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Identification of conserved polar residues important for salt tolerance by the Na^+/H^+ exchanger of *Schizosaccharomyces pombe*

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Abstract

The Na^+/H^+ exchanger is a ubiquitous protein that transports Na^+ and H^+ in opposite directions across cell membranes. In fission yeast, the Na^+/H^+ exchanger sod2 plays a major role in the removal of excess detrimental intracellular sodium. The effect of mutagenesis of conserved polar amino acids of sod2 was examined by expressing 10 different mutant forms of sod2 in sod2 deficient *S. pombe* and characterizing salt tolerance. Asp145, 266, 267, and Glu173 were critical for proper function of sod2. Asp241 had an intermediate effect on sod2 function while mutation of Asp178 did not impair sod2 function. Simultaneous mutation of the Asp266, 267 pair impaired sod2 function. Mutation of each individual residue demonstrated that both were critical for sod2 function. Conservative mutations (Asp to Glu) of Asp266 and 267 failed to restore sod2 function. The results suggest that acidic residues associated with transmembrane segments are important in function, possibly being important in binding and coordinating cations. (Mol Cell Biochem **268:** 83–92, 2005)

Key words: Na^+/H^+ exchanger, *Schizosaccharomyces pombe,* cation binding, salt tolerance

Introduction

Drought and salinity are the two major environmental factors that limit agricultural production. Excess salinity has been an important factor in the decline of ancient agrarian societies [1]. The most direct way of dealing with excess sodium is to remove it from the cytosol. In plants one method of sodium removal is by plasma membrane Na^+/H^+ exchangers of varying type. The energy of transport comes from the proton gradient generated by the plasma membrane H^+-ATPase (reviewed in [2]). In yeast salt tolerance is mediated in a similar fashion. In fission yeast, the Na^+/H^+ exchanger (sod2) plays a major role in removal of sodium from the cytosol. When deleted, *S. pombe* are acutely sensitive to external sodium or lithium [3]. The *sod2* gene was previously cloned using selection for increased LiCl resistance [3]. Analysis of the cloned DNA sequence predicted that *sod2* encodes an integral membrane protein of 12 transmembrane segments (Fig. 1) and is approximately 47 kDa in size [4]. Disruption of the *sod2* gene results in a reduced ability to extrude cytoplasmic Na^+ and to take up external protons in exchange for internal sodium ions [3]. Sod2 catalyzes sodium and lithium tolerance by removal of these ions from the cytoplasm at the expense of the proton gradient (ΔpH) created by the plasma membrane ATPase. We have mutated all the histidine residues in sod2 and shown that His 367 functions as a pH sensor and that the acidic Asp pair 266, 267 are critical for proper function [5]. These acidic residues are similar in location to those of several other Na^+/H^+ antiporters (Fig. 2, [6]).

While a number of studies have described the basic cloning of Na^+/H^+ exchangers of plants and yeast and have demonstrated their activity, aside from our own initial work, there have been few studies that examine how yeast Na^+/H^+ exchangers function and few that have determined the amino acids that are critical for function. A good understanding of

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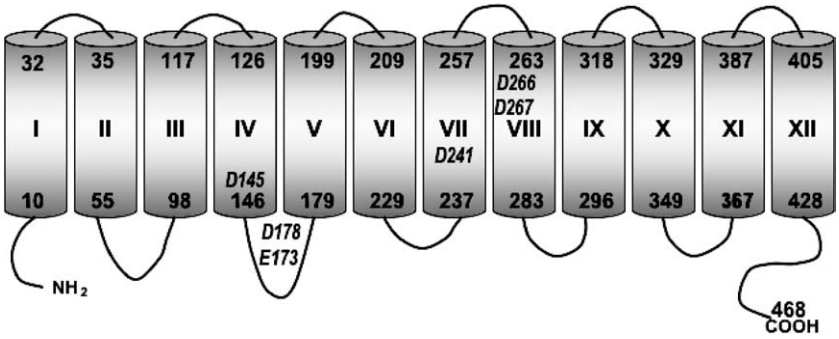

Fig. 1. Topology model of sod2. The amino acid sequence of sod2 was analyzed utilizing TopPredII [13] and predicted transmembrane segments are indicated. The positions of several polar residues potentially important in transport are indicated.

