Mutagenesis analysis of the yeast Nha1 Na^+/H^+ antiporter carboxy-terminal tail reveals residues required for function in cell cycle

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Abstract **The yeast Nha1 Na^+,K^+/H^+ antiporter may play an important role in regulation of cell cycle, as high-copy expression of the *NHA1* gene is able to rescue the blockage at the G_1/S transition of cells lacking Sit4 protein phosphatase and Hal3 activities. Interestingly, this function was independent of the role of the antiporter in improving tolerance to sodium cations, it required the integrity of a relatively large region (from residues 800 to 948) of its carboxy-terminal moiety, and was not performed by the fission yeast homolog antiporter Sod2, which lacks a carboxy-terminal tail. Here we show that a hybrid protein composed of the Sod2 antiporter fused to the carboxy-terminal half of Nha1 strongly increased sodium tolerance, but did not allow growth at high potassium nor did rescue growth of the *sit4 hal3* conditional mutant strain. Deletion of Nha1 residues from 800 to 849, 900 to 925 or 926 to 954 abolished the function of Nha1 in cell cycle without affecting sodium tolerance. A screening for loss-of-function mutations at the 775–980 carboxy-terminal tail of Nha1 has revealed a number of residues required for function in cell cycle, most of them clustering in two regions, from residues 869 to 876 (cluster A) and 918 to 927 (cluster B). The later is rather conserved in other related antiporters, while the former is not.**

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Key words: H^+/Na^+ antiporter; Cell cycle; Saline tolerance; Mutagenesis analysis; *Saccharomyces cerevisiae*

1. Introduction

Na^+/H^+ antiporters are an important family of proteins present in the membranes of most cell types, from human to bacteria, which play a key role in maintaining cation and pH homeostasis [1]. In yeast cells, the Na^+/H^+ antiporters serve to remove intracellular sodium cations utilizing the external H^+ accumulation generated by the plasma membrane H^+-ATPase (for a review, see [2]). A number of genes encoding plasma membrane Na^+/H^+ antiporters have been cloned within the last few years from different yeast species, such as *Schizosaccharomyces pombe* [3], *Zygosaccharomyces rouxii* [4,5], *Saccharomyces cerevisiae* [6] or *Candida albicans* [7], thus allowing a preliminary characterization of this type of proteins. As their mammalian counterparts, they are composed in many cases by a hydrophobic, membrane-bound region, comprising 10–12 transmembrane domains, followed by a hydrophilic carboxy-terminal tail. One of the few exceptions is the *S. pombe* Sod2 antiporter, which contains only the transmembrane moiety. While the primary sequences corresponding to the transmembrane region are rather similar, the divergence at the carboxy-terminal tail is quite high, and only a few short regions are conserved [8].

Na^+/H^+ antiporters from different yeast species also differ in their substrate specificity for alkali cations: for instance, while Sod2 transports only Na^+ and Li^+, the antiporters from *S. cerevisiae* (Nha1) and *C. albicans* (Cnh1) are able to recognize also K^+ and Rb^+ as substrates. In the case of the budding yeast *S. cerevisiae*, Nha1 has only a modest role in mediating sodium tolerance, particularly at moderately acidic and neutral pH [6,9]. Nha1 has been proposed to regulate intracellular pH [9,10], to mediate sodium and potassium efflux [9], and to promote growth at low K^+ concentrations, probably through regulation of Trk1-dependent K^+ uptake [11].

The budding yeast Ser/Thr phosphatases Sit4 and Ppz1 are positive and negative effectors, respectively, of the G_1/S transition at the cell cycle [12–14]. *HAL3* encodes a negative regulator of Ppz1 [15] and, consequently, a *sit4 hal3* mutant cannot grow because of a severe blockage in G_1/S [16–18]. In a recent paper [18], we described the construction of a conditional *sit4 hal3* mutant strain (JC002) and its use to carry out a high-copy screening for suppressors of the *sit4 hal3* synthetically lethal phenotype. This screening yielded, among other genes, the *NHA1* antiporter gene, pointing out to a possible role of Nha1 in cell cycle regulation. The positive effect of Nha1 was not dependent of its ability to improve tolerance to Na^+ cations, and could not be mimicked by overexpression of the powerful Na^+-ATPase *ENA1* (a major determinant of salt tolerance in *S. cerevisiae*) or the *S. pombe* Sod2 antiporter. Budding yeast Nha1 is a 985 amino acid protein in which approximately the first 450 residues correspond to the transmembrane domain, followed by a large carboxy-terminal tail. By deletion mapping, we were able to show [18] that a region near the carboxy-terminal end of Nha1, comprising from residue 800 to 948, was required for the antiporter to effectively overcome the cell cycle blockage, and contained residues important to modulate the transport of potassium. Here we extend these findings and present a detailed mutational analysis of this important region of Nha1 that reveals a number of residues necessary for Nha1 function in cell cycle.

