Site directed mutagenesis reduces the Na^+ affinity of HKT1, an Na^+ energized high affinity K^+ transporter

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Abstract HKT1 encodes a high affinity Na^+ coupled K^+ transporter expressed in the cortical cells of *Triticum aestivum* roots. To identify regions of the protein involved in the binding and transport of Na^+ and K^+, mutations were introduced into a domain of HKT1 containing 16 amino acids that are highly conserved across a range of putative K^+ transport proteins from different phyla. Two mutations had a significant effect on the functional characteristics of the transporter. A yeast growth assay showed that concentrations of NaCl between 2.5 to 50 mM stimulated the growth of yeast expressing HKT1 containing the E464Q substitution, but not the growth of yeast expressing HKT1. Kinetic analysis confirmed that the E464Q mutation lowered the affinity of HKT1 for Na^+ but did not affect its affinity for K^+. A second mutation in the same region F463L was created that also lowered the affinity of the transporter for Na^+. The importance of these highly conserved amino acid residues is highlighted by the fact that they have remained conserved through evolution. The results of this mutational analysis suggest that this domain in HKT1 plays a role in the binding and transport of Na^+.

Key words: Transporter; Potassium; Sodium; High affinity; Plant; Site-directed mutagenesis

1. Introduction

HKT1 is expressed in the roots of *Triticum aestivum* and encodes a high affinity K^+ symport that is energized by Na^+ [1–3]. Na^+ energized K^+ uptake mechanisms have been characterized in lower plants such as the fresh water charophyte alga, *Chara corallina* [4]. HKT1 is the first Na^+ energized transporter to be cloned from higher plants, where cotransport is usually coupled to a H^+ gradient. The HKT1 transporter has been characterized in heterologous expression systems, but there is as yet no published characterization of the activity of this transporter in planta. In addition to being a high affinity K^+ transporter, HKT1 has been shown to be a high conductance pathway for Na^+ when present in excess of K^+, and hence HKT1 may be an important mechanism for Na^+ uptake during salinity stress in higher plants [2].

HKT1 provides an excellent model system for studying the structure and function of Na^+ energized transporters since its functional characteristics can be studied in a yeast mutant deficient in K^+ uptake [5] and in *Xenopus* oocytes [1–3]. These heterologous expression systems allow for the isolation of functional mutations [2] and the high resolution characterization of these mutations [6]. Random mutagenesis and expression in yeast were used to select mutations that alter the function of HKT1 [2]. Two HKT1 mutants were isolated that enhanced the NaCl tolerance of the K^+ uptake deficient yeast strain, by screening a randomly mutagenized HKT1 cDNA library. These mutations are located in a region which may be involved in K^+/Na^+ discrimination.

In this study we report on the identification of a domain that may be involved in the energization of K^+ transport systems that are homologous to HKT1. We used site-directed mutagenesis and the yeast heterologous expression system to test the importance of this domain in the function of HKT1. This work was directed by the sequence similarity between HKT1 and other putative K^+ transporters in the GenBank database. Databases contain a large amount of data on genes of unknown function that can be used to search out homologous domains which may be important in the function of particular proteins. We used this approach to identify one specific region of HKT1 that is highly conserved in proteins from fungi and bacteria. We have identified, through mutational analysis, at least two amino acid residues in this conserved region that are important in determining the functional characteristics of HKT1.

2. Materials and methods

2.1. Site directed mutagenesis

Mutagenesis was conducted on a 600-bp fragment excised from the HKT1 cDNA and subcloned in pBluescript (Stratagene) using overlap extension PCR [7]. The fragment was completely sequenced after PCR to confirm the presence of mutations and the absence of PCR errors, and then cloned back into HKT1 in pYES2 (Invitrogen). pYES2 containing the mutated form of HKT1 was transformed [8] into the CY162 yeast strain deficient in high and low affinity K^+ transporters [5]. The control yeast genotype used in all experiments was the CY162 strain transformed with pYES2 containing HKT1 and this yeast strain is referred to as HKT1.