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Sod2 Sp 140 CITSTDPVLSA(4) 170 LIAESGCNDGM(5) 236 KYRLIDAISYY(7) 261 TIIGVDDLLMS(8)
Sod2 Zr 139 CITATDPVLAQ(5) 168 LSCESGCNDGL(6) 235 GKRIIDRESFL(7) 260 SMLGVDDLLVS(8)
NhaA Pa 140 CVTATDPVLAA(5) 170 LTAESGCNDGM(6) 237 KKGLIDRESFL(8) 262 SMLGTDDLLVS(9)
Nha1 Sc 140 CITATDPILAQ(5) 169 LSAESGCNDGM(6) 236 KKNIIDRESFL(7) 261 SILGVDDLLVS(8)
Nhx  Sc 196 TLSATDPVTIL(6) 222 IFGESLLNDAI(7) 287 IRRYPQIESCL(8)
NhaA Ec 128 IPAATDIAFAL(4)  60 KNMLLWINDAL(2)                     158 ALAIIDDLGAI(5)
NhaA Va 120 IPAATDIAFAL(4)  49 MSVSHWINDGL(2)                     148 ALAIIDDLGVV(5)
NHE1 Hu 234 IISAVDPVAVL(6) 259 VFGESLLNDAV(7)
NHE2 Rt 214 LISAVDPVAVL(6) 240 VFGESLLNDPV(7)
NHE3 Rt 184 LIAAVDLVAVL(6) 210 VFGESLLNDAV(7)
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Fig. 2. Alignment of Na^+/H^+ exchangers. Numbers preceding sequences indicate the number of the first amino acid in the segment. Assignment of the transmembrane segments was according to the indicated reference. For *S. pombe* the assignment was made as described in Fig. 1. Dark shading indicates identity with sod2, light shading indicates a conserved substitution. Underlined residues indicate residues examined in the present study. Ec, *E. coli*; Hu, human; Pa, *Pichia anomala*; Rt, Rat; Sc, *S. cerevisiae*; Sp, *S. pombe*; Va, *Vibrio alginolyticus*; Zr, *Zygosaccharomyces rouxii*. Citations are: sod2 Sp [3]; sod2 Zr, [29]; NhaA Pa, [30]; Nha1 Sc [31]; Nhx Sc [32]; NhaA Ec [33]; NhaA Va [34]; Va, [35]; NHE1 Hu, [36]; NHE2 Rt [37]; NHE3 Rt [38].

these proteins is critical to an understanding of their activity, of salt tolerance and of Na^+/H^+ exchanger activity in general. For the *E. coli* protein NhaA, significant insights into Na^+/H^+ exchanger activity have been made (reviewed in [7] and [8]) but sod2 has different fundamental properties such as a nonelectroneutral stoichiometry. The mammalian Na^+/H^+ exchanger has also been studied by us [9, 10] and has differences from the plant and yeast proteins (Fig. 2). For both mammals and *E. coli* it is apparent that polar residues and some nonpolar residues play critical roles in the function of these membrane proteins. We have earlier suggested that polar conserved residues could form a coordination sphere that binds substrate cations in a crown ether-like cluster. Also, that it may be that a relatively small amount of critical conserved residues are important in this coordination in the Na^+/H^+ exchanger family. These conserved residues might exist across Na^+/H^+ exchanger isoforms that might not necessarily have long stretches of conventional homology between isoforms [6]. In this paper, we examine the importance of polar, conserved acidic residues in the function of the yeast protein sod2. Our results are the first such extensive characterization of this yeast, eukaryotic Na^+/H^+ exchanger.

Experimental procedures

Strains, media and growth conditions

We used the *S. pombe sod2::ura4* [3] that is deficient in endogenous Na^+/H^+ exchanger (sod2), for complementation and phenotype characterization. *S. pombe* strains were grown on yeast extract adenine (YEA) or low sodium Edinburg Minimal medium adenine (EMMA), with appropriate supplements where necessary [11]. For yeast deficient in sod2, replacing Na_2HPO_4 with K_2HPO_4 at the same concentration made a low sodium minimal medium. Na_2SO_4 was omitted from the 50X salts stock and KOH was used to adjust the pH to 5.5. For growth in liquid cultures, yeasts were incubated at 30 °C, and at 200 rpm. Transformation of *S. pombe sod2::ura4* was using lithium acetate [11] or by electroporation [12] as described earlier [5].

Bacterial manipulation

E. coli DH5α were used for routine transformation and propagation of plasmid DNA. *E. coli* cells were cultured at 37 °C

Table 1. Oligonucleotides used for site-directed mutagenesis

Mutation	Oligonucleotide sequence	Restriction site
D145N	5′-TGATCGCAGGATGTATAAC**g**TC**g**ACT**a**ATCCTGTTCTATCAGCATTG-3′	SalI
E173Q	5′-CGGTCTTTATTGATCGCT**c**AGTC**cG**GATGTAATGATGGAATGGC-3′	BspEI
D178N	5′-TGATCGCTGAGTC**cG**GATGTAAT**a**ATGGAATGGCGGTTCCTTTT-3′	BspEI
D241N	5′-GCGGAAGGGAATAAT**A**g**C**TAATAGCAT**t**AATTAAACGGTAT-3′	AluI
D266, 267N	5′-GGAACTATTATTGGA**G**TT**aAca**ACCTGTTGATGTCCTTTTTTGC-3′	HincII
D266N	5′-GGAACTATTATTGGA**G**TT**aAc**GACCTGTTGATGTCCTTTTTTGC-3′	Hinc II
D267N	5′-GGAACTATTATTGGA**G**T**cG**A**ca**ACCTGTTGATGTCCTTTTTTGC-3′	SalI/HincII/AccI
D266E	5′-GGAACTATTATTGGAGTTGA**aG**A**tC**TGTTGATGTCCTTTTTTGC-3′	BglII
D267E	5′-ACTATTATTGGAGTTGAT**G**A**gC**T**c**TTGATGTCCTTTTTTGCTGG-3′	SacI (SStI)
D266, 267E	5′-ACTATTATTGGAGTTGA**gG**A**gC**T**c**TTGATGTCCTTTTTTGCTGG-3′	SacI (SStI)

Mutated nucleotides are in lower case letters and bold. Mutated amino acid residues are indicated using single letter notation and new restriction sites are underlined. All oligonucleotides encoded for the complementary DNA strand.

in LB media with appropriate antibiotic selection. Plasmids were isolated and purified using standard techniques.

