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Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions

Taro Nakamura[1], Yusen Liu[1,2], Dai Hirata[1], Hiromitsu Namba[1], Shin-ichi Harada, Takeshi Hirokawa[3] and Tokichi Miyakawa[4]

Department of Fermentation Technology and [3]Department of Applied Physics and Chemistry, Faculty of Engineering, Hiroshima University, Higashi-Hiroshima, 724 Japan

[2]Present address: Laboratory of Molecular Genetics, Gerontology Research Center, National Institute on Aging, 4940 Eastern Avenue, Baltimore, MD 21224, USA
[4]Corresponding author
[1]Authors contributed equally to this work

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To assess the physiological function of Ca^{2+}-dependent protein phosphatase (PP2B) in the yeast *Saccharomyces cerevisiae*, the phenotypes of PP2B-deficient mutants were investigated. Although PP2B was dispensable for growth under normal conditions, the mutations did, however, cause growth inhibition under certain stress circumstances. The growth of the mutants was inhibited by NaCl and LiCl, but not by KCl, $CaCl_2$, $MgCl_2$ or nonspecific osmotic stresses. Upon shift to high NaCl medium, intracellular Na^+ levels of both wild type yeast and the mutants initially increased at a comparable rate. However, internal Na^+ in wild type cells started to decline more rapidly than the mutant cells during cultivation in high NaCl medium, indicating that PP2B is important in maintaining a gradient across the membrane. The protection against salt stress was achieved, at least in part, by the stimulation of Na^+ export. The maintenance of a high level of internal K^+ in high NaCl medium was also PP2B-dependent. In the presence of the immunosuppressant FK506, the growth behaviour and intracellular Na^+ and K^+ of wild type cells in high NaCl medium became very similar to those of the PP2B-deficient mutant in a manner dependent on the presence of the FK506 binding protein.

Key words: FK506/Na^+ transport/PP2B/salt tolerance/yeast

Introduction

Protein phosphatase 2B (PP2B), also called calcineurin, is one of four classes of phosphoserine- and phosphothreonine-specific protein phosphatase that requires Ca^{2+} for enzyme activation. Mammalian PP2B is a heterodimer composed of a 59-61 kDa calmodulin-binding catalytic subunit and a 19 kDa calcium-binding regulatory subunit which has a structural similarity to calmodulin (for a review see Klee *et al.*, 1988). Ca^{2+} can stimulate protein phosphatase activity by binding to the regulatory subunit and calmodulin. PP2B has been identified in various mammalian tissues, and cDNA encoding the catalytic and the regulatory subunits has been isolated. The physiological function of PP2B is only beginning to be understood. Since the identification of calcineurin as a component of T cell receptor-mediated signalling pathway that leads to the induction of interleukin-2 transcription, calcineurin is recognized as a key component that couples cell membrane associated and nuclear events (Liu *et al.*, 1991a; Clipstone and Crabtree, 1992; O'Keefe *et al.*, 1992). Liu *et al.* (1991a) also identified calcineurin as the molecular target for the immunosuppressants cyclosporin A and FK506, and suggested that the inhibition of the phosphatase activity is the mechanism by which the immunosuppressant exerts its effect.

To investigate the physiological functions of PP2B in the yeast *Saccharomyces cerevisiae* by a genetic approach, we have previously isolated two homologous genes *CMP1* and *CMP2* encoding the catalytic subunit of PP2B (Liu *et al.*, 1991b). The deduced amino acid sequences of the Cmp1 and Cmp2 proteins were 64% identical. The same genes, designated *CNA1* and *CNA2*, were independently cloned and characterized by Cyert *et al.* (1991). Yeast cDNA and genomic DNA for the regulatory subunit of PP2B were also cloned and characterized (Kuno *et al.*, 1991; Cyert and Thorner, 1992). Because the identities of the deduced amino acid sequences between mammalian PP2B subunits and the yeast counterparts are >50%, it was considered that the enzyme may play important roles in the regulation of basic cellular processes. However, single or double disruption of *CMP1* and *CMP2* had no significant effect on cell viability, morphology, growth rate or sporulation, indicating that these genes are not essential for normal growth (Cyert *et al.*, 1991; Liu *et al.*, 1991b). Similarly, null mutation of *CNB1* caused no distinct phenotypes under normal growth conditions (Cyert and Thorner, 1992). Only the phenotype of the PP2B mutants observed is a defect in the ability to recover from G_1 arrest induced by the mating pheromone α-factor to re-enter the cell cycle (Cyert *et al.*, 1991; Cyert and Thorner, 1992). In the present study, we investigated the changes in growth behaviour of yeast containing null mutations in the genes encoding the PP2B subunits under various stress conditions. Mutations in the catalytic or the regulatory subunits of PP2B caused defect of salt tolerance, hypersensitivity to vanadate and slow growth in alkaline pH medium, indicating that yeast PP2B is involved in the regulation of ion homeostasis under certain stress circumstances that affect internal ion conditions. The PP2B-mediated regulation of salt tolerance was sensitive to FK506 in a manner strictly dependent on the FK506 binding protein (FKBP) yFKBP-12.

