Transmembrane Segment XI of the Na⁺/H⁺ Antiporter of *S. pombe* is a Critical Part of the Ion Translocation Pore

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The Na⁺/H⁺ exchanger of the plasma membrane of *S. pombe* (SpNHE1) removes intracellular sodium in exchange for an extracellular proton. We examined the structure and functional role of amino acids 360–393 of putative transmembrane (TM) segment XI of SpNHE1. Structural analysis suggested that it had a helical propensity over amino acids 360–368, an extended region from 369–378 and was helical over amino acids 379–386. TM XI was sensitive to side chain alterations. Mutation of eight amino acids to alanine resulted in loss of one or both of LiCl or NaCl tolerance when re-introduced into SpNHE1 deficient *S. pombe*. Mutation of seven other amino acids had minor effects. Analysis of structure and functional mutations suggested that Glu361 may be involved in cation coordination on the cytoplasmic face of the protein with a negative charge in this position being important. His367, Ile371 and Gly372 were important in function. Ile371 may have important hydrophobic interactions with other residues and Gly372 may be important in maintaining an extended conformation. Several residues from Val377 to Leu384 are important in function possibly involved in hydrophobic interactions with other amino acids. We suggest that TM XI forms part of the ion translocation core of this Na⁺/H⁺ exchanger.

Na⁺/H⁺ exchangers are a family of integral membrane proteins that exist in plants, yeast and mammalian cells1. In mammalian cells, they function to remove excess intracellular protons in exchange for external sodium and are important in the pathology of several diseases including ischemic heart disease and breast cancer2,3. In plants and yeast, plasma membrane members of this family of proteins also catalyze the exchange of sodium for protons, but to serve a different end. They function in salt tolerance. Excess intracellular sodium is toxic and one way in which yeast and plants deal with sodium load is to extrude the ion through these plasma membrane transporters. In plants, different types of plasma membrane Na⁺/H⁺ antiporters remove sodium. The energy of transport comes from a proton gradient that is generated by the plasma membrane H⁺-ATPase4. Overexpression of plasma membrane salt tolerance proteins can improve salt tolerance in plants5.

In yeast, plasma membrane Na⁺/H⁺ antiporters also serve to mediate salt tolerance by removal of intracellular sodium in exchange for extracellular protons. The fission yeast *Schizosaccharomyces pombe* is an excellent system in which to study plasma membrane salt tolerant proteins. In *S. pombe*, the Na⁺/H⁺ exchanger SpNHE1 (previously known as sod2) plays the major role in salt removal from the cytosol and in salt tolerance. NHE1 of *S. pombe* can convey salt tolerance to plants and can function at the plasma membrane6 and SpNHE1 clusters with plant plasma membrane salt tolerance proteins in phylogenetic analysis7. Also, there are limited other salt tolerance mechanisms in *S. pombe* so the disruption of SpNHE1 yields a salt sensitive phenotype8. This makes SpNHE1 in *S. pombe* an ideal system for characterizing the protein’s activity and we have earlier9,10 used this system to study the effect of specific mutations of SpNHE1 on transport.

Mechanisms of transport of plasma membrane Na⁺/H⁺ exchangers and of ion transporting proteins in general, are an important fundamental scientific problem. The structures of a few Na⁺/H⁺ exchangers such the *E. coli* sodium transporter NhaA11 and those of *Thermos thermophilus* (NapA)12, *Methanococcus jannaschii* (MjNhaP1) *Pyrococcus abyssi* (PaNhaP)13 have been characterized, but most, including humans and those of

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higher eukaryotes, are not well studied. In the well studied Na\(^+\)/H\(^+\) exchangers, one key feature that has been noted is the critical nature of transmembrane (TM) segments IV and XI. These two segments create a characteristic fold. Both are discontinuous helices and at their crossing point are unfolded, accommodating charged and polar residues that neutralize the dipoles of the helices within the lipid bilayer. This region may harbor the ion translocation center. This fascinating arrangement and the details of these transmembrane segments have not been well demonstrated in other Na\(^+\)/H\(^+\) exchangers of higher species such as in vertebrate, plant or yeast Na\(^+\)/H\(^+\) exchangers.

In this study, we examined the structure and function of TM XI of SpNHE1. We used alanine scanning mutagenesis to characterize functional residues of this region of the protein. Additionally, we examined the structure of this TM segment by NMR spectroscopy. This region appears similarly structured to other Na\(^+\)/H\(^+\) exchangers, with a putative helix-extended region-helix in dodecylphosphocholine (DPC) micelles. A number of regions of the TM segment are important in function, possibly contributing to cation coordination or maintaining structure of this TM segment or in interaction with other transmembrane segments. Our results are the first examination of the structure and function of this region of the membrane protein. They support the hypothesis that SpNHE1 has a "Na\(^+\)/H\(^+\) exchanger"-like fold within the protein and that amino acids 360–393 are a critical part of this fold.