2.2. Yeast growth study

Yeast growth studies were conducted in 125×11-mm glass test tubes filled with 2 ml of arginine phosphate medium [9] containing 100 μM KCl on a shaker at 28°C. The growth of the yeast strains expressing the different forms of HKT1 was compared at high (0, 50, 100, 200 and 300 mM) and low (0, 2.5, 10, 25 and 50 mM) NaCl concentrations in several different experiments. The optical density of the yeast cultures was measured at 600 nm.

2.3. $^{22}Na^+$ uptake assays

Liquid cultures of yeast strains were grown to mid-log phase in arginine phosphate medium [9] supplemented with 30 mM KCl and 2 mM NaCl. Cells were then pelleted and starved of K^+ and Na^+ for 5 h in arginine phosphate medium deficient in Na^+ and K^+. After starvation, cells were washed twice and resuspended in uptake buffer containing 10 mM MES, 0.1 mM $MgCl_2$, 2% galactose, 2% sucrose and adjusted to pH 6.0 with $Ca(OH)_2$ [3]. For experiments conducted at pH 4.5 and 8.0, 1 mM $CaCl_2$ was added and the pH of the solution was adjusted with Tris. At pH 8.0 bicine was used instead of MES.

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KCl and $^{22}Na^+$ labeled NaCl were added to 1-ml aliquots of these cells to achieve a range of final concentrations. The cells were then incubated for 4 min with intermittent shaking, collected on 0.45-μm nylon membrane filters and washed with 10 ml of 50 mM $CaCl_2$ to remove extracellular Na^+. The cellular Na^+ was measured by a scintillation counter after addition of a liquid scintillation cocktail.

3. Results

3.1. Identification of a domain highly conserved across phyla

A search was conducted of the entire deduced amino acid sequence of HKT1 with peptide sequence databases using the search algorithm BLASTP ([10]; Table 1). Those sequences that were significantly related to HKT1, with a probability of being randomly aligned with this protein of <0.02 (P sum values) are listed in Table 1 and are considered to be homologous [11]. The search revealed that HKT1 is related to K^+ transport proteins from *Saccharomyces* and *Schizosaccharomyces* spp. (sum P values $\leq 10^{-21}$), but not to the K^+ transporter HAK1 of the yeast *Schwanniomyces occidentalis* [12], which is homologous to the newly discovered family of K^+ transporters cloned from *Arabidopsis* [13]. Of the protein sequences listed in Table 1, only the deduced amino acid sequences HKT1 from wheat, TRK1 and TRK2 from *S. cerevisiae*, and TKHp from *S. pombe* have been functionally characterized and shown to transport K^+ [1,5,14,15]. Also related to HKT1 is the NtpJ protein from *Enterococcus hirae* which was originally designated to be a subunit of the Na^+-ATPase by Takase et al. [16]. Subsequently, the NtpJ protein was shown to be a component of the K^+ transport system of *E. hirae*, that appears to be important for survival at more alkaline pH [17]. Since no functional evidence has been published on the two proteins that were similar to NtpJ (accession nos. sp P47564 and gi 1653100), we presume these were classified as Na^+-ATPases based on the earlier designation of NtpJ being an Na^+-ATPase subunit. Of the proteins listed in Table 1, only HKT1 has been shown to be an Na^+-coupled K^+ transporter. Lichtenberg-Frate et al. [15] showed that TKHp functions as a H^+ coupled K^+ symport. No detailed functional data are available to establish if the other proteins listed in Table 1 are Na^+ or H^+ coupled transporters.

The percent identity of each deduced amino acid sequence with HKT1 (Table 1) was determined using the GAP algorithm (GCG Genetics Computer Group, Wisconsin Package 8.1-UNIX 1995). Low percent identity (20–30%) was observed between HKT1 and the deduced amino acid sequences that are related to HKT1. Whilst there are some small regions of identity (3–7 amino acids) between HKT1 and homologous proteins, the largest stretch of identity is the region of 16 amino acids. This region is highly conserved across a range of putative K^+ transport proteins derived from plants, fungi and bacteria.

