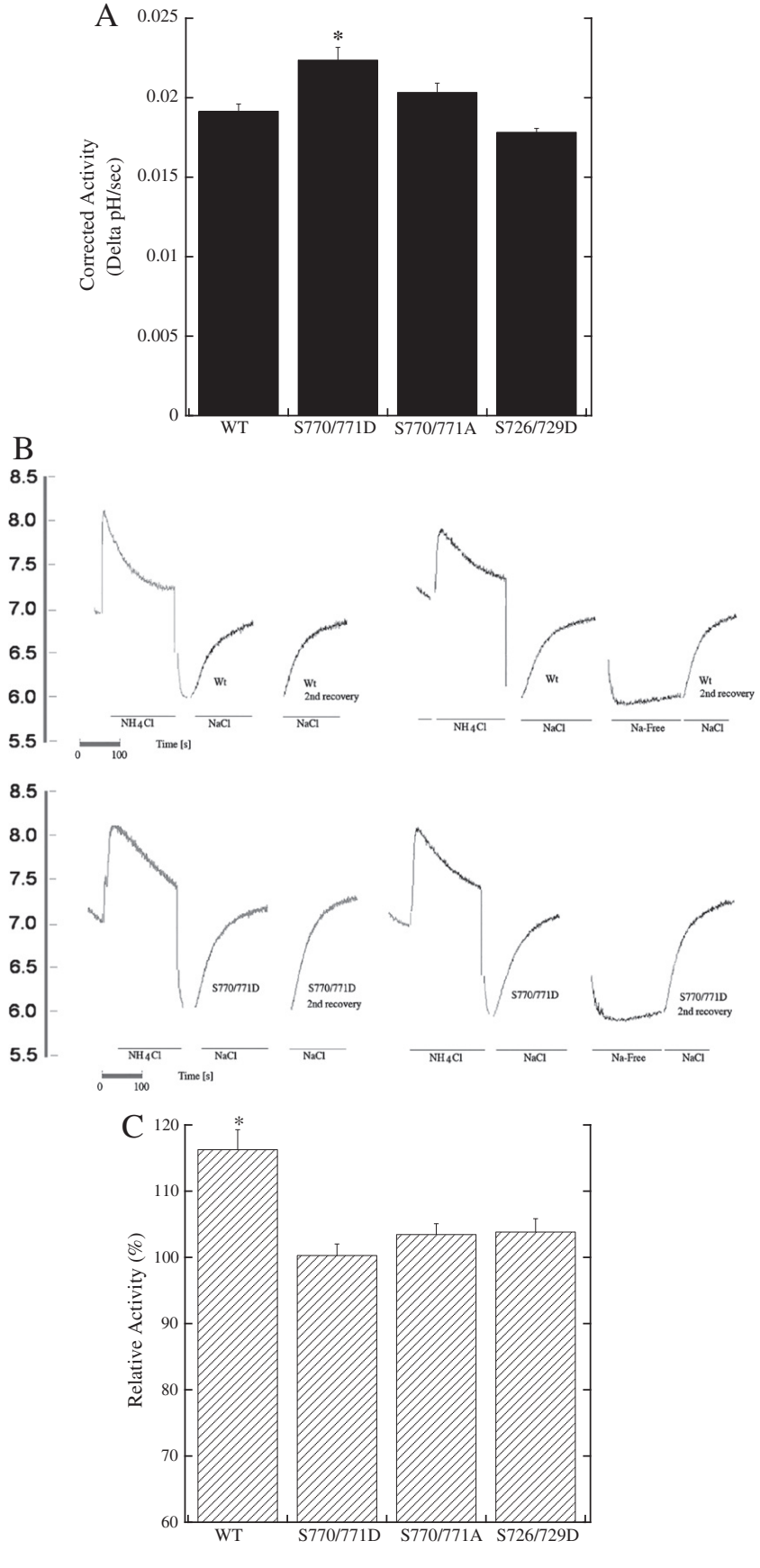


Fig. 1. Characterization of expression of NHE1 mutant proteins. A. Comparison of expression levels of NHE1 protein mutants. Western blot analysis of whole cell extracts of WT NHE1 and mutants. Each lane contained 80 μg of total protein. The specific mutations of the serine amino acids are indicated. NHE1 expression levels were examined using anti-HA antibody. Numbers underneath the lanes indicate the mean value (±SE) of the sum of densitometric scans of both 110 kDa and 95 kDa bands relative to wild-type NHE1 for at least three experiments. B. Surface localization of NHE1 proteins with mutations in the C-terminal regulatory region. Sulfo-NHS-SS-biotin treated cells were lysed and their proteins were solubilized and subsequently treated as described in the "Materials and methods". Equal samples of total lysates (T) and unbound (representing intracellular) lysates (U) were resolved on SDS-PAGE and probed with anti-HA antibody to identify NHE1 protein. The amount of surface localized fully glycosylated NHE1 was calculated from densitometric analysis by taking the (total protein) − (unbound protein) = (membrane surface localized protein). The percent of the total NHE1 protein localized to the plasma membrane is indicated. Results are mean ± SE, n = at least 6 experiments.

(pDest14-WT/Mutant) as a template. The forward primer (FP2) and the reverse primer (FP2) are shown in Table 1 and were used for cloning into the pMPLB vector (kindly provided by Dr. Howard Young, Department of Biochemistry, University of Alberta). The vector contains cDNA coding for maltose binding protein as an N-terminal tag for purification. A six glycine coding sequence (bold) was added in the



N-terminal to enhance the TEV cleavage. DNA sequencing confirmed the fidelity of the PCR product. Protein purification was using immobilized amylose affinity chromatography following the manufacturer's instructions. Further purification was using immobilized metal (nickel) affinity chromatography essentially as described earlier [23]. Fusion proteins for cleavage were incubated in cleavage buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM DTT) at room temperature for 36 h with TEV and His-tagged proteins were finally purified with Ni-NTA affinity chromatography.

