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ARTICLE

Characterization of human mutations in phosphorylatable amino acids of the cytosolic regulatory tail of SLC9A1[1]

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Abstract: The NHE1 isoform of the mammalian Na^+/H^+ exchanger is a ubiquitous plasma membrane protein that regulates intracellular pH in cells by removing one intracellular proton in exchange for one extracellular sodium. Genetic defects in NHE1 have been shown to affect the growth and motor ability of mice, but mutations in humans have not been studied. NHE1 has a cytosolic C-terminal regulatory domain of approximately 300 amino acids. We investigated the functional effects of two human mutations found in the regulatory phosphorylatable amino acids Ser^{703} and Ser^{771}. A Ser703Pro mutant protein had essentially the same activity, expression, and targeting as the wild type NHE1 protein. In contrast, the Ser771Pro protein had reduced activity and expression of NHE1 protein, though cell surface targeting was normal. In dual pulse assays the Ser771Pro mutant was not further activated by sustained intracellular acidosis but displayed an unusual activation by brief pulses of acidosis. The results demonstrate that the Ser771Pro human genetic mutation has significant and detrimental physiological effects on the activity of the NHE1 protein, SLC9A1.

Key words: Na^+/H^+ exchanger, pH regulation, genetic mutation, phosphorylation.

Résumé : L'isoforme NHE1 de l'échangeur Na^+/H^+ de mammifère consiste en une protéine membranaire plasmatique ubiquiste qui régule le pH intracellulaire en enlevant un proton intracellulaire en échange d'un sodium extracellulaire. Il a été montré que des défauts génétiques de NHE1 affectaient la croissance et les capacités motrices chez la souris, mais les mutations chez l'humain n'ont pas été étudiées. NHE1 possède une extrémité C-terminale cytosolique régulatrice d'environ 300 acides aminés. Les auteurs ont examiné les effets fonctionnels de deux mutations trouvées chez l'humain sur les acides aminés Ser^{703} et Ser^{771} cibles de la phosphorylation. Une protéine mutante Ser703Pro possédait essentiellement les mêmes activité, expression et ciblage que la protéine NHE1 sauvage. En revanche, la protéine Ser771Pro possédait une activité et une expression réduites, alors que le ciblage à la surface cellulaire était normal. Lors de dosages réalisés à la suite d'impulsions doubles, le mutant Ser771Pro n'était pas activé par une acidose intracellulaire soutenue, mais il présentait une activation inhabituelle par des brèves impulsions acides. Les résultats démontrent que la mutation humaine Ser771Pro exerce un effet physiologique significatif et néfaste sur l'activité de la protéine NHE1, SLC9A1. [Traduit par la Rédaction]

Mots-clés : échangeur Na^+/H^+, régulation du pH, mutation génétique, phosphorylation.

Introduction

In mammalian cells, the maintenance of pH homeostasis in both normal and neoplastic cells is chiefly regulated by the Na^+/H^+ exchanger isoform one (NHE1). NHE1 is an integral plasma membrane protein consisting of a membrane domain that facilitates ion movement and a cytoplasmic C-terminal domain that regulates activity. NHE1 has been shown to play an important role in promoting cell growth, proliferation, differentiation, and apoptosis. In Na^+/H^+ exchange, one intracellular proton is exchanged for a single extracellular sodium ion. Transport is driven by the transmembrane Na^+ gradient, and NHE1 is ubiquitously expressed in mammalian cells. NHE1 is allosterically regulated. At low intracellular pH (pH_i), protons allosterically activate NHE1, facilitating proton extrusion and a return to homeostatic pH_i, at which point the NHE1 protein becomes inactive. NHE1 therefore maintains pH_i, but its activity is altered by hormonal regulation, which alters the proton sensitivity of the protein (Amith and Fliegel 2013; Fliegel 2005).

Regulation of NHE1 activity is critical and tightly controlled by multiple signaling pathways initiated by growth factors and hormonal stimulation (Amith and Fliegel 2013). Much of the regulation of NHE1 is controlled by the phosphorylation state of its C-terminal domain (Amith and Fliegel 2013; Malo and Fliegel 2006). Several different amino acids have been implicated as being critical to NHE1 regulation in various tissues, and this includes Ser^{703}, Ser^{770}, and Ser^{771}. We have shown that amino acids Ser^{770} and Ser^{771} are required for Erk 1/2-dependent activation of NHE1 by sustained acidosis in CHO cells (Malo et al. 2007), isolated cardiomyocytes (Coccaro et al. 2009), and in kidney cells (Odunewu and Fliegel 2013). A number of studies have also implicated Ser^{703} as being critical to NHE1 function and as being important in activation by growth factors (Takahashi et al. 1999) in 293 cells. Furthermore, phosphorylated Ser^{703} was important in binding of the adaptor protein 14-3-3 to NHE1 (Lehoux et al. 2001; Maekawa et al. 2006).