Recombinant DNA techniques

DNA manipulation was by standard protocols. DNA sequencing was by the University of Alberta Department of Biochemistry Core Facility using a Beckman Coulter CEQ™ 2000XL DNA Analysis System. Mutagenesis of amino acid residues of sod2, was performed using the Transformer Site-Directed Mutagenesis Kit (ClonTech, Version 2) essentially as described earlier [5]. For mutagenesis reactions, the trans oligonucleotide ScaI/StuI (Clonetech) was used as a selection primer. The mutations were designed to create a new restriction enzyme site that could easily be detected in subsequent analysis. Primers used are summarized in Table 1. The mutagenesis template was pSK-sod2. This contains pBluescript and the sod2 coding sequence along with the 187 nt upstream and 692 nt downstream flanking regions of the gene as a 2.3 kb HindIII insert [3]. Positive clones from the site-specific mutagenesis were confirmed by DNA sequencing. To avoid the possibility of introducing unwanted mutations we excised a minimal fragment of sod2 containing the desired mutation and replaced it into a plasmid that had not undergone mutagenesis. The fragments were used to replace the corresponding sequences in nonmutated pSK-sod2. The various mutants were subcloned as 2.4 kb HindIII fragments into the *S. pombe* expression vector, pWH5, in some cases mutants were cloned into expression vectors so as to contain a hemagluttin (HA) tag as described earlier [5].

Salt tolerance

The salt tolerance of various *S. pombe* strains was determined using liquid Na^+-free minimal medium, KMA. KMA medium contained (per liter): 3 g potassium hydrogen phthalate, 3 g dibasic potassium phosphate, 7 g yeast nitrogen base without amino acids, 20 g glucose, 200 mg adenine. The pH of the medium was buffered with 50 mM citrate/Mes and adjusted to 5.0 with KOH. After autoclaving, the medium was supplemented with the indicated amounts of LiCl or NaCl. To assess growth, 2×10^6 cells were inoculated into 2.5 ml of medium and incubated at 30 °C, 200 rpm, for the indicated times. Growth was assessed by measuring the absorbance of the cell suspension at 600 nm. The pH of the culture medium was assessed at the end of each experiment to show that there was no significant acidification of the medium. The ΔpH was less than 0.15 pH units in all experiments. Results are mean $\pm$ S.E. of at least three trials for each strain and at least two isolates of each mutant were examined. To assess growth of yeast strains on solid media five μl aliquots from saturated cultures of wild-type and mutant sod2-expressing cells were diluted 10-fold serially and spotted onto Na^+ free minimal media plates. For these plates potassium phosphate was used instead of sodium phosphate and salt stocks were without sodium sulphate. The pH's were at pH 5.5 and pH 7.0 and the media was supplemented with NaCl at 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.5 M or LiCl at 2, 5, 10, 20 mM and incubated at 30 °C for 2.5 days.

Results

Figure 1 illustrates a model of the transmembrane topology of sod2. It is based on predictions using the program Topopredict[II] [13]. At present the topology of sod2 is not known with any certainty and some algorithms have suggested that amino acids 80–98 and 169–189 are transmembrane segments and that amino acids 237–257 are not [3]. This means that the hydrophilic amino acids 173 and 178 could also be within a transmembrane segment or within an

intracellular loop as shown. For the time being, it was decided to portray these amino acids as being within intracellular loop 2, though this location is not certain. In earlier experiments we have noted that it was necessary to permeabilize cells to get an immunofluorescence signal when the carboxyl terminus had a hemagglutinin tag ([4] and unpublished observations). This suggested that the carboxyl terminus was within the cell and the present model was therefore chosen.

We have earlier hypothesized that polar amino acids, within the transmembrane segments are good candidates for coordination of cations transported by Na^+/H^+ exchangers [6]. We initially examined the sequences of sod2 in comparison to several related proteins in a variety of species. Figure 2 shows an alignment of Na^+/H^+ exchangers from yeast, *E. coli* and some mammalian Na^+/H^+ exchangers. Several representative types of Na^+/H^+ exchangers were chosen from different species and from both plasma membrane and endosomal locations. Acidic polar amino acids either within or associated with transmembrane segments are underlined for *S. pombe*. For Z. *rouxii, P. anomala,* and Nha1 of *Saccharomyces cerevisiae* there is extensive homology throughout the four regions. In all cases the underlined acidic residues, including a double Asp motif, are conserved. Nhx of *S. cerevisiae* is localized to the endosomal membrane of *S. cerevisiae* [14]. It contained some regions that were similar to sod2 of *S. pombe* but less than plasma membrane Nha1 of *S. cerevisiae*. The TDPV sequence was conserved as well as the ES and ND sequences of TM 5 of sod2. The double Asp motif and D241 of sod2 were not conserved. NhaA of *E. coli* and *V. alginolyticus* showed only small putative regions that were identical to acidic residues in TM IV and TM V of sod2. However, a double Asp motif was present in both these transporters. For the mammalian Na^+/H^+ exchangers a DPV sequence was found in TM VI and the ES and ND sequences were found in TM VII of these transporters. Overall, it was clear that the yeast plasma membrane transporters had a higher degree of conservation to sod2 of *S. pombe,* than Na^+/H^+ exchangers of mammals or *E. coli*. Nevertheless, *E. coli, Vibrio alginolyticus* and mammalian Na^+/H^+ exchangers had some discrete regions of conservation that could be important in Na^+/H^+ exchanger activity.