2.9. Fluorescence measurements

To examine the endogenous fluorescence of the C-terminal regulatory region of NHE1 in response to pH we used tryptophan emission spectra recorded with a Photon Technologies Incorporated Deltascan spectrofluorometer using an excitation wavelength of 295 nm and examining emission from 310 to 400 nm. Time-dependent traces were recorded with an excitation wavelength of 295 nm and an emission wavelength of 338 nm. The incubation buffer contained 20 mM HEPES, 140 mM KCl, 1 mM K_2HPO_4, 2 mM $MgSO_4$, and 3.0 μM of proteins were incubated at 25 °C while being stirred. Samples were stirred and incubation continued for 1 min, and the emission spectra were then measured. The emission was measured for each protein sample at each pH value. To alter the pH in some experiments 3.8 M Tris or 3.7% HCl was added [24]. For measurement of spectra, the comparable spectra of a blank buffer containing solution were subtracted from all measurements.

2.10. Native PAGE

Pre-casted native PAGE gels were purchased from Bio-Rad. For native PAGE the expressed and purified C-terminal 239 (WT) amino acids (577–815) or the phosphomimetic (S770D/S771D) protein were run essentially following the manufacturer's instruction. The running buffer was either pH 4.0 (0.1 M glycine–HCl buffer) or 8.3 (0.1 M Tris–glycine buffer).

2.11. HDX mass shift analysis

Quadruplicate experiments were performed for each state of wild type or mutant protein (amino acids 577–815). In each experiment, an aliquot of protein stock solution (7 μl of 5 μM protein in 10 mM MOPS, 0.5 mM NaCl, pH 7.3) was labeled with 3 μl D_2O at 20 °C for 2 min. The solution was quenched by addition of 25 μl 0.1 M glycine–HCl, pH 2.3. It was then immediately injected into the chilled injection chamber of a LEAP H/D-X PAL system (4 °C), containing an immobilized pepsin column. The digest was captured on a short C18 reversed-phase monolithic column and separated using a 5–90% steep linear gradient of acidified acetonitrile (0.1% formic acid, v/v). The effluent was directly infused into a 5600 Triple TOF mass spectrometer (AB Sciex) for a total analysis time of 12 min per experiment (digestion to end of gradient). Analysis involved: (a) identifying all detectable peptides using MS/MS data searched against the wild-type and mutant proteins, in Mascot v2.3; (b) retrieving a set of features from the identification data (peptide sequence, m/z and retention times) for submission to Mass Spec Studio (developed in house), along with all replicate data sets, for extraction of peptide-level deuteration data and; (c) performing statistical analysis to identify differences in deuteration between the two protein states at the peptide level, using previously described methods [25].

3. Results

3.1. Characterization of phosphomimetic mutations in full length NHE1 protein

We initially characterized the effect of phosphomimetic mutations on activity and conformation of mutants of the full length NHE1 protein. We have earlier demonstrated that Ser^{770} and Ser^{771} are important in regulation of NHE1 activity via Erk-dependent pathways [11] and that Ser^{726} and Ser^{729} mediate activation of NHE1 by apoptosis in CHO cells [14]. The double mutants S770D/S771D, S770A/S771A and S726D/S729D were made and tested for expression, activity and targeting in AP-1 cells. Fig. 1 shows the characterization of expression of these mutants relative to the wild type. Western blot analysis (Fig. 1A) shows that the mutants all expressed the NHE1 protein. As shown earlier [20], the protein was present as a fully glycosylated mature protein and a partial or de-glycosylated protein. The level of mutant protein was increased in some cases, though it was not clear if this was a function of the mutation or variability between the stable cell lines [20].

The surface localization of the wild type and mutants was investigated. Previously we have found that mutations in NHE1 may interrupt the synthesis and transport of the protein, which causes intracellular retention of mutant proteins [26]. Surface targeting was carried out as described in the "Materials and methods". The total and unbound (intracellular) fractions of cell lysates were examined by SDS-PAGE and western blotting. The amount of fully glycosylated and partially/non-glycosylated NHE1 were measured. The results are shown in Fig. 1B. Approximately 50% of the wild type and mutant proteins were targeted to the cell surface. The mature fully glycosylated NHE1 protein generally targeted more efficiently to the membrane surface however significant amounts of partially- or unglycosylated NHE1 protein were also present on the cell surface similar to results we have observed earlier [27].

The cation transport activity of NHE1 wild type and mutant stable cell lines was examined by assessing their ability to recover from a transient intracellular acidification. The results are shown in Fig. 2. When we examined NHE1 activity after a single ammonium chloride pulse, slight but significant increases in activity were found for mutation of Ser^{770} and Ser^{771} to Asp. In contrast, mutation of Ser^{726} and Ser^{729} to Asp caused a slight decrease in activity while mutation of Ser^{770} and Ser^{771} to Ala did not stimulate activity (Fig. 2A). To further characterize the functional effects of the mutations, we examined the ability of sustained acidosis to stimulate NHE1 activity. We [11,12] and others [28] have shown that prolonged intracellular acidosis activates NHE1 through an Erk-dependent pathway that involves phosphorylation of amino acids Ser^{770} and Ser^{771}. Mutant and wild type NHE1 activity was measured after repetitive ammonium chloride pulses as described earlier [11,12]. The results (Figs. 2B, C) showed that wild type NHE1 protein was activated by sustained acidosis, however the mutant proteins were not. In one set of experiments U0126 (10 μM) was included in the second pulse of two pulse assays. We compared the rate of recovery of the second pulse to that of the first pulse and examined wild type NHE1 and S770D/S771D containing cells with the second recovery after 30 s of Na^+-free medium. We found that U0126 caused a significant 16% ($p = 0.01347$) decrease in the rate of recovery of wild type NHE1, but did not affect recovery of the S770D/S771D-NHE1 containing cells (not shown).