**Results**

**SpNHE1 alignment and modeling.** We examined amino acids of the putative TM segment XI of SpNHE1, which we hypothesized is important in activity S. pombe. Figure 1A is an alignment of the putative TM XI region of SpNHE1 with several other members of the Na\(^+\)/H\(^+\) exchanger family of proteins. Supplementary Fig. 1 illustrates the entire alignment of the protein. Multiple members of the family were included. Boxed and red amino acids highlight regions of conservation. There was little conservation beyond amino acid 383 of SpNHE1. This was a general trend in the protein, with regions intervening between transmembrane segments showing little conservation except in some more closely related species (Supplementary Fig. 1). Figure 1B illustrates the putative structure of SpNHE1, which is based on molecular modeling of the protein described earlier. A helical structure was predicted for residues 360–368 and residues 374–385, while an extended region was predicted between these two helical segments (Fig. 1B). According to the previous model of SpNHE1, Leu\(^{360}\) to Ser\(^{403}\) form an extended extracellular loop linking to putative TMXII. There was little conservation of this region of SpNHE1 with other family members.

**NMR Resonance Assignment and Analysis.** Well-resolved homonuclear \(^1\)H-\(^1\)H TOCSY and NOESY experiments and a natural abundance \(^1\)H-\(^13\)C HSQC experiment were acquired in DPC micelles for a synthetic TM XI peptide. On the basis of these data, we were able to unambiguously sequentially assign all residues (chemical shifts are deposited in the BMRB, accession # 27092) Beyond the exhibition of appropriate spin-system properties for a given amino acid type, these assignments were developed on the basis of unambiguous sequential (i to i+1) and medium range (i to i+2 through i+4) NOE contacts (Supplementary Fig. 2).

Through consideration of NOE contacts characteristic of helical structuring alongside the observed \(\Delta \delta\) values of \(\mathrm{H}_\text{N}, \mathrm{C}_\alpha, \text{ and } \mathrm{C}_\gamma\) nuclei \(^{17-19}\) calculated relative to random coil chemical shifts in a low dielectric environment, a helical segment is clear over residues 18Ala-Lys 21 (Fig. 2, corresponding to amino acids 375–386 of SpNHE1). In the N-terminal portion of the TM XI peptide, Lys to Val (containing amino acids 360–365) showed some degree of helicity according to \(\Delta \delta\)-based predictions (Fig. 2), but this was not continuous. Similarly, DANGLE, which predicts secondary structuring based upon comparison of \(\Delta \delta\) relative to random coil values in 8 M urea \(^{22}\) to database inferences for a given sequence and structuring, predicted a long helical stretch in the C-terminal half of the TM IX segment and a short helical stretch in the N-terminal portion (Fig. 2). Unambiguous canonical helical NOE contacts were not observed in the N-terminal region, as chemical shift overlap was more problematic within this portion of the peptide.

**Mutagenesis.** We made two sets of mutants in the putative TM XI to analyze the importance of amino acids in this region. The first set was an alanine scan of residues 360 to 393. The second set involved particular mutations on specific residues of this region, plus amino acid Ser\(^{420}\), which we hypothesized was associated with TM translocation center11,14. This fascinating arrangement and the details of these transmembrane segments have not been well demonstrated in other Na\(^+\)/H\(^+\) exchangers of higher species such as in vertebrate, plant or yeast Na\(^+\)/H\(^+\) exchangers.

We (Supplementary Figure 3) then examined the ability of the wild type or mutant SpNHE1 protein to restore growth in salt containing medium. Supplementary Figure 3A–D illustrate the growth curves in NaCl containing medium. Wild type SpNHE1 protein restored growth to the knockout strain. Cells were able to grow robustly in solution containing 500 mM NaCl. In contrast, the knockout strain showed no growth in 500 mM NaCl and compromised growth in 200 mM NaCl. Mutation of several amino acids caused impairment of growth in high concentrations of NaCl. This was most notable with the K360A mutation (Supplementary 3A), which was almost comparable to the knockout strain. Mutations H367A and K383A also caused growth to be largely compromised in 500 mM NaCl. Several other mutants had more minor effects on the ability to tolerate higher concentrations of NaCl including E361A (summarized in Table 1). In the second round of mutagenesis, with mutations to other

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amino acids, the proteins with mutations K360R, E361D and H367W conveyed NaCl resistance at levels equivalent or similar to that of the wild type protein. There was also a minor effect with K360E. The mutant proteins I371R, K383R and S420A did not convey sodium tolerance (Supplementary Figure 3E).

We also examined the same set of mutants for their ability to grow on solid media supplemented with NaCl. Figure 4A,B illustrates the results of growth on solid media with NaCl for the wild type and mutants, and the results are summarized in Table 1. The pattern of effects of the mutations on the ability to confer salt tolerance was largely the same as in liquid media. Mutant K360A and K383A proteins were unable to convey NaCl tolerance. Additionally, the H367A and L386A mutant proteins were largely ineffective in conferring NaCl tolerance. More minor effects were shown by the F364A, I371A, G372A, Y377A, M378A, F380A, L384A, and I391A (Table 1).