Results

CMP1 and CMP2 are the only genes that encode the catalytic subunit of PP2B

Because the mutant containing null mutations in both *CMP1* and *CMP2* genes exhibited no significant phenotype under normal growth conditions, we first examined the possibility

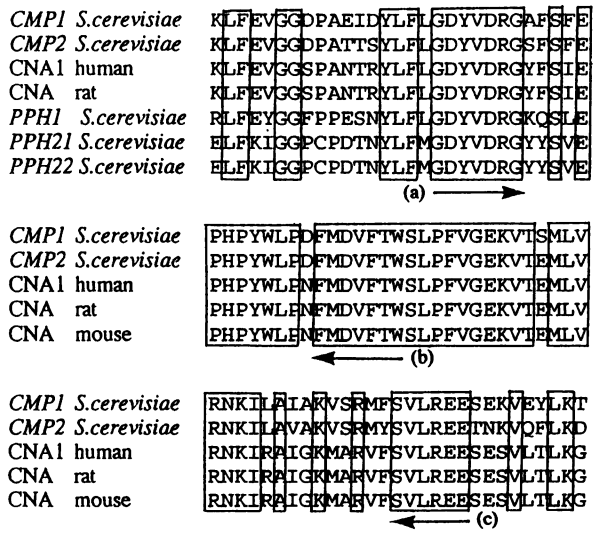

Fig. 1. Primers used for PCR analysis of the homologous genes of *CMP1* and *CMP2*. Sense strand primer (a) was synthesized according to the deduced amino acid sequences highly conserved in all of the three classes of serine/threonine protein phosphatases, PP1, PP2A and PP2B. Antisense primers (b and c) were synthesized according to the deduced amino acid sequences highly conserved within the PP2B-specific region of the phosphatase. Conserved amino acids are boxed. The direction of each primer is indicated by an arrow.

of the presence of additional homologous gene(s) by PCR analysis. To amplify DNA sequences of the genome having features of the genes encoding the catalytic subunit of PP2B, three degenerate oligonucleotide primers were designed and synthesized on the basis of the amino acid alignments of known sequences (Figure 1). The sense primer contains the codons for GDYVDRG which is conserved in the deduced sequences in all of the three classes of serine/threonine-type protein phosphatases and the restriction site for *Xho*I at the 5′ end. Two antisense primers contain the codons corresponding to the sequences (F/Y)SVLREE or FMDVFTW which are conserved from yeast to mammals in the PP2B-specific region of the deduced sequences and a *Bam*HI site at the 5′ end. DNA amplification was carried out using the genomic DNA prepared from a *cmp1* null mutant strain YLL34-1b (*cmp1::LEU2 CMP2*, Liu *et al.*, 1991b) as template. A YCp50-based yeast genomic library was screened with the purified PCR products as probe. Southern blot analysis of a total of 45 clones obtained by the screening demonstrated that all the PCR products contained the sequence derived from *CMP2* and did not contain other amplified products (data not shown). These results indicate that *CMP1* and *CMP2* are the only genes that encode the catalytic subunit of PP2B, supporting our previous biochemical observation that PP2B activity was not detectable in the *cmp1 cmp2* strain (Nakamura *et al.*, 1992).

Genetic analysis of the physiological function of PP2B

To assess the function of PP2B, we investigated the phenotypes caused by null mutations in *CMP1*, *CMP2* or *CNB1* under various stress conditions. For this purpose, *S.cerevisiae* strain W303, a diploid heterozygous at each of the two *CMP* loci (*MATa/MATα cmp1::LEU2/CMP1 cmp2::HIS3/CMP2*) or at the *CNB1* locus (*MATa/MATα cnb1::HIS3/CNB1*) were constructed. The strain W303 was chosen as a host of gene disruption because this strain is sensitive to the immunosuppressant FK506, while most other *S.cerevisiae*

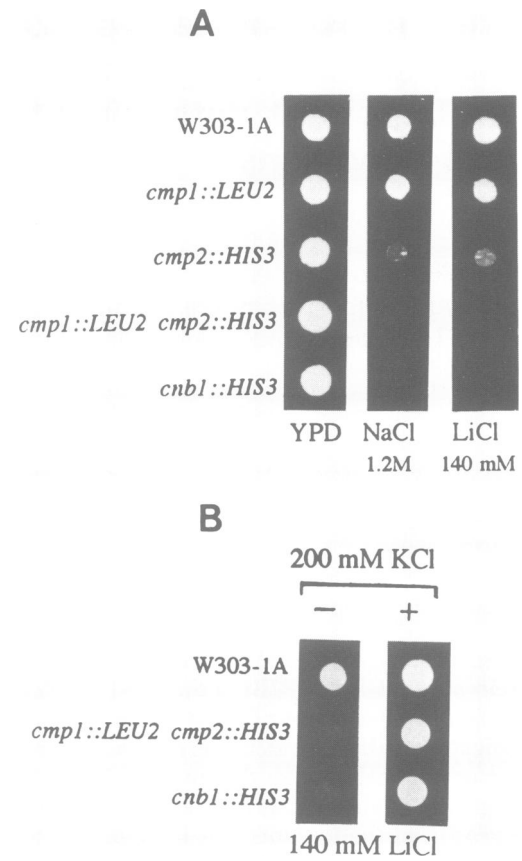

Fig. 2. Growth properties of various PP2B null mutants with different genotypes on YPD plates containing a high concentration of salt. (**A**) Isogenic haploid derivative of strain DHT22-1b with various mutations were grown on YPD medium containing 1.2 M NaCl or 140 mM LiCl. The strains were DHT22-1b (wild type), DHT22-1c (*cmp1::LEU2*), DHT22-1d (*cmp2::HIS3*), DHT22-1a (*cmp1::LEU2 cmp2::HIS3*) and DHT14 (*cnb1::HIS3*). (**B**) The growth of DHT22-1a and DHT14 on 140 mM LiCl in the presence or absence of 200 mM KCl was compared. Isogenic strain DHT22-1b was used for the growth comparison.

strains were relatively insensitive to the immunosuppressant (Brizuela *et al.*, 1991). The diploid strain was subjected to sporulation and tetrad segregation. Tetrad segregants with the desired genotypes *CMP1 CMP2* (DHT22-1b), *cmp1::LEU2 CMP2* (DHT22-1c), *CMP1 cmp2::HIS3* (DHT22-1d) and *cmp1::LEU2 cmp2::HIS3* (DHT22-1a) for the *CMP1* and *CMP2* loci, and *cnb1::HIS3* (DHT14) for the *CNB1* locus were selected and used for the experiments.