To determine if the mutant NHE1 proteins had a change in conformation, we examined their sensitivity to limited proteolysis with trypsin. Initial experiments were done on lysates of whole cells (Fig. 3A). In

Fig. 2. Characterization of NHE1 activity of mutant and wild type NHE1 proteins. A. Summary of NHE activity of mutant NHE1 proteins. The activities of NHE1 wild type Wt control, and mutants were determined as described in "Materials and methods". Results are mean ± SE (n = at least 8 determinations). Absolute levels of activity were correct for protein expression and targeting levels. B, C. Two pulse assay examining the effects of sustained intracellular acidosis on NHE1 activity. B, Example of activity traces of wild type and S770D/S771D mutant NHE1 protein. For clarity, only the pHi recovery after acidosis is illustrated for the second pulse assay. C. Summary of NHE activity of wild type and mutant NHE proteins with two pulse assay. The recovery from the first pulse was set at 100%. The rate of recovery of the second pulse was compared to that of each individual first pulse and is shown (hatched rectangle, results are mean ± SE, n = at least 8 determinations).

all cases, to minimize variation, digestion with trypsin was done in paired experiments run simultaneously with the wild type NHE1. Analysis was by using western blotting against the HA tag on the C-terminal of the protein. We found that the NHE1 proteins with the mutation S726D/S729D and S770A/S771A showed no difference in the digestion pattern or sensitivity to digestion compared with the wild type. However, in multiple experiments the S770D/S771D mutant always appeared to be more sensitive to digestion with trypsin, which was especially notable at the higher trypsin concentrations (Fig. 3A). To examine this in more detail we made a crude membrane fraction of the cells which was enriched in NHE1 protein relative to the cell lysate. Samples were subjected to limited tryptic digestion and analyzed by western blotting. The results (Figs. 3B, C) were similar to that shown with the cell lysate. The S770D/S771D NHE1 mutant protein was more sensitive to tryptic digest than the wild type protein. Treatment with either 10 or 20 ng of trypsin, virtually eliminated the full length NHE1 protein, while much more wild type NHE1 protein remained.

3.2. Effect of phosphomimetic mutations on C-terminal regulatory region trypsin sensitivity

In order to examine if a change in conformation occurred with phosphorylation of the C-terminal regulatory region, we produced the protein in *E. coli*, with or without phosphomimetic mutations in this region. Wild type human NHE1 protein (amino acids 577–815) was produced as described in the "Materials and methods". The same protein was produced with the mutation of amino acids S703D, S726D/S729D and S770D/S771D. The partially purified proteins are shown in a coomassie blue stained gel in Fig. 4A. It was noted that the mutation of amino acids S726D/S729D and S770D/S771D caused a slight shift in the mobility of the intact protein. Mutation of Ser^{703} to Asp did not cause such a shift. To discern in more detail any changes in the conformation of the proteins we tested their sensitivity to tryptic digestion (Figs. 4B–D). Wild type protein was digested in parallel experiments with each of the mutant proteins with varying concentrations of trypsin. Samples were run on SDS-PAGE and examined by coomassie blue staining. There was no apparent difference in the digestion of the S703D protein vs. wild type protein. There were only some minor differences in the digestion of the S726D/S729D mutant protein vs. the wild type NHE1 protein. In contrast, there appeared to be major differences in the degradation patterns of wild type NHE1 vs. the S770D/S771D mutant protein (Fig. 4D). We also examined the peptides produced by tryptic digestion of the wild type and mutant proteins. Samples of wild type and mutant (S770D/S771D) protein were digested with trypsin for 15 min as described above, and intact masses of protein fragments were identified at the Institute for Biomedical Design core facility, University of Alberta as described in the