Figure 1. Alignment and molecular modeling of TM XI of SpNHE1. (A) Alignment of sequences of yeast, fungi, plant, mammalian and bacterial plasma membrane Na\(^+\)/H\(^+\) exchangers. Conserved amino acids are colored red and conserved regions are boxed. Representative included are; yeast and fungi group S. pombe NHE1 (NP_592782.1), S. pombe Sod22 (NP_594194.1), Candida albicans CNH1 (XP_710352.1), C. albicans Cnh1p (AA124468.1), Debaryomyces hansenii Nha1p (CAI45290.1), Saccharomyces cerevisiae Nha1p (NP_013239.1), Zygosaccharomyces rouxii Sod22 (XP_002497045.1), Z. rouxii Nha1 (XP_002497801.1), Yarrowia lipolytica Nha1p (XP_501299.1), Y. lipolytica Nha2p (XP_503447.1); Methanocaldococcus jannaschii NhaP1 (NP_247021.1), Pyrococcus Abyssii NhaP (CAB50204), Thermus thermophiles NapA (YP_144738), and Escherichia coli NhaA (WP_000681354) are included as archaeh and bacterial members. From plant groups Arabidopsis thaliana SOS1 (AAL32824), Brassica napus SOS1 (AGA37213.1), and Theobromia cacao SOS1 (XP_007045406) are included. Finally, from mammals, Homo sapiens NHE1 (NP_003038.2), Pan troglodytes NHE1 (XP_016812591) and Rattus norvegicus NHE1 (AAA98479) are included. See supplementary Figure 1 for full alignment. (B) Molecular model of SpNHE1. The model of SpNHE1 is shown in green. Amino acids 360–393 are shown in purple. Amino acids 169–188 were removed to allow visualization of TM XI. (C) Amino acids 360–393 of SpNHE1 model. Asterisk indicates residues found to be important in the present study (see below).
Proteins with the mutation K360E, I371R, K383R and S420A were also defective in conveying salt tolerance on solid media, similar to the effect in liquid media.

The sensitivity of the mutants to LiCl was also examined in liquid and solid media (Supplementary Figure 4, Fig. 4C,D). Many mutants, with alanine mutations that were sensitive to NaCl, were also sensitive in similar degrees to LiCl. This was the case for F364A, H367A, Y377A, K383A and L386A. However, some mutants showed acute differences in their ion sensitivity. This was most notable for the K360A mutant strain, which lost virtually all NaCl tolerance, but was able to grow relatively well in the presence of external LiCl. The adjacent amino acid E361A had sensitivity to both LiCl and to NaCl. The cells with the mutant protein containing the amino acids I371A, G372A, M378A, and L381A were all more sensitive to LiCl than NaCl. Of the second group of mutations, proteins with the I371R, K383R and S420A did not confer resistance to LiCl, similar to what occurred with NaCl. The K360E mutant protein, conferred reasonable resistance to LiCl, in contrast to its weaker ability to do the same for NaCl (Summarized in Table 1).

**Discussion**

Na\(^+\)/H\(^+\) exchangers were earlier reported to have a unique fold that is hypothesized to be critical in transport by these proteins and is believed to part of the ion translocation core. The key features that have been noted are in the critical TM segments IV and XI. Both segments have been shown to be discontinuous helices that cross over
each other in anti-parallel fashion with their crossing point within the lipid bilayer. Other amino acids neutralize the dipoles of the helices within the bilayer \(^{11,14}\). This arrangement was initially reported in NhaA of \(E.\ coli\) \(^{11}\), and has subsequently been shown in \(Thermos\ thermophilus\) (NapA) \(^{12}\), in the archaeal Na\(^{+}\)/H\(^{+}\) exchanger of \(Methanococcus\ MjNhaP1\) \(^{25}\) and in \(Pyrococcus\ abyssi\) (PaNhaP) \(^{13}\). In PaNhaP and NapA, this structure is formed by helices 5 and 12 of a 13 TM segment protein, in contrast to the 12 TM segment protein NhaA.

While significant progress has been made in these species, in others there is not as much known about other NHE’s. Notably, whether the same mechanisms of transport and protein conformation are maintained across species is not known. The \(S.\ pombe\) protein \(Sp\)NHE1 is thought to be electroneutral in contrast to NhaA, which would certainly require some structural and functional differences\(^{8,11}\). From the sequence alignment, we noted that this yeast plasma membrane NHE also has some unique substitutions in TM XI. For example, Pro 370 is exclusively present in TMXI of \(Sp\)NHE1 and yeast. Additionally, a conserved histidine (His\(^{367}\)) in TMXI segment is