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2. Materials and methods

2.1. Growth of Escherichia coli and yeast strains

E. coli strains DH5α or DH5 were used as a host for DNA cloning. Bacterial cells were grown at 37°C in Luria–Bertani (LB) medium containing 50 μg/ml ampicillin for plasmid selection. *S. cerevisiae* cells were grown at 28°C in YPD medium or, when indicated, in complete minimal (CM) synthetic medium lacking uracil [19]. Strain JC002 (*MATa sit4::TRP1 tetO:HAL3*) derives from JA100 [15] and the salt-hypersensitive strain B31 (*MATα ena1Δ::HIS3::ena4Δ nha1::LEU2*) derives from W303-1B [9].

2.2. Recombinant DNA techniques and plasmid constructions

E. coli cells were transformed by using standard calcium chloride treatment [20] or by electroporation [21] when high efficiency was needed. Yeast cells were transformed following a modification of described methods [22]. Restriction reactions, DNA ligations, and other standard recombinant DNA techniques were carried out as described elsewhere [20].

The construction of pES071, which allows high-copy number expression of a version of *NHA1* carrying a C-terminal 3×HA-tag, was as follows. The 4.3 kbp *Xba*I/*Sph*I fragment of plasmid VHS5A/Ura [18], containing the *NHA1* gene was cloned into the same sites of pUC19, yielding pES018. An artificial *Sal*I site right in front of the stop codon (which introduces the residues VD) was then created by sequential polymerase chain reaction (PCR) [23], and the amplification fragment used to replace the 1.1 kbp *Apa*I/*Sph*I fragment of pES018, to yield pES070. A 3×HA-tag, with added *Sal*I sites, was amplified by PCR from plasmid pCM113 [24], and cloned in the appropriate orientation in pES070. The resulting 3×HA-tagged version of *NHA1* (pES099) was then transferred to plasmid YEplac195 [25] for expression in yeast, producing plasmid pES071.

To generate plasmid pES149, which allows expression of the *S. pombe sod2*$^{+}$ gene under the control of the strong *ADH1* promoter, the *Bam*HI 3.4 kbp fragment from plasmid pDBSod2 [18] was cloned into the same site of plasmid YEplac195.

A version of Sod2 carrying the carboxy-terminal tail of *S. cerevisiae NHA1* was constructed as follows. The 1.4 kbp intronless *sod2*$^{+}$ fragment described in [18] was cloned into the *Bam*HI site of plasmid pGEM3Z+ (Promega) to produce pES035. A *Xho*I site was introduced between residues S^{451} and D^{452} of Sod2 by sequential PCR, and the resulting fragment used to replace the original 0.76 kbp *Nhe*I/*Eco*RI fragment present in pES035. The new construct (pES116) was digested with *Bam*HI and the approximately 1.4 kbp fragment, containing the Sod2 open reading frame (ORF) plus the added *Xho*I site, cloned into the *Bgl*II site of plasmid pDB20BglII [26] to generate pES121. This construct was digested with *Bam*HI to release a 3.4 kbp fragment containing the Sod2 ORF (with the added *Xho*I site) flanked by the *ADH1* promoter and terminator elements, which was cloned into the *Bam*HI site of YEplac195, to yield pES134. The carboxy-terminal half of *NHA1* (from N^{458} to the stop codon, including the 3×HA-tag at its 3′-end), was amplified from pES071 by PCR, with added *Xho*I sites. The 1.7 kbp amplification fragment was cloned into pBS-SK (Stratagene), sequenced, released by *Xho*I digestion and cloned into the artificial *Xho*I site of plasmid pES134 in the appropriate orientation to produce plasmid pES136. Therefore, this construct bears a fusion of *S. pombe* Sod2 and the C-terminal half of *S. cerevisiae* Nha1, carrying a triple HA-tagging, under the control of the *ADH1* transcriptional promoter.

All PCR-produced fragments were fully sequenced using an ABI 3100 Genetic Analyzer (Applied Biosystems) and the big dye 3.0 terminator kit, to detect unwanted changes in sequence and/or verify the desired mutations.

2.3. Mutagenesis of the carboxy-terminal half of Nha1

Internal deletions were generated by sequential PCR as follows. The VHS5A/Ura genomic clone [18] was used as template and the amplified fragments, once its sequence was verified, used to replace the equivalent ones in pES018. In the case of the deletion between residues 800 and 849 (inclusive), a 1.98 kbp *Hpa*I/*Sph*I fragment containing the desired deletion was amplified and used to replace the original fragment. Deletions between residues 900–925 and 926–954 were made by removing the 1.06 kbp *Apa*I/*Sph*I region and replacing it with PCR-amplified DNA fragments containing the indicated deletions. In all cases, the entire *Xba*I/*Sph*I fragment, containing the modified versions of the antiporter, was recovered from pES018 and cloned back into YEplac195 for high-copy expression in yeast.