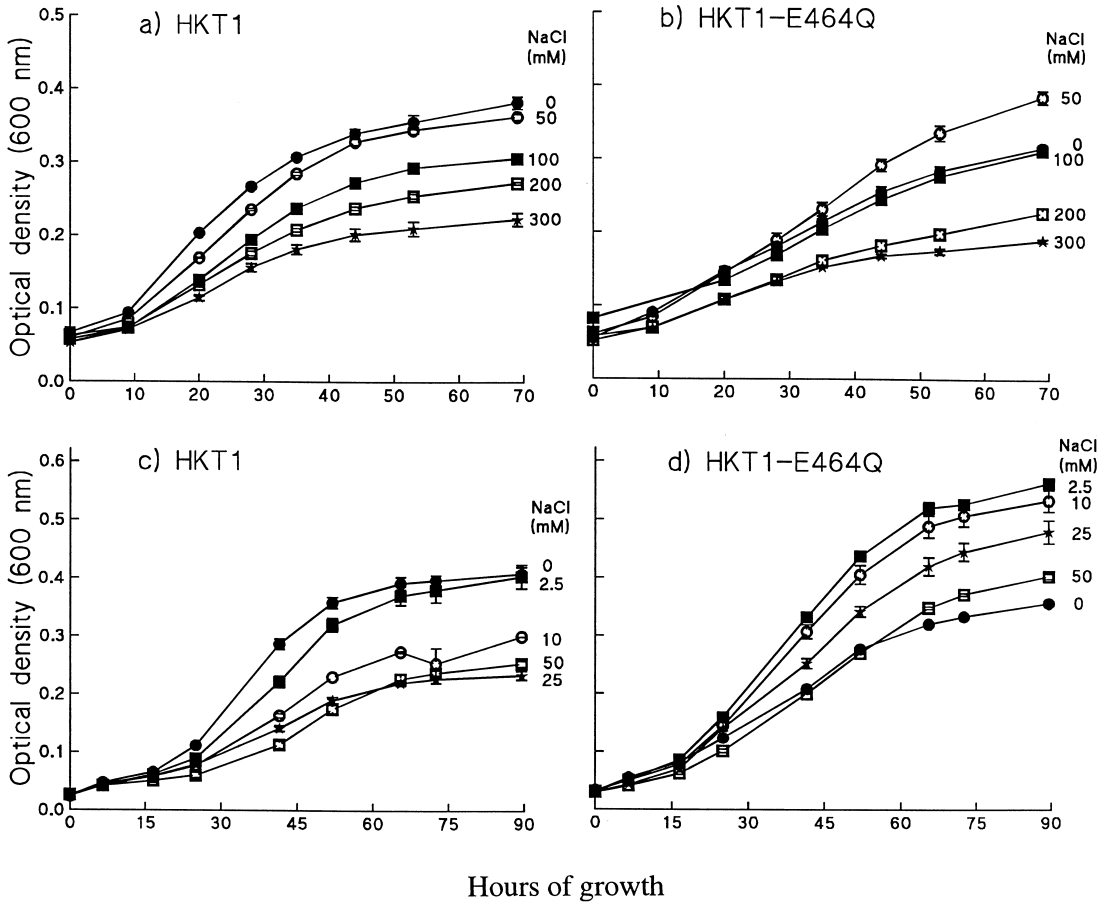

Fig. 1. Growth of HKT1 and HKT1-E464Q expressing yeast strains in a range of NaCl concentrations. Yeasts were grown in liquid arginine phosphate medium [9] containing 100 μM K^+ with (a,b) high and (c,d) low NaCl concentrations. HKT1 and HKT1-E464Q were both expressed in the CY162 yeast mutant [5] deficient in K^+ uptake. Vertical bars indicate standard error of the mean of 3 replicates, where these are larger than the symbol.