Natural mutations in the NHE1 gene (encoded by SLC9A1) have not been well characterized, particularly in humans. Mice with absent NHE1 exhibit ataxia and recurrent seizures by 2 to 3 weeks of age. They also exhibit early death (Bell et al. 1999; Cox et al. 1997). Mice with deleted NHE1 also show neuronal death in the cerebellum and brainstem (Cox et al. 1997).

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Recently, (Genomes Project et al. 2010) the 1000 Genomes Project characterized human genome sequence variation by sequencing large numbers of individuals and describing the location and alleles of many polymorphisms. We examined the polymorphisms association with the SLC9A1 gene. We noted that of the polymorphisms in NHE1, several occurred in the regulatory amino acids Ser^{703}, Ser^{770}, and Ser^{771}. In this study, we examined the effect of these mutations on the expression, targeting, and activity of the NHE1 protein. We demonstrate that the Ser771Pro mutant is defective in expression and has unusual activation by sustained intracellular acidosis. To our knowledge, this is the first characterization of the effect of human mutations on the SLC9A1 gene.

Materials and methods

Materials

Sulfo-NHS-SS-biotin was acquired from Pierce and synthetic DNA was from IDT (Coraiveille, Iowa, USA). 2′,7-bis(2-carboxyethyl)-5(6) carboxyfluorescein acetoxymethyl ester (BCECF-AM) was from Molecular Probes, Inc. (Eugene, Oregon, USA). All the other chemicals used were of analytical grade and were acquired from Fisher Scientific (Ottawa, Ontario, Canada), Sigma-Aldrich (St. Louis, Missouri, USA), or BDH (Toronto, Ontario, Canada). The plasmid pYN4+ expresses cDNA for the human NHE1 protein with a HA (hemagglutinin) tag, as described earlier (Li et al. 2004).

Cell culture and stable transfection

AP-1 cells are a mutant cell line derived from Chinese hamster ovarian cells that do not express NHE1. They were used to characterize the activity of the wild type vs. mutant Na^+/H^+ exchanger (Sardet et al. 1989). We stably transfected AP-1 cells using LIPOFECTAMINE™ 2000 Reagent (Invitrogen Life Technologies, Carlsbad, California, USA) (Slepkov et al. 2005). The NHE1 expression plasmid, pYN4 +, allows selection of stably transfected cells using a neomycin resistance gene, as described earlier (Ding et al. 2006). Cell lines were regularly re-established from frozen stocks at passage numbers between 5–11. Results are typical of at least 2 independently made stable cell lines. In some experiments we used the wild type and mutant plasmids for transient transfection of AP-1 cells using LIPOFECTAMINE™ 2000 Reagent.

Site-specific mutagenesis

For mutagenesis of NHE1 we used the plasmid pYN4+ that contains cDNA for the HA tagged, human protein (Slepkov et al. 2004). The mutations were made as described earlier (Slepkov et al. 2005). Both strands of oligonucleotides of the sequence ATGTCTCGGGCCCGCATCGGCcCgGAtCCACTGGCCTATGAGCCGAAG and GCAAGGAGACTTCGcCCCCgGGAACCGACGATGTC were used to make the mutations Ser703Pro and Ser771Pro, respectively. Both mutations created a restriction enzyme site for use in screening for mutants. DNA sequencing confirmed the mutation and the fidelity of DNA amplification.

Cell surface expression

Targeting of the NHE1 protein to the cell surface was measured as described earlier (Slepkov et al. 2004). The cell surface proteins were labeled with sulfo-NHS-SS-biotin and cells were solubilized. Cell surface proteins (and the cell surface Na^+/H^+ exchanger) were removed with immobilized streptavidin resin. Equal amounts of unbound and total protein were run on SDS-PAGE and examined by western blotting for immunoreactive (HA-tagged) NHE1 protein. The levels of the NHE1 protein on western blots were estimated using Image J 1.35 software (National Institutes of Health, Bethesda, Maryland, USA). It was not possible to efficiently and reproducibly elute proteins bound to immobilized streptavidin resin because of the high affinity of streptavidin for biotin. We compared both the upper and lower HA-immunoreactive species of NHE1 for the plasma membrane targeting estimations.