To examine if conserved acidic residues were important in sod2 function we examined the activity of 10 mutant forms of sod2 that had mutations to these conserved acidic residues. Table 1 illustrates the series of mutations made. All mutant cDNA's were transfected into the sod2::ura4 knockout strain and assayed for salt tolerance in liquid medium in the presence of NaCl or LiCl. We have previously shown that the sod2::ura4 strain requires an active sod2 protein for growth in salt containing medium [3, 5]. Figure 3 illustrates the growth of the mutant strains and various controls in liquid medium. Figure 3A shows the growth of wild type *S. pombe* contained the endogenous sod2 gene. LiCl was added to the medium in concentrations of up to 10 mM. LiCl was used because it is more toxic than NaCl, which allows it to be used at relatively low concentrations that would have little or no osmotic effects [5]. The wild type *S. pombe* grew well in concentrations of LiCl up to 10 mM. Similarly, sod2::ura4 transformed with wild type sod2, grew well in these concentrations of LiCl (Fig. 3B). In contrast, the sod2::ura4 knockout grew in Li^+ free medium and in medium with 2 mM LiCl, but did not grow in medium with 5 and 10 mM LiCl. The sod2 mutant DNAs D145N and E173Q did not restore salt tolerance to sod2::ura4 (Fig. 3D and 3E). Sod2::ura4 transformed with the D178N (Fig. 3F) mutant grew in all LiCl concentrations up to 10 mM. Similarly, sod2::ura4 transformed with the D241N mutant grew in all LiCl concentrations (Fig. 3G) but growth was variable in 5 and 10 mM LiCl, at times some lines grew well and other transformants grew quite weakly in these higher LiCl concentrations.

We extensively analyzed the double Asp pair at amino acid position 266 and 267. Changing both residues to Asn eliminated the ability of sod2 to restore salt tolerance (Fig. 3H). Surprisingly, substitution of Asp 266 and Asp 267 with the negatively charged Glu residue, also did not restore the ability of sod2 to confer salt tolerance (Fig. 3I). Individual mutations of the acidic pair were examined to determine if either of these amino acids alone was critical for activity. Mutation of Asp 266 alone to either Asn or Glu resulted in an inability of sod2 to restore salt tolerance to sod2::ura4 (Fig. 3J,K). For the mutant D267N, there was a small restoration of activity. Growth was restored partially with 5 mM LiCl, but was variable. There was no restoration of the ability to grow in 10 mM LiCl (Fig. 3L). The D267E mutant, was similar to the D267N mutant but showed somewhat less growth than D267N in 5 mM LiCl.

To confirm the results that were shown in liquid medium we examined the growth of the mutant strains on solid medium. Figure 4A illustrates that sod2::ura4 transformed with wild type sod2 (WT) grows at high concentrations of LiCl and NaCl while sod2::ura4 itself only grew in the absence of NaCl and LiCl. Both the D145N and the E173Q mutants did not grow well in the presence of NaCl or LiCl on the pH 5.5 solid medium. In contrast the D178N mutant strain grew well in all concentrations of LiCl and in high NaCl containing medium in agreement with the results seen in liquid medium. Figure 4B illustrates that all of the D266 and D267 mutants, whether single or double mutants, were impaired in growth. Only the D267N mutant showed significant growth in NaCl containing medium above 0.2 mM, which was similar to the observation seen in liquid medium. The D241N mutant, grew irregularly in this assay at pH 5.5 and was omitted from this analysis at this pH.

Figure 4C illustrates examples of the growth of *S. pombe* on plates of pH 7.0 containing either wild type sod2, or the sod2 mutants D145N, D178N, D241N, D266, 267N or