To generate a library of clones containing the *NHA1* ORF enriched for mutations at the 775–980 region, we proceeded as follows. A 1.65 kbp fragment was amplified by sequential PCR so that a *Spe*I restriction site was introduced at position +2298 from the initiating ATG codon (the changes introduced did not modify the amino acid sequence). Then, a 1.26 *Hpa*I/*Apa*I fragment, including the artificial *Spe*I site, was used to replace the equivalent fragment of plasmid pES070. The resulting construct (pES153) was digested with *Xba*I/*Sph*I and the 4.3 kbp insert was cloned into the same sites of YEplac195 (pES154). pES153 was used as a template to amplify a 0.68 kbp fragment, spanning from the *Spe*I to the *Sal*I artificial sites introduced in the *NHA1* ORF, in four independent reactions, as described in [27] with some modifications. The four reaction products were mixed, purified, digested with *Spe*I and *Sal*I, and used to replace the equivalent 0.66 kbp fragment by ligation into the *Spe*I/*Sal*I sites of pES154. The ligation products were then introduced into *E. coli* competent cells by electroporation. Plasmid DNA from at least 30 000 independent clones was recovered, amplified, and the library used for the Nha1 loss-of-function screening.

2.4. Screening for loss of function of Nha1 in cell cycle

Strain JC002 (*sit4::TRP1 tetO:HAL3*) was transformed with the above-mentioned library (approximately 30 000 clones) and plated in CM plates lacking uracil. After 48 h, colonies (200–1000/plate) were replicated on CM (lacking uracil) plates containing 20 μg/ml doxycycline and these plates were incubated for 48–72 h. Clones unable to grow in the presence of doxycycline were picked out from the master plates, and the plasmids recovered in *E. coli* and tested for integrity of the insert by restriction mapping. The constructs were then reintroduced into strain JC002 (and tested again for loss of growth in the presence of doxycycline), as well as into strain B31 (and tested for growth in high sodium). Clones negative for growth in doxycycline were subjected to DNA sequencing, covering the entire *Spe*I/*Sal*I 0.66 kbp fragment subjected to mutagenesis, to identify possible mutations responsible for the loss of function. In some cases, when two different mutations were encountered in this region and they were separated by the *Apa*I site (residues 866–867), it was possible to evaluate the effect of the mutation downstream this restriction site by rescuing the 1.06 kbp *Apa*I/*Sph*I fragment of the mutated clone and using it to replace the equivalent region of pES153. Then, the 4.3 kbp *Xba*I/*Sph*I insert was cloned into the same sites of YEplac195 and the existence of a single mutation confirmed by DNA sequencing.

2.5. Other techniques

Immunoelectrophoretic analysis was performed as follows. JC002 cells carrying the different constructs were grown up to an OD_{660} of approximately 2, collected by centrifugation, and disrupted by vortexing with the aid of glass beads in the presence of a buffer containing 100 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), plus an antiprotease mixture (0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 0.5 mM benzamidine, 1 μg/ml pepstatin). The mixture was centrifuged at 750×*g* for 10 min at 4°C to remove glass beads and cellular debris, and the supernatant recovered (total cell extract). 50 μl of this extract was centrifuged at 16 100×*g* for 15 min at 4°C, the supernatant was recovered (16 K supernatant), and the pellet resuspended in the same volume of the disruption buffer described above (16 K pellet). Samples (40 μg of protein) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (8% acrylamide) and proteins were transferred to Immobilon-P membranes (Millipore), blocked, and incubated with a monoclonal anti-HA antibody (Roche). Immunoreactive proteins were identified with luminescence substrates (ECL, Amersham).

Growth on plates (drop tests) was assessed as described in [28]. Tolerance to cations was determined in liquid cultures as described [29].

3. Results

In a recent paper [18], we demonstrated that high levels of expression of the *S. cerevisiae* Nha1 antiporter were able to