| Type     | NaCl (L) | LiCl (L) | NaCl (S) | LiCl (S) | Group | NMR | model |
|----------|----------|----------|----------|----------|-------|-----|-------|
| WT       | +++      | +++      | +++      | +++      |       |     |       |
| KO       | −        | −        | −        | −        | −     |     |       |
| K360A    | −        | +++      | +        | ++       | *     | h   | H     |
| E361A    | +        | −        | +        | +        | ***   | h   | H     |
| L363A    | +++      | +++      | +++      | +++      | h     | H   |       |
| F364A    | ++       | ++       | ++       | +        | #     | h   | H     |
| Y365A    | +++      | +++      | +++      | +++      | h     | H   |       |
| G366A    | +++      | +++      | +++      | +++      | h     | H   |       |
| H367A    | +        | +        | +        | +        | ***   | E   | H     |
| F368A    | +++      | +        | +        | +        | #     | E   | H     |
| G369A    | +++      | ++       | +++      | ++       | E     | H   |       |
| P370A    | +++      | ++       | +++      | ++       | E     | E   |       |
| I371A    | ++       | −        | +        | −        | **    | E   | E     |
| G372A    | ++       | −        | +        | −        | **    | E   | E     |
| Y373A    | ++       | +++      | +++      | +        | E     | E   |       |
| C374A    | +++      | +++      | +++      | +++      | E     | H   |       |
| Y376A    | +++      | +++      | +++      | +++      | H     | H   |       |
| Y377A    | ++       | ++       | ++       | +        | #     | H   | H     |
| M378A    | +++      | −        | +        | +        | **    | H   | H     |
| E380A    | ++       | ++       | ++       | +        | #     | H   | H     |
| L381A    | +++      | −        | +++      | +        | **    | H   | H     |
| K382A    | +        | +        | −        | +        | ***   | H   | H     |
| L384A    | +++      | −        | +++      | +        | #     | H   | H     |
| L385A    | +++      | +++      | +++      | +++      | H     | H   |       |
| L386A    | ++       | ++       | +        | +        | #     | H   | E     |
| S387A    | +++      | ++       | +++      | +        | −     | E   |       |
| P388A    | ++       | ++       | +++      | +++      | −     | E   |       |
| D389A    | ++       | +++      | +++      | ++       | −     | E   |       |
| E390A    | +++      | +++      | +++      | ++       | −     | E   |       |
| I391A    | +++       | +        | ++       | +        | −     | E   |       |
| E392A    | +++      | +++      | +++      | ++       | −     | E   |       |
| K393A    | ++       | ++       | +++      | +        | #     | −     | E |
| K360R    | +++      | ++       | +++      | +        | #     | −     | E |
| K360E    | ++       | ++       | +        | +        | *     | −     | E |
| E361D    | +++      | ++       | +++      | +        | −     | E   |       |
| H367W    | ++       | +++      | +++      | ++       | #     | −     | E |
| I371R    | −        | −        | −        | −        | −     | *** |       |
| K383R    | +        | +        | +        | ++       | *** | −     | E |
| S420A    | −        | −        | −        | +        | *** | −     | E |

**Table 1.** Summary of growth of yeast strains containing wild type, or mutant \(Sp\)NHE1 in liquid (L) or solid (S) media containing NaCl or LiCl. Mutations to amino acids are indicated. The measured or modeled propensity of an amino acid to be in a helix or extended conformation is indicated. *Cells with increased relative sensitivity to NaCl than to LiCl; **cells with increased relative sensitivity to LiCl than to NaCl; ***cells with sensitivity to both LiCl and NaCl; # cells with minor changes in sensitivity to LiCl and/or NaCl; h, NMR results suggest possible helicity; H, in a helical conformation; E, predicted or shown to be in an extended conformation. −, No growth; +, +++, ++++, increasing amount of growth with ++++, indicating growth equivalent to wild type. −, not applicable or not measured.

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unique to the yeast plasma membrane NHEs. Also, a hydrophobic amino acid (Ile 371) is present in yeast plasma membrane NHE1, which is Arg in other NHE1’s. \( \text{Sp} \)NHE1 may therefore have a novel or altered mechanism of \( \text{Na}^+ \) transport. \( \text{Sp} \)NHE1 works to bring \( \text{Na}^+ \) out of the cell in contrast to mammalian NHE1. These conserved unique substitutions may play a key role in function.

We therefore examined the role of putative transmembrane segment XI in \( \text{Sp} \)NHE1 which we hypothesized was critical in function. In this species, this protein is the key sodium tolerance protein. Its deletion causes salt sensitivity that makes it ideal to assay \( \text{Sp} \)NHE1 function in live cells. We have earlier studied amino acids of this protein and defined several residues critical or important for function in earlier studies\(^{15}\). The 27 amino acids KEALFVGHFGPIGVCAVYMAFLAKLLL comprise a hydrophobic segment that was chosen for study. There were varied degrees of similarity of this segment to \( \text{Na}^+/\text{H}^+ \) exchangers of other species (Fig. 1A), with other yeast species showing the greatest degree of similarity. In \textit{Thermos thermophilus} (NapA), this segment of \( \text{Sp} \)NHE1 aligned with TM 12, which is thought to be part of the ion translocation core. This was also the case with TM XI of \textit{E. coli} NhaA, though in neither case was the similarity very high. In the case of MjNhaP1 and PaNhaP, the alignment with the corresponding regions of the ion translocation core is stronger.

In an earlier study we generated a \( \text{Sp} \)NHE1 homology model (Fig. 1B) based on the \textit{E. coli} NhaA structure and a comparative sequence alignment\(^{15}\). This model was validated using mutagenesis and structural analysis of TMIV\(^{15}\). Based on that model, a helical prediction of TMXI segment was made for residues 360–368 and residues 374–385, with an intervening non-helical segment (Fig. 1B, Table 1). To examine the structure of this transmembrane segment directly, we characterized a 27 amino acid peptide corresponding to residues 360–386 of \( \text{Sp} \)NHE1 using solution-state NMR spectroscopy. It has been previously demonstrated that isolated peptides of TM segments contain most of the required structural information needed to form their native structures in membranes. For example, the solution structure of isolated segments of bacteriorhodopsin corresponded very well to the crystal structure of the protein\(^{26–28}\). Our results suggest that amino acids Lys\(^{360}\) to Val\(^{365}\) exhibited chemical shifts predominantly consistent with helicity, while a continuous helical segment was strongly indicated over residues Ala\(^{375}\) and Lys\(^{380}\). These results are summarized in Table 1. This was essentially in agreement with the earlier modeling of the protein\(^{15}\), and is consistent with a break in helical character around the central proline residue of the segment. A proline in the midst of a transmembrane segment of \( \text{Na}^+/\text{H}^+ \) exchangers has earlier been suggested to cause an extended region within the lipid bilayer. Overall, these results suggest that this TM segment retains the basic structure of the ion translocation domain. That is, a helix-extended region-helix motif. While the helical propensity of the N-terminal region of the segment was less pronounced, it was nonetheless present. It may be that interactions with hydrophobic regions of other transmembrane segments or lipids could stabilize a slightly more helical conformation.