SDS-PAGE and immunoblotting

Expression of NHE1 in stably or transiently transformed AP-1 cell lines was confirmed by immunoblotting, using antibodies against the HA tag of the NHE1 protein. Samples of cell lysates were separated on 10% SDS-PAGE gels and were transferred to nitrocellulose membranes. Equal amounts of protein of wild type and experimental lysates (S703P and S771P) were run in triplicate on immunoblots for quantitative comparative purposes. Protein concentrations were measure using the BioRad D/C™ Protein Assay kit. The primary antibody to identify tagged NHE1 was anti-HA monoclonal antibody. This was followed by peroxidase-conjugated goat anti-mouse antibody (Bio-Can, Mississauga, Canada) for signal detection. The reactive protein was detected using X-ray film with the Amersham enhanced chemiluminescence western blotting and detection system.

Intracellular pH measurement

BCECF was used to measure Na^+/H^+ exchanger activity and pH_i. Recovery was measured after an acute acid load was induced, as described earlier (Slepkov et al. 2005). For these experiments, cells were grown to approximately 80%–90% confluence on coverslips, and fluorescence was measured using a PTI Deltascan spectrofluorometer. Acute acidosis was induced with ammonium chloride (50 mmol/L × 3 min) addition followed by removal. The first 20 s of recovery in NaCl-containing medium was measured as ΔpH/s. Calibration of pH_i fluorescence was done for each sample, as described earlier (Slepkov et al. 2005). Results are the mean ± SE of at least 6 experiments. Individual buffering capacity and proton flux of each stable cell type was determined, as described earlier (Murtazina et al. 2001; Silva et al. 1997).

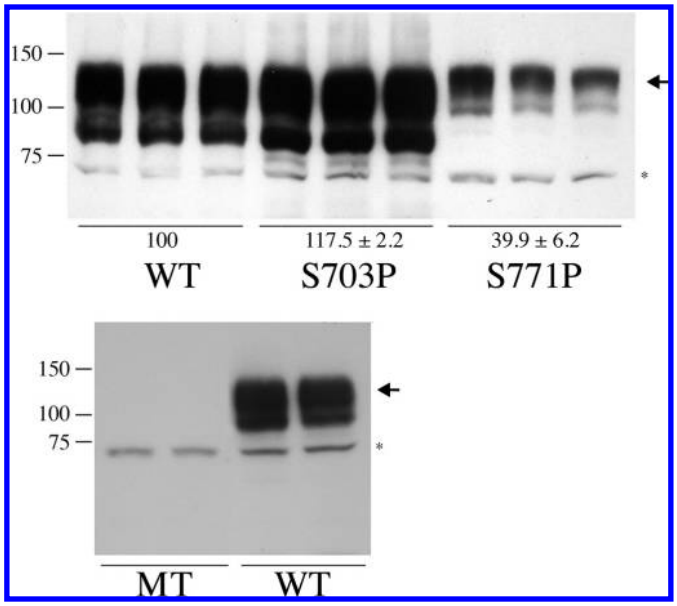
In one series of experiments we examined cells subjected to a two-pulse acidification assay, as previously described (Coccaro et al. 2009; Odunewu and Fliegel 2013), to induce a sustained intracellular acidosis (SIA). NH_4Cl prepulse was used to acidify the cells both times as described above. After the first pulse, acidification of cells by NH_4Cl removal was followed by ~30 s incubation in a Na^+-free buffer. After this, the cells recovered in a normal Na^+-containing buffer. The second pulse was the same as the first, but acidification induced by NH_4Cl withdrawal was for 3 min in Na^+-free buffer. A recovery in normal Na^+-containing buffer followed. SIA was not introduced in the second pulse for control cells where indicated.

Results

An analysis of the data of the 1000 genomes project revealed a number of polymorphisms in the SLC9A1 gene. Two of these were mutations to amino acids that are sites of phosphorylation and regulation of NHE1. They were mutation of Ser^{703} and Ser^{771} (ID's ESP_1_27427697 and ESP_1_27426936 respectively). Curiously, both were single bp mutations to proline (Ser^{703}, TCA-CCA; Ser^{771}, TCC-CCC).