Decreased tolerance of PP2B mutants to NaCl. Various null mutants with alterations in the PP2B subunits (*cmp1::LEU2*, *cmp2::HIS3*, *cmp1::LEU2 cmp2::HIS3* and *cnb1::HIS3*) were plated onto YPD medium containing various salts and their growth was compared with that of the wild type strain. High concentrations of each salt without severe growth inhibition of wild type cells were used. Wild type cells could grow on the plate that contained up to 1.5 M NaCl. In contrast, the growth of *cmp2*, *cmp1 cmp2* and *cnb1* mutants was severely inhibited in the presence of 1.2 M NaCl (Figure 2A). The growth of the *cmp1 cmp2* and *cnb1* mutants was inhibited by 1.0 M NaCl and the *cmp2* mutant by 1.5 M NaCl. The *cmp1* mutant grew similarly to the wild type strain on NaCl plates. Sodium ions, but not chloride, were responsible for sensitivity because the mutant was sensitive

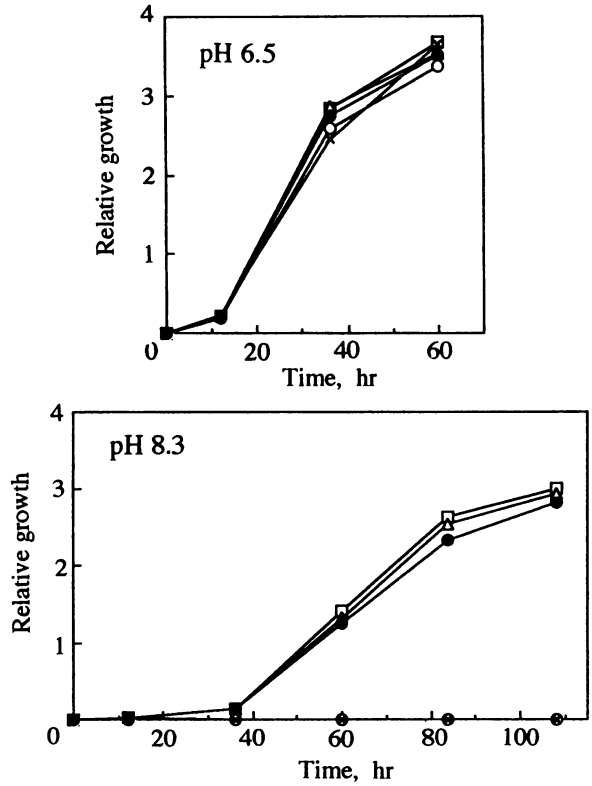

Fig. 3. The growth of PP2B null mutants with different genotypes in YPD medium adjusted to different pHs. The growth of various mutants in the medium adjusted to pH 6.5 (top) and pH 8.3 (bottom) was monitored by optical density at 660 mμ. Symbols are as follows: wild type (DHT22-1b) (●); DHT22-1c (*cmp1::LEU2*) (□); DHT22-1d (*cmp2::HIS3*) (△); DHT22-1a (*cmp1::LEU2 cmp2::HIS3*) (○); and DHT14 (*cnb1::HIS3*) (×).

to 0.5 M sodium acetate, but no difference was noted for the growth of the mutants on the plate containing either 2 M KCl or 0.5 M $CaCl_2$. The mutants also showed sensitivity to Li^+ on the plate containing 140 mM LiCl (Figure 2A). Li^+ is an analogue of Na^+ and presumably transported by Na^+ transporters. LiCl was ~10-fold more toxic to cell growth than NaCl at the molar concentration basis. Due to high osmolarity, the growth rate of wild type cells on the plate containing 1.5 M NaCl was much slower when compared with growth on the plate containing 140 mM LiCl. Therefore, we chose the medium containing LiCl instead of NaCl for most of the experiments designed to examine salt tolerance. The growth of the mutants on the plate containing 1.2 M NaCl or 140 mM LiCl was restored by the addition of 200 mM KCl (Figure 2B). KCl also improved the growth of wild type cells on NaCl and LiCl plates.

Slow growth of PP2B mutants under alkaline pH conditions. The growth rate of PP2B mutants was measured in liquid medium adjusted to various pHs (between 3 and 9) and was compared with that of wild type cells (Figure 3). In the media adjusted to 'low to neutral' pHs (3.0−6.5), the growth curves of the mutants were very similar to that of wild type strain (the growth curve obtained at pH 6.5 is shown in Figure 3). However, the growth rate of the *cmp1 cmp2* strain was severely inhibited at pHs >8.0. The growth of the mutants containing a null mutation in either of the *CMP* genes was apparently normal. The *cnb1* mutant exhibited growth behaviour at various pHs similar to that of the

