Functional Analysis of Acidic Amino Acids in the Cytosolic Tail of the Na^+/H^+ Exchanger†

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ABSTRACT: The mammalian Na^+/H^+ exchanger is a membrane protein with a C-terminal regulatory cytosolic domain and an N-terminal membrane domain. Na^+/H^+ exchanger isoform 1 (NHE1) possesses a conserved amino acid sequence of seven consecutive acidic residues in the distal region of the cytosolic tail. We examined the structural and functional role of this acidic sequence. In human NHE1, varying mutations of the sequence ^{753}EEDEDDD^{759} resulted in defective NHE1 activity. Mutation of the core acid sequence, ^{755}DED^{757}, or of the entire sequence caused a decrease in the activity of NHE1 in response to acute acid load. This was not due to changes in Na^+ affinity but rather due to decreased maximum velocity of the protein and delayed activation. Mutation of the target sequence did not affect the ability of the cytoplasmic domain to bind carbonic anhydrase II or tescalcin but did affect calmodulin binding. Mutation of the acidic domain also caused altered sensitivity to trypsin and changes in size of the protein in gel-filtration chromatography and sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Our results demonstrate that the acidic sequence is critical in maintaining proper conformation of the cytosolic domain, calmodulin binding, and in maintenance of Na^+/H^+ exchanger activity.

The mammalian Na^+/H^+ exchanger isoform 1 (NHE1)[1] is a ubiquitously expressed integral membrane protein that removes one intracellular proton in exchange for a single extracellular sodium ion (*1*), thereby protecting cells from intracellular acidification (*2*, *3*). Its regulation is of great importance, and stimulation of NHE1 activity promotes cell growth and differentiation (*2*) and regulates sodium fluxes and cell volume after osmotic shrinkage (*4*). The Na^+/H^+ exchanger also plays a vital role in several clinically important diseases. Activation of NHE1 is important in hypertension (*5*, *6*), and recently, the protein has been demonstrated to play a critical role in some types of heart hypertrophy (*7*−*10*). In ischemic heart disease, damage occurs to the human myocardium during ischemia and reperfusion, and it has been shown that inhibition of the exchanger has beneficial effects on the myocardium under these conditions (*11*). Amiloride and its derivatives are inhibitors of the NHE1 isoform that can prevent myocardial hypertrophy in several animal models (*7*−*10*, *12*, *13*), and a new generation of Na^+/H^+ exchanger inhibitors is being developed for clinical treatment of heart disease (*14*, *15*).

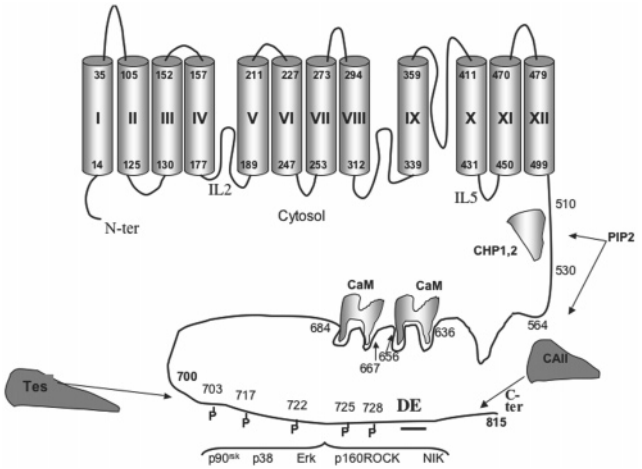

FIGURE 1: Model of the NHE1 isoform of the Na^+/H^+ exchanger. The orientation of the transmembrane segments is based on ref *33*. Features of the cytosolic tail shown include the presence of binding sites for the proteins calmodulin (CaM) (*21*, *34*), CHP (*22*), tescalcin (Tes) (*23*), and carbonic anhydrase II (CAII) (*18*). The distal tail and some of the kinases and sites of phosphorylation are also indicated (*35*). DE indicates the approximate location of an acidic sequence of seven amino acids. IL2 and IL5 are intracellular loops 2 and 5, respectively.

NHE1 is composed of two general domains. A 315 amino acid C-terminal regulatory domain (*1*, *11*) (Figure 1) is one component that modifies the activity of the second, 500 amino acid N-terminal membrane domain. It does so by interactions with a number of proteins including calcineurin homologous protein (CHP) (*16*), calmodulin (*17*), carbonic anhydrase II (*18*), and heat shock protein (*19*). In addition, the Na^+/H^+ exchanger is subject to regulation by phosphorylation that stimulates transport activity (*20*). Within the

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[1] Abbreviations: AtoN, acid to neutral; BSA, bovine serum albumin; CaM, calmodulin; CAII, carbonic anhydrase II; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GST, glutathione *S*-transferase; HA, hemagglutinin; His 182, histidine-tagged protein with the C-terminal 182 amino acids of Na^+/H^+ exchanger isoform 1; HRP, horseradish peroxidase; IL, intracellular loop; MALDI, matrix-assisted laser desorption ionization; NHE1, Na^+/H^+ exchanger isoform 1; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tes, tescalcin.

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Human NHE1     745-SRDPAK VAEEDEDDDG GIMM-764 (36)
Rat NHE1       750-RGPRTT PEEEEEDEDG VIMI-769 (37)
Mouse NHE1     750-RGPRVT PEEEEEDEDG IIMI-769 (38)
Rabbit NHE1    746-REPRVA EEAAEEDEDG GIVM-765 (39)
Pig NHE1       748-PGRATE EEDDDEDHDG GLVM-767 (40)
Bovine NHE1    745-SRDPGR LAEEEEDDNG PRWL-764 (41)
Hamster NHE1   751-DPTRLT RGEEDEDEDE DGVI-770 (42)
Trout NHE1     695-PDAVSL EEEEEEVPKR PSLK-714 (43)
Amphiuma NHE1  743-EEKKSL PTEEEEEEEE GIVM-762 (44)
Human NHE5     695-AVILTV ESEEEEEESD SSET-714 (45)
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FIGURE 2: Sequence alignment of Na^+/H^+ exchangers. Twenty amino acids of the cytosolic tail of vertebrate NHE1 isoforms of the Na^+/H^+ exchanger were aligned manually, with shading indicating acidic residues. An alignment with the human NHE5 isoform is also shown.

C-terminal regulatory domain are protein phosphorylation sites, an autoinhibitory site, and binding sites for the regulatory proteins (*21*, *22*). Earlier, we examined the structure of the last 182 amino acids the C-terminal domain and found that it reveals a very small amount of α-helical structure but much more *β*-sheet and *β*-turn (*23*). A close examination of the human NHE1 cytosolic tail amino acid sequence reveals an unusual acidic sequence of amino acids, ^{753}EEDEDDD^{759}. This sequence is highly conserved among different species within the NHE1 isoform of the protein (Figure 2) despite a lack of conservation in the tail as a whole in comparison to the membrane domain (*24*). The acidic sequence is present in all the vertebrate NHE1 isoforms, including trout and *Amphiuma*. All human, rat, mouse, rabbit, pig, bovine, hamster, trout, and *Amphiuma* isoforms had a sequence of at least five continuous acidic residues and most had six or seven, with a maximum of nine in the hamster. The sequence was not present in NHE1-like isoforms of *Drosophila* or *Caenorhabditis*, which had greatly shortened cytoplasmic domains (not shown). Though the acidic sequence was conserved in vertebrate NHE1 isoforms, it was not conserved in other isoforms of the Na^+/H^+ exchanger with the possible exception of NHE5. Similar acidic sequences occur in other cation transport proteins including those of rice, *Arabidopsis*, and *Saccharomyces cerevisiae*. It has recently been suggested that these acidic sequences can contribute to the capture and selection of cations (*25*) and they are unlikely to have occurred randomly.