Table 1
Motif of amino acids conserved across transport proteins from a range of organisms

Species	Description in data base	Highly conserved region (Deduced amino acid sequence)	Accession No.	Sum P value	% identity to HKT1[a]
Triticum aestivum	HKT1	462 I F E V I S A Y G N A G L S T G 477	pir S47582		
Saccharomyces cerevisiae	TRK1 high-affinity K^+ transporter	1115 L F E I V S A Y G T V G L S L G 1130	sp P12685	10^{-28}	21.7
Saccharomyces uvarum	TRK1 high-affinity K^+ transporter	1121 L F E I V S A Y G T V G L S L G 1136	sp P28569	10^{-26}	23.3
Saccharomyces cerevisiae	TRK2 low-affinity K^+ transport protein	781 L F E V V S A Y G T V G L S L G 796	sp P28584	10^{-24}	27.4
Schizosaccharomyces pombe	K^+ transport protein homolog (TKHp)	743 L F E V V S A Y G T V G L S T G 758	pir S50225	10^{-22}	22.2
Schizosaccharomyces pombe	Putative K^+ transport protein	793 L F E V I S G Y G T V G L S L G 808	sp Q10065	10^{-21}	22.4
Enterococcus hirae	NtpJ protein	381 A F E V F S A F G T V G L T M G 396	pir G53610	10^{-9}	29.9
Mycoplasma genitalium	Hypothetical protein MG322	486 L F E T T S A F G T V G L S S G 501	sp P47564	10^{-4}	23.8
Synechocystis spp.	Na^+-ATPase subunit J	379 A F E A V S A F A T V G L S L G 394	gi 1653100	10^{-4}	26.1
	Consensus sequence	L F E V V S A Y G T V G L S - G			

Residues that are identical to the consensus sequence are indicated by a black background, and conserved substitutions are indicated by shaded boxes.
[a]% identity relates to the entire sequence.

3.2. Topological analysis of HKT1

Five different computer modeling programs were used in an attempt to derive a consensus model of the 2D structure of the HKT1 polypeptide ([18–21] and SOSUI www.tuat.ac.jp/cgi/~mitaku/sosui/). The number of transmembrane spanning domains was predicted to be between 8 and 10. Four of the five programs predicted that the amino and carboxyl termini are located endofacially, and the 16 amino acid motif conserved across putative K^+ transporters from different phyla (Table 1) is located exofacially either in the loop between transmembrane spanning domains 9 and 10, or in the carboxyl terminus.

3.3. Growth of yeast expressing the HKT1-E464Q mutant

In the following sections, HKT1 refers to the yeast mutant CY162 (see Section 2) expressing the HKT1 cDNA, HKT1-E464Q and HKT1-F463L refers to the same yeast mutant expressing HKT1 containing either the E464 to Q464 or the F463 to L463 substitution.

We chose to neutralize the glutamate residue E464 to the uncharged glutamine (Q464) because it was the only charged residue in this highly conserved region and charged residues are known to be important in the function of other cation transporters. Initial experiments examined the effects of NaCl concentrations from 0 to 300 mM on the growth of the yeast mutant CY162 expressing HKT1 and HKT1-E464Q (Fig. 1a,b). In the absence of added NaCl, the growth of HKT1-E464Q was slightly lower than that of HKT1 (Fig. 1a,b). At 50 mM NaCl, the growth of HKT1-E464Q was enhanced by approximately 23% at 70 h compared to 0 mM NaCl (Fig. 1b), whereas the growth of HKT1 was slightly decreased (Fig. 1a). Above 50 mM NaCl, the growth of both strains was reduced. To further investigate this growth stimulation by NaCl of the yeast mutant expressing HKT1-E464Q, the growth of the same two yeast strains was measured in the presence of less than 50 mM NaCl (Fig. 1c,d). The observed growth enhancement of HKT1-E464Q by NaCl was much larger in the presence of lower concentrations of NaCl (Fig. 1d). For example, the final density of HKT1-E464Q at 2.5 mM NaCl was 57% higher than the final density measured in the absence of added NaCl (Fig. 1d). In contrast to the growth stimulation observed in HKT1-E464Q cells, the growth of yeast cells expressing HKT1 was reduced by the addition of low concentrations of NaCl (Fig. 1c) above 1 mM NaCl. A slight growth stimulation was observed at 1 mM NaCl in the yeast strain expressing HKT1. The growth enhancement of the yeast mutant expressing the HKT1-