![Figure 3. (A–G) Expression and localization of various \( \text{Sp} \)NHE1 proteins. (A–F) Western blot analysis of expression of \( \text{Sp} \)NHE1 proteins was from cell extracts from \textit{S. pombe} strains expressing either wild type \( \text{Sp} \)NHE1 or various \( \text{Sp} \)NHE1 mutants. It was blotted with anti-GFP antibody as described in the materials and methods. Arrow indicates approximate location of full length \( \text{Sp} \)NHE1 with the GFP tag. (A–E) First round of mutagenesis to alanine. (F) Second round of mutagenesis to amino acids other than Ala. (G) Confocal microscopy of wild type \( \text{Sp} \)NHE1 and selected \( \text{Sp} \)NHE1 mutants in \textit{S. pombe}. Exponentially grown cells were harvested and used directly for live cell imaging of GFP fluorescence.](https://www.nature.com/scientificreports/)
We next asked the question, what is the functional importance of the individual amino acids of this transmembrane segment? Alanine scanning mutagenesis was used. Alanine has a small side chain that can substitute for most amino acids without disrupting the protein and at the same time alter the side chain. This kind of scan has been used earlier on SpNHE1 and other proteins21,33. All of the amino acids of this TM segment were mutated to alanine, aside from those that were already present as alanine. We then examined the ability of the expressed protein to rescue salt tolerance in the yeast knockout strain. Mutation to alanine (or other amino acids) did not affect the level of SpNHE1 protein expression. This result is consistent with what we observed earlier when amino acids of TM IV were mutated 15. In contrast, when the mammalian NHE1 protein is mutated, many mutations affect both expression levels and targeting34. It appears as though that, at least for this class of proteins, in yeast, expression and targeting of the protein is more robust than for the mammalian NHE1 protein.

Figure 4. Growth of wild type and mutant SpNHE1 containing S. pombe transformants on solid media. Samples of the various strains were from stationary phase cultures and were serially diluted 10-fold repeatedly. They were then spotted onto minimal media plates supplemented with NaCl (A,B) and LiCl (C,D) at the indicated concentrations. Plates were incubated at 30°C for 3 days. (A) Growth of controls and alanine scanning mutants on NaCl containing media. (B) Growth of controls and other mutants on NaCl containing media. Panels illustrating growth on plates were supplemented with NaCl at the concentrations indicated. (C) Growth of controls and alanine scanning mutants on LiCl containing media. (D) Growth of controls and other mutants on LiCl containing media. Panels illustrating growth on plates were supplemented with LiCl at the concentrations indicated. Sod2:ura4 refers to S. pombe with the SpNHE1 knockout described earlier9. WT refers to the sod2::ura4 with wild type SpNHE1 expressed from pREP-41sod2GFP as described earlier23. Other designations refer to SpNHE1 expressed from the plasmid pREP-41sod2GFP with the indicated mutation. Results are typical of at least 3 experiments.
We consider the effects of the amino acid substitutions in the light of the SpNHE1 model (Fig. 1B,C) and some of the amino acid interactions predicted in this model. It should be noted, that the model has yet to be definitely proven, nevertheless, some insights may be gained by this analysis. Nine of the mutations to alanine had more major effects on conferring resistance to Li, Na or both cations. Beginning from the N-terminus, there were two amino acids, Lys360 and Glu361, that, when mutated, affected Na and Li tolerance. According to the previously hypothesized model, these two amino acids could be on or near the intracellular face of the membrane. Mutation of these residues affecting ion specificity could be because they are involved in coordination of the internal cation, especially for negatively charged glutamic acid. Figure 5A illustrates their position on the surface of the protein. Replacement of the acidic Glu361 with aspartic acid largely restored activity, supporting this hypothesis. It is also notable that this amino acid is largely conserved across many species (Fig. 1), though in some species it is replaced by an aspartate residue. Notably though, T. thermophilus NapA and E. coli NhaA do not contain an acidic residue at this location. A predicted topological assignment of this residue is at the cytoplasmic side and is located in close proximity to Glu385 and Asp355. Asp355 is conserved among the yeast species while Glu165 is not conserved at all. Therefore, it is more likely that Glu361 is directly involved in proton transport together with Asp355 among the yeast group.

The side chain of the positively charged Lys360 is predicted to point away from the central cavity (Fig. 5A). Its mutation affected ion specificity and this may be through an affect on the coordination sphere indirectly through alterations with other amino acids, that affect the sphere structure. Replacement of Lys360 with a positively charged arginine largely restored function. Replacement of Lys360 with a negatively charged glutamic acid

Figure 5. Molecular models of selected regions of SpNHE1 based on. TM XI shown in yellow. Other segments in green. (A) Illustration of position of Lys360 and Glu361 on the external face of SpNHE1. (B) Position of Ser420 relative to His367. (C) Position of Ile371 relative to residues Leu148, Phe415, and Leu418. (D) Position of M378A, L381A, L384A and L386A relative to other hydrophobic resides. (E) Putative association of Asp241 and Lys383.
had an intermediate effect, demonstrating that the charge requirement is not absolute (Table 1). This amino acid is largely conserved across species (Fig. 1), though often replaced with arginine. A basic amino acid is present in NhaP1, NhaP2, and NapA, though NhaA of *E. coli* is different, which may reflect specific differences in function and activity of this protein.