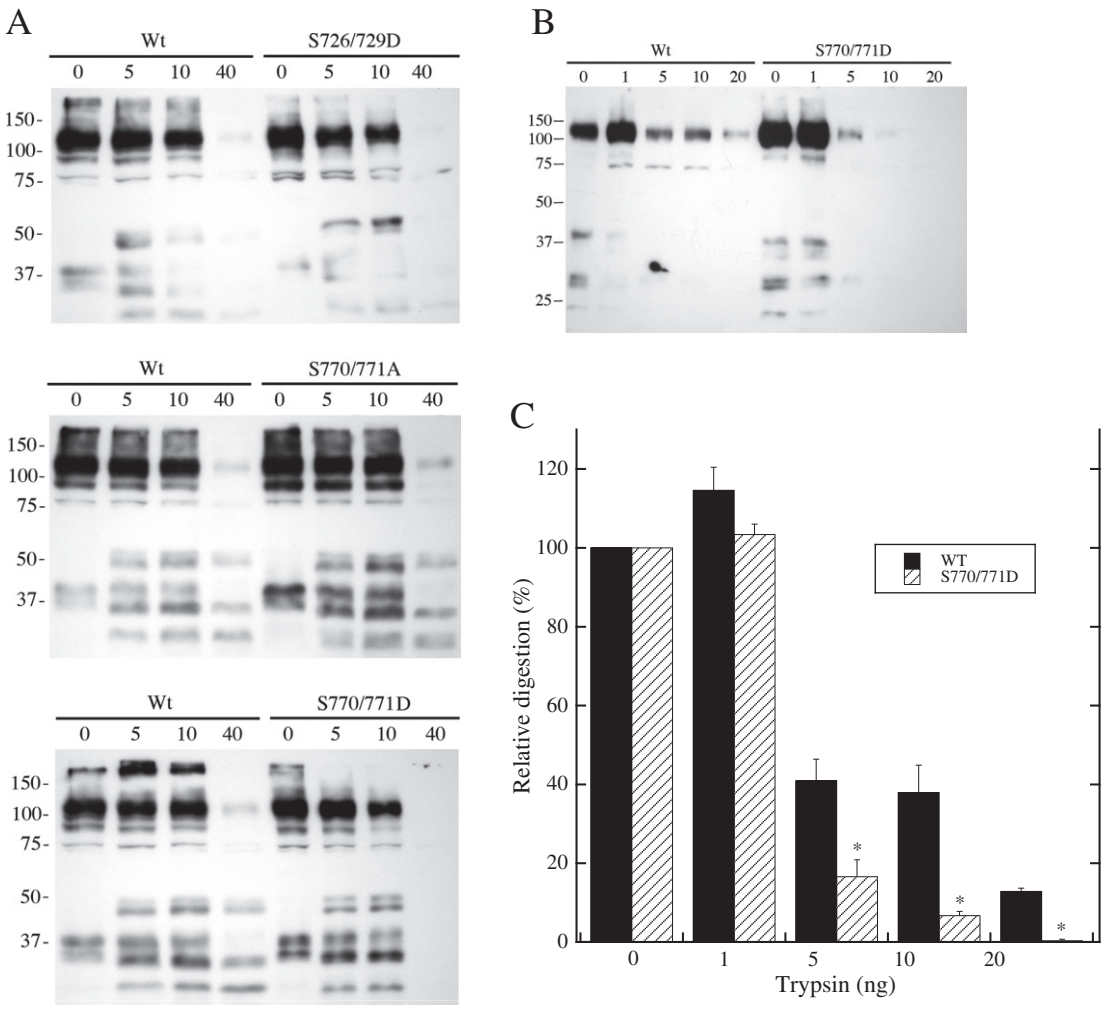


Fig. 3. Limited trypsin digestion of wild type (WT) mutant NHE1 proteins. A, Western blot of cell lysates treating with varying amounts of trypsin. A, 80 μg of cell lysate was treated with 5–40 ng trypsin at 30 °C for 10 min. The pattern of NHE1 digestion was detected using anti-HA antibodies. Similar results were reproduced at least three times. B, Crude membrane fractions (20 μg) of wild type and S770/771D mutant protein were examined by limited digestion with trypsin (1–20 ng). The pattern of NHE1 digestion was detected using anti-HA antibodies. C, Summary of results of limited tryptic digestion of crude membrane fractions of wild type NHE1 and S770/771D mutant proteins. *, significantly ($p < 0.05$, $n = 3$) different from WT at the same time point.

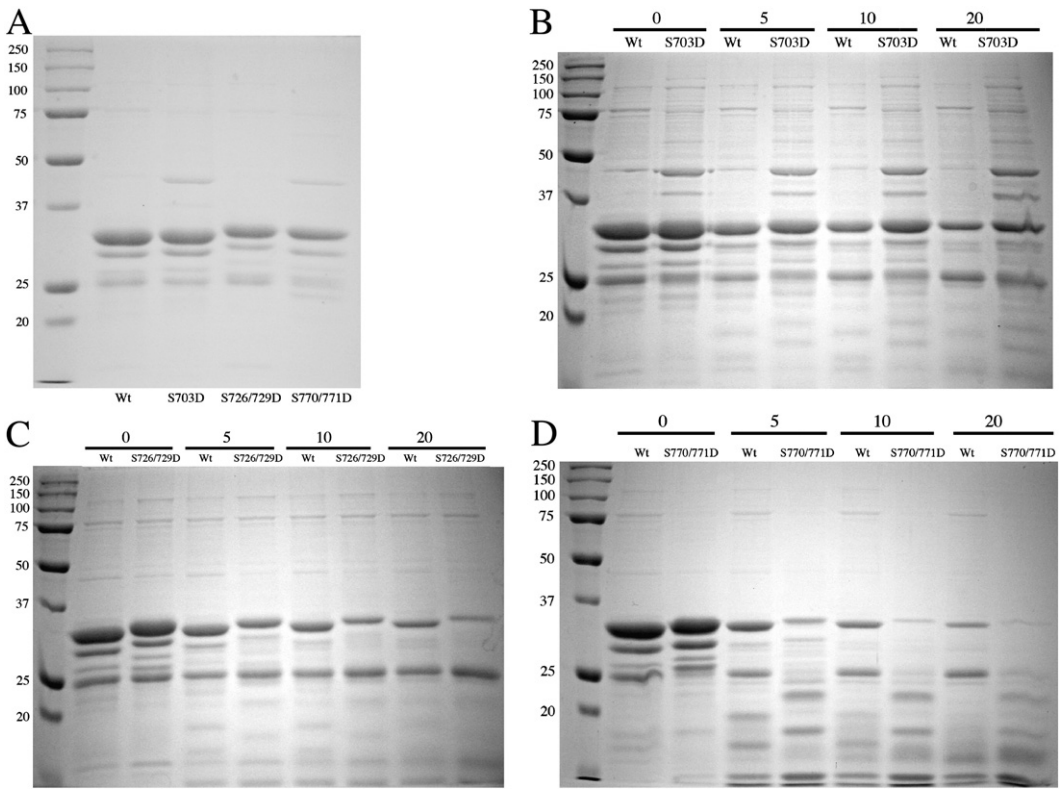


Fig. 4. Expression and characterization of NHE1 C-terminal regulatory region proteins. Amino acids 577–815 of the human NHE1 protein were expressed and partially purified as described in the "Materials and methods". Wild type NHE1 protein and NHE1 with the mutations S703D, S726/S729D and S770D/S771D were produced and purified. A, Partially purified mutant NHE1 proteins. B, Parallel tryptic digestion of wild type NHE1 protein (amino acids 577–815) and S703D NHE1 protein. Trypsin (0–20 ng) was added as indicated. C, Parallel tryptic digestion of wild type NHE1 protein (amino acids 577–815) and S726D/S729DNHE1 protein. D, Parallel tryptic digestion of wild type NHE1 protein (amino acids 577–815) and S770D/S771D NHE1 protein.

"Materials and methods". We found several peptides were generated in the mutant protein digested with trypsin, that were not found in the wild type protein digest. Cleavage with trypsin occurred uniquely in the phosphomimetic protein after amino acids Lys^{16} and Arg^{78} (numbering Lys^{583} and Arg^{645} in the full length NHE1 protein).

3.3. Phosphomimetic mutations affect pH dependent changes on the C-terminal regulatory region

We examined the pH dependent changes in conformation in the C-terminal regulatory region of the NHE1 protein. We expressed and purified the C-terminal 239 amino acids, (577–815) with and without the phosphomimetic S770D/S771D mutations. One Trp residue is present in the C-terminal regulatory region that we expressed, and it is located at amino acid 663 (of the full length protein). We examined the steady state emission spectrum of the proteins at pH 6 or 8.5 and the time dependent changes that occurred with alterations in pH. The results are shown in Fig. 5. In Figs. 5A and B we examined the emission curve of equal amounts of wild type or phosphomimetic C-terminal regulatory region at acidic or basic pH. The phosphomimetic protein consistently displayed an increased fluorescence relative to the wild type protein (Fig. 5A) however this only occurred at the acidic pH. At pH 8.5, the wild type and phosphomimetic proteins were equivalent in their fluorescence spectra. There was a slight but reproducible blueshift of the peak at pH 6.0 (336–337 nm) vs. pH 8.5 (337–338 nm). We then used a time dependent assay to further confirm the changes in fluorescence that occurred with the different pH values, examining the change in emission at 338 nm, with excitation at 295 nm. The results are shown in Figs. 5C, D. Changing the pH from 6 to 8.5 results in a decrease in fluorescence in both the wild type and the phosphomimetic mutant. However, the decrease was more substantial in the phosphomimetic mutant than in the wild type protein.