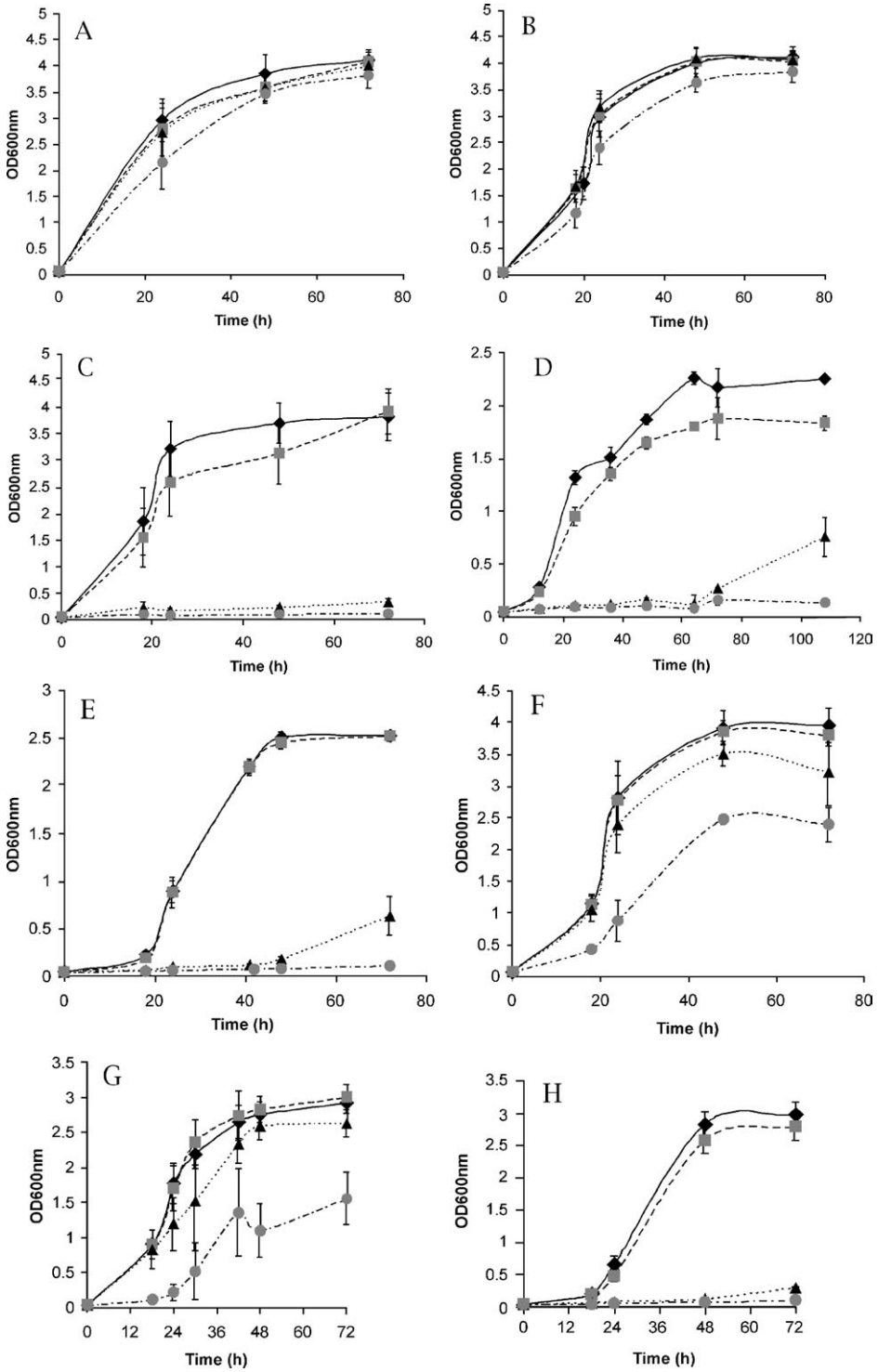

Fig. 3. Growth of *S. pombe* containing wild type or mutant sod2. Growth of *S. pombe* was assessed by inoculating 2×10^6 cells into 2.5 ml of medium and incubating medium at 30 °C, 200 rpm, for up to 100 h. Growth was assessed by measuring the absorbance of the cell suspension at 600 nm at the time intervals indicated. Results are mean ± S.E. of at least three determinations using at least two independent isolates of each mutant. *S. pombe* were grown in the presence of 0 mM [♦], 2 mM [■], 5 mM [▲] or 10 mM [●] LiCl as described in the "Materials and Methods". A, wild type *S. pombe* containing native sod2; B, sod2::ura4 strain containing pWH5 plasmid with wild type sod2; C, sod2::ura4 strain (endogenous sod2 is disrupted); D-M sod2::ura4 strain containing pWH5 plasmid with sod2 derivative carrying the respective mutations; D, D145N; E, E173Q; F, D178N; G, D241N; H, D266, 267N; I, D266, 267E, J, D266N; K, D266E; L, D267N, M, D267E.
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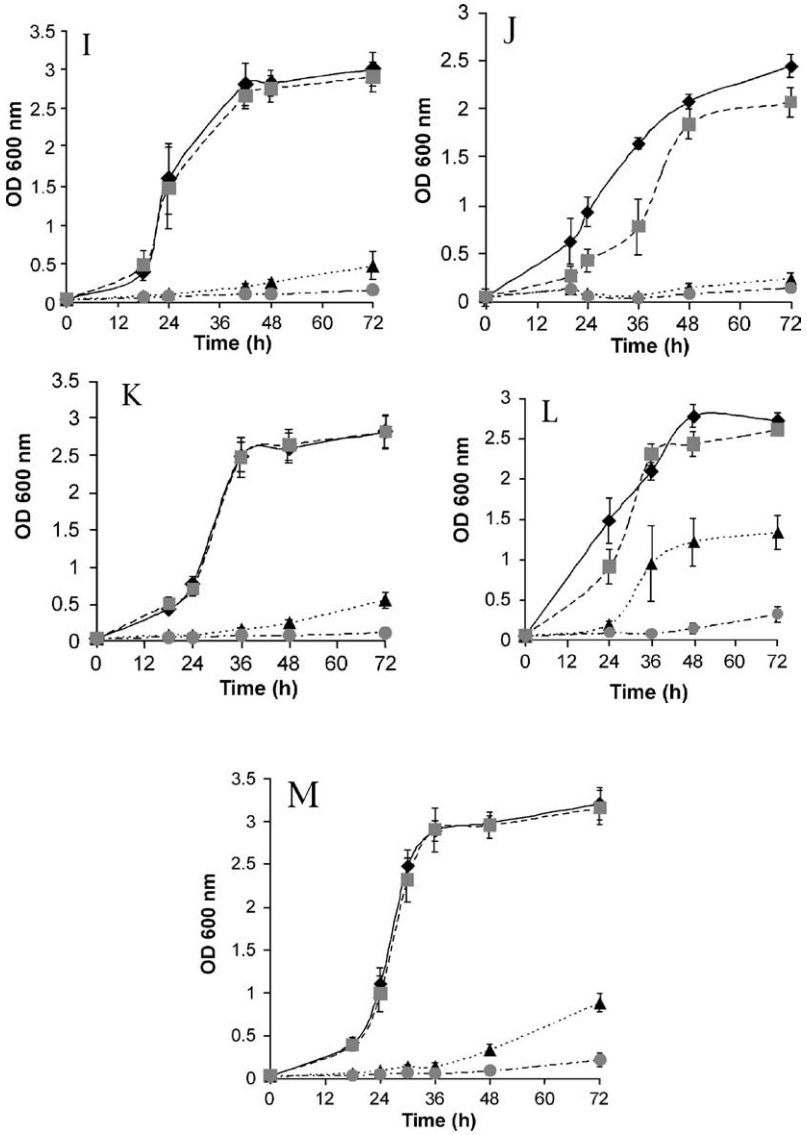