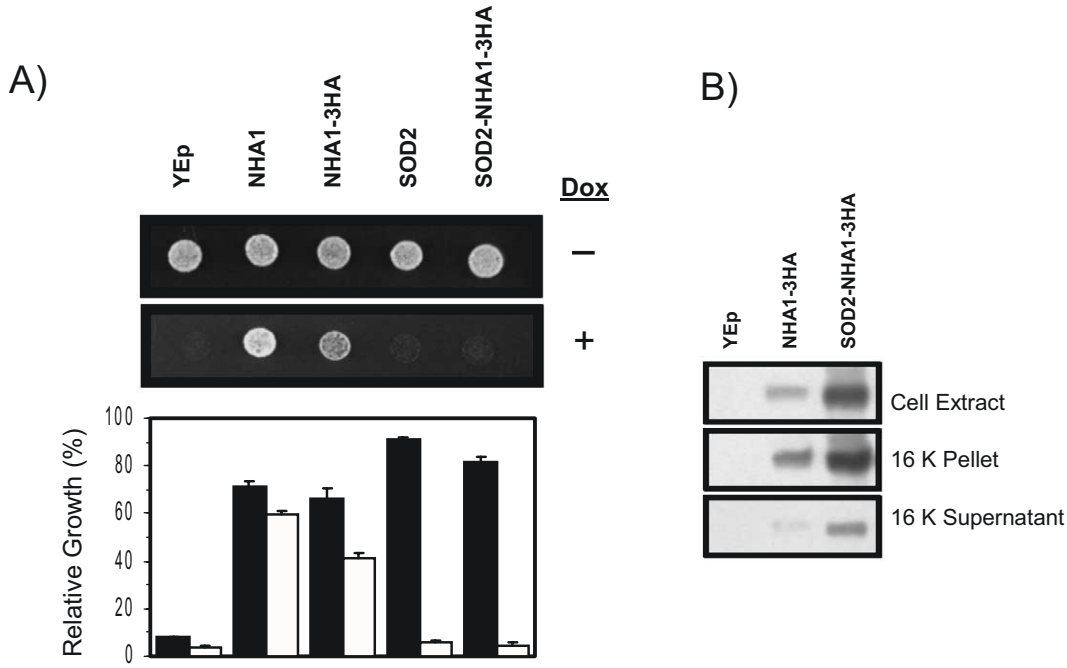

Fig. 1. Functional analysis of a chimerical Sod2/Nha1 protein. A: Upper panel: Strain JC002 was transformed with plasmids YEplac195 (YEp), VHS5A/Ura (NHA1), pES071 (NHA1-3HA), pES149 (SOD2) or pES136 (SOD2-NHA1-3HA). 3 μl of a diluted suspension of cells (OD_{660} 0.03) were spotted on CM plates lacking uracil in the absence (−) or the presence (+) of 20 μg/ml doxycycline (Dox). Growth was monitored after 3 days. Lower panel: The above-mentioned plasmids were introduced into strain B31 and diluted cell suspensions (OD_{660} 0.015) grown for 15 h on YPD (pH 5.5), in the presence of 0.4 M sodium chloride (closed bars) or 1 M potassium chloride (empty bars). Relative growth was calculated as the ratio between growth in the presence and growth in the absence of the added salt, and represented as percentage. Data are means ± S.E.M. from three to six independent clones. B: Total cells extracts were obtained from strain JC002 bearing the indicated plasmids, and 16 K pellets and supernatants were prepared as indicated in Section 2. Samples (40 μg of protein) were electrophoresed, transferred to membranes and probed with monoclonal anti-HA antibodies to detect the expressed tagged proteins.

relieve G_1/S cell cycle blockage, while overexpression of the *S. pombe* homolog Sod2, which lacks a carboxy-terminal tail, was not. As it was also found that Nha1 required its carboxy-terminal half to carry out this function, we considered the possibility that the failure observed for Sod2 could be due to the absence of the necessary carboxy-terminal tail. To test this possibility, we constructed a fusion protein comprising the first 451 residues of *S. pombe* Sod2 (transmembrane domain) followed by the last 528 residues of Nha1 (that is, starting shortly after the last transmembrane domain of Nha1, where the sequence similarity between both proteins is lost). This construct also contained a triple HA carboxy-terminal tag, to allow immunological detection of the expressed protein. As shown in Fig. 1A, the tagged version of Nha1 retained its ability to allow growth of the JC002 strain (although perhaps with somewhat less potency). This version of Nha1 was fully functional in terms of providing high-sodium tolerance to a B31 strain, but slightly less effective than the non-tagged protein in allowing growth in high potassium. As documented previously, overexpression of Sod2 did not recover growth of a *sit4 tetO:HAL3* strain (JC002) on non-permissive conditions (that is, in the presence of doxycycline), nor increased tolerance to high potassium. Interestingly, the Sod2 version carrying the carboxy-terminal tail of Nha1 also failed to promote growth when subjected to these tests. It is important to note that the hybrid protein was successfully expressed, as deduced from the observation that B31 cells containing this construct showed a very high tolerance to sodium ions. Furthermore, immunological analysis (Fig. 1B) showed that most of the expressed protein was recovered in

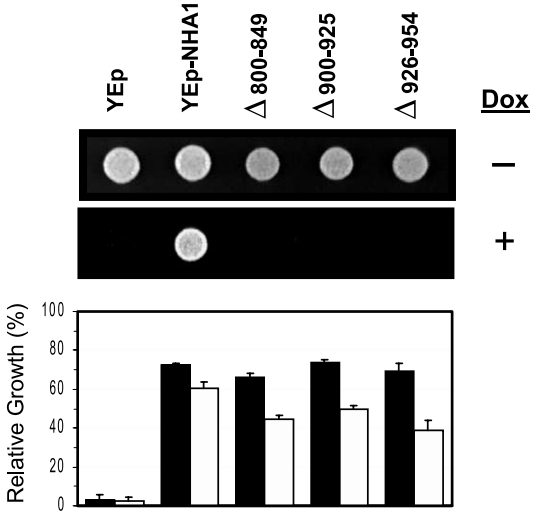

Fig. 2. Functional mapping of the Nha1 antiporter by selective deletions within its 800–954 carboxy-terminal region. Upper panel: Strain JC002 (*sit4::TRP1 tetO:HAL3*) was transformed with high-copy plasmid YEplac195 (YEp), as well as with the same plasmid bearing the native *NHA1* gene (VHS5A/Ura) or different *NHA1* versions in which the indicated regions had been deleted, and tested for growth as described in Fig. 1. Growth was monitored after 3 days. Lower panel: Strain B31 (*ena1Δ::HIS3::ena4Δ nha1::LEU2*) was transformed with the indicated constructs and the cells tested for growth as indicated in Fig. 1. Data are means ± S.E.M. from three independent clones.

particulate fractions, as expected from a protein associated to membranes.