3.4. Changes in mobility of the C-terminal regulatory region with phosphomimetic mutations

As noted above, we noticed that in SDS-PAGE there was a slight change in the mobility of the C-terminal regulatory region protein, when comparing the phosphomimetic (S770D/S771D) protein to the wild type protein (Fig. 4). To examine possible changes in conformation of the protein in more detail we used a non-denaturing gel system and compared the mobility of the WT and the three phosphomimetic proteins. The results are shown in Figs. 6A, B. We examined the relative motilities at acidic (pH 4.0) (Fig. 6A) and alkaline pH (pH 8.3) (Fig. 6B). In all cases we found that the mobility of the phosphomimetic proteins was greater than that of the wild type protein. This indicated a more compact, less extended structure was induced by the phosphomimetic mutations. It was not clear if the amount of difference between the WT and phosphomimetic protein varied with pH. We noted that the mobility change of the S703D was at least as large, or larger, than that of the other mutant proteins, suggesting that the change in mobility was not due to a simple effect of inducing a charge into the protein. In preliminary experiments we used gel filtration to compare the size of the wild type and mutant protein and found similar results, with the mutant having a smaller size than the wild type protein (not shown).

3.5. Changes in accessibility of the C-terminal regulatory region with phosphomimetic (S770D/S771D) mutations

To confirm that there was a difference in the conformation of the phosphomimetic (S770D/S771D) protein relative to the wild-type, we

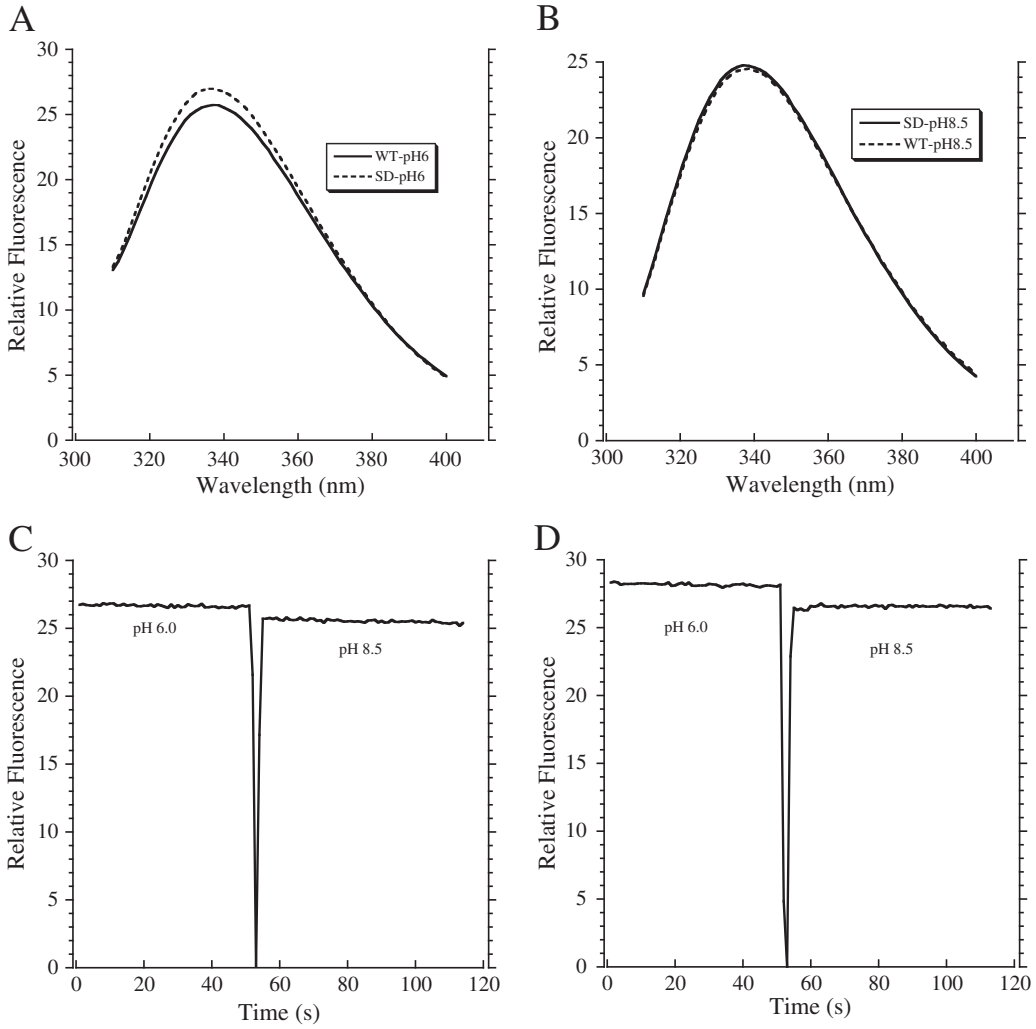


Fig. 5. Comparison of emission spectra of wild type (WT) and phosphomimetic C-terminal regulatory region of the human NHE1 protein. A, B Steady-state emission spectra of purified WT, and phosphomimetic (S770D/S771D, SD) proteins of amino acids 577–815 of the human NHE1 protein. Purified proteins were incubated at a protein concentration of 3 μM in a reaction mixture containing 20 mM HEPES, 140 mM KCl, 1 mM K_2HPO_4, 2 mM $MgSO_4$ at pH 6.0 or pH 8.5. (A, pH 6.0 WT and SD, B, pH 8.5, WT and SD). Results are typical of at least four repetitions. C, D, Time and pH dependent changes in the emission of WT and SD NHE1 C-terminal proteins. Excitation was at 295 nm and emission measurements were at a wavelength of 338 nm. Discontinuities were caused by addition of solutions to alter pH.

examined the two proteins using a mass shift analysis method based on H/D exchange. In this method, proteins that are deuterated under neutral conditions may be quenched and digested at low pH and temperature. Rapid mass analysis with a mass spectrometer permits localizing deuteration levels to specific regions of structure. These deuteration levels provide measures of local conformational stability, which arise through