In this paper we examine the functional role of the acidic amino acid sequence in the cytoplasmic tail of the NHE1 isoform of the Na^+/H^+ exchanger. We demonstrate that these amino acids are crucial for optimal NHE1 function and that they affect the structure of the cytosolic domain. Our observations are the first analysis of this sequence in this critical protein.

MATERIALS AND METHODS

Materials. PWO DNA polymerase for DNA amplification was from Roche Molecular Biochemicals, Mannheim, Germany. Lipofectamine 2000 and related Gateway cloning items were from Life Technologies, Inc. (Rockville, MD). Synthetic oligonucleotides were from MWB Biotech AG, High Point, NC. Glutathione−Sepharose 4B was from Pharmacia Biotech AB (Uppsala, Sweden). Ni−NTA agarose resin was from Qiagen (Valencia, CA). Trypsin, trypsin inhibitor, and carbonic anhydrase II were from Sigma−Aldrich Canada (Oakville, Ontario).

Site-Directed Mutagenesis. To examine the physiological function of acidic amino acids, mutations were made to an expression plasmid containing a hemagglutinin- (HA-) tagged human NHE1 isoform of the Na^+/H^+ exchanger. The plasmid pYN4+ contains the cDNA of the entire human NHE1 coding region plus a hemagglutinin (HA) tag on the C-terminus (*26*). The amino acid sequence ^{753}EEDEDDD^{759} was subjected to site-directed mutagenesis by amplification with PWO DNA polymerase followed by use of the Stratagene (La Jolla, CA) QuikChange site-directed mutagenesis kit as recommended by the manufacturer. Mutations were designed to create new restriction enzyme sites for use in screening transformants. DNA sequencing confirmed the accuracy of the mutations. Table 1 illustrates the mutations made to the sequence.

Cell Culture and Stable Transfection. To examine Na^+/H^+ exchanger expression and activity, cells that lack an endogenous Na^+/H^+ exchanger were used (*27*). These AP-1 cells, a derivative of CHO cells, were used to make stable cell lines containing mutant and wild-type Na^+/H^+ exchangers. Transfection was with Lipofectamine 2000 reagent according to the manufacturer's protocol as described earlier (*26*) or with calcium phosphate as described earlier (*27*). Transfected cells were selected by use of Geneticin (G418, 800 *μ*g/mL), and stable cell lines for experiments were regularly reestablished from frozen stocks at passage numbers between 5 and 10.

SDS−PAGE and Immunoblotting. To confirm NHE1 expression, immunoblot analysis was used on samples from total cell lysates of stable cell lines. For Western blot analysis,

Table 1: Oligonucleotides Used for Site-Directed Mutagenesis[a]

mutation[a]	name[b]	primer[c]	introduced site
^{753}EEDEDDD^{759}	Wt		
^{753}QQNQNNN^{759}	AtoN	CGGGATCCTGCAAAGGTGGCT**c**AG**c**AG**a**AC**c**AG**a**AC**a**AC**a**ATGGGGG-CATCATGATGCGG AGCAAG	*Xcm*I
^{753}EENQNDD^{759}	DED	GTGGCTGAGGA**aa**AC**c**AG**a**ACGACGATGG	*Xcm*I
^{753}QQDEDNN^{759}	E2D2	AAAGGTGGCT**c**AG**c**AGGACGAGGAC**a**AC**a**A TGGGGGCAT	*Blp*I
^{753}QQDEDDD^{759}	E2	AAAGGTGGCT**c**AG**c**AGGACGAGGACGACG ATGGGGGCAT	*Blp*I

[a] Mutated amino acid residues are indicated in single-letter notation and are underlined. [b] AtoN, acidic to neutral mutant; Wt, wild type. [c] Mutated nucleotides are in lowercase letters and boldface type. New restriction sites in the synthetic oligonucleotides are underlined. The forward direction of the primer pair is illustrated.

equal amounts of cell lysates (up to 100 μg) were resolved on SDS−12% polyacrylamide gels (*26*). The gel was transferred onto a nitrocellulose membrane and immunoreactive material was revealed by use of anti-HA monoclonal antibody (Boehringer Mannheim, Laval, Quebec, Canada) and peroxidase-conjugated goat anti-mouse antibody (Bio/Can, Mississauga, Ontario, Canada). To visualize immunoreactive proteins, the Amersham enhanced chemiluminescence Western blotting and detection system was used.

Cell Surface Expression. To measure cell surface expression, cells were labeled with sulfo-NHS−SS−biotin (Pierce Chemical Co., Rockford, IL) and immobilized streptavidin resin was used to remove cell surface labeled Na^+/H^+ exchanger (*26*). Equal amounts of the total and unbound proteins were analyzed by SDS−PAGE and Western blotting as described above. The relative amount of NHE1 on the cell surface was calculated by comparing the 110 kDa species of NHE1 in Western blots of the total and unbound fractions. Results are shown as mean ± SE.

Na^+/H^+ Exchange Activity. Na^+/H^+ exchange activity was measured on a Shimadzu RF 5000 spectrofluorometer as described earlier (*18*, *23*). The initial rate of Na^+-induced recovery of cytosolic pH (pH_i) was measured after acute acid load by use of 2′,7-bis(2-carboxyethyl)-5(6)carboxyfluorescein-AM (BCECF-AM; Molecular Probes Inc., Eugene, OR). Ammonium chloride (50 mM × 4 min) was used to transiently induce an acid load and the recovery in the presence of 135 mM NaCl was measured as described previously (*27*). The rate of recovery over the first 10 s, second 10 s, or the entire initial 20 s after acid load was measured. There were no differences in buffering capacities of stable cell lines as indicated by the degree of acidification induced by ammonium chloride. Results are shown as mean ± SE.

For some experiments, the amount of acidification induced in Na^+/H^+ exchanger-expressing cells was varied by using 10−40 mM ammonium chloride for 2−5 min. Curve fitting was done with SigmaPlot, by use of the Hill plot with three parameters. In other cases we examined the effect of varying the Na^+ concentration on the Na^+/H^+ exchanger. For these experiments, NaCl concentration was varied between 5 and 100 mM. At reduced NaCl concentrations, osmolality was maintained by addition of *N*-methylglucamine.

Immunocytochemistry. To determine the intracellular localization of the Na^+/H^+ exchanger, immunocytochemistry was used. Stably transfected cells expressing wild-type or mutant NHE1 were grown on coverslips to 70−80% confluence. Coverslips containing cells were washed twice with 1× PBS, pH 7.4, followed by fixation with methanol for 15 min at −20 °C. Cells were then washed twice for 10 min in 1 × PBS. They were subsequently permeabilized by incubating in TA-PBS [0.1% BSA and 0.2% Triton X-100 in phosphate-buffered saline (PBS)] for 15 min and then washed three times in TA-PBS. The coverslips were blocked with 5% (v/v) goat serum in TA-PBS for 20 min. After being washed three times with TA-PBS, coverslips were incubated with mouse monoclonal anti-HA antibody (1:300 in TA-PBS; Boehringer Mannheim, Laval, Quebec, Canada) for 1 h at room temperature. The cells were washed three more times with TA-PBS and reacted with the secondary antibody, Alexa Fluor 488-conjugated goat anti- (mouse IgG) Ig (1:300 in TA-PBS; Molecular Probes Inc., Eugene, OR). Cells were finally washed three times with PBS, and the coverslips were mounted by use of Vectasheild (Vector Laboratories Inc., Burlingame, CA) mounting medium with 4′,6-diamidino-2-phenylindole (DAPI). Cells were examined on a Leica DMIRB fluorescent microscope.