Our previous work revealed that a region spanning from residues 800 to 948 of the presumably cytoplasmic carboxy-terminal half of Nha1 was necessary for function in cell cycle. To gain more insight on the structural elements involved in this effect, we prepared versions of Nha1 carrying different deletions within this region. As shown in Fig. 2, when these versions were tested on strain JC002 under non-permissive conditions, none of the versions was able to allow growth. In contrast, when introduced into the salt-hypersensitive strain B31 (*ena1-ena4Δ nha1*), all of them conferred a tolerance to high concentrations of sodium chloride similarly to that produced by a native version of the antiporter. Interestingly, all three mutated versions were less effective than the native *NHA1* clone in supporting growth in liquid cultures containing 1 M potassium chloride. This growth defect was not due to an osmotic effect, as growth in the presence of 2 M sorbitol was essentially identical in all cases (not shown).

The observation that structural elements important for cell cycle-related function were located in different segments of the region spanning from residues 800 to 954 of Nha1 prompted us to undertake a detailed mutagenesis analysis of this region using a random mutagenesis PCR approach. Functional analysis of about 30 000 clones yielded 121 candidate clones, unable to allow growth of JC002 cells. However, in many cases failure to growth was due to deletions (45 clones) or insertions (one clone) that would change the reading frame. In addition, eight clones contained premature stop codons, defining six different stop positions at codons 788, 797, 811, 822, 827 and 890. All these versions were tested for growth in high potassium, sodium and lithium. It could be observed that the version carrying a stop codon at position 890 was the only one conferring a slightly improved tolerance to sodium and a very strong tolerance to lithium to B31 cells (data not shown).

The final output of this screening was the identification of 18 single point mutations that would block the ability of Nha1 to allow growth of JC002 cells in the presence of doxycycline. As it can be observed (Fig. 3, upper panel), most mutations cluster into two regions. The first region (cluster A) spans from S^{869} to T^{876} and contains four mutations. Two of the changes (L870F and T874A) resulted in partial loss of function, as they allowed growth of the JC002 strain, albeit rather slow. On the contrary, mutation T876A completely blocked growth of strain JC002, even after 7 days of incuba-