To determine the effects of mutations on the NHE1 protein, we made these mutations in NHE1 and expressed the protein in AP-1 cells that are devoid of their own endogenous NHE1 protein. We examined expression, targeting, and activity of the mutant proteins relative to the wild type protein. The NHE1 protein contains a HA tag for detection. We have earlier determined that this tag does not affect NHE1 function (Slepkov et al. 2005). Initially, we examined the expression of the wild type and mutants in AP-1 cells using western blot analysis. Figure 1 (upper panel) shows the results of blotting vs. the HA tag. Wild type NHE1 protein was expressed as principally two bands. One of approximately 110–120 kDa and a second smaller band, which has been earlier shown to be partially or de- glycosylated NHE1 protein (Slepkov et al. 2005). The results demonstrated that the Ser703Pro mutant NHE1

Fig. 1. Analysis of expression wild type (WT) NHE1 and mutant proteins. (Upper panel) Western blot of whole cell lysates of stable cell lines expressing WT Na^+/H^+ exchanger or S703P and S771P mutants. (Lower panel) Western blot of whole cells lysates of cells mock transfected (MT) or transfected with wild type (WT) NHE1 protein. 100 μg of total protein was loaded in each lane. The primary antibody was anti-HA tag antibody. The arrow indicates the position of full-length glycosylated NHE1 protein. * indicates a non-specific protein present in all samples reacting with antibodies. WT are cell lysates from cells transfected with wild type NHE1 protein. S703P and S771P are cell lysates for stable cell lines expressing these mutant NHE1 proteins. Numbers indicate the expression levels of mutants in comparison to wild type protein, mean ± SE, *n* = at least 3 determinations.



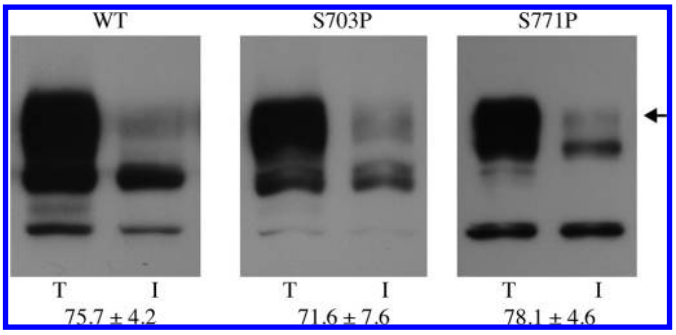
protein was expressed in similar amounts to the wild type NHE1 protein. In contrast, expression of the Ser771Pro mutant was greatly reduced to about 40% of the level of the wild type NHE1 protein. A separate immunoblot (Fig. 1, lower panel) confirmed that the antibody reacted specifically with the NHE1 protein, with the exception of a minor immunoreactive band of approximately 66 kDa in size.

In other studies, we have made mutations of the NHE1 protein to study its structure and function (Ding et al. 2006; Slepkov et al. 2005). Some of these resulted in protein mistargeting, with increased retention of intracellular NHE1 protein. To determine if the Ser703Pro and Ser771Pro mutations resulted in aberrant targeting of the NHE1 protein, we examined cell surface targeting of the mutant protein in comparison to the wild type (Fig. 2). Approximately 75% of the wild type and of each of the mutant proteins was targeted to the cell surface. There were no significant differences in targeting between the wild type and the mutant proteins.

To further characterize the effect of the mutations on the NHE1 protein, we used a fluorometric assay to determine the activity of the NHE1 mutant protein relative to that of the wild type NHE1 protein. Figure 3A illustrates examples of NHE1 activity of a stable cell line expressing either wild type or the mutant proteins. Figure 3B shows a summary of the results. The activity of the Ser703Pro mutant was not different from the wild type Na^+/H^+ exchanger. In contrast, the activity of the Ser771Pro mutant protein was substantially reduced from that of the wild type protein.

To ensure that the differences we observed in the activity of NHE1 were not due to differences in the buffering power of the stable cell lines, we measured buffering capacity of each of the cell lines and calculated proton flux of the different cell lines. There

Fig. 2. Surface localization of NHE1 in AP-1 cells expressing wild type (WT) and mutant NHE1 proteins. Equal amounts of total cell lysate (T) and unbound intracellular lysate (I) were examined by western blotting with anti-HA antibody to identify NHE1 protein. WT and S703P and S771P are cell lysates from cell lines stably expressing wild type NHE1 and mutant NHE1 proteins. Numbers indicate the mean ± SE, *n* = at least 3 determinations for the amount of protein targeted to the cell surface. The arrow indicates the position of full-length glycosylated NHE1 protein.



was no difference in the buffering power of the different cell lines (not shown). The proton flux of the different cell lines is shown in Fig. 3C. The results are very similar to the measurement of change in pH_i. The Ser703Pro mutant had essentially the same activity as the wild type NHE1 protein. Once again, the Ser771Pro mutant protein exhibited less activity than the wild type NHE1 protein. Correcting for the levels of protein expressed and the surface targeting, partially, but not completely, reversed this trend.