The next critical residue in Sp*NHE1* function was amino acid His367. Mutation of this residue to Ala severely impaired the ability of the protein to rescue salt tolerance, confirming an earlier result. We have previously also shown that the H367R mutant is unable to transport Na+, whereas the H367D mutant was also defective shifting the pH optimum to a more alkaline range. Analyzing the predicted Sp*NHE1* structure suggests that the imidazole ring can make a hydrogen bonding interaction with hydroxyl group of Ser420 (or Thr), which is present in TM XII of Sp*NHE1* (Fig. 5B). We therefore mutated Ser420 to an alanine residue and examined the effect on protein function. The results demonstrated that this mutant protein is defective (Table 1). This supports the suggestion that there is an association that affects function. We also noted that in plant SOS1 protein, the equivalent position of His367 is replaced with a tryptophan. Therefore, we examined if the H367W mutant can retain the protein’s activity. We found that the H367W containing cells are not sensitive to Na+ and Li+, supporting the idea that that the charge distribution on the imidazole nitrogen of histidine is responsible for the activity. Tryptophan has a nitrogen-containing ring in its indole side chain, which may perform a similar role to the imidazole side chain of histidine. Interestingly this residue is located in the discontinuous helix region.

More towards the center of the TM segment, both the I371A and G372A mutants had similar effects on salt tolerance, causing defects in both Na+ and Li+ tolerance, with a more pronounced effect on Li+ tolerance. Both of these residues are within the extended region of the TM segment (Fig. 2). Ile371 is present in yeast plasma membrane NHEs and *E. coli* NhaA and is within the sequence GPI(G). Sequence alignment (Fig. 1) identified the corresponding sequence motif in human NHE1, with an Ile to proline substitution as G369GGLR (or Thr), which is present in TM XII of Sp*NHE1* (Fig. 5B). We therefore mutated Ser420 to an alanine residue and examined the effect on protein function. The results demonstrated that this mutant protein is defective (Table 1). This supports the suggestion that there is an association that affects function. We also noted that in plant SOS1 protein, the equivalent position of His367 is replaced with a tryptophan. Therefore, we examined if the H367W mutant can retain the protein’s activity. We found that the H367W containing cells are not sensitive to Na+ and Li+, supporting the idea that that the charge distribution on the imidazole nitrogen of histidine is responsible for the activity. Tryptophan has a nitrogen-containing ring in its indole side chain, which may perform a similar role to the imidazole side chain of histidine. Interestingly this residue is located in the discontinuous helix region.

The K383A mutation had a dramatic effect on activity of the protein and the K383R substitution only partially recovered activity. The larger size of this side chain may have affected activity with other amino acids. This amino acid is conserved across the yeast and plant antiporters but not in some other species. Examination of the position of Gly386 in the model of Sp*NHE1* suggests that this residue may interact with Asp241 (Fig. 5E). We have previously shown that mutation of Asp241 to Asn interferes with Sp*NHE1* function.7 This supports the suggestion that there is an interaction between these amino acids.

Amino acid residues 387 to 393 showed no large effects with mutation to alanine. We earlier showed that the mutations E390Q and D389N did not affect salt tolerance, confirming an earlier result9. We have previously also suggested in mid membrane centered around amino acid Leu457.

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Another difference between human NHE1 and SpNHE1 is that, SpNHE1 TM-XI does not contain an Arg (or Lys) at the center of the segment whereas human NHE1-TMXI contains Arg358 there. The side chain of such a residue could be attracted to phospholipid head groups and could aid in conformational switching26. In the absence of an arginine, SpNHE1 TM-XI contains His359, which is located at the N terminus of the discontinuous region, and can attain a positive charge over certain pH ranges. The H367R mutant is incapable of transport confirming this is an important residue. However, as noted above, this residue might also interact with other amino acids such as Ser420. Three important residues L457, I461, and L465 in human NHE1-TMXI, have also been identified based on cysteine mutagenesis and are located either at the discontinuous region or at the C-terminal half of the TM35. An analogous effect of mutagenesis was observed for the C-terminal half of SpNHE1 where the mutations of the residues to alanine are moderately or highly affected protein activity.