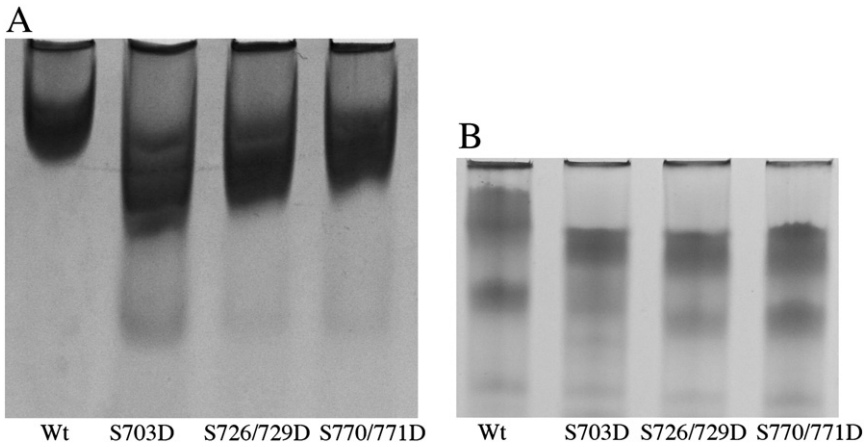


Fig. 6. Native PAGE of purified wild type and phosphomimetic proteins of amino acids 575–815 of the human NHE1 protein. A, Wild type and phosphomimetic proteins run on native PAGE with pH 4.0 running buffer. B, Wild type and phosphomimetic proteins run on native PAGE with pH 8.3 running buffer. WT, wild type.

perturbations of secondary and tertiary structures. The effects of the phosphomimics on the local deuteration profiles are shown in Fig. 7. (Supplementary Table 1 lists additional supportive data.) Positive shift values in three peptides indicate that the wild type is much less stable in a region of sequence between residues M764 and T779. That is, the phosphomimics appear to strongly stabilize all or part of this region, implying the adoption of local secondary and/or tertiary structure. Conversely, the negative shift values in three peptides of the N-terminal region (residues Y577–L588) indicate that the phosphomimetic is less stable in this segment of structure.

4. Discussion

4.1. Critical amino acids Ser^{770} and Ser^{771} specifically activate NHE1 and change conformation of the C-terminal regulatory region

In this study we examined the mechanisms by which phosphorylation activates the NHE1 isoform of the Na^+/H^+ exchanger protein. Initial experiments examined the effects of mutation of amino acids Ser^{770} and Ser^{771} and Ser^{726} and Ser^{729} on activity. Mutating amino acids 770 and 771 to Asp had a stimulatory effect on activity. In contrast, mutation of amino acids 726 to 729 did not stimulate activity and had a slight inhibitory effect. The intact wild type protein was activated by sustained intracellular acidosis, while the mutants were not. This was likely due to their being present in an already activated state, that was unable to be further activated. We have previously demonstrated that Ser^{770} and Ser^{771} are important in activation of NHE1 by sustained intracellular acidosis in two cell types [11,12]. The present experiments confirm these results, but also demonstrate the novel results, that the phosphomimetic protein is in an activated state that is not able to be further stimulated by sustained intracellular acidosis.

We hypothesized that phosphorylation of amino acids induces a change in the conformation of the NHE1 tail, that leads to activation of the membrane domain. To investigate this hypothesis we used several approaches. Initially, we obtained full length NHE1 protein from mammalian cells, and there appeared to be a difference in the sensitivity to trypsin of the phosphomimetic protein, relative to the wild type. In this case, our analysis was based only on the use of a HA tag which was present on the C-terminal of the full length protein. Thus there may have been unobserved novel peptide fragments generated that were not seen because there were not linked to the HA tag.

To gain more details into possible conformational changes induced by phosphorylation of the NHE1 C-terminal regulatory region, we produced and purified this region of the NHE1 protein with and without phosphomimetic mutations. We produced three phosphomimetic proteins containing the mutation S703D, S726D/S729D and S770D/S771D. Interestingly, only the S770D/S771D mutant caused a differential sensitivity to trypsin, relative to the control. There was both a greater sensitivity to digestion, and a modified pattern of digested products, indicating differences in conformation of the proteins.

It is interesting to note that the all three phosphomimetic proteins (S703D, S726D/S729D and S770D/S771D) appear to cause a change in conformation of the C-terminal regulatory region, as indicated by native PAGE. The sensitivity to trypsin varied between the proteins, indicating that the changes in conformation vary. Changes in conformation in response to phosphorylation, may represent a specific mechanism of mediating regulation of the NHE1 protein.

4.2. The S770D/S771D phosphomimetic protein exhibits pH dependent changes in conformation

To further examine putative differences in the conformation of the wild type C-terminal regulatory region vs. the S770D/S771D phosphomimetic protein we used Trp fluorescence. Here we found a pH-dependent difference between the two proteins. Changing the pH from 6 to 8.5 results in a decrease in fluorescence in both the wild type and the phosphomimetic mutant. However, the decrease was more substantial in the phosphomimetic mutant than in the wild type protein. The changes we found were not large, but were reproducible. This region of the NHE1 protein has only one Trp residue which is found at amino acid 663. This may account for the relatively weak change in signal that we observed. However, since our mutation was at amino acids 770 and 771, this indicates that the mutation has affected the conformation of the NHE1 protein over 100 amino acids upstream.