Fig. 3. (*Continued*).

D266, 267E. Growth of *S. pombe* containing the other mutants (E173Q, D266N, D266E, D267N, D267E) was essentially the same as the D266, D267N strain and is not illustrated. The Sod2::Ura4 mutant containing the wild type sod2 showed limited growth in the absence of NaCl and LiCl, and also showed growth in the presence of 2 mM LiCl. Similar to results in liquid medium, the D178N mutant, was able to restore limited salt tolerance that was comparable to the wild type sod2. The D241N mutant behaved in a similar fashion. The D145N, and all mutants of the D266,D267 pair, were not able to grow on salt containing solid medium at this pH. Cells not transfected with sod2 behaved similar to D145N and D266, 267N transfected mutants (not shown).

Discussion

Sod2 is the primary mechanism of salt removal across the plasma membrane of *S. pombe*. When the sod2 protein is deleted, this yeast is unable to grow in the presence of external Na^+ or Li^+ [3, 5]. To gain a better understanding of the mechanism of salt tolerance, and Na^+/H^+ exchange in eukaryotic species, we examined amino acids that might be critical to sod2 function. We have earlier postulated that conserved polar amino acids could be important in coordinating cations transported by Na^+/H^+ exchangers [6]. A comparison of Na^+/H^+ exchanger isoforms shows that some polar residues are present in many isoforms. The highest degree of conservation was present in the more closely related species

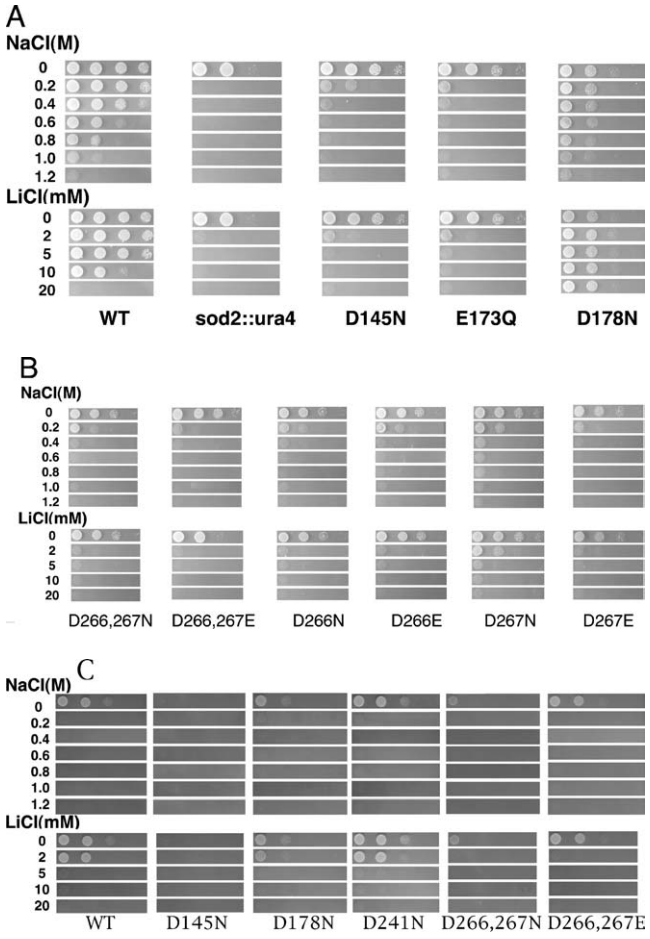

Fig. 4. Growth of wild type and mutant *S. pombe* transformants on solid media. Aliquots of yeast strains from saturated cultures of wild-type and mutant sod2-expressing cells were diluted 10-fold serially and spotted onto Na^+-free minimal media plates supplemented with NaCl and LiCl at the concentrations indicated. Plates were incubated at 30 °C for 2.5 days. Panel **A** illustrates the growth of various indicated yeast strains at pH 5.5, WT = sod2::ura4 strain containing the pWH5 plasmid with the wild type sod2; sod2::ura4 = strain with endogenous sod2 is disrupted; D145, E173Q and D178N indicate yeast strains containing the pWH5 plasmid with the indicated mutant. NaCl and LiCl concentrations are indicated. Panel *B*, growth at pH 5.5 as with panel A. Yeast strains contain the pWH5 plasmid with the indicated mutants. Panel *C*, growth of selected examples of yeast strains on solid medium at pH 7.0.