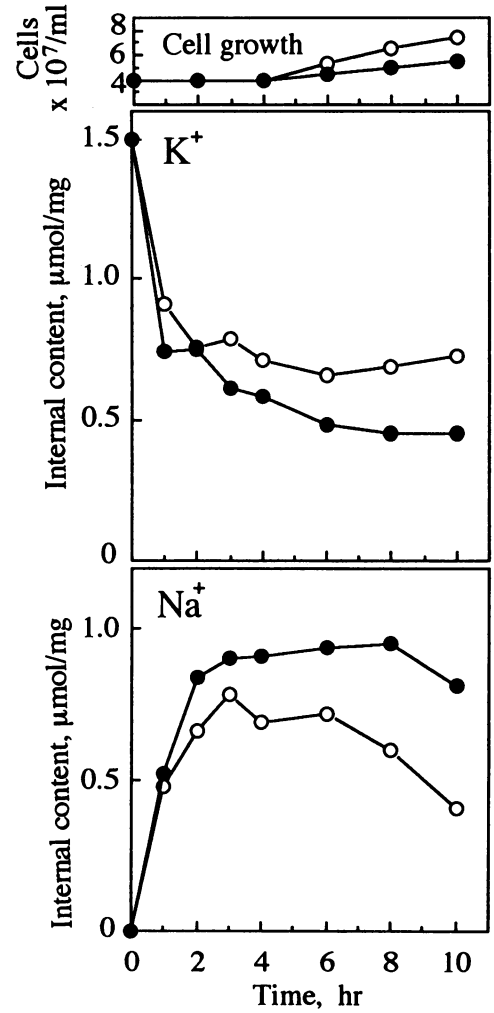

Fig. 4. Changes in the intracellular Na^+ and K^+ of WHU3B (*CMP1 CMP2*) (○) and DHT23-1b (*cmp1::URA3 cmp2::HIS3*) (●) cells upon shift to YPD medium containing 0.85 M NaCl. The samples were prepared and Na^+ and K^+ concentrations were determined as described in Materials and methods. The result is expressed as the amount (μmol) of the ions per mg of total cellular protein. The experiment was done five times and the results were reproducible. The result presented is from a representative single experiment. The change in cell number during the incubation in high NaCl medium is also shown. Symbols are as used in the above experiment.

cmp1 cmp2 mutant. The growth inhibition of the mutants under alkaline pH conditions was not rescued by the addition of 200 mM KCl (data not shown).

Alteration of Na^+ and K^+ transport in PP2B-deficient mutants

To examine the possibility that the decreased tolerance of the PP2B-deficient mutants to Na^+ is due to a defect in the regulation of ion transport through the membrane, the transport activity was measured by a change in intracellular Na^+ concentrations after a shift to medium containing 0.85 M NaCl. At this concentration of NaCl, the mutant is still viable and able to grow at a slower rate than the wild type. Sodium and potassium contents of the cell extract were determined by isotachophoresis, a capillary-type electrophoresis system for ion analysis. The data were expressed as μmol Na^+ or K^+ per mg total cellular protein. The uptake profiles of Na^+ by wild type and the *cmp1 cmp2* double mutant cells are shown in Figure 4. Upon

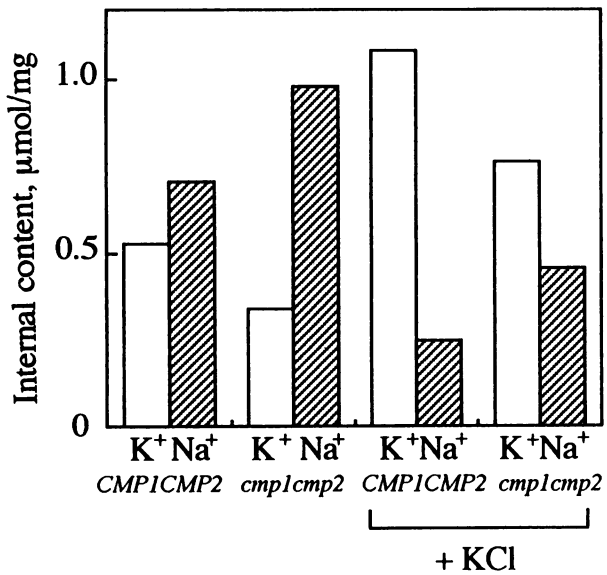

Fig. 5. Effect of KCl on intracellular Na^+ and K^+ levels. The concentrations of the cell extract prepared from WHU3B (*CMP1 CMP2*) and DHT23-1b (*cmp1::URA3 cmp2::HIS3*) cells grown for 6 h in 0.85 M NaCl in the presence or absence of 200 mM NaCl. The experiment was done three times and similar results were obtained. The result presented is from a single experiment.

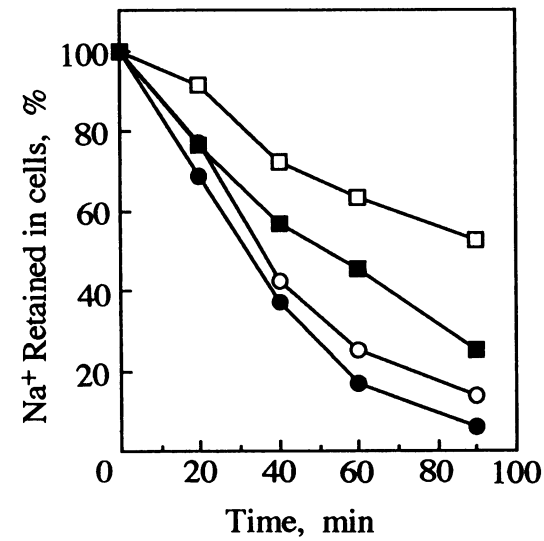

Fig. 6. Sodium efflux from WHU3B (*CMP1 CMP2*) and DHT23-1b (*cmp1::URA3 cmp2::HIS3* cells. Wild type (○,●) and the mutant (□,■) cells were preloaded with Na^+ in high NaCl medium and cellular Na^+ after shift to YPD medium containing LiCl instead of NaCl in the presence (●,■) or absence (○,□) of 200 mM KCl were determined as described in Materials and methods. The experiment was done three times and the results were reproducible. The result presented is from a single representative experiment.