Production and Purification of Proteins. To examine inter- and intramolecular protein−protein interactions, we produced the cytosolic domain and several other regions of the Na^+/H^+ exchanger, plus several Na^+/H^+ exchanger-binding proteins. The carboxyl-terminal 182 amino acids of the human Na^+/H^+ exchanger (NHE1) was expressed as a fusion protein with a C-terminal hexahistidine tag (His-182) by use of the plasmid pDest 14 and the Gateway Cloning System as described earlier (*18*). Mutant His182, E2D2, and DED proteins were produced the same way. Tescalcin was produced as an amino-terminal hexa-His-tagged fusion protein by use of the Gateway cloning system as described earlier (*23*). Calmodulin was produced with an S-tag as described earlier (*23*). Intracellular loops (IL) 2 and 5 of NHE1 were produced by use of the Gateway cloning system. For IL2 and IL5, amino acids ^{174}GYFLPLRQFTENLGTI^{189} and ^{431}GLTWFINKFRIVKLTPKDQF^{450} were produced (respectively) with in-frame N-terminal GST fusions. These proteins were purified via glutathionine−Sepharose 4B affinity chromatography as described earlier (*28*). The identity of the purified fusion proteins was confirmed by MALDI mass spectrometry at the Institute of Biomedical Design at the University of Alberta. For unknown reasons, similar attempts to produce IL4 were not successful.

Affinity Blotting of Na^+/H^+ Exchanger and Interacting Proteins. To examine binding to the Na^+/H^+ exchanger proteins immobilized on nitrocellulose, 20 μg of ovalbumin or GST and the His182 proteins were separated on SDS−12% PAGE and then transferred to nitrocellulose membranes. To ensure equivalent amounts of protein had transferred, nitrocellulose membranes were stained with Ponceau S stain and the amount of transferred proteins was quantified. Nitrocellulose membranes were then blocked with 10% (w/v) skim milk powder in Tris-buffered saline (TBS; 20 mM Tris, pH 7.4, and 137 mM NaCl) overnight at 4 °C. They were then incubated with 20 μg/mL putative interacting proteins with 1% (w/v) skim milk powder in TBS for 5 h at 4 °C. Membranes were washed with TBS for 4 × 15 min at room temperature. The nitrocellulose was then incubated with appropriate antibodies against the interacting protein in TBS with 1% skim milk powder for 1 h at room temperature, followed by washing for another hour with TBS. Further amplification was achieved by a subsequent incubation with goat anti-rabbit−HRP antibodies. Reactive bands were visualized by the Amersham enhanced chemiluminescence system. For calmodulin, affinity blotting was done in the presence of 0.1 mM $CaCl_2$. The IL2 and IL5 blots were probed with a GST fusion of these proteins, and the first antibody for detection of interactions was rabbit anti-GST protein.

Trypsin Treatments of His182 Proteins. To compare the conformation of wild-type His182 and mutant His182 with the AtoN (acid to neutral) mutations, we treated samples with varying times and concentrations of trypsin, followed by analysis of tryptic fragments. Target proteins were diluted to a protein concentration of 1 mg/mL in 1 mM EDTA, adjusted to pH 7.4 with Tris-HCl. After preincubation of

20 μg of protein solution for 5 min at 37 °C, trypsin was added to give a trypsin/protein ratio of 1:2000 or 1:200. After incubation at varying times, the reaction was terminated by addition of 2 μL (7 mg/ mL) of trypsin inhibitor (Sigma) followed by addition of sample buffer for SDS−PAGE.

Gel-Filtration Chromatography. Gel-filtration chromatography was used to compare the apparent size of wild-type and mutant His 182 proteins. A Bio-Rad Bio-Sil SEC 125-5 column was used of size 300 mm × 7.8 mm. The mobile phase was 0.1 M sodium phosphate ane 150 mM KCl, pH 6.8, and the flow rate was 1.0 mL/min, with sample detection at 280 nM. The protein standards were thyroglobulin, IgG, ovalbumin, myoglobin, and vitamin B12.

Protein Identification by In-Gel Tryptic Digestion. Stained bands (spots) were excised and automated in-gel tryptic digestion was performed on a Mass Prep Station (Micromass). The gel pieces were destained, reduced with DTT, alkylated with iodoacetamide, and then digested with trypsin (Promega sequencing grade modified). The resulting peptides were extracted from the gel and analyzed via liquid chromatography and mass spectroscopy (LC/MS). LC/MS was performed on a CapLC HPLC (Waters) coupled with a Q-ToF-2 mass spectrometer (Micromass). Tryptic peptides were separated by use of a linear water/acetonitrile gradient (0.2% Formic acid) on a Picofrit reversed-phase capillary column (5 μm BioBasic C18, 300 Å pore size, 75 μm i.d. × 10 cm, 15 μm tip) (New Objectives, MA), with an in-line PepMap column (C18, 300 μm i.d. × 5 mm) (LC Packings, CA) used as a loading/desalting column. Protein identification from the generated MS/MS data was done by searching the NCBI nonredundant database with Mascot Daemon (Matrix Science). Search parameters included carbamidomethylation of cysteine, possible oxidation of methionine, and one missed cleavage per peptide.

RESULTS

Acidic Residues in the Na^+/H^+ Exchanger. Figure 1 shows a general model of the NHE1 isoform of the Na^+/H^+ exchanger. Putative binding sites on the C-terminal cytosolic tail for many protein regulators of the Na^+/H^+ exchanger are illustrated. The location of an acidic sequence of amino acids present in the distal region of the tail is indicated by DE. Figure 2 illustrates an alignment of 20 amino acids of the cytosolic tail that surround and include this acidic region. All vertebrate forms of NHE1 had an acidic region, including trout and salamander. Nonvertebrate forms of NHE1, such as *Drosophila* and *Caenorhabditis elegans*, did not have this region, but their cytosolic tails were usually much shorter than the vertebrate NHE1 isoforms and did not extend to this region (not shown). In most cases the acidic sequence consisted of at least seven continuous acidic residues, though in the rabbit seven acidic residues were interrupted by two alanines. Human NHE5 also had a similar acidic sequence within its tail.