In summary, based on NMR analysis, the deduced structure of a peptide of TMXI of SpNHE1 was that of a putative helix-extended region-helix. The functional data suggested that there were several more important regions on the transmembrane segment, where alteration to Ala compromised the protein’s activity. When correlating effects of the mutations with molecular modeling of the protein, we came to the following putative roles of these amino acids on the protein. Glu361 may be involved in cation coordination on the cytoplasmic face of the protein. His359, may be in association with Ser420 and this is an important functional association. Ile371 and Gly372 are important in function. Ile371 may have important hydrophobic interactions with other residues on transmembrane segments and Gly372 may be important in maintaining an extended conformation of this region. A unique feature of SpNHE1 that we demonstrated, was that His367 and Ile371 are important functional residues of SpNHE1 and are conserved across related species. Residues from Val377 to Leu384 are important in function and may be important in hydrophobic interactions with other amino acids. Lys383 of this region is also important in function and may be important in other ionic-electrostatic interactions with other amino acids. We suggest that TM XI of SpNHE1 has a similar structure and function to TM XI of E. coli, forming part of the ion translocation pathway that is characteristic of Na+/H+ exchangers. It is possible that some mutations affect SpNHE1 indirectly, disrupting protein structure. Interpretations such as Glu361 acting through an effect on cation coordination are based on molecular modeling, which remains to be confirmed by determination of the three dimensional structure of the protein.

Methods

Materials. Restriction enzymes were obtained from New England Biolabs, Inc. or In vitrogen. PWO DNA polymerase was from Roche Applied Science (Roche Molecular Biochemicals, Mannheim, Germany). Synthetic DNA for mutagenesis was from Integrated DNA Technologies. A synthetic peptide corresponding to the putative TMIX (sequence Acetyl-KKKEALFGVGPIGVCAYMAFLAKLLKKK-amide) was purchased from the Alberta Proteomics and Mass Spectrometry Facility and was purified by HPLC and the identity verified by Mass Spectrometry. Deuterium oxide (D2O; 99.9% D); D2O with 1% sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS); and, deuterated dithiothreitol (DTT-d6; 98% D) and DPC-d38 (98% D) were purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). CD3COOH (>99% D) was purchased from Sigma Aldrich (Oakville, Ontario, Canada).

Strains and media. S. pombe with the NHE1 gene disrupted (sod2::ura4) was used as a host to reintroduce wild type and mutant SpNHE1 protein9. The sod2::ura4 strain was maintained on low sodium minimal KMA medium or yeast extract adenine (YEA) as we described earlier89. KMA medium contains potassium hydrogen phthalate, 3 g; K2HPO4, 3 g; yeast nitrogen base without amino acids, 7 g; glucose, 20 g; and adenine, 200 mg (per 1 liter). Leucine at 200 mg/l was added to maintain the sod2::ura4 leu1-32 strain wherever indicated and all media was buffered using 50 mM MES/Citrate and pH adjusted to 5.0 with sodium free KOH. Wherever indicated NaCl or LiCl was added to the media at the indicated concentrations. The pREP-41sod2GFP plasmid without mutations23 was used as a control. 

Transformation of the plasmid (and mutant forms) was into the sod2::ura4 strain by electroporation89. Briefly, cells were grown in 100 ml of KMA (with Leucine) media at 30 °C with vigorous shaking until the OD600 reaches to 0.5 to 1.2. Cells were then incubated in ice for 15 min and harvested by centrifuging at 3500 × g for 5 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 200 ml of ice cold water. Washed cells were collected by centrifugation as above and further washed with 50 ml of ice-cold 1.0 M sorbitol twice. Finally, the cell pellet was resuspended in 1 ml of 1.0 M sorbitol. Resuspended cells were divided into 200 μl of aliquots and mixed with 0.1 ng of purified DNA. The cell-DNA mixture was transferred to a 0.2 cm electroporation cuvette pre-incubated in ice. After 5 min incubation an electric pulse was applied according to the Gene Pulser II (Bio-Rad) specifications. Immediately after pulsing, cells were resuspended in 800 μl of 1.0 M sorbitol and incubated at 30 °C without shaking for 1 hr. Cells were then centrifuged and spread in KMA agar containing 1.0 M sorbitol without leucine for growth and selection. Colonies appeared after 3–4 days.

Growth of transformed strains was in liquid and solid media. For growth curves in liquid media 5 × 106 cells were taken from an overnight exponentially growing culture. This was inoculated into 2.5 ml of fresh liquid media. S. pombe containing the pREP-41sod2GFP plasmid and mutants were routinely grown in medium in the absence of thiamine. Cultures were grown at 30 °C in a rotary shaker with constant agitation. The A600 was determined at the indicated times. Growth curves were determined a minimum of three times and results are the mean ± SE.

Growth on plates was examined in agar with KMA medium containing leucine supplemented with either NaCl or LiCl at the indicated concentrations. The pREP-41sod2GFP plasmid without mutations25 was used as a control.
Site-Directed Mutagenesis. Mutations to SpNHE1 were made by PCR amplification of the pREP-41sod2GFP plasmid. The mutations were designed to create or remove a restriction enzyme site as described earlier. DNA sequencing was used to confirm the accuracy of the mutations and the fidelity of DNA amplification. Supplementary Table 1 summarizes the mutations made to SpNHE1.