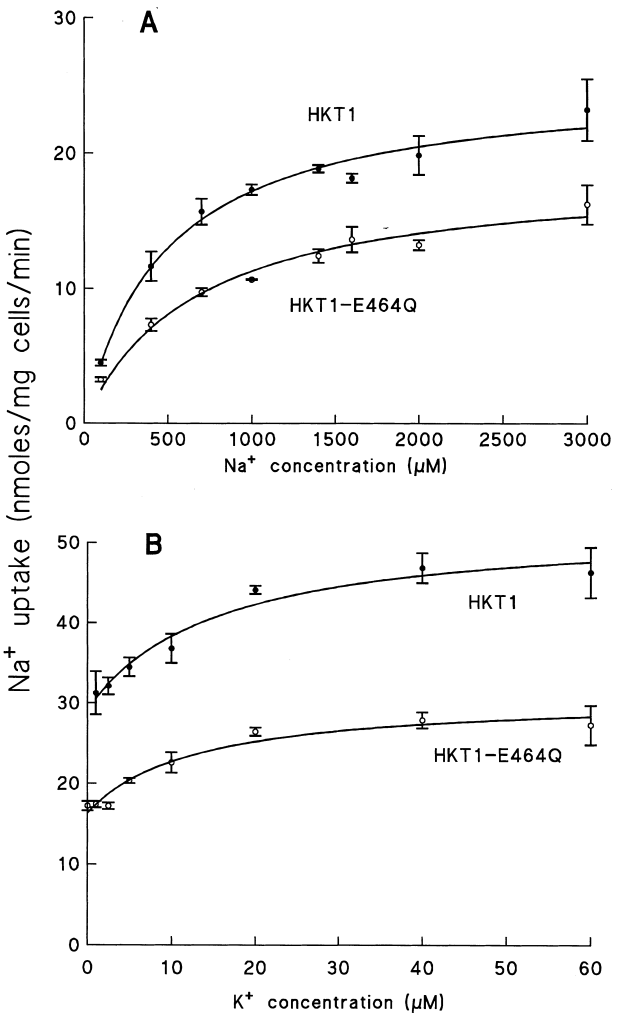

Fig. 2. Michaelis-Menten curves fitted to the Na^+ uptake by HKT1 (closed symbols) and HKT1-E464Q expressing (open symbols) yeast strains as a function of (A) Na^+ and (B) K^+ concentrations in solution. Error bars indicate standard error of the mean of 3 replicates.

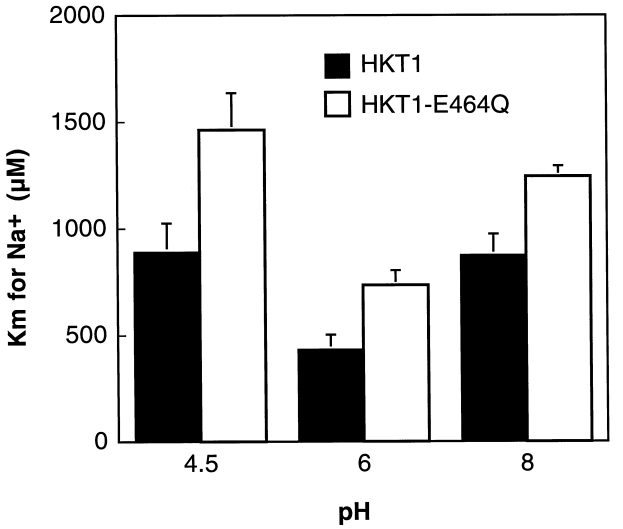

Fig. 3. The mean apparent K_m for Na^+ (±S.E.) for the CY162 yeast mutant expressing HKT1 and HKT1-E464Q at three different external pHs. Error bars indicate standard error of the mean of 3–5 replicate experiments.