Functional Role of Acidic Residues. To examine the functional role of this acidic sequence in the Na^+/H^+ exchanger we made several mutants of the protein. Table 1 illustrates the different mutants constructed. We transfected expression plasmids containing these different Na^+/H^+ exchanger DNAs and made stable cell lines of each. From Figure 3A it can be seen that the mutant and wild-type

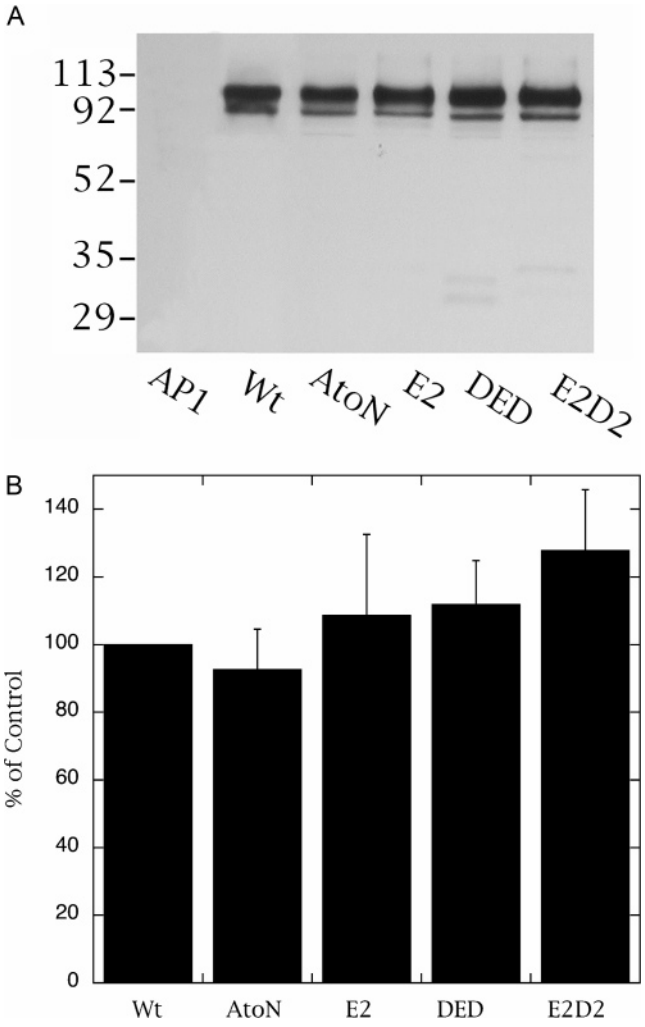

FIGURE 3: Western blot analysis of cell extracts from control and stably transfected AP-1 cells. Cell extracts were prepared from control (AP-1) cells and from cells stably transfected with cDNA coding for HA-tagged exchanger with the sequence of the following at amino acids 753−759: wt, wild-type; NHE1, EEDEDDD; AtoN, QQNQNNN; E2, QQDEDDD; DED, EENQNDD; E2D2, QQDEDNN. Sixty micrograms of total protein was loaded in each lane. (A) Western blot of samples; (B) summary of five experiments (results are mean ± SE).

exchangers displayed the same pattern of immunoreactive bands. There was a larger band at approximately 110 kDa that represents the glycosylated form of the mature Na^+/H^+ exchanger and a smaller band at approximately 95 kDa that represents an immature form of the exchanger that is not fully glycosylated (*29*). There was no evidence of increased amounts of the 95 kDa isoform of the exchanger, which sometimes occurred with mutations that result in protein mistargeting (*27*). In several experiments we noted that the level of expression of the mutant Na^+/H^+ exchangers was similar to that of the wild type (Figure 3). For these experiments, cells in a similar degree of confluence were lysed and equal amounts of lysate protein were used for Western blot analysis.

To verify correct targeting of NHE1 within the cell we used two methods. Figure 4A illustrates the immunocytochemical localization of the Na^+/H^+ exchanger showing the predominant plasma membrane localization. Panel A shows cells transfected with the wild-type NHE1 and panels B−E show the AtoN, DED, E2D2, and E2 mutants, respectively.

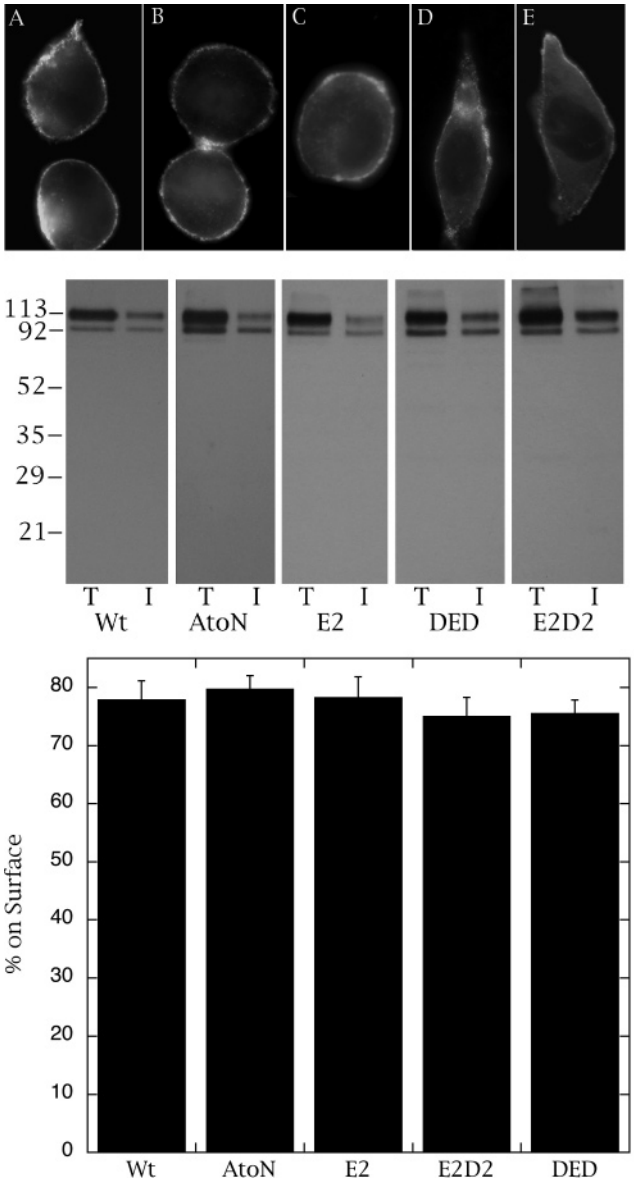

FIGURE 4: Localization of wild-type and mutant Na^+/H^+ exchanger-expressing cell lines. (Top panel) Immunocytochemical localization of the Na^+/H^+ exchanger protein demonstrating plasma membrane localization. Transfected cells were prepared for immunocytochemical analysis with anti-HA antibody as described under Materials and Methods. (A) Wild-type NHE1; (B) AtoN; (C) DED; (D) E2D2; (E) E2. (Middle panel) Representative Western blots for measurement of the subcellular localization of the Na^+/H^+ exchanger. Cells were treated with sulfo-NHS−SS−biotin and solubilized, and biotin-labeled proteins were bound to streptavidin agarose beads as described under Materials and Methods. A sample of the total cell lysate (T) and an equivalent amount unbound lysate (I, intracellular) were run on SDS−PAGE. Western blotting was with an anti-HA antibody and identifies the NHE1 protein. Wt, Wild-type NHE1; AtoN, E2, DED, and E2D2, mutant Na^+/H^+ exchanger proteins as described in Table 1. (Bottom panel) Summary of results of subcellular localization of the Na^+/H^+ exchanger by biotin labeling. The results are mean ± SE of at least three determinations.