To confirm the effects we found in the stable cells lines containing the mutant Ser771Pro protein, we used transient transfection of the NHE1-deficient cells with plasmids containing the wild type NHE1 and the Ser771Pro mutant DNA. The results are shown in Fig. 4. Transient transfection of equal amounts of the wild type and Ser771Pro mutant DNA containing plasmid led to expression of reduced amounts of the Ser771Pro mutant protein (Fig. 4A). Additionally the activity of the Ser771Pro mutant protein was greatly reduced, compared with that of the control (Figs. 4B and 4C).

We have previously shown that the amino acid Ser^{771} is involved in activation of the NHE1 protein by SIA (Coccaro et al. 2009; Malo et al. 2007; Odunewu and Fliegel 2013). We therefore examined the ability of the Ser771Pro NHE1 mutant to be activated by sustained acidosis, in comparison with the control. The results (Fig. 5) demonstrated that there was an unusual defect in the activation of the NHE1 protein with the Ser771Pro mutation. In this assay, cells are subjected to one acid pulse, followed by a second acid pulse in which cells are maintained at low pH_i for 3 min (SIA). The control for the experiment is cells subjected to two acid pulses but without the second acidification being maintained for 3 min. The results are plotted as the change in the rate of recovery from the second pulse, compared with the first. For wild type NHE1 protein, we found the same effect as documented earlier (Coccaro et al. 2009; Malo et al. 2007; Odunewu and Fliegel 2013). Cells subjected to two acid pulses, without sustained acidosis, showed no difference in the rate of recovery of the second pulse from the first. When cells were subjected to two acid pulses, with the second pulse having a SIA, the second rate of recovery was significantly increased relative to the first one. In contrast, the results with the S771P mutant NHE1 were different. Both the dual pulse without SIA and the dual pulse with SIA exhibited an increased rate of recovery from the second acid pulse. There was no difference in the second rate of recovery between cells treated with or without SIA.