shift to high NaCl medium, intracellular Na^+ in the wild type strain initially increased at a rate similar to that of the mutant, then the rate of increase slowed down and finally the Na^+ level started to decrease after 3 h following the shift. The number of wild type cells remained unchanged for the first 4 h and then started to increase when internal Na^+ began to decrease, suggesting that the high internal Na^+ is toxic to the growth (Figure 4). The decrease of the intracellular Na^+ concentration in the mutant was much slower than that in the wild type cell. As a result, the level of intracellular Na^+ in the mutant in high NaCl medium was significantly higher than that in wild type cells and the growth rate of the mutant was lower. Upon shift to high NaCl medium, nearly half of initial internal K^+ of wild type cells was lost within the first hour. After this decrease, the K^+ level stayed at a steady level (Figure 4). The K^+ content of the mutant initially decreased similarly to the wild type cell and it further decreased at a slower rate. As a result, the internal K^+ of the mutant after 10 h following the shift was 60% of that in wild type cells. The intracellular levels of Na^+ and K^+ in *cnb1* mutants were very similar to those of the *cmp1 cmp2* mutant (data not shown). The effect of KCl on intracellular Na^+ and K^+ of the cells growing for 6 h in high NaCl medium was examined (Figure 5). By the presence of exogenously added KCl, the internal level of Na^+ in DHT23-1b cells was lowered to 46% of the control, whereas the K^+ level was elevated to 2.3-fold of the control. A similar effect with KCl was observed in the intracellular ion concentrations of wild type cells. These results are consistent with the improved growth of the mutant and wild type cells in high NaCl medium in the presence of KCl (Figure 2).

The higher Na^+ level of the mutant could be explained by the lack of either or both of the inhibition of Na^+ import and/or the stimulation of ion export. We examined whether export activity is altered in the mutant. The export rate was followed by measuring decrease of preloaded Na^+. Wild type and mutant cells were preloaded with Na^+ by cultivation for 4 h in YPD medium containing 0.85 M NaCl. The cells were harvested by centrifugation, suspended in the medium containing 1.7 M sorbitol and 70 mM LiCl instead of 0.85 M NaCl, and further cultivated with shaking. Intracellular Na^+ was measured at 1 h intervals. The decrease in the rate of intracellular Na^+ export in wild type cells was faster than that of the mutant cell, suggesting that the mutant has a defect in the Na^+ export capacity (Figure 6). In the presence of KCl (200 mM) in the medium, Na^+ export was stimulated both in the mutant and the wild type cells (Figure 6). The presence of LiCl was essential for efficient efflux of Na^+ from the preloaded cells.

Effect of FK506 on salt tolerance and intracellular Na^+ and K^+ concentrations

We next examined if the PP2B-mediated salt tolerance of wild type cells is inhibited by the immunosuppressant FK506. The growth of wild type, *cmp1 cmp2* and *fkb1* strains was compared on YPD plates containing LiCl, FK506 and LiCl and FK506 (Figure 7A). FK506 (1 μg/ml) alone had no effect on the growth of these strains. In the presence of FK506, the wild type strain was unable to grow on the plate containing 160 mM LiCl. In contrast, the *fkb1* mutant DHF1, lacking the FK506 binding protein yFKBP-12, could grow on the same medium. These results indicate that the adaptation of wild type cells to high salt medium is sensitive to the immunosuppressant and yFKBP-12 is the receptor mediating the effect of FK506. Next, the concentration dependency of salt tolerance on FK506 was examined. The growth of wild type and PP2B-deficient mutant cells in liquid medium containing 50 mM LiCl and various concentrations of FK506 was monitored by the absorbance of the medium after 48 h of cultivation (Figure 7B). To minimize the influence of auxotrophic requirements on the FK506 sensitivity (Heitman *et al.*, 1991), a Ura^+ His^+ transformant

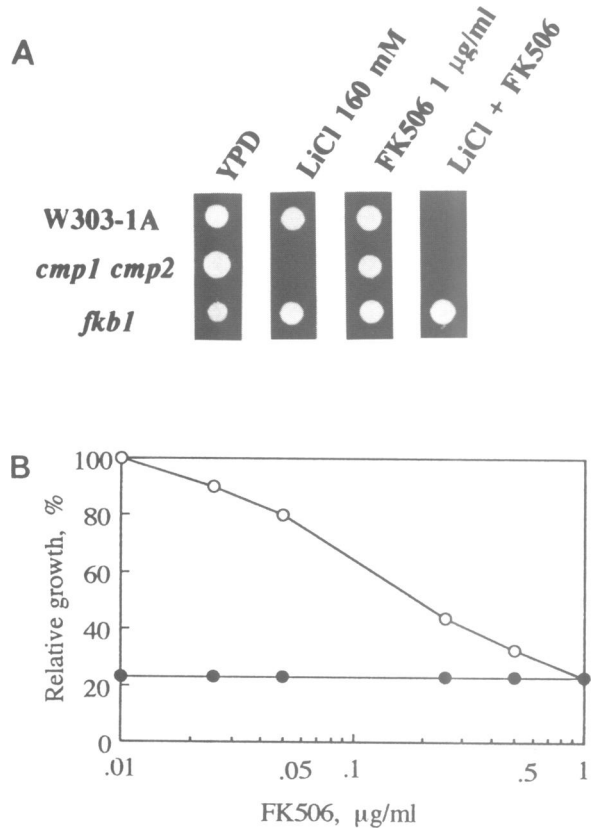

Fig. 7. The effect of FK506 on salt tolerance of various strains. (**A**) The growth of isogenic strains of DHT22-1b (wild type), DHT22-1a (*cmp1::LEU2 cmp2::HIS3*) and DHF1 (*fkb1::HIS3*) was compared on YPD plates containing LiCl (160 mM), FK506 (1 μg/ml) and LiCl (160 mm) + FK506 (1 μg/ml). (**B**) The growth of WHU3B (Ura^+His^+ transformant of DHT22-1b) (○) and DHT23-1b (*cmp1::URA3 cmp2::HIS3*) strain (●) in YPD containing 50 mM LiCl and various concentrations of FK506 was monitored by the absorbance at 660 nm after 48 h of incubation.