with plasma membrane isoforms of the Na^+/H^+ exchanger such as sod2 of *Z. rouxii* and Nha1 of *S. cerevisiae*. Many types of Na^+/H^+ exchanger had an acidic residue that was comparable to D145 of sod2 (Fig. 2). This was often followed by a proline and a valine residue. Asp145 was critical for sod2 function while in the mammalian Na^+/H^+ exchanger NHE1, we found that the analogous residue, D238 was not necessary for function [9]. In contrast in *Vibrio alginolyticus* the residue D125 was shown to be critical to function [15]. Similarly, in the *E. coli* Na^+/H^+ exchanger NhaA, residue D133 has been shown to be critical to activity [16]. The topology of NhaA is known, with residue D133 present in the midst of TM IV [8]. The homology between NhaA of *E. coli* and sod2 is weak. Nevertheless, it is clear that an acidic residue in the midst of an early TM segment is critical to function in the yeast and *E. coli* Na^+/H^+ exchangers. It is, however, also apparent that the presence of the DPV sequence, does not necessarily mean that this sequence is critical to function. We have earlier, mutated both D238 and P239 of NHE1 and neither amino acid was critical for function [9].

Amino acids Glu173 and Asp178 were also examined to determine if they affect sod2 function. These acidic residues were contained within the sequence ESGCND. This sequence was predicted to be either an intracellular loop connecting TM IV and V, (Fig. 1) or was predicted to be a TM segment by some algorithms (not shown). Within the yeast Na^+/H^+ exchangers, this region was quite conserved (Fig. 2) and the equivalent of Glu173 was predicted to be at the membrane aqueous interface while the Asp178 equivalent is predicted to be within TM VI. *E. coli* had an ND sequence, but the ES sequence was absent. The mammalian Na^+/H^+ exchangers had an ES and ND sequence with the same spacing as with sod2. These were predicted to be centrally within TM VII [17]. In this study we found that E173 was critical to sod2 function while mutation of D178 did not affect the ability of sod2 to restore salt tolerance. In the mammalian protein we found that both residues were essential for transport [9]. Thus, similar to the case with Asp145, there was functional divergence between the mammalian and the yeast Na^+/H^+ exchanger. In *E. coli,* D68 has been shown to be critical for function and is located in the midst of TM II [8]. Though there was a low degree of conservation between NhaA of *E. coli* (and *V. alginolyticus*, Fig. 2), if these residues are indeed comparable, it is clear that the yeast Na^+/H^+ exchanger is functionally different from NhaA of *E. coli* and *V. alginolyticus.*

We also examined Asp241. In earlier preliminary studies [5, 18] yeast transformed with this mutant displayed somewhat conflicting results. Growth by this strain was impaired, however, it demonstrated both sodium transport and proton flux ability. We, therefore, examined the ability of yeast carrying this mutant sod2 protein to grow in more detail. We found that it demonstrated an intermediate and curious phenotype (Figs. 3 and 4). Growth in liquid medium was restored in 10 mM LiCl, but the growth was at a lower level than that restored by wild type sod2 and was variable. We also found that growth on solid media at pH 7.0 was similar to the wild type. These results together suggest that D241 is not essential for activity but play a supportive role in activity. The sequence near Asp241 is conserved within yeast Na^+/H^+ exchangers, but a similar sequence was not evident in *E. coli* or mammalian Na^+/H^+ exchangers. As mentioned above, some algorithms predict that D241 is within the membrane while

others suggest that it is within an extramembrane region. In the case of NHE1, we showed that acidic residues with a TM segment may or may not play an important role in activity [9]. In addition, polar residues in extramembrane segments were shown to either contribute to Na^+/H^+ exchanger function or not [9]. In this case, the D241 residue clearly has some limited contribution to sod2 function.

An interesting sequence is found within putative segment TM VIII. It is a double Asp motif that is conserved among several types of yeast and is found in the bacterial but not mammalian Na^+/H^+ exchanger (Fig. 2). Previously we had shown that mutation of both these residues together impairs sod2 function [5]. In the present study, we demonstrate that mutation of each individual residue also affects sod2 function. Residue 266 appears to be more critical to function than 267, but both affect the ability of sod2 to confer salt tolerance. Surprisingly, substitution with an acidic residue did not restore the ability of sod2 to confer salt tolerance. This is in contrast to a similar experiment in the mammalian Na^+/H^+ exchanger. In this case, mutation of E262, D267 and E391 to neutral residues eliminated activity while the E262D, D267E and E391D mutations restored activity [9]. The rigorous requirement for these particular residues in sod2 suggests that not only is a negative charge required, but that its exact location within the protein or coordinating a cation is critical.