Cells transfected with wild-type Na^+/H^+ exchanger (NHE1) or the mutants all showed strong plasma membrane localization. Figure 4B illustrates a different method of confirming correct intracellular localization of cells, use of quantitative measurement with sulfo-NHS−SS−biotin. Cells were treated, lysed, and solubilized and then labeled proteins were bound to streptavidin agarose beads. An equal amount of the total cell lysate and unbound lysate was separated by size by SDS−PAGE, followed by Western blotting with anti-HA antibody to identify tagged NHE1 protein. It was not possible to reliably elute the biotin-labeled protein from the streptavidin agarose beads, likely due to its high affinity for the beads. Control and Na^+/H^+ exchanger with mutations showed equivalent amounts of plasma membrane localization. In other experiments (not shown) we noted that nonspecific binding of proteins to streptavidin agarose beads was approximately 10%; therefore, our values of NHE1 on the cell surface could be overestimated by this amount. It is interesting to note that both the glycosylated and unglycosylated Na^+/H^+ exchanger appeared to be present on the cell surface. The significance of this observation is not yet clear; however, it has been shown that immature forms of intracellular membrane proteins can sometimes leak to the cell surface (*30*). A summary of a series of experiments on the 110 kDa isoform is shown in Figure 4C. (Results did not vary substantially when the 95 kDa isoform was included.)

To determine the physiological significance of the acidic sequence in NHE1 function, we examined the activity of cell lines containing wild-type and mutant Na^+/H^+ exchangers with the changes to the acidic sequence. Because we found that mutations in this sequence caused unusual time-dependent effects, we examined the rate of recovery from acid load both in the initial 10 s of recovery and in the following 10 s. Figure 5A,B illustrates a summary of the effect of mutations in the acidic sequence on the initial rate of recovery from an acute acid load that was induced by ammonium chloride. In the first 10 s of recovery, the activity of the AtoN mutant was reduced by more than two-thirds (Figure 5A). The activity of the DED mutant was reduced by approximately one-third, while the E2 and E2D2 mutants were near normal. For the following 10 s, the activity of all the mutants was reduced by about half. Figure 5C illustrates an example of the rate of recovery from an acid load, in the wild-type NHE1 and the AtoN mutant. The mutant typically started recovering from the acid load slowly and then the rate of recovery accelerated. This was in contrast to wild-type NHE1 that activates rapidly from the onset at the initial, most acidic pH.

Because the A to N mutants displayed abnormal activity with ammonium chloride-induced activation, we examined the kinetic characteristics of the wild-type and mutant (AtoN) Na^+/H^+ exchanger. We acidified both wild-type and mutant NHE1 to varying degrees to examine the effect of varying proton concentration. The activity of the AtoN mutant was very low and variable in the first 10 s, making accurate kinetic characterization difficult. Figure 6A,B illustrates the effects of modifying the amount of acidification on the rate of recovery in the second 10 s. The wild-type and AtoN mutant proteins illustrated positive Hill coefficients of 1.24 and 1.44, respectively (Figure 6 C,D). Half-maximal activation was at pH 6.9 and 7.2, respectively. In all cases the maximum velocity of the AtoN mutant was much less than that of the wild type. The wild-type and mutant activity were 2 and 1 (ΔpH/min), respectively. Further analysis of the kinetics of activation of the AtoN mutant was done by examining the effect of different Na^+ concentrations on activity of the exchanger. Figure 6C shows that at every NaCl concentration the wild-type Na^+/H^+ exchanger had more activity than the mutant. When plotted as a percent of maximum activity (Figure 6D), it is evident that the activation

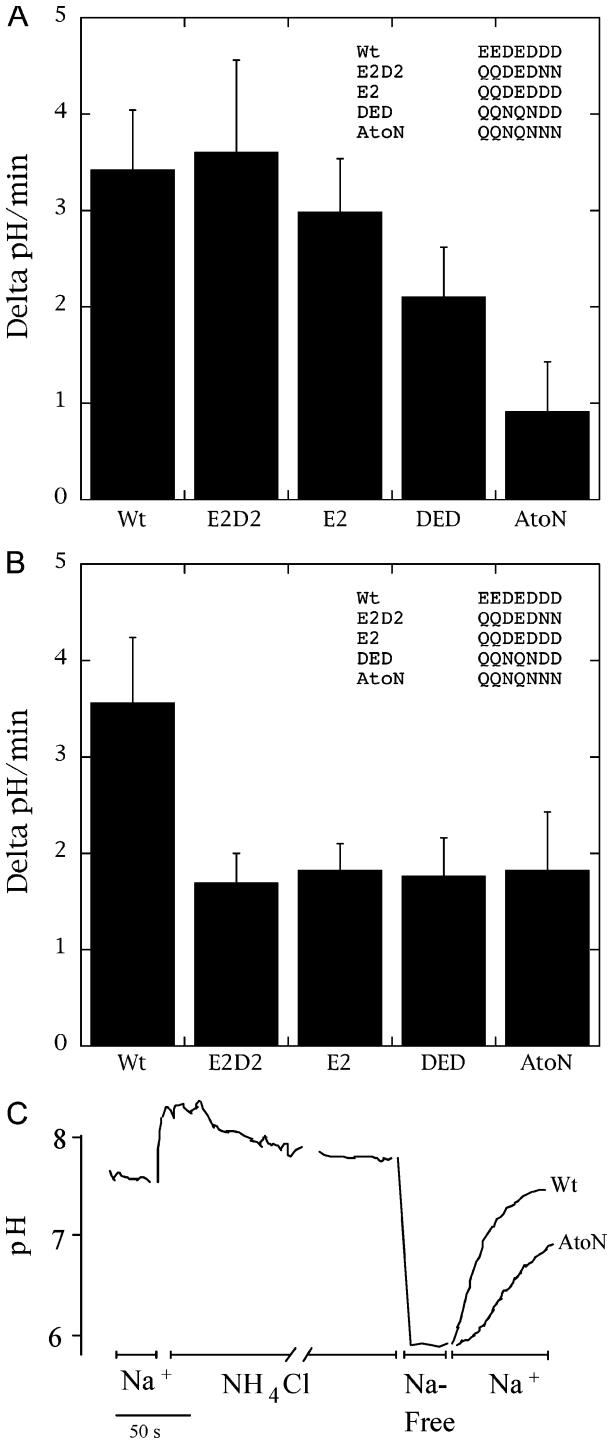

FIGURE 5: Initial rate of recovery from an acute acid load by AP-1 cells containing Na^+/H^+ exchanger mutants. Wt, Wild-type NHE1; AtoN, E2, DED, and E2D2, mutant Na^+/H^+ exchanger proteins as described in Table 1. Cells were prepared by stable transfection with mutant Na^+/H^+ exchanger DNA, and the initial rate of recovery from an NH_4Cl-induced acid load was measured as described under Materials and Methods. (A) Summary of the initial rate of recovery during the first 10 s after reintroduction of NaCl, following the Na^+-free medium. **(**B) Summary of the rate of recovery during the second 10 s after reintroduction of NaCl, following the Na^+-free medium. (C) Example of the rate of recovery from an acute acid load of wild type (Wt) and the acid to neutral (AtoN) mutant, with only one example of NH_4Cl loading of cells illustrated. Na^+, time of NaCl present; NH_4Cl, 50 mM NH_4Cl present; Na-free, NaCl free buffer.

by NaCl is the same in the mutant Na^+/ H^+ exchanger as in the wild type.