(WHU3B) of DHT22-1b was used as a control. In the presence of 50 mM LiCl, the growth of WHU3B and DHT23-1b (*cmp1 cmp2*) was inhibited by 40 and 85%, respectively. As a result, the growth of the mutant in LiCl-containing medium was inhibited by 77% when compared with the wild type strain. The growth of WHU3B cells in LiCl medium was inhibited by FK506 in a dose-dependent manner. The sensitivity of the cell to LiCl in the presence of 1 μg/ml of FK506 was very similar to that of the PP2B null mutant (Figure 7B). FK506 had no effect on Li^+ sensitivity of the PP2B-deficient mutant DHT23-1b. The salt tolerance of the mutant DHF1 lacking yFKBP-12 was not affected by FK506 under these conditions (data not shown). Fifty percent inhibition of salt tolerance of wild type cells was achieved by 0.1 μg/ml FK506 (Figure 7B). The concentrations of FK506 required for the inhibition of salt tolerance were much lower than those (~10 μg/ml) for growth inhibition (Brizuela *et al.*, 1991) and were similar to those responsible for the inhibition of growth recovery from G_1 arrest induced by α-factor (Foor *et al.*, 1992). Similar effects of FK506 were obtained in the medium containing NaCl instead of LiCl and also in high pH medium (data not shown).

We next examined the effect of FK506 on intracellular Na^+ and K^+ levels in the cells growing in high NaCl medium. In the presence of FK506 in high salt medium, the internal level of Na^+ in the wild type cell was elevated to a level similar to that of the PP2B-deficient mutant in high salt medium and the maintenance of the K^+ level was also inhibited by the immunosuppressant (Figure 8A). We next examined the effect of FK506 on internal ion concentrations of the yFKBP-12-deficient mutant DHF1 (Figure 8B). Unexpectedly, the intracellular K^+ levels in DHF1 were lower than those of wild type strain. The altered intracellular ion concentrations may explain the lower growth rate of the *fkb1* mutant in YPD medium. Although the profile of the changes in the internal Na^+ and K^+ levels in the *fkb1* mutant were different from those in wild type cells, the intracellular ion levels of the mutant were not affected by FK506, suggesting that FK506 inhibits PP2B through Fkb1 protein. The stimulation of Na^+ export of wild type cells was not obtained in the presence of 1 μg/ml of FK506 (data not shown).

Discussion

We have shown here that PP2B of *S.cerevisiae* is an important determinant for adaptation to certain stress circumstances that affect internal ion conditions. The mutants having null alleles of the two genes encoding the catalytic subunit (*cmp1 cmp2*) or the regulatory subunit (*cnb1*) of PP2B were unable to grow in the medium containing 1.2 M NaCl or 140 mM LiCl. The growth of the mutants was specifically inhibited by certain ions, but not by nonspecific osmotic stresses.

An important feature of the PP2B-mediated salt response in yeast is its high sensitivity to the immunosuppressant FK506. In the presence of FK506, the growth properties and intracellular Na^+ and K^+ levels in wild type cells in high NaCl medium became very similar to those of the PP2B-deficient mutants in high salt medium. In T lymphocytes, biochemical and genetic evidence has shown that FK506 binds to cytosolic proteins termed FKBP and inhibits protein phosphatase activity of PP2B. PP2B, which catalyses a critical dephosphorylation event leading to the transcriptional activation, is recognized as a key signalling enzyme in T lymphocyte activation (Liu *et al.*, 1991a; Clipstone and Crabtree, 1992; O'Keefe *et al.*, 1992). The inhibition of the adaptation of wild type yeast to salt stress in the presence of FK506 strictly depends on the presence of FK506 binding protein (yFKBP-12) encoded by *FKB1* (Heitman *et al.*, 1991; Wiederrecht *et al.*, 1991). It was recently reported that the immunosuppressant inhibits the growth recovery of *S.cerevisiae* from G_1 arrest induced by α-factor (Foor *et al.*, 1992). They further demonstrated that yFKBP-12 bound to FK506 forms a complex with PP2B (Foor *et al.*, 1992). Therefore, the regulatory elements that inhibit the signal transduction pathway of salt response appear to be strikingly similar to those for the inhibition of T cell activation (Liu *et al.*, 1991a) and the regulatory mechanism is conserved throughout the eukaryotes (Luan *et al.*, 1993). An important question that has to be clarified about the yeast signalling pathway of salt tolerance is whether the PP2B-mediated salt response is achieved at the transcriptional level as in T cell activation. If this is the case, the yeast system would become a good model for the study of PP2B-mediated signalling pathway and its regulation by the immunosuppressant–immunophilin complex. The phenotypes of the PP2B mutants characterized in the present study will