E464Q mutation, at low NaCl concentrations, suggests that E464Q may play an important role in the binding or transport of Na^+.

3.4. Na^+ uptake by yeast expressing the HKT1-E464Q mutant

To determine how the E464Q mutation altered the Na^+ transport properties of HKT1, experiments were conducted that examined the uptake of Na^+ by HKT1 and HKT1-E464Q expressing cells. The K^+ concentration in these experiments was held constant at 300 μM. The apparent affinity constants for Na^+ (K_m) and maximum velocity rates (V_m) were derived from the Michaelis-Menten curves from six separate experiments with 3–4 replicates in each experiment, at each concentration. Data from a representative experiment are presented in Fig. 2A. Consistent with the growth phenotype, the Na^+ affinity for HKT1-E464Q expressing yeast ($K_m = 734 \pm 69$ μM (±S.E.)) was significantly lower ($P < 0.05$) than the Na^+ affinity of HKT1 expressing yeast ($K_m = 433 \pm 69$ μM (±S.E.)). In two of the experiments with the yeast strain expressing HKT1-E464Q, linear relationships between Na^+ concentration in solution and Na^+ uptake were observed and therefore no estimate of apparent K_m or V_m was calculated. Considerable variation (5-fold) was observed in V_m calculations for the HKT1 and HKT1-E464Q expressing yeast (7–38 nmol Na^+/mg/min). However, in 3 out of the 4 experiments where V_m was estimated, it was lower for HKT1-E464Q than for the yeast strain expressing HKT1.

To determine the effects of external pH on the Na^+ affinity of HKT1 and HKT1-E464Q, experiments were also conducted at pH 4.5 and 8.0. Those experiments showed that the apparent affinity of Na^+ for HKT1-E464Q was 30–40% lower at pH 4.5 and 8.0, which was consistent with the differences in apparent K_m at pH 6.0 (Fig. 3). At the acidic pH, the apparent affinity for Na^+ is reduced in both HKT1 and HKT1-E464Q which may indicate competition between H^+ and Na^+ binding. At pH 8.0 the affinity for Na^+ was also reduced, but this may be due to an inaccurate estimation of the absolute K_m which was caused by a 3–4-fold decrease in V_{max}. In an attempt to increase the absolute uptake rates of cells expressing HKT1 and HKT1-E464Q at pH 8.0, experiments were repeated using techniques to reduce the time yeast cells spent in the alkaline buffer since it is known that transport processes are affected by high pH in *S. cerevisiae* [2]. Those methods did not increase uptake rates through HKT1 or HKT1-E464Q. The results of the kinetic analysis show that the HKT1-E464Q mutation lowered the affinity of HKT1 for Na^+ across a range of external pH.