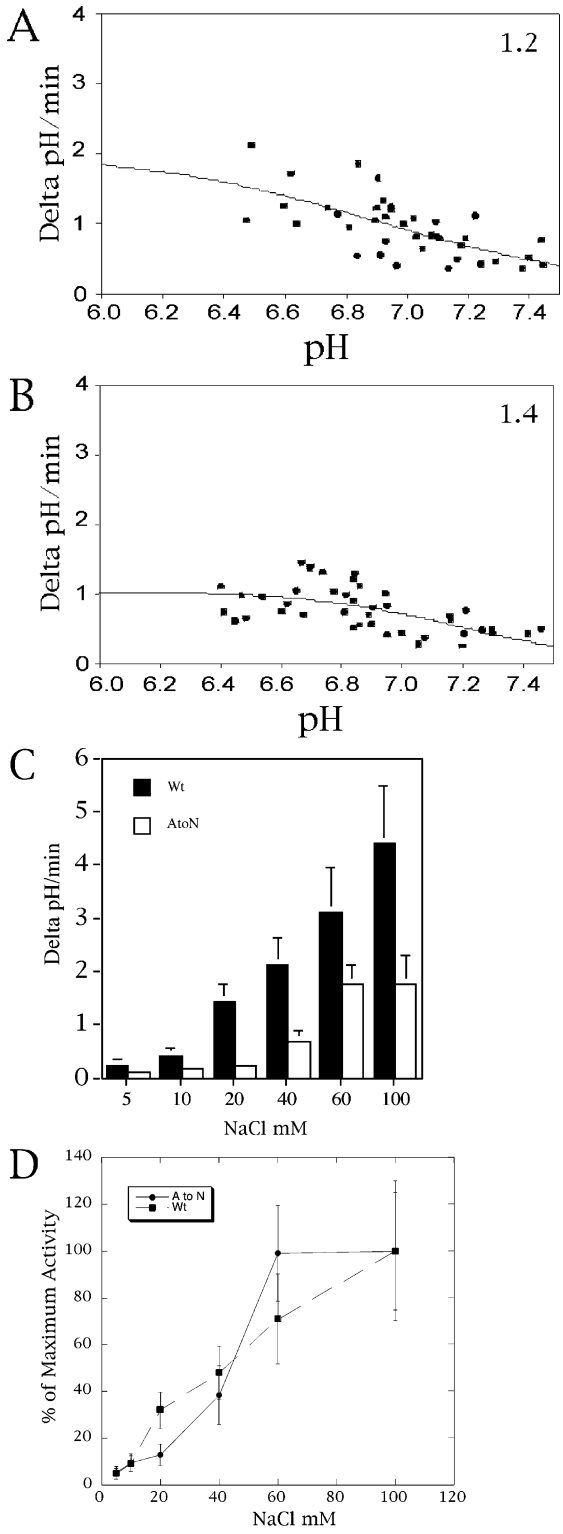

FIGURE 6: Analysis of kinetic characteristics of cells containing wild-type and acid to neutral (AtoN) mutant Na^+/H^+ exchanger. (A, B) Dependence of Na^+/H^+ exchanger activity on intracellular pH. Cells were preincubated with different concentrations of NH_4-Cl for varying times. Recovery was determined in Na^+-containing buffer (135 mM) in the second 10 s after acidification (time 10−20 s) as described under Materials and Methods. (A) Cells containing wild-type Na^+/H^+ exchanger; (B) cells containing Na^+/H^+ exchanger with the acid to neutral (AtoN) mutation. Insets contain the values of the Hill coefficients. (C, D) Dependence of Na^+/H^+ exchanger activity on extracellular sodium concentration. Cells were acidified and activity was measured in the presence of varying extracellular sodium concentrations as described under Materials and Methods.

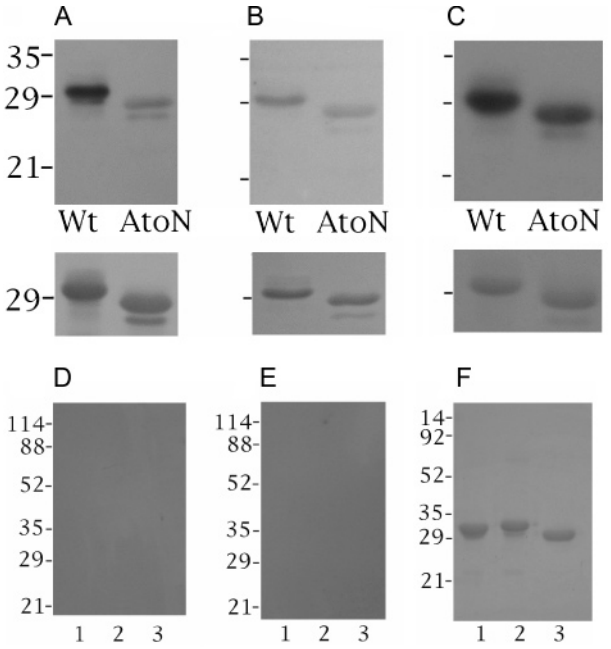

FIGURE 7: Affinity blotting assays of Na^+/H^+ exchanger carboxyl-terminal protein of the cytoplasmic domain of the Na^+/H^+ exchanger. (A−C) Aliquots (20 μg) of the wild-type (Wt) and acidic to neutral mutant His182(AtoN) proteins were separated by SDS−PAGE and transferred to nitrocellulose. Affinity blotting assays with (A) calmodulin, (B) carbonic anhydrase II, and (C) tescalcin are shown. (D, E) In lanes 1−3, respectively, 20-μg aliquots of His182, His182(AtoN), and control (ovalbumin) were separated by SDS−PAGE and transferred to nitrocellulose. They were probed with either IL2 (D) or IL5 (E). (F) Coomassie blue-stained gel with samples of purified IL2 (lane 1), IL5 (lane 2), and GST (lane 3).

Effect of Acidic Residues on Protein−Protein Interactions. We examined whether the ^{753}EEDEDDD^{759} sequence affected protein−protein interactions of the cytosolic tail and whether this region mediates its function via interaction with intracellular loops of NHE1. Initial experiments examined whether changing the sequence to neutral residues affected the ability of interacting, regulatory proteins to bind to the NHE1 cytoplasmic domain. His182 protein encodes the C-terminal 182 amino acids of the Na^+/H^+ exchanger and includes binding sites for calmodulin, tescalcin, and carbonic anhydrase II (*18*, *23*). Figure 7A−C examines the ability of calmodulin, carbonic anhydrase II, and tescalcin (respectively) to bind to wild-type and mutant His182(AtoN) protein. The mobility of the mutant His182 protein was somewhat greater than that of the wild-type His182 (see later discussion). For tescalcin and carbonic anhydrase II, the mutant His182(AtoN) bound similar amounts of these proteins in comparison to the wild type. Comparison of the intensity of the bound NHE binding proteins with the intensity of the full-length His182 and mutant His182 showed no apparent differences in protein binding (not shown). However, we found that the binding of calmodulin was significantly reduced in the mutant His182(AtoN) in contrast to the control (Figure 7A). Analysis of the level of calmodulin binding relative to protein showed that the His182(AtoN) mutant had less than half (43%) the level of wild-type binding ($n = 8$), which was a significant reduction ($P < 0.05$) in comparison to the controls.