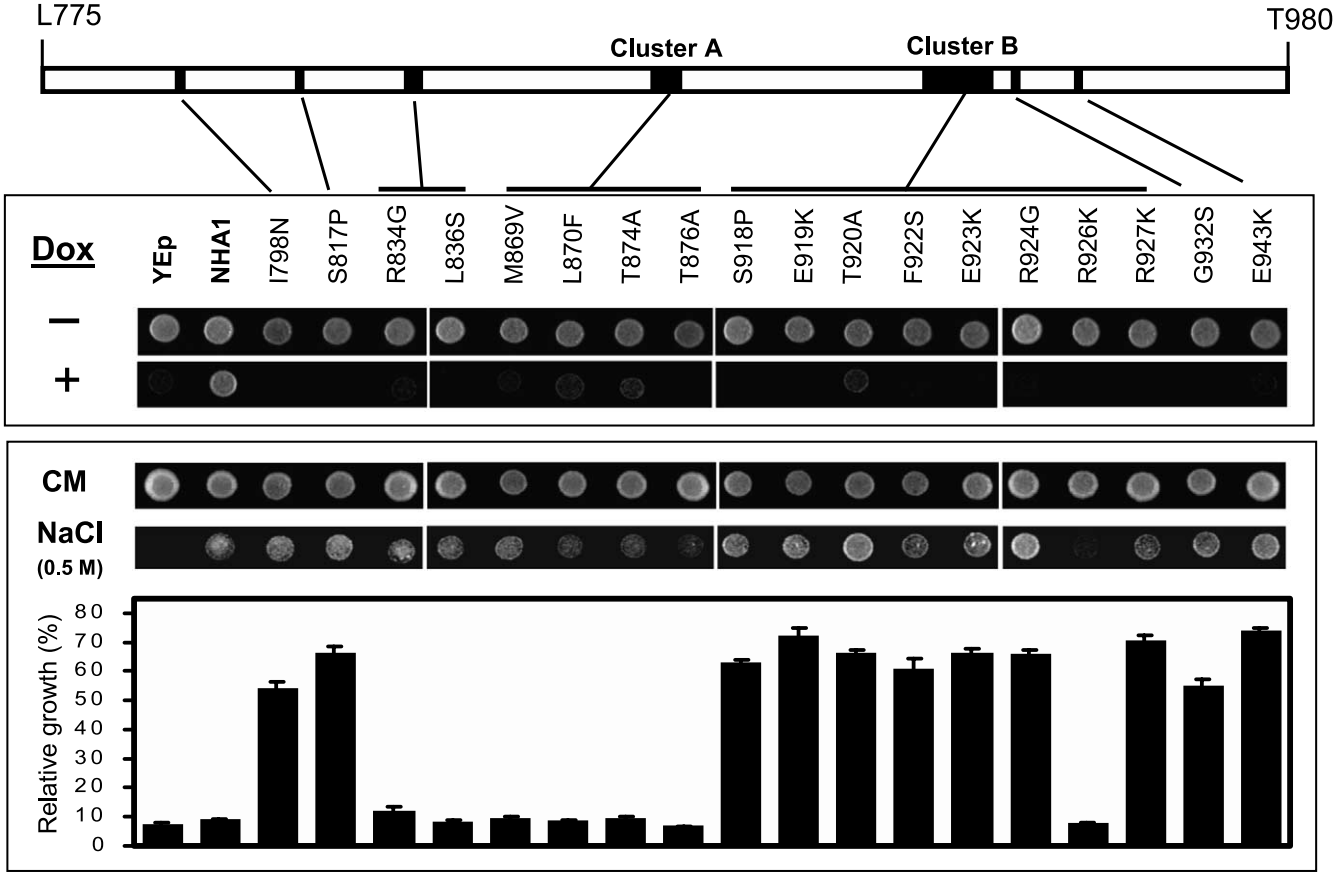

Fig. 3. Localization and phenotypic effects of point mutations impairing the function of Nha1 in cell cycle. Upper panel: Strain JC002 was transformed with YEplac195 (YEp), plasmid VHS5A/Ura (NHA1) or the same high-copy plasmid bearing versions of the Nha1 antiporter carrying the indicated point mutations. Growth was monitored after 4 days. The approximate location of each mutation within the region 775–980 is depicted on the top, including location of clusters A and B (see main text). Lower panel: Strain B31 was transformed with the above-mentioned plasmids and tolerance to sodium tested on CM plates (lacking uracil) in the absence or the presence of 0.5 M NaCl. Growth was monitored after 4 days. In all cases strains were spotted in a single plate for each condition tested and are aligned in the figure for descriptive purposes. The same strains were grown in liquid YPD medium (pH 5.5) in the presence or absence of 20 mM LiCl. Relative growth was calculated as the ratio between growth in the presence and growth in the absence of the added salt, and represented as percentage. Data are means ± S.E.M. from four experiments.

tion (when normally this strain recovers somewhat from the effect of doxycycline). The second region (cluster B) includes amino acids 918–927 and in this short segment, composed of 10 residues, mutations for all but two of them (E^{921} and Q^{925}) have been found. Several of these mutations had a very severe effect on growth, similarly to that of the T876A change, while one mutation (T920A) still allowed growth on doxycycline, although very slowly. In addition, a pair of close mutations, affecting residues R834 and L836, has been identified. Four additional changes resulting in loss of function were found upstream (I798N and S817P), or downstream (G932S and E943K) of the mentioned groups.

All mutated versions of Nha1 were also tested for the ability to confer tolerance to high potassium, sodium or lithium to strain B31. We observed that most mutations in cluster B slightly reduced cell growth in the presence of high concentrations of potassium chloride, similarly to that observed for the versions carrying deletions (data not shown). When tolerance to sodium cations was tested, we found (Fig. 3, lower panel) that with the exception of the L836S and M869V changes, mutations on the residues of cluster A produced a slight decrease in tolerance to sodium. Mutations in cluster B yielded a non-homogeneous phenotype: while some of them, such as T920A and R924G, conferred higher tolerance than native Nha1, a conserved change, R926K, resulted in a rather strong decrease in the ability of this Nha1 version to improve tolerance to sodium cations. The evaluation of lithium tolerance in strains overexpressing the mutated forms of Nha1 yielded rather interesting results. At low LiCl concentrations (10 mM), differences in tolerance were small (not shown). However, when higher concentrations were tested (20 mM), at which overexpression of wild-type Nha1 cannot significantly improve growth, we found that mutations at positions 798 and 817, and particularly all mutations in cluster B (but none in cluster A) were able to strongly improve growth. The only exception was the conserved change R926K, which did not improve tolerance and, in fact, when tested at lower LiCl concentrations, resulted in slightly lower tolerance than that conferred by wild-type Nha1.

4. Discussion

In contrast to what is known for certain fungi, such as *S. pombe* or *Z. rouxii*, in which Na^+/H^+ antiporters are major elements for sodium and lithium cation tolerance [3,30], in the budding yeast *S. cerevisiae* the Na^+/H^+ antiporter Nha1 represents a relatively minor component of the mechanisms involved in tolerance to sodium cations. Alternatively, other cellular roles, such as regulation of cell volume, K^+ concentration or intracellular pH have been proposed for this protein [9–11].

Recently, we showed that Nha1 might be involved in the regulation of cell cycle progression, as high-copy expression of this gene (from its own promoter) was able to rescue the G_1/S cell cycle blockage of a conditional *sit4 hal3* mutant [18]. This ability was not related to the capacity of the antiporter to increase tolerance to sodium cations, but required the presence of the transmembrane antiporter region and the integrity of a relatively large region of its carboxy-terminal tail, comprising approximately from residue 800 to 948. The requirement for this carboxy-terminal element was indicative that it might be important for regulation of the Nha1 function in cell cycle. Therefore, we undertake its functional characterization aiming to get further insight into the regulation and function of this protein.