3.5. K^+ uptake kinetics of yeast expressing the HKT1-E464Q mutant

To determine whether the HKT1-E464Q mutation affected the K^+ transport characteristics of the mutant protein, the K_m for K^+ was measured. $^{22}Na^+$ was used to measure the transport activity of HKT1 and HKT1-E464Q in preference to the commonly used tracer for K^+ which is $^{86}Rb^+$, since K^+ uptake rates by yeast expressing HKT1 were approximately 15-fold higher than Rb^+ uptake rates [3], indicating that Rb^+ may not be a suitable tracer of K^+ uptake in HKT1. The K_m for K^+ was measured in the presence of 200 μM NaCl as used by Rubio et al. [2]. The K_m was calculated using a modified Michaelis-Menten equation containing an offset value to account for the K^+ independent Na^+ uptake (data from one representative experiment shown in Fig. 2B). The apparent affinity constant for K^+ was not significantly different between HKT1 (15 ± 2 μM) and HKT1-E464Q (17 ± 2 μM) (mean ± S.E., $n = 3$ separate experiments each with 3 replicates). The mean maximum velocity of Na^+ transport by HKT1 (39 ± 6 nmol Na^+/mg/min) was approximately twice that of HKT1-E464Q (21 ± 6 nmol Na^+/mg/min). The significant Na^+ uptake measured in the absence of added K^+ was not due to K^+ contamination, because the solutions were found to contain < 0.3 μM K^+ as determined by inductively coupled plasma mass spectrometry. This low level of K^+ contamination along with separate experiments (data not presented) showed that the kinetic curves in Fig. 2B do not go through the origin because of significant Na^+ transport in the absence of added K^+. We found that K^+ independent Na^+ transport could be reduced to almost zero when approximately 6 μM Na^+ was present (data not presented). Results from the kinetic analysis suggest that the HKT1-E464Q mutation specifically affects how HKT1 binds Na^+, reduces Na^+

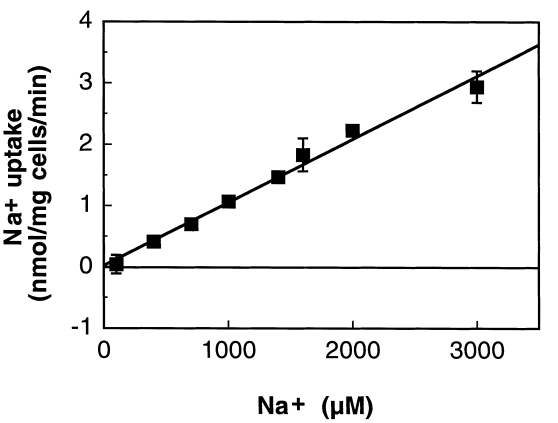

Fig. 4. Mean sodium uptake rate (±S.E.) across a range of Na^+ concentrations by CY162 cells expressing HKT1-F463L. Error bars indicate standard error of the mean of 3 replicates.

flux rates through the transporter and has little or no effect on K^+ binding.

3.6. Linear rate of uptake in F463L mutant

To confirm that this region of HKT1 containing the 16 highly conserved amino acids is involved in Na^+ transport, a mutation was created adjacent to E464. The single amino acid substitution F463L had a striking effect on the kinetics of Na^+ uptake. Rates of Na^+ uptake in yeast cells expressing this site directed mutation (F463L) in HKT1 were linear ($r^2 = 0.99$) up to 3 mM external Na^+ (Fig. 4). Experiments to measure the K_m of this site directed mutant were not conducted because it has been previously shown that Na^+ competes with K^+ binding in HKT1 [3] which would invalidate the kinetic analysis as higher concentrations of Na^+ were added to solutions containing 300 μM K^+.

4. Discussion

The proteins that are most similar to HKT1 are putative K^+ transporters isolated from microorganisms such as yeast and bacteria. These HKT1 related proteins are between 20–30% identical to HKT1. Despite the relatively low overall amino acid identity between these proteins and HKT1, a 16 amino acid motif was identified that is highly conserved across all these proteins. This high level of conservation across phyla in a specific region suggests that this domain may be important in the function of these related proteins, some of which have been characterized to be K^+ transporters [1,5,15]. Based on five different computer modeling programs, the location of this conserved region in HKT1 is predicted to occur on the exofacial side of the membrane and most probably lies between transmembrane spanning domains 9 and 10. Since the models predict that this conserved region is located outside the membrane it may therefore be important in the recognition and binding of Na^+ or K^+ prior to being transported across the membrane.