We suggest that there is a larger change in the conformation of the phosphomimetic protein when exposed to more acidic pH values. The increase in fluorescence intensity is possibly due to removal of quenching by amino acids such as Asp or Glu residues [29]. Negative charges that are induced by phosphorylation would be good candidates to repel Asp or Glu residues. Several groups of negatively charged residues are present in the upstream region of the NHE1 protein. The slight blueshift that we observed with decreased pH could be indicative of a change to a less polar environment, but this is not always the case and a shift to more exposure to water can result in

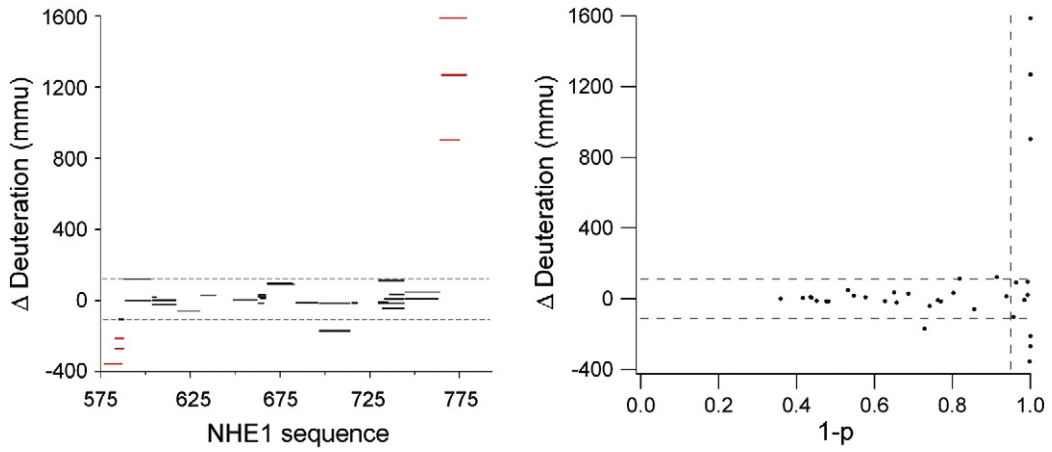


Fig. 7. H/D mass shift analysis of wild type and phosphomimetic (S770D/S771D) proteins of amino acids 577–815 of the human NHE1 protein. (A) Sequence plot where positive shift values represent higher deuteration levels in the wild-type relative to the phosphomimetic, and negative shift values represent lower deuteration levels in the wild-type relative to the phosphomimetic. Significant shift values are shown in red and insignificant values in black, with bar length and position representing the peptide measured in the shift experiment. (B) The corresponding scatterplot showing statistical significance. Horizontal dashed lines in both plots represent ±2 s.d. of shift noise and the vertical dashed line in (B) represents a $p = 0.05$. All deuteration values are shown in millimass units (mmu).

either a red shift or a blue shift [29]. It should be noted however that experiments with native gel electrophoresis indicated that the phosphomimetic protein has a smaller size than the native protein. Together these results indicate that the phosphorylation of amino acids Ser^{770} and Ser^{771} leads to a change to a more compact conformation of the protein. This conformation appears to be more pH responsive than the wild type, unphosphorylated protein.

4.3. Conceptual model of phosphorylation induced changes in conformation

Fig. 8 illustrates a conceptual model of changes in the structure of the C-terminal regulatory region. The distal region is intrinsically disordered [17], though it adopts regions of transient structure. This is consistent with the extensive deuterium labeling we observed in the tail. Amino acids 577–588 (labeled region A) were shown by HDX-MS to be less accessible in the un-phosphorylated protein, while amino acid region C (764–779) was more accessible in the un-phosphorylated protein. We suggest that upon phosphorylation the newly introduced negative charges cause the protein to associate with an unknown upstream sequence (which we indicate as region B). This alters and reduces the apparent size of the protein as our studies showed. This also reduces the accessibility of region C, and has the effect of displacing region A and increasing its accessibility. We demonstrated that Trp^{663} has a slightly different environment in the phosphomimetic protein. This is also indicated in the diagram. Changes in sensitivity to digestion by trypsin were noted and this would be caused by a change in structure of the protein. Specifically, the residues Lys^{583} and Arg^{645} were more accessible to trypsin digestion in the phosphomimetic protein, which is also illustrated. Additionally, with phosphorylation, the resulting change results in an overall change in size. It is not clear that region B associates directly with both regions A and C. A and C could associate with different regions, and interactions of C with another region could have the effect of displacing A from B. We suggest that this may be a dynamic equilibrium, the balance of which is shifted by phosphorylation or by other factors such as cellular pH.

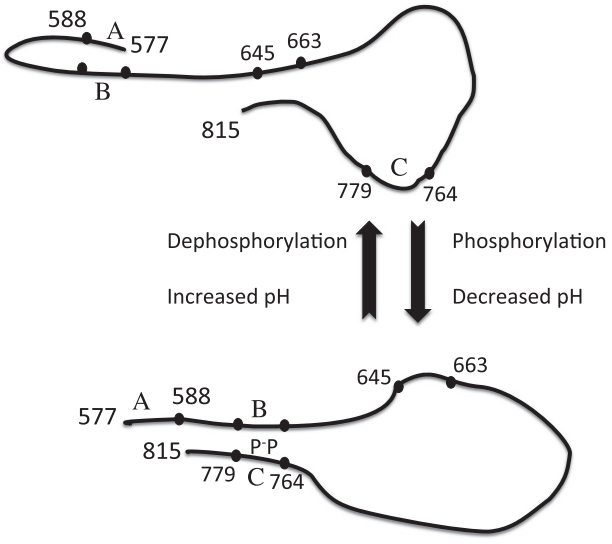


Fig. 8. Model of the effect of phosphorylation on the structure of the NHE1 C-terminal regulatory region. Schematic diagram indicating putative changes in conformational equilibrium of NHE1 cytosolic domain that occur with phosphorylation. Phosphorylation causes a change to an equilibrium favoring a more compact conformation, amino acids 764–779 become less accessible while at the same time amino acids 577–588 become more accessible. There is also a shift in the relative position of Trp 663.

4.4. Summary

Overall, our work has shown that phosphorylation causes a change in the structure of the NHE1 C-terminal regulatory region. This is reflected by changes in size, in accessibility to trypsin, and by changes in accessibility of specific peptides of this region of the protein. Conformational changes in the C-terminal regulatory region of the protein likely affect the membrane domain and activity of the NHE1 protein. Future studies will examine this mechanism.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2013.04.007.

Disclosures

None.