One possibility is that the tail of the Na^+/H^+ exchanger modulates NHE1 function by binding to intracellular loops of the membrane domain. Furthermore, mutations in the ^{753}EEDEDDD^{759} sequence could affect this phenomenon. To examine whether mutations in the acidic sequence could be acting in this way, we produced intracellular loops 2 and 5 as GST fusion proteins as outlined under Materials and Methods. His182 and His182(AtoN) were run on SDS−PAGE and transferred to nitrocellulose membranes. They were probed with either IL2 or IL5 at either pH 6.4 or 7.4. In both cases we were not able to show significant binding to IL2 or IL5 regions (Figure 7D,E).

In gel overlay experiments, we noticed that the mutant His182(AtoN) always migrated with a greater mobility than the wild-type His182 protein. To examine this property in more detail, we produced purified His182 protein, His182-(AtoN), and His182 with the E2D2 and DED mutations (Table 1). Figure 8A,B illustrates differences in mobility of these proteins. The mutant His 182(AtoN) protein had a greater mobility than the wild type, with the E2D2 protein having an intermediate effect and the DED mutant being only mildly affected. For His182 and His182(AtoN), the change in apparent mobility was almost 1 kDa while the calculated change in molecular weight was only 7 Da. We confirmed the identity of the mutant and wild-type His182 proteins by cutting bands out of SDS−PAGE gels and subjecting proteins to MALDI mass spectroscopy. The relatively large difference in apparent mobility in SDS−PAGE, combined with the small difference in actual mass of the proteins, suggested that there might be a significant change in the conformation of the protein induced by neutralizing the acid sequence. To examine this possibility we used two different approaches: comparison of the two proteins' sensitivity to proteases and gel-filtration chromatography. Figure 8C shows the products of digestion of the two proteins with trypsin. There were several differences in the digestion pattern of the His182 protein and the His182(AtoN) mutant. Trypsin digests C-terminal to lysine and arginine residues; therefore, the site of digestion is not the mutations in the acidic residues.

We used another independent method to confirm that mutation of the acidic sequence in His182 results in a change in conformation of the protein: size-exclusion chromatography (Figure 9). Samples of purified His182 and His182-(AtoN) were size-fractionated as described under Materials and Methods. The apparent size of the wild-type His182 protein was 4.7 kDa. The His182(AtoN) protein was 3.2 kDa, 1.5 kDa smaller than the size of the wild-type protein. The identity of the peaks was confirmed by mass spectroscopy. Other peaks represented degradation products.

A final test was used to compare the accessibility of the mutant His182 with that of the wild type. Figure 8C illustrates the results of partial trypsinolysis of the wild-type and mutant proteins. The asterisks indicate different size products produced by the different proteins. The wild-type and mutant products indicated by the asterisks were excised and subject to in gel digestion with trypsin, followed by analysis via mass spectroscopy. In the wild-type His182 we were able to identify four peptides that corresponded to amino acids 652−669, 691−698, 701−711, and 751−765. In the mutant His182(AtoN), three peptides corresponding to amino acids 691−698, 701−711, and 751−765 were identified but the 652−669 peptide was absent. The absence of this peptide indicated either that it was not present in the original peptide fragment from the gel or that it was not released by further digestion of the peptides with trypsin.

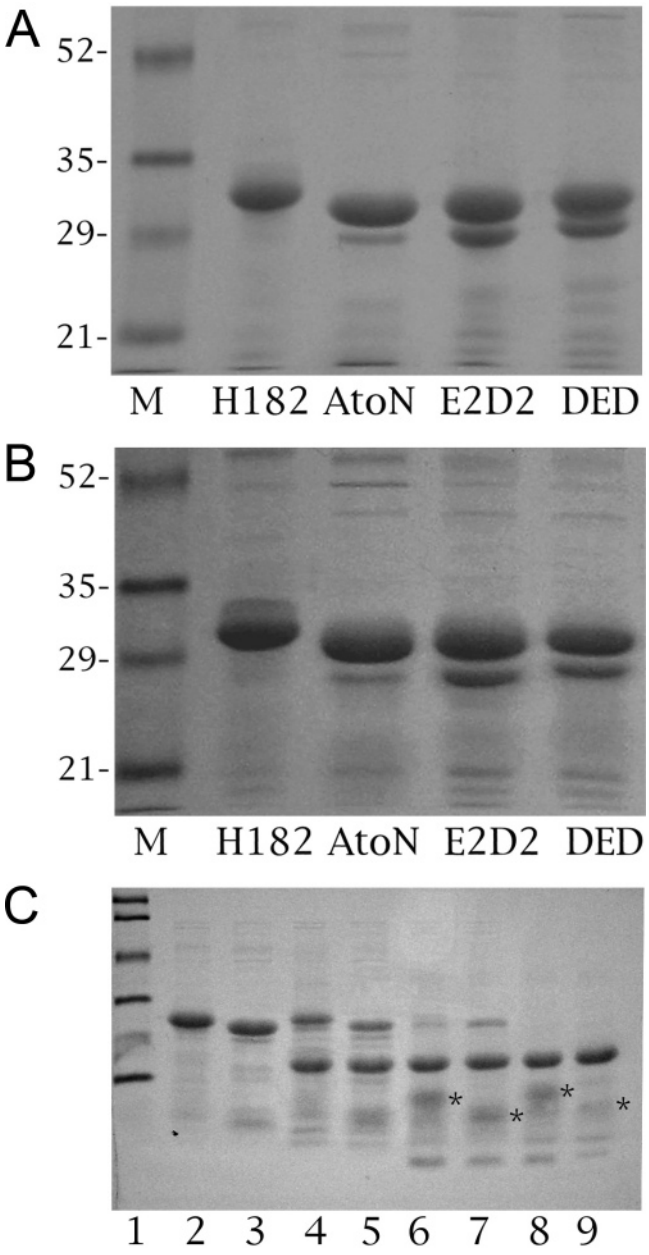

FIGURE 8: Characterization of electrophoretic mobility and trypsin sensitivity of His182 and His182 mutants. (A, B) Samples of partially purified His182 (H182) and mutated His182 (His182AtoN, His182E2D2, and His182DED) were separated by SDS−PAGE (15% polyacrylamide in panel A, 12% polyacrylamide in panel B), followed by staining with Coomassie blue. (C) His182 and His182-(AtoN) were treated with trypsin as described under Materials and Methods and separated by SDS−15% PAGE, followed by staining with Coomassie blue. Lane 1: protein markers of mass 113, 88, 52, 35, 29, and 21 kDa. Lanes 2, 4, 6, and 8: wild-type His 182 protein. Lanes 3, 5, 7, and 9: His182(AtoN) protein. Lanes 2 and 3, no treatment with trypsin; lanes 4 and 5, treatment with 1:2000 trypsin/protein for 1 min; lanes 6 and 7, treatment with 1:200 trypsin/protein for 1 min; lanes 8 and 9, treatment with 1:200 trypsin/protein for 10 min. Asterisks indicate the location of protein bands with different apparent mobility when His182 is compared to His182(AtoN).