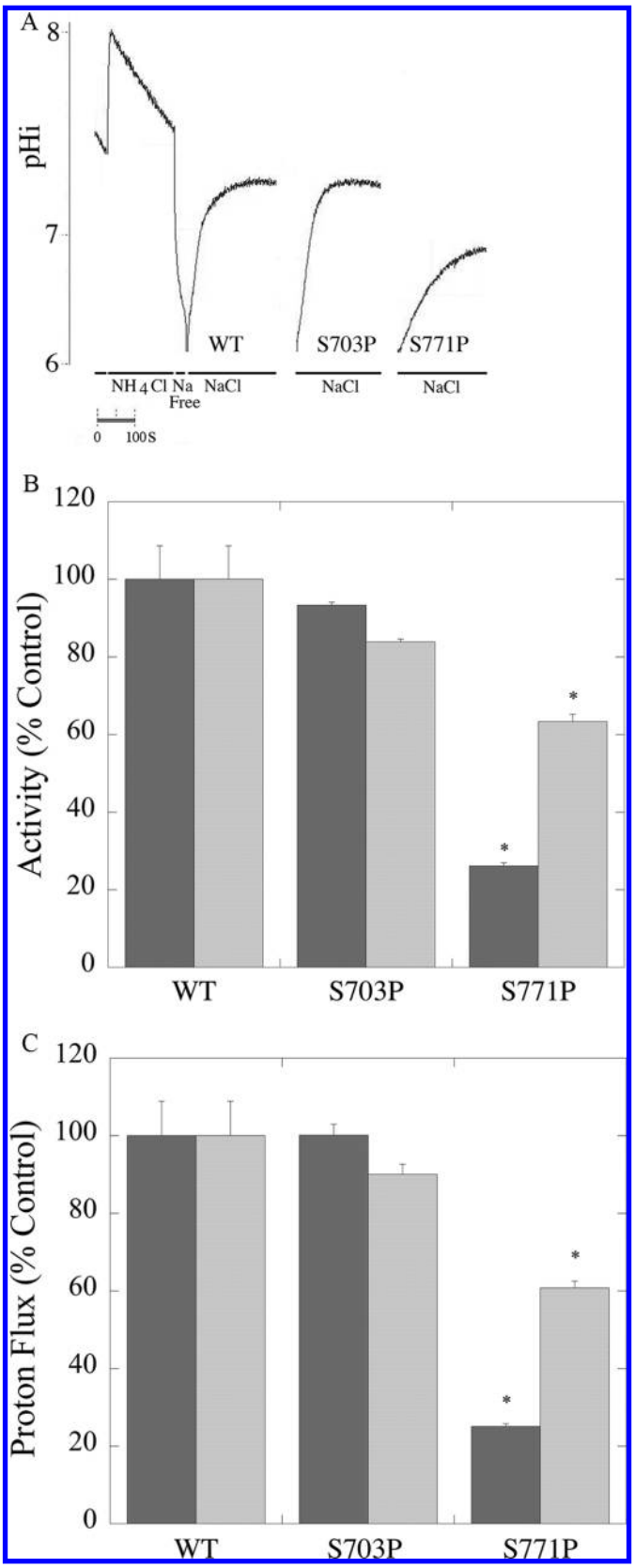


Fig. 3. Analysis of NHE1 activity of wild type (WT) and mutant NHE1 proteins. (A) Example of Na^+/H^+ exchanger activity of stable cell lines containing wild type NHE1 and S703P and S771P NHE1 mutant proteins. NHE1 protein activity was assayed in stably transfected AP-1 cells as described in Materials and methods. For ease of viewing, only the recovery from acidosis is shown for the mutant NHE1 proteins. NH_4Cl, treatment with ammonium chloride. After NH_4Cl treatment there was a brief "Na Free" treatment to induce acidosis. NaCl, recovery from acidosis was in NaCl containing buffer. (B) Summary of activity of WT and mutant NHE proteins in stably transfected cells. Activity was measured after ammonium chloride prepulse. Results are change in intracellular pH/s. * indicates significantly different from wild type at $P < 0.001$, $n > 6$. Dark bar indicates uncorrected activity relative to the wild type protein. Light bars indicate activity corrected for differences in expression and surface processing. The mean value of the control NHE1 activity was 0.021 ΔpH/s. (C) Summary of proton efflux of WT and mutant NHE proteins in stably transfected cells. Buffering capacity of the various cell lines was measured and proton efflux calculated as described in Materials and methods. The mean value of the proton flux for the wild type was 565 mmol/L H^+/min.

Discussion

Genetic defects in membrane proteins are responsible for a variety of diseases, including cystic fibrosis and muscular dystrophy. While some diseases are relatively common and have a profound effect on those affected, many other diseases occur with more subtle phenotypes and in lower frequency. Improvements in DNA sequencing technology have allowed large-scale sequencing of the human genome to discover mutations that are associated with specific diseases or traits including diseases or phenotypes not widely known or previously identified.

The SLC9A1 gene, the Na^+/H^+ exchanger isoform one, is a critical membrane protein that is involved in pH regulation, cell growth, and hypertrophy (Amith and Fliegel 2013; Fliegel 2005). It is also involved in ischemic heart disease, heart hypertrophy (Fliegel 2009), and in metastasis of several tumorigenic cell types including breast cancer cells (Amith and Fliegel 2013; Boedtkjer et al. 2012; Reshkin et al. 2014). Because of its critical role in cellular homeostasis and in human disease, we examined the polymorphisms associated with this gene. Regulation of NHE1 is a critical component of its cellular function. Elevation of activity of the NHE1 protein is responsible for, or contributes to, cell growth, cell hypertrophy, cell metastasis, and to cardiovascular disease (Malo and Fliegel 2006). Previous studies have determined that the amino acids Ser^{703} and Ser^{771} are important in regulation of the protein, being subject to protein kinase mediated phosphorylation that modulates NHE1 activity (Lehoux et al. 2001; Maekawa et al. 2006; Malo et al. 2007; Takahashi et al. 1999). For this reason, when we noted that mutations of these regulatory amino acids were present, we examined the effect of these mutations on function.

Our results demonstrate that mutation of Ser^{703} to proline had no apparent effects on basal NHE1 activity, expression, and surface targeting of the NHE1 protein. In contrast, mutation of Ser^{771} to proline had a marked effect on the expression of the NHE1 protein and an inhibitory effect on NHE1 activity. Surprisingly, surface targeting of the protein was not affected. We have earlier made mutations in the membrane domain of NHE1. Several mutations, which affected activity, led to defective targeting of the NHE1 protein (Ding et al. 2006; Slepkov et al. 2005; Tzeng et al. 2010). Therefore it was surprising that a mutation that affected activity did not alter targeting of the protein. However, the difference in this case may be that the mutation was present in the cytosolic regulatory tail of the NHE1 protein as opposed to the membrane domain.

Ser^{703} is involved in regulation of NHE1 in several cell types. Ser^{703} is a substrate for the protein kinase $p90^{rsk}$, and phosphorylation of this amino acid was reported to mediate growth factor activation in vascular smooth muscle cells (Takahashi et al. 1999). Also, expression of constitutively active ErbB2 leads to Ser^{703} phosphorylation and activation (Lauritzen et al. 2012).