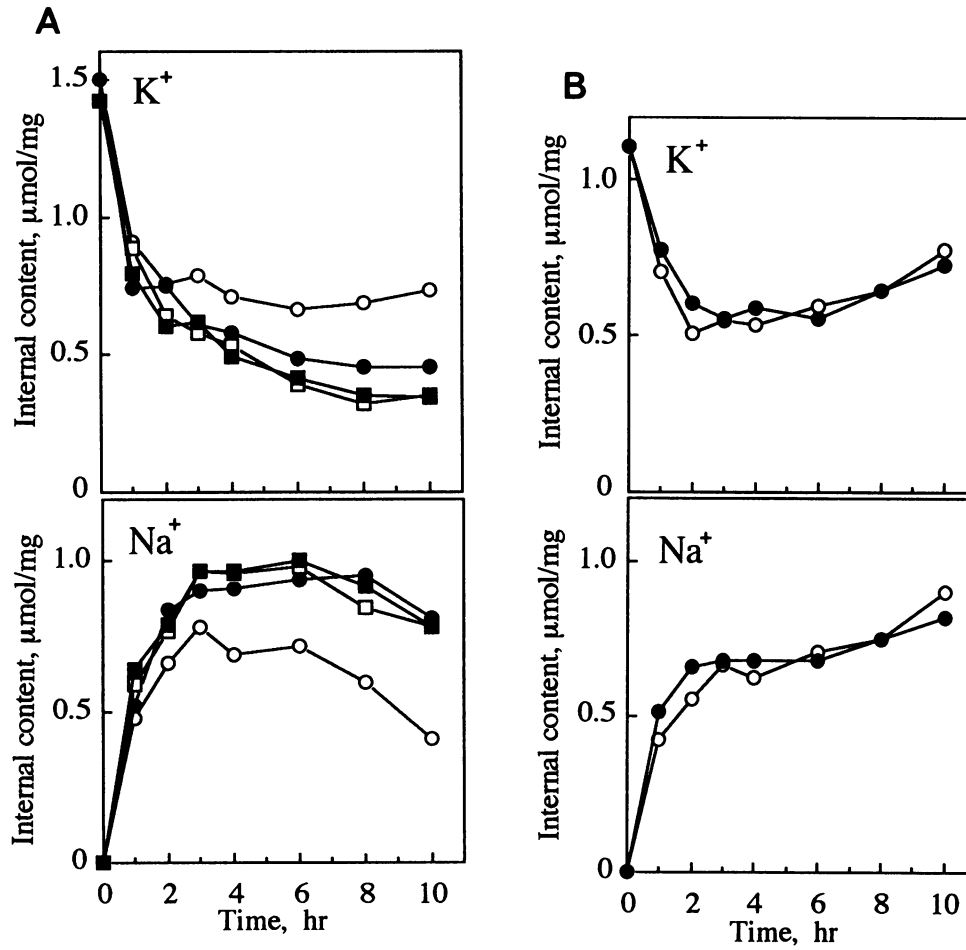

Fig. 8. The effect of FK506 on intracellular Na^+ of various strains. (**A**) Internal Na^+ and K^+ concentrations of WHU3B (wild type) (○,●) and DHT23-1b (*cmp1::URA3 cmp2::HIS3* (□,■) cells after shift from YPD medium to the medium containing 0.85 M NaCl with (●,■) or without (○,□) concentrations 1 μg/ml of FK506 were determined. (**B**) Internal Na^+ and K^+ concentrations of yFKBP-12-deficient mutant DHF1 (*fkb1::HIS3*) cells in YPD medium containing 0.85 M NaCl in the presence (●) or absence (○) of 1 μg/ml FK506 were determined as described above. Both experiments were done three times and the results were reproducible. The results presented are from a representative single experiment.

offer a direct approach towards understanding the functional mechanism of the signalling system.

We studied the kinetics of Na^+ and K^+ transport in PP2B-deficient mutant and wild type strains. Upon shift to high salt medium, intracellular Na^+ levels of both wild type and the mutant cells increased initially at a similar rate and the cells stopped growing. However, the elevation of the intracellular Na^+ concentration in wild type cells growing in high NaCl medium began to be suppressed after 3 h and then it started to decrease after 6 h of shift. The decrease in concentration of intracellular Na^+ was faster in wild type cells compared with the mutant cells, indicating that the phosphatase is important in maintaining a gradient across the membrane in high NaCl medium. The cells resumed growth after 4 h when the intracellular Na^+ level started to decrease, indicating that the high intracellular Na^+ level is toxic to growth and the PP2B-mediated regulation of intracellular Na^+ concentration is critical for adaptation to the medium containing high NaCl. Further study suggested that PP2B is required for the enhancement of a mechanism responsible for lowering the level of intracellular Na^+. It is not clear, however, whether this mechanism involves the regulation of translational events or post-translational modification of the proteins of the machinery responsible for the regulation of intracellular Na^+ levels.

In mammalian cells, transport of Na^+ and K^+ ions is mainly achieved by the ouabain-sensitive Na^+, K^+-ATPase which utilizes energy of ATP to import K^+ and export Na^+ through the plasma membrane. Phosphorylation and dephosphorylation play a role in the regulation of the activity of Na^+, K^+-ATPase (Bertorello *et al.*, 1991). Recently, it was demonstrated that the regulation of α-adrenergic stimulation of Na^+, K^+-ATPase of renal tubule cells is mediated by PP2B, indicating the involvement of the phosphatase in the regulation of Na^+ excretion (Aperia *et al.*, 1992). In yeasts, however, an ouabain-sensitive Na^+, K^+-ATPase which is similar to that of mammalian cells has not yet been identified. A PP2B-regulated mechanism which is distinct from that of the mammalian ion transporting system may operate in ion homeostasis in yeast. Since the regulatory mechanism for intracellular Na^+ in yeast is not clear yet and the molecular basis for Na^+ transport is only beginning to be understood, the mechanism by which PP2B may achieve ion homeostasis is only speculative at present, but several models are plausible. Haro *et al.* (1991) have demonstrated that the P-type ATPase encoded by *PMR2/ENA1* of *S.cerevisiae* is important in Na^+ export in high NaCl medium. The PP2B-mediated salt tolerance in yeast could be achieved by the enhancement of Na^+ export by the activation of the *PMR2/ENA1* ATPase