We show here that expression of a chimera in which the transmembrane region of Nha1 has been replaced by the equivalent (43% of identity, 58% conserved residues) element of the *S. pombe* antiporter Sod2 is able to increase tolerance to sodium cations, even more efficiently than that native Nha1 does. While this might reflect the highest transport capacity of the Sod2 antiporter, as previously demonstrated [31], in our case it may just result from a different expression level, somewhat higher in the case of Sod2 (it should be noted that the promoter elements in both constructs were not equivalent). The hybrid protein was not able to allow growth at high potassium, suggesting that the inability of *S. pombe* Sod2 to transport potassium [31] is an intrinsic property of the transmembrane domain and not due to the lack of a carboxy-terminal moiety. In any case, this hybrid protein failed to sustain growth of strain JC002 under non-permissive conditions, indicating that while the 800–948 carboxy-terminal region of Nha1 is required for function in cell cycle, specific elements at the amino-terminal transmembrane region, not present in Sod2, must be also important.

A more detailed analysis of the carboxy-terminal region was initiated by generating several deletions within the region 800–954 (Fig. 2). All of them resulted in loss of the suppressor effect when these versions of Nha1 were tested in JC002 cells, indicating that residues important for this function are dispersed within this relatively large region. These deletions also resulted in a small, but significant reduction in the growth rate of the cells at high potassium, suggesting the alterations in this region of the protein may affect the capacity for potassium transport. This agrees with our previous findings [18], although it must be noted that other authors have reported that high-copy expression of versions of Nha1 carrying truncation of most of its carboxy-terminal tail still support growth on high potassium [32]. This apparent discrepancy may result from the fact that we determine the effect of high potassium in liquid culture, which allows a more sensitive monitoring of growth.

The possibility that residues important for cell cycle function could be dispersed within the 800–954 region prompted us to develop a strategy to identify point mutations within this segment of the protein that could abolish the ability of Nha1 to rescue growth of JC002-blocked cells. Among the mutations identified, six corresponded to premature stop codons, at positions 788, 797, 811, 822, 827 and 890, thus confirming our previous finding in that truncations prior residue 923 eliminate the function [18]. In addition, the version truncated at position 890 was able to increase tolerance of B31 cells, moderately to sodium and strongly to lithium cations. This is in agreement with the previous finding that the original genomic clone isolated, which was truncated at position 888, was more efficient in increasing the tolerance to these cations than the entire protein [6,9].

In addition, 18 single additional mutations resulting in change of amino acid were identified. These mutations were located between residues 798 and 943, and affected all three short segments deleted within the 800–954 region, thus explaining the phenotype previously observed (Fig. 2). However, it is remarkable that most mutations (12 out of 18) clustered into two very short regions (clusters A and B, Figs. 3 and 4),

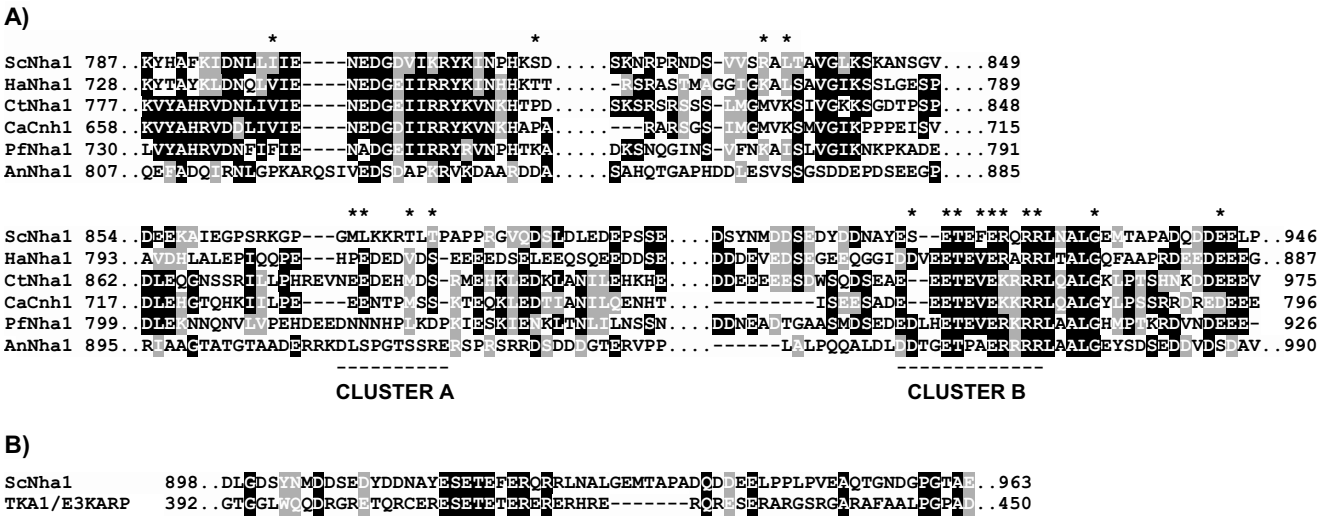

Fig. 4. Comparative analysis of residues important for Nha1 cell cycle function in other yeast antiporter sequences. A: The complete amino acid sequences of the Na^+/H^+ antiporters from *S. cerevisiae* (ScNha1, GenBank, DDBJ accession number Z73310), *Hansenula anomala* (HaNha1, AB073976), *Candida tropicalis* (CtNha1, AB073975), *C. albicans* (CaCnh1, AF375984), *Pichia farinosa* (PfNha1, AJ496431) and *Aspergilus nidulans* (AnNha1, AB073977) were aligned using Clustal W (version 1.8). The regions corresponding to the mutagenized segment of *S. cerevisiae* Nha1 are represented. Very poorly conserved regions, not comprising point mutations described in this paper (showed as asterisks), have been eliminated and are represented by several dots. Conserved residues are denoted by black-shaded boxed, while conservative changes are shaded in gray. B: Alignment (Clustal W 1.8) of the region surrounding cluster B in *S. cerevisiae* Nha1 and the carboxy-terminal end of isoform 3 of human TKA1/E3KARP (GenBank, DDBJ Z50150).