Fig. 4. Analysis of NHE1 activity of transiently transfected AP-1 cells containing wild type (WT) and S771P NHE1 proteins. Plasmids containing either wild type (WT) NHE1 protein or the S771P mutant were transiently transfected into AP-1 cells. (A) Western blot analysis of NHE1 protein expression. (B, C) Na^+/H^+ exchanger activity, (B) example and (C) summary of $n = 8$ experiments. * indicates significantly different from wild type, * $P < 0.001$.

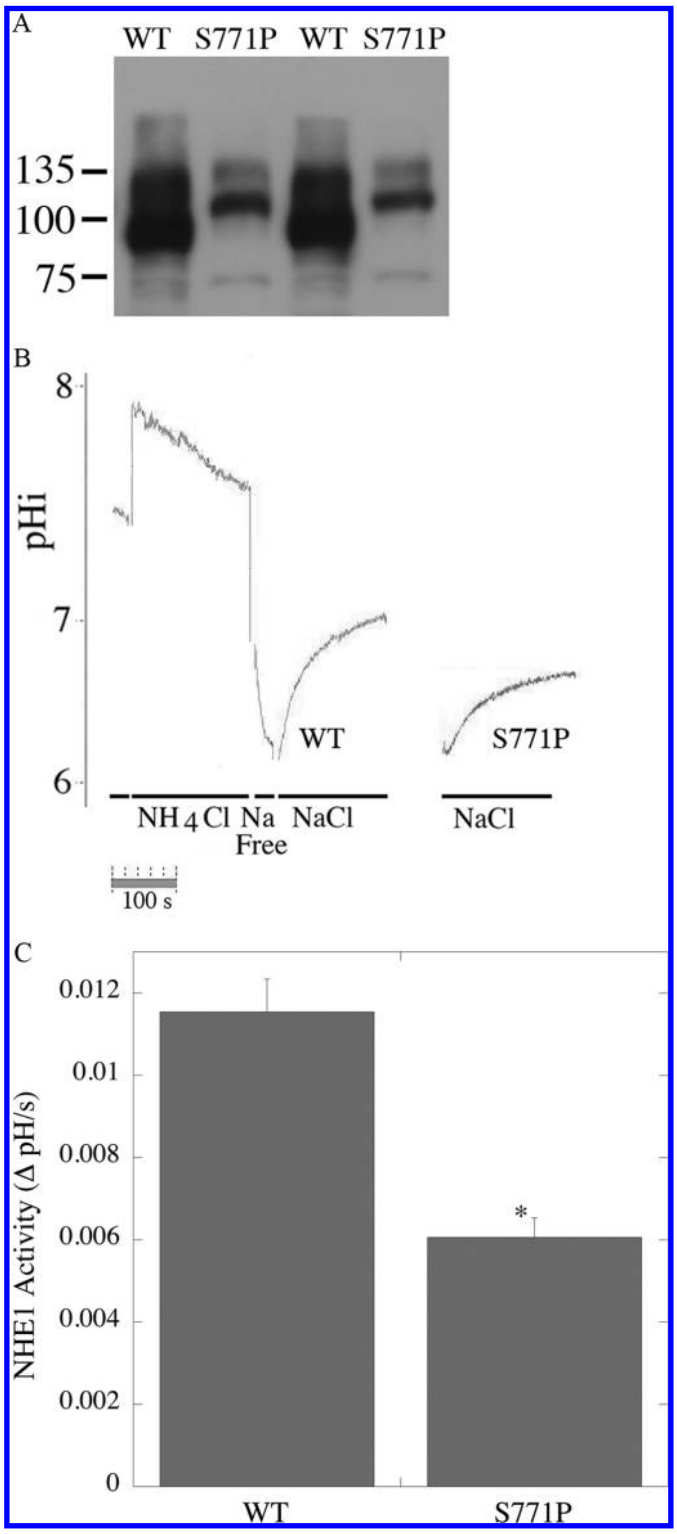
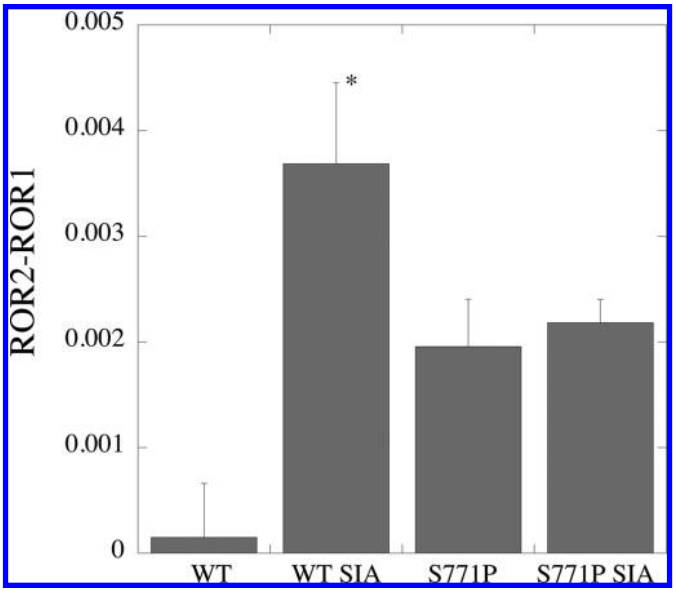