Western Blotting of SpNHE1. Western blot analysis was used to compare levels of SpNHE1 expression in wild type and mutant SpNHE1 protein. Cell lysates were made from 50 ml of cultures of yeast transformants. Yeast cells were grown at 30 °C in KMA medium to an OD600 of 2. Cells were pelleted (3500 × g, 10 min) and were then washed with double distilled water and resuspended in a lysis buffer consisting of 50 mM Tris-Cl, pH 8.0, 5 mM EDTA, 1 mM dithiothreitol, and a protease inhibitor cocktail. Cells were then lysed using a Bullet Blender® using 0.5 mm zirconium oxide beads, speed of 10 × 40 minutes. In some cases they were lysed by passage through an emulsiflex homogenizer at a pressure of 25000 psi. Unbroken cells were pelleted by centrifugation at 3500 × g for 5 min, and the supernatant was centrifuged (14000 × g × 10 min). Enriched membranes of the supernatant were then pelleted at 100000 × g for 1 h, and were resuspended in a small volume of 50 mM Tris-Cl pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1.0% (v/v) deoxycholate and 0.1% (w/v) SDS. Equal amounts of up to 25 μg of each sample were resolved on SDS/polyacrylamide gels (10%). Western blotting of nitrocellulose transfers used a primary antibody of anti-GFP polyclonal antibody (a generous gift of Dr. Luc Berthiaume, Dept. of Cell Biology, University of Alberta). The secondary antibody was peroxidase-conjugated goat anti-mouse antibody (Bio-Can, Mississauga, Canada). Protein reactivity was detected using X-ray film via Berthiaume, Dept. of Cell Biology, University of Alberta). The secondary antibody was peroxidase-conjugated goat anti-mouse antibody (Bio-Can, Mississauga, Canada). Protein reactivity was detected using X-ray film via

SpNHE1 sequence Alignment. SpNHE1 multiple sequence alignment was using MAFFT. Representatives of different groups included, sequences of yeast, fungi, plant, mammalian, and bacterial plasma membrane Na+/H+ exchangers were included. From yeast and fungi: S. pombe NHE1 (NP_592782.1), S. pombe Sod22 (NP_594194.1), Candida albicans CNH1 (XP_710352.1), C. albicans Cnh1p (AAU24468.1), Debaryomyces hansenii Nha1p (CAJ45290.1), Saccharomyces cerevisiae Nha1p (NP_013239.1), Zygosaccharomyces rouxii Sod22 (XP_002497045.1), Z. rouxii Nha1 (XP_002497801.1), Yarrowia lipolytica Nha1p (XP_501299.1), Y. lipolytica Nha2p (XP_503447.1) are included. Methanocaldococcus jannaschii NhaP (NP_247021.1), Pyrococcus Abyssii NhaP (CAB50204), Thermus thermophiles NapA (YP_144738), and Escherichia coli NhaA (NP_000813534) are included as archaean and bacterial members. Plant Arabidopsis thaliana SOS1 (AAL32824), Brassica napus SOS1 (AGA37213.1), and Theobroma cacao SOS1 (XP_007045406.1) are included. Finally mammalian Homo sapiens NHE1 (NP_003038.2), Pan troglodytes NHE1 (XP_016812591), and Rattus norvegicus NHE1 (AAA98479) are also included. The alignment was prepared using ESPript.

Nuclear magnetic resonance spectroscopy. The NMR sample was prepared by dissolving 1 mM synthetic peptide in a 95% H2O 5% D2O solution containing 20 mM CD3COO−, 150 mM DPC-d38, pH was then adjusted to 5.00 ± 0.05. All NMR experiments (natural abundance 1H-13C HSQC, 1H-1H TOCSY and 1H-1H NOESY) were acquired at 30 °C using an Avance III 700 MHz spectrometer equipped with a 5 mm triple resonance inverse cryoprobe with a z-axis gradient (Bruker Canada) at the Biomolecular Magnetic Resonance Facility, National Research Council, Halifax, NS. Supplementary Table 1 lists NMR experimental details. Spectra were processed using TopSpin 3.1, with 1H frequencies referenced to DSS (0 ppm). Spectra were analyzed using CcpNmr Analysis 2.4.2. Sequential assignment and all 1H chemical shifts (H N, H β, H α, H γ, H δ, H ε, H α′, H β′, H γ′, H δ′, H ε′, H α″, H β″, H γ″, H δ″, H ε″) through 1H- 13C HSQC cross-peaks. H N, H α, C α, and C δ assignments were compared to expected chemical shifts for random coil peptides in dimethyl sulfoxide (DMSO)62. Secondary chemical shifts (Δδ) for H N, C δ and C δ were used for Cα calculation63,64, using the optimized cut-offs for α-helices in the DMSO environment. For comparison, the Dihedral Angles from Global Alignment were used to analyze the α-helical content. The NMR sample was made in CcpNmr Analysis.

Microscopy and Indirect Immunofluorescence. Confocal imaging of S. pombe containing GFP tagged SpNHE1 was performed on an Olympus IX18 microscope equipped with a spinning-disk optimized by Quorum Technologies (Guelph, ON, Canada). Images were acquired using the software Velocity (Improvision Inc., Lexington, MA) with a 60 × objective on a Hamamatsu EM-CCD camera (Hamamatsu, Japan). Yeast cells were either immobilized with 1% gelatin or for live cell imaging of the GFP tag, confocal microscopy was essentially as described earlier.

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Author Contributions
D.D. was responsible for carrying out all mutagenesis experiments and subsequent analysis of activity and proteins. He also contributed molecular modeling, in preparing Figs 1, 3, 4, and 5 and in the writing the paper. K.S. carried out N.M.R. experiments and their analysis and in preparing Fig. 2. J.R. supervised N.M.R. experiments and their analysis and assisted in editing and in preparing Fig. 2. L.F. conceived the manuscript, experiments and wrote and edited the manuscript and contributed to preparing Figs 1 and 3–5.

Additional Information
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