through the action of the phosphatase (directly or indirectly) in response to salt stress. An alternative model in which a Na^+/H^+ antiporter is involved for Na^+ export may also be possible. In *S.cerevisiae*, an Na^+/H^+ antiporter of the plasma membrane has been suggested to be responsible for Na^+ export (Rodriguez-Navaro and Ortega, 1982). In fission yeast *Schizosaccharomyces pombe*, overexpression of the *sod2* gene encoding a putative Na^+/H^+ antiporter increased Na^+ export capacity and conferred Na^+ and Li^+ tolerance, suggesting that the antiport is rate-limiting in salt tolerance (Jia *et al.*, 1992). However, the antiporter of *S.cerevisiae* has not yet been characterized at the molecular level. If the Na^+/H^+ antiporter is involved in the export of Na^+ under as high salt conditions in *S.cerevisiae* as in *S.pombe*, H^+ extrusion by plasma membrane H^+-ATPase which acts in concert with the Na^+/H^+ antiporter is required for efficient transport of Na^+. Yeast plasma membrane contains an ATPase activity responsible for transmembrane H^+ transport (for reviews see Goffeau and Slayman, 1981; Serrano, 1991). In *S.cerevisiae*, two genes encoding putative P-type H^+-ATPase designated *PMA1* (Serrano *et al.*, 1986) and *PMA2* (Schlesser *et al.*, 1988), which is highly homologous with *PMA1*, have been identified. Another P-type ATPase that could act in this process is *PMR2/ENA1* ATPase (Rudolph *et al.*, 1989). Although this ATPase has been demonstrated to be involved in Na^+ transport at neutral to high pHs, the ion species transported by this ATPase has not been defined yet. If *PMR2/ENA1* ATPase is a proton-transporting ATPase, Na^+ can be exported by the Na^+/H^+ antiporter which operates in concert with H^+ exported by the *PMR2/ENA1* ATPase at neutral to high pHs. Whether or not the antiporter is involved in the mechanism of salt tolerance, *PMR2/ENA1* ATPase seems to play a major role in Na^+ export and PP2B may be involved in the enhancement of the Na^+ export mechanism through the regulation of *PMR2/ENA1* ATPase. Correlation of PP2B and *PMR2/ENA1* ATPase in salt tolerance is suggested by the observation that the null mutant of the ATPase (*ena1 ena2* double mutant) exhibited phenotypes very similar to those of the PP2B-deficient mutants, such as being defective in Na^+ export, and hypersensitive to high NaCl and high pH conditions (Haro *et al.*, 1991). Furthermore, the sensitivity of PP2B-deficient mutants and *ena1 ena2* double null mutants to high pHs was further increased by the presence of KCl (Haro *et al.*, 1991; H.Namba, unpublished data). In addition to the similarity of the phenotypes of the ATPase-deficient and PP2B-deficient mutants, our observation that overexpression of *PMR2/ENA1* can complement NaCl and LiCl sensitivity of the PP2B mutant is consistent with this hypothesis (H.Namba, unpublished data). Further analyses of the phenotypes of the mutants deficient in PP2B and *PMR2/ENA1* will clarify whether *PMR2/ENA1* ATPase is regulated through the action of PP2B. The increase in internal Na^+ levels in PP2B-deficient mutants in high NaCl medium ceased 3 h after shifting, reaching a plateau that continued for ~4 h and then the Na^+ level gradually decreased. Therefore, a mechanism that acts to lower intracellular Na^+ in a PP2B-independent manner also seems to operate for Na^+ homeostasis in yeast. In fact, PP2B-deficient mutant growing in high NaCl medium had a significant activity in exporting preloaded Na^+.

The change of intracellular K^+ level following the shift to high salt medium may also provide insights into the mechanism of salt tolerance. Upon shift to high NaCl medium, intracellular K^+ level of both wild type and PP2B-deficient mutant cells decreased very rapidly. The decrease of K^+ level in wild type cells was stopped in 1–2 h and a steady level was maintained during growth in high salt medium. In contrast, the concentration of intracellular K^+ in the mutant further continued to decrease at a lower rate, being ~60% of the wild type level after 10 h following the shift. Yeast cells exchange K^+ for cellular H^+, accumulating K^+ against a steep gradient. Although the mechanism for this exchange is not clearly established, an indirect electric coupling has been suggested (for a review see Serrano, 1991). Externally added KCl restored the growth of PP2B-deficient mutant in high NaCl or LiCl medium. By adding KCl, intracellular K^+ was elevated, Na^+ export was stimulated and, as a result, the intracellular Na^+/K^+ ratio became similar to that of wild type cells. These observations raise the possibility that PP2B regulates (directly or indirectly) the K^+ transport system and the PP2B-deficient mutant may have a defect in the K^+ import system. The internal Na^+/K^+ ratio has been suggested to be important for the growth of yeast and Na^+ becomes toxic to growth when the ratio is high (Camacho *et al.*, 1981). The intracellular Na^+/K^+ ratio and growth properties of wild type and mutant cells under salt stress conditions was consistent with this hypothesis. In YPD medium, both the wild type and mutant cells had a similar Na^+/K^+ ratio of >1:20. The K^+ level of wild type cells in high salt medium was maintained below 1.1. In contrast, cellular Na^+ of the mutant exceeded K^+ after 2 h following the shift to high salt medium and the Na^+/K^+ ratio increased thereafter. The Na^+/K^+ ratios of wild type and mutant cells after 10 h of shift were 0.6 and 1.8, respectively. A high level of K^+ accumulation occurs in *S.cerevisiae* overexpressing *HAL1*, a rate-limiting, functionally unknown determinant of salt tolerance (Gaxiola *et al.*, 1992). The salt-inducible Hal1 protein is