Site-directed mutagenesis was used to determine the role of this highly conserved region in the transport of Na^+ and K^+. The E464Q substitution resulted in a growth enhancement by low concentrations of added NaCl suggesting that this region of HKT1 may be involved in binding or transport of Na^+. To further test this hypothesis we determined the affinity of HKT1 and HKT1-E464Q for Na^+. For HKT1, the apparent K_m for Na^+ was 433 ± 69 μM in the presence of 300 μM K^+. In contrast, Rubio et al. [2] measured the K_m for Na^+ to be 175 ± 50 μM, but in those experiments 15 μM Rb^+ was used in contrast to the 300 μM K^+ used in our studies. The Na^+ affinity of the HKT1-E464Q mutant ($K_m = 734 \pm 69$ μM) was significantly lower than the Na^+ affinity measured for HKT1. A lower affinity in HKT1-E464Q was consistently measured across a range of external pH. This supports the hypothesis based on the yeast growth data, that the HKT1-E464Q mutation alters the transporter's affinity for Na^+. To further test the hypothesis that this region is involved in Na^+ transport we mutated the highly conserved F463 residue to L463. This substitution led to a reduction in the transporter's affinity for K^+. The results of the mutational and kinetic studies suggest that the region containing the 16 conserved amino acids in HKT1 plays a role in the energization of K^+ transport.

To test whether the E464Q mutation altered the overall conformation of the protein such that K^+ transport was also affected, we measured the K_m for K^+ in both the HKT1 and the HKT1-E464Q expressing yeast strains. These experiments showed that the HKT1-E464Q mutation did not alter the K_m for K^+ leading to the conclusion that the conserved region in HKT1 containing E464 appears to be important in the binding/transport of Na^+ and not K^+. Therefore, we propose that this region is located in an area of high-affinity Na^+ binding in HKT1. Our results support the model proposed by Gassmann et al. [22] suggesting that HKT1 has distinct and separate binding sites for Na^+ and K^+.

The changes in function upon neutralization of a charged residue and the substitution of a phenylalanine for a leucine suggests that the 16 amino acid region that is highly conserved across phyla is involved in determining the Na^+ transport characteristics of HKT1. The importance of negatively charged residues has been demonstrated in other cation selective carriers and channels such as the Na^+/H^+ antiporter from *E. coli* [23] and a Na^+ channel from rat skeletal muscle [24]. In an Na^+-ATPase from *Proprionigenium modestum* there is evidence that a negatively charged amino acid, E65 in subunit c is an essential residue involved in the binding of Na^+ [25]. In HKT1 the neutralization of E464 not only reduced the Na^+ affinity, but appeared to reduce the flux rates of Na^+ and K^+. This is consistent with studies on cardiac Ca^{2+} channels where the neutralization of conserved glutamate residues reduced the flux and the channel's affinity for Ca^{2+} [26,27].

Amino acids that contain aromatic rings have previously been shown to be important in the functional characteristics of cation [28,29] and anion [30] transport proteins. In K^+ channels, the high degree of selectivity for K^+ over Na^+ is determined by an interaction between phenylalanine-glycine residues [28]. It is interesting to note that the 16 amino acid region that we chose for mutagenesis also contains a number of highly conserved glycine residues that may be important for determining specific functional characteristics. Random mutagenesis of HKT1 led to the substitution of a phenylalanine for a leucine and this increased the K^+/Na^+ selectivity of the transporter [2]. It is perhaps not surprising that the conserved phenylalanine residue in this region plays an important role in transporting the energizing cation in HKT1, but further work will be needed to determine precisely how this amino acid containing an aromatic ring interacts with other components of the protein to form a domain that may be involved in the binding and transport of the cation that provides the energization for high affinity K^+ transport.

In summary this computational and mutational analysis identified a region in a high affinity K^+ transport protein (HKT1) that is highly conserved across phyla and demonstrated that it is functionally important in determining Na^+ binding and transport.

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