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References

[1] Fliegel L, Wang H. Regulation of the Na^+/H^+ exchanger in the mammalian myocardium. J Mol Cell Cardiol 1997;29:1991–9.
[2] Fliegel L. Regulation of myocardial Na^+/H^+ exchanger activity. Basic Res Cardiol 2001;96:301–5.
[3] Counillon L, Pouyssegur J. The expanding family of eukaryotic Na^+/H^+ exchangers. J Biol Chem 2000;275:1–4.
[4] Malo ME, Fliegel L. Physiological role and regulation of the Na^+/H^+ exchanger. Can J Physiol Pharmacol 2006;84:1081–95.
[5] Putney LK, Denker SP, Barber DL. The changing face of the Na^+/H^+ exchanger, NHE1: structure, regulation, and cellular actions. Annu Rev Pharmacol Toxicol 2002;42:527–52.
[6] Putney LK, Barber DL. Expression profile of genes regulated by activity of the Na–H exchanger NHE1. BMC Genomics 2004;5:46–59.
[7] Wang H, Singh D, Fliegel L. The Na^+/H^+ antiporter potentiates growth and retinoic-acid induced differentiation of P19 embryonal carcinoma cells. J Biol Chem 1997;272:26545–9.
[8] Fliegel L. Regulation of the Na^+/H^+ exchanger in the healthy and diseased myocardium. Expert Opin Ther Targets 2009;13:55–68.
[9] Wakabayashi S, Bertrand B, Shigekawa M, Fafournoux P, Pouyssegur J. Growth factor activation and "H^+-sensing" of the Na^+/H^+ exchanger isoform 1 (NHE1). J Biol Chem 1994;269:5583–8.
[10] Moor AN, Fliegel L. Protein kinase mediated regulation of the Na^+/H^+ exchanger in the rat myocardium by MAP-kinase-dependent pathways. J Biol Chem 1999;274:22985–92.
[11] Malo ME, Li L, Fliegel L. Mitogen-activated protein kinase-dependent activation of the Na^+/H^+ exchanger is mediated through phosphorylation of amino acids Ser^{770} and Ser^{771}. J Biol Chem 2007;282:6292–9.
[12] Coccaro E, Karki P, Cojocaru C, Fliegel L. Phenylephrine and sustained acidosis activate the neonatal rat cardiomyocyte Na^+/H^+ exchanger through phosphorylation of amino acids Ser770 and Ser771. Am J Physiol Heart Circ Physiol 2009;297:H846–58.
[13] Khaled AR, Moor AN, Li A, Kim K, Ferris DK, Muegge K, et al. Trophic factor withdrawal: p38 mitogen-activated protein kinase activates NHE1, which induces intracellular alkalinization. Mol Cell Biol 2001;21:7545–57.
[14] Grenier AL, Abu-ihweij K, Zhang G, Ruppert SM, Boohaker R, Slepkov ER, et al. Apoptosis-induced alkalinization by the Na^+/H^+ exchanger isoform 1 is mediated through phosphorylation of amino acids Ser726 and Ser729. Am J Physiol Cell Physiol 2008;295:C883–96.
[15] Takahashi E, Abe J, Gallis B, Aebersold R, Spring DJ, Krebs EG, et al. p90(RSK) is a serum-stimulated Na^+/H^+ exchanger isoform-1 kinase. Regulatory phosphorylation of serine 703 of Na^+/H^+ exchanger isoform-1. J Biol Chem 1999;274:20206–14.
[16] Lehoux S, Abe J, Florian JA, Berk BC. 14-3-3 binding to Na^+/H^+ exchanger isoform-1 is associated with serum-dependent activation of Na^+/H^+ exchange. J Biol Chem 2001;276:15794–800.
[17] Norholm AB, Hendus-Altenburger R, Bjerre G, Kjaergaard M, Pedersen SF, Kragelund BB. The intracellular distal tail of the Na^+/H^+ exchanger NHE1 is intrinsically disordered: implications for NHE1 trafficking. Biochemistry 2011;50:3469–80.
[18] Slepkov ER, Chow S, Lemieux MJ, Fliegel L. Proline residues in transmembrane segment IV are critical for activity, expression and targeting of the Na^+/H^+ exchanger isoform 1. Biochem J 2004;379:31–8.
[19] Slepkov ER, Rainey JK, Li X, Liu Y, Cheng FJ, Lindhout DA, et al. Structural and functional characterization of transmembrane segment IV of the NHE1 isoform of the Na^+/H^+ exchanger. J Biol Chem 2005;280:17863–72.

[20] Ding J, Rainey JK, Xu C, Sykes BD, Fliegel L. Structural and functional characterization of transmembrane segment VII of the Na^+/H^+ exchanger isoform 1. J Biol Chem 2006;281:29817–29.
[21] Michalak M, Fliegel L, Wlasichuk K. Isolation and characterization of calcium binding glycoproteins of cardiac sarcolemmal vesicles. J Biol Chem 1990;265:5869–74.
[22] Murtazina R, Booth BJ, Bullis BL, Singh DN, Fliegel L. Functional analysis of polar amino-acid residues in membrane associated regions of the NHE1 isoform of the mammalian Na^+/H^+ exchanger. Eur J Biochem 2001;268:4674–85.
[23] Li X, Liu Y, Kay CM, Muller-Esterl W, Fliegel L. The $Na^{(+)}/H^{(+)}$ exchanger cytoplasmic tail: structure, function, and interactions with tescalcin. Biochemistry 2003;42:7448–56.
[24] Kozachkov L, Padan E. Site-directed tryptophan fluorescence reveals two essential conformational changes in the Na^+/H^+ antiporter NhaA. Proc Natl Acad Sci U S A 2011;108:15769–74.
[25] Bennett MJ, Barakat K, Huzil JT, Tuszynski J, Schriemer DC. Discovery and characterization of the laulimalide-microtubule binding mode by mass shift perturbation mapping. Chem Biol 2010;17:725–34.
[26] Lee BL, Li X, Liu Y, Sykes BD, Fliegel L. Structural and functional analysis of transmembrane XI of the NHE1 isoform of the Na^+/H^+ exchanger. J Biol Chem 2009;284:11546–56.
[27] Tzeng J, Lee BL, Sykes BD, Fliegel L. Structural and functional analysis of transmembrane segment VI of the NHE1 isoform of the Na^+/H^+ exchanger. J Biol Chem 2010;285:36656–65.
[28] Haworth RS, McCann C, Snabaitis AK, Roberts NA, Avkiran M. Stimulation of the plasma membrane Na^+/H^+ exchanger NHE1 by sustained intracellular acidosis. Evidence for a novel mechanism mediated by the ERK pathway. J Biol Chem 2003;278:31676–84.
[29] Vivian JT, Callis PR. Mechanisms of tryptophan fluorescence shifts in proteins. Biophys J 2001;80:2093–109.