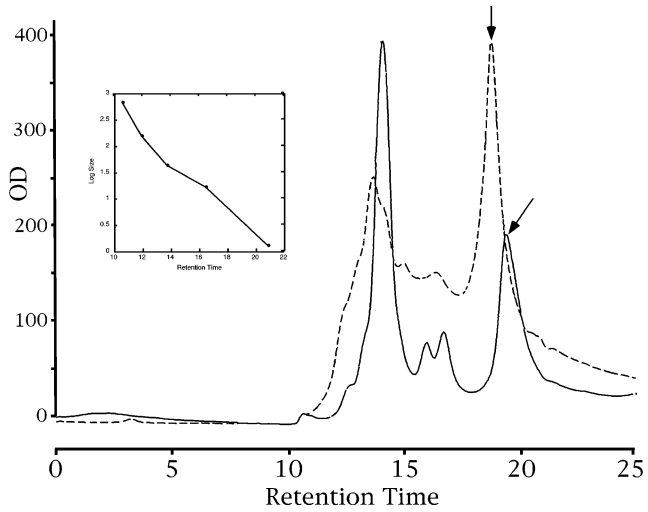

FIGURE 9: Size-exclusion chromatography of His182 and His182-(AtoN). Wild-type and mutant His182 proteins were size-fractionated as described under Materials and Methods. A calibration curve of standards of known molecular weights (inset) was used to compare the apparent size of the two proteins. Arrows indicate the peak fractions containing the wild-type and mutant His182. Dashed line, wild-type His182; solid line, His182(AtoN).

DISCUSSION

The cytosolic regulatory domain of the Na^+/H^+ exchanger is responsible for regulation of activity of the membrane domain. It binds regulatory proteins and is phosphorylated by several different protein kinases (*16*−*18*, *20*). We examined the sequence of the regulatory domain and found that in all vertebrate NHE1 isoforms there was a conserved series of acidic residues present. Given that NHE1 functions in cation transport and that acidic sequences may contribute to the capture and selection of cations in some transporters (*25*), an investigation of the function of this sequence was merited. Mutation of the acidic region caused up to 45% decreases in the rate of recovery from an acute acid load. The effect of mutation of the sequence was not due to low expression of the protein or to aberrant targeting of the Na^+/H^+ exchanger. By examination of various mutants of the acidic sequence, it was clear that mutation of amino acids ^{755}DED^{757} was sufficient to cause a debilitation of the activity of the exchanger in the first 10 s of recovery. Other mutations to this region caused a reduced activity in the second 10 s after acidification.

The mechanism by which the acidic sequence mediates NHE1 activity was investigated by examining the kinetic parameters of the mutant and wild-type protein. Activity of the mutant Na^+/H^+ exchanger protein was peculiar in that the cells had a delay or lag period in their activation, while wild-type Na^+/H^+ exchanger was activated immediately and rapidly upon acid treatment (Figure 5C). Analysis of the kinetic characteristics of the mutant NHE1 confirmed that proton activation was altered in comparison to the wild-type protein. During the first 10 s after acidification, mutant NHE1 activity was impaired (Figure 5A). During the next 10 s period, the activity of the wild-type NHE1 and the AtoN mutant both had positive Hill coefficients, though the wild type NHE1 had much more activity. Activation of the AtoN mutant occurred at a higher pH, indicating increased proton affinity; however, the activation appeared to be incomplete. Even at very acidic intracellular pH, the AtoN mutant never completely activated in comparison to the wild-type Na^+/H^+ exchanger. These results suggest that the acidic sequence contributes to proton activation of the Na^+/H^+ exchanger and that its mutation results in abnormal activation of the protein. The lack of complete activation of the Na^+/H^+

exchanger results in greatly reduced activity, despite an earlier activation at a more basic intracellular pH.

We examined whether regulatory protein binding to the C-terminal was impaired by mutation of the acidic residues. While we were unable to detect binding of the cytosolic tail to intracellular loops of the Na^+/H^+ exchanger, we were able to examine the binding of the regulatory proteins calmodulin, CAII, and tescalcin. CAII and tescalcin binding were not impaired; however, binding of the regulatory protein calmodulin was decreased by over 50%. The functional implications of a decrease in calmodulin binding agree with the results we obtained. Calmodulin binds to amino acids 637−656 and it prevents the action of an autoinhibitory domain, which affects the intracellular proton affinity (*17*). Defective calmodulin binding would likely lead to increased ability of the autoinhibitory domain to affect activity of the Na^+/H^+ exchanger. This would cause a decrease in proton affinity, similar to what we observed.

The region that we mutated, amino acids 753−759, is over 100 amino acids downstream of the sequence shown to bind calmodulin. Therefore, the question arises as to how it could mediate an effect on calmodulin binding. Likely the answer is that the acidic sequence is necessary for maintenance of a proper conformation in the cytosolic tail. Disruption of the sequence caused a substantial change in the size of the C-terminal region protein that was detected both on SDS−PAGE and by gel-filtration chromatography. In addition, mutation of the acidic sequence caused an alteration in the pattern of digestion by trypsin that was visible on SDS−PAGE.

Additional evidence that the acidic sequence affects conformation came from further analysis of the products of trypsin digestion, and this analysis gave more evidence of differences in protein conformation. The unique products of trypsin digestion that were visible on SDS−PAGE were excised and subjected to further tryptic digestion and mass spectroscopic analysis. A tryptic fragment of His182 gave four detectable peptides including one from amino acids 652 to 669 that contains part of the calmodulin-binding domain. However, tryptic fragments of the mutant His182(AtoN) did not contain the fragment with the calmodulin-binding domain. It is likely that this domain was not accessible to trypsin either in the tryptic digestion prior to SDS−PAGE or in the tryptic digestion of the gel fragments. These results suggest that the acidic sequence somehow affects trypsin accessibility of the calmodulin-binding domain. It is interesting to note that amino acids 613−651 contain eight basic residues that could potentially interact with the acidic domain, though further experiments are necessary to examine this possibility. Overall, our results suggest that mutation of the acidic sequence mediates its effects on activity by altering the conformation of the tail, which in turn decreases calmodulin binding and results in reduced affinity for protons.

We found that mutation of the acidic sequence of the cytosolic tail resulted in slower activation of the mutant Na^+/H^+ exchanger. Sluggish activation of the NHE3 isoform has been reported; however, this further activation of the protein occurred after several minutes of acidification. Therefore, the slow activation was likely due a to change in conformation of the exchanger rather than protein aggregation or cytoskeletal interactions. NHE1 did not show a comparable slow activation (*31*). While it is uncertain how the reduced rate of activation of the Na^+/H^+ exchanger is caused in our study, it could be due to a diminished rate of change to the optimal conformation necessary for transport. This is possibly mediated by the autoinhibitory calmodulin-binding domain. Knowledge of exactly how the tail modulates the membrane domain awaits further experimentation, possibly studies on the crystallization of the full-length protein.

It is interesting to note that in plant cation exchangers, acidic residues contribute directly to a vestibule or filter for cation selection (*25*). While in the plant cation exchanger this type of sequence is found in intracellular loops (*25*), in our case the acidic residues were found in a more distal region of the tail. It is well-known that in the nicotinic acetylcholine receptor a different polypeptide contributes to the formation of the cytosolic vestibule on the channel that may regulate ion flow (*32*). Therefore, it is clear that a part of a polypeptide need not be on an intracellular loop to contribute to cytosolic vestibules and regulation of ion flux. Whether the acidic sequence could directly contribute to cation binding or selection in the Na^+/H^+ exchanger is not known at this time. Such a contribution would require that the acidic domain normally contribute to cation binding and transport and that the calmodulin-binding domain is a negative modulator of this function. This hypothesis awaits further experimentation.

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