Fig. 5. Summary of NHE1 activity in dual pulse assay of wild type and NHE1 S771P mutants. Dual pulse assays were performed as described in experimental procedures. In controls, two ammonium chloride pulses were performed, and the second pulse was the same as the first. In experimentals, the second pulse contained an extended period of sustained acidosis (SIA). A summary of the difference between the rate of recovery of the first and second pulse is shown. * indicates significantly different from the first pulse at $P < 0.01$, $n > 6$. ROR, rate of recovery.



Phosphorylated Ser^{703} is also the site of binding of the regulatory protein 14-3-3 (Lehoux et al. 2001), and mutation of Ser^{703} to alanine interferes with 14-3-3 binding and with regulation. In the present study, we found no effect on basal NHE1 activity with mutation of Ser^{703} to proline. It is of note that we recently demonstrated that Ser^{703} is not involved in activation of NHE1 through sustained acidosis that was mediated by Erk kinase (Karki et al. 2010). Also, mutation of Ser^{703} to Ala did not decrease basal activity of NHE1 expressed in cardiomyocytes (Karki et al. 2010). Nevertheless, one study showed that overexpression of NHE1 with the Ser703Ala mutation acted as a dominant negative against wild type NHE1 and protected H9c2 (rat embryonic cardiac myocyte) cells from deleterious effects of anoxia (Maekawa et al. 2006). Clearly Ser^{703} has some significant physiological roles in some specific settings. We therefore cannot rule out that the Ser703Pro mutation has a fine regulatory effect that was not detected in our assays or under different physiological conditions.

Ser^{771} has been shown to be the subject of Erk-dependent phosphorylation. Either SIA or hormonal stimulation result in the phosphorylation of this amino acid and in stimulation of activity of NHE1 (Coccaro et al. 2009; Malo et al. 2007; Odunewu and Fliegel 2013). In our study, we found reduced expression of the NHE1 protein with mutation of Ser^{771} to proline. While the cause of the reduced expression is not known, we speculate that it could be due to an aberrant conformation of the protein that leads to less production or more rapid degradation. While the C-terminal regulatory tail of NHE1 is intrinsically disordered, it contains regions of structure (Norholm et al. 2011). We recently (Li et al. 2013) determined that phosphorylation of the tail in the region of Ser^{771} alters the conformation of this region in a pH-dependent manner. Prolines are known to create kinks in helices of both soluble and membrane proteins (Von Heijne 1991; Wilman et al. 2014). As this region appears to have a structural role in regulation of NHE1, it may be that insertion of a proline in this location leads to an abnormal conformation that impeded synthesis of NHE1 or pro-

moted its degradation. Molecular determinants of NHE1 expression and targeting are not well characterized. However, it has been shown that calcineurin B homologous protein binds to the cytosolic tail and promotes its targeting to the cell surface by altering its half-life at the cell surface (Zaun et al. 2008). It is possible that the Ser771Pro mutation acted through a similar mechanism.

We also found that the Ser771Pro mutant was not activated by SIA. The dual pulse acidification assay demonstrated no difference in the activation of this protein between dual control pulses and a dual pulse acidification with SIA in the second pulse. It was peculiar that the Ser771Pro mutant protein appeared to be partially activated by dual pulses with or without SIA. The mechanism of this activation is unknown, however we speculate that a change in the conformation of the tail region caused by this amino acid change leads to this activation.

There were no records kept of alterations in phenotype in the 1000 genome project (Genomes Project et al. 2010). Therefore it is unclear what the effect of these mutations is in humans. We suggest that there would be subtle but significant effects that might manifest themselves at certain times. This would include times when regulation of NHE1 was critical, such as in ischemia/reperfusion damage to the myocardium, and in cases where cell growth was critical, possibly in tumor development. Further experiments are necessary to explore these possibilities.

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