suggesting that these regions are particularly important in function. Cluster B corresponds to a short region highly conserved between most fungal antiporters (Fig. 4), defined as C6 in [8], while cluster A contains a non-conserved sequence, specific for *S. cerevisiae*. This probably explains why the *C. albicans* antiporter Cnh1, which does not contain a similar sequence, was unable to replace Nha1 [18] despite their overall similarity (54% of identity). In addition, we have identified mutations, such as L870F, T874A or T920A with relatively weak effect on cell cycle, while others (for instance, most mutations in cluster B) appear to aggravate the growth defect, resulting in a dominant negative phenotype. This would be compatible with the notion of this region acting as a regulatory one.

The availability of these mutations allowed us to evaluate their effect on the ability of the antiporter to increase tolerance to alkaline cations. Perhaps the most interesting finding is the observation that 11 out of 18 mutations strongly increase tolerance to lithium cations. Two aspects are remarkable: (1) the position of the mutated residues is not random, as none of the six mutations from residues 834 to 876 results in increased tolerance, and (2) all mutations in cluster B, with the only exception of the R926K change, improve lithium tolerance. It is reasonable to assume that the lack of positive effect of this mutation is due to the conservative nature of the change, although it must be noted that a similar change in the following position (R927K) does improve tolerance. In fact, with this only exception, all mutations from position 918 to the carboxy-terminal end of Nha1 result in increased lithium tolerance, suggesting that this region of the protein contains a negative element for lithium transport. The observation that Nha1 versions truncated at positions 888 and 890 also increase lithium tolerance ([9] and this work) suggests that this negative element might extend at least 20 residues upstream in the protein sequence. It should be noted that the relevance of residues 920–928 in Nha1 with respect to lithium tolerance has been pointed out in a recent report [32], showing that truncation at residues 920, 923 and 928 resulted in increased tolerance to lithium. Therefore, our data confirm and expand these results, by showing that residues at positions 817, 834, 932 and 943 also play an important role in regulating the ability of Nha1 to extrude lithium.

The identification of sequences important for Nha1 to play a role in cell cycle (as well as in saline homeostasis) reinforces the notion that a possible role for the carboxy-terminal tail of Nha1 would be to regulate specific functions of this antiporter. It is worth noting that we do not observe here a correlation between cell cycle and saline phenotypes, supporting the idea that both are probably unrelated events. In mammalian cells, the carboxy-terminal tail of Na^+/H^+ exchangers has been identified as target for regulation (see [33] for review), including phosphorylation by different protein kinases. It is suggestive that five of the mutated residues identified in this work correspond to Ser or Thr residues that are located in a favorable context [34] for phosphorylation by casein kinase-2 (S^{817}, S^{918} and T^{920}), cAMP-dependent protein kinase (T^{874}) or calcium-calmodulin kinase II (T^{876}). In addition, at least in the case of mammalian NHE3, the existence of interacting proteins (named NHERFs, for Na^+/H^+ exchanger regulatory factors) that can serve as adapters for specific protein–protein interactions has been demonstrated. It is suggestive that, while in the overall the carboxy-terminal tail sequence of Nha1 does not display evident sequence similarity with other proteins, a structural element quite similar to the conserved cluster B in Nha1 (Fig. 4B) can be found at the carboxy-terminal end of one of the variants of the regulatory factor NHERF2 (TKA1/E3KARP, accession number Z50150). The possibility of this element playing a regulatory role is being investigated in our laboratory.

We have recently carried out a two-hybrid approach using the carboxy-terminal tail of Nha1 as bait to search for proteins able to interact with this region, which yielded no detect-

able interactions. However, very recently, a different approach based on affinity precipitation and mass spectrometry analysis has revealed a possible interaction between Nha1 and the product of the YHL010c ORF [35]. This gene encodes a 585-residue protein of unknown function, which is remarkably similar (31% identity, 47% conserved residues) to BRAP2, a human protein able to bind to nuclear localization signal motifs [36] found in BRCA1, a breast cancer susceptibility gene product that contributes to homologous recombination, DNA repair and transcriptional regulation [37]. A data bank survey reveals that YHL010c may contain several interesting structural domains, such as a RING finger domain (C3HC4-type), a Zn finger found in ubiquitin hydrolases and other proteins, and an ERM-like binding domain. These structural elements are known in many cases to mediate protein–protein interactions, often involved in signal transduction and cell proliferation. Therefore, it would be most interesting to investigate if the Nha1 residues essential for cell cycle function identified in this work are involved in binding and/or signalling through YHL010C.

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