A pair of polar residues in other Na^+/H^+ exchanger proteins is also important in function. D344 and T345 have been shown to be critical for cation exchange mediated by NhaD, the Na^+/H^+ exchanger of *Vibrio cholerae*. The pair of acidic residues in *Zygosaccharomyces rouxii* (D265, 266) was also vital for function in this antiporter [19]. *E. coli* NhaA has a pair of acidic residues (D163, 164) that precede a Leucine and are present in the middle of TM V. These acidic residues have been shown to be critical for the function of this bacterial transporter and mutation of either completely eliminated activity [20]. The mammalian Na^+/H^+ exchangers do not have paired acidic residues within a transmembrane segment. Several polar residues occur together in NHE1 in a membrane-associated segment between TM IX and TM X; however, we have shown that none of these are essential for activity [9]. Only residue Glu391 partially affected activity. Thus, overall, it is clear that a pair of critical transmembrane acidic residues is a common feature of some bacterial and yeast antiporters, but is not an important feature of the mammalian Na^+/H^+ exchangers.

Polar negative charges in cation transporters have been shown to be important in a number of other examples including the melibiose permease [21, 22], bacteriorhodopsin [23, 24], the Na^+/Ca^{+2} exchanger [25] and the Ca^{2+}-ATPase of the sarcoplasmic reticulum [26]. Some of these residues are believed to interact with the cations during transport [6, 21–26]. Our study, suggests that the yeast Na^+/H^+ exchanger sod2 possesses a number of critical residues that could be involved in cation coordination. Residues Asp145, Glu173, D266, D267 and possibly D241 are involved in cation binding and transport. Figure 5 illustrates a possible model in which these amino acids are involved in cation coordination and transport by sod2. TM segments IV and VIII are suggested to line the pore permeation pathway while TM VII may contribute to a lesser degree or in a supportive manner.

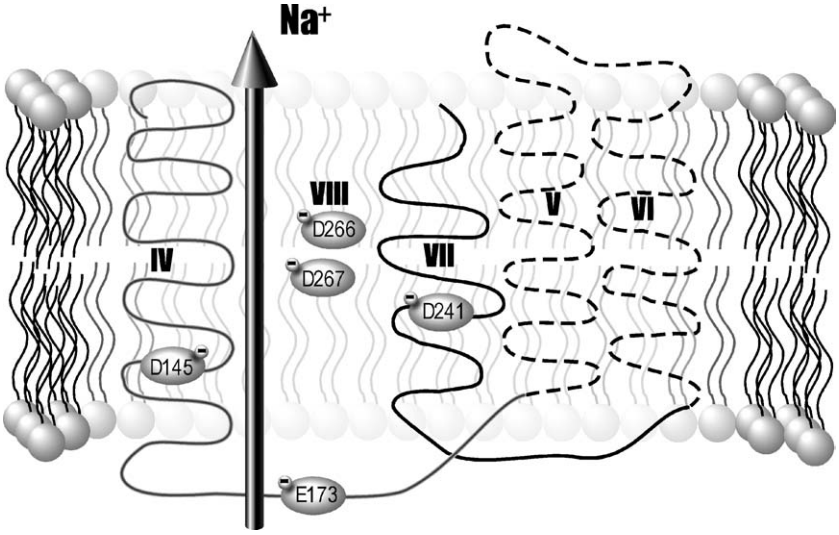

Fig. 5. Model of sod2 with amino acids critical for transport. Transmembrane segments IV–VIII are illustrated within a lipid bilayer. Residues that were demonstrated to be important in transport, D145, E173, D241, D266, D267 are shown surrounding a putative permeation pathway for Na^+ that includes TM IV, VIII and VII.

Some similarities and differences were found between *S. pombe* sod2 and bacterial and mammalian exchangers. For the residues examined, sod2 was more similar to NhaA than to mammalian Na^+/H^+ exchangers. Sod2 was similar to bacterial NhaA in that it had pair of essential Asp residues, followed by a Leu. In addition Asp125 was similar to Asp145 of sod2 in function. However, sod2 was functionally different from NhaA of *E. coli* in that in *E. coli*, residue Asp68 is essential for function, while a similar residue of sod2, Asp178, was not critical for function.

When comparing essential residues of sod2 with the mammalian Na^+/H^+ exchanger, mostly sod2 was quite divergent in the residues important in function. There was no functional equivalent of a double Asp motif that was present in sod2. In addition, while both sod2 and mammalian Na^+/H^+ exchangers have a DPV sequence, the Asp residue was critical for sod2 function but was not important in NHE1 function. Finally, Asp178 was not critical for sod2 function, but the equivalent (Asp267) was important for NHE1 function. The main similarity we found was that Glu173 was critical for sod2 function while the NHE1 equivalent Glu262 was also critical for function. An analysis of the primary structure of sod2 has shown that it has minimal overall similarity with mammalian Na^+/H^+ exchangers [27]. Yeast Na^+/H^+ exchangers have also been suggested to be closer to the *E. coli* protein than to mammalian Na^+/H^+ exchangers [28]. Our results suggest that, at least by a comparison of conserved polar residues, this may be true. This is surprising in view of the fact that both sod2 and the mammalian Na^+/H^+ exchangers are electroneutral while NhaA is electrogenic. Verification of this hypothesis will await further experimentation including an examination of the structure of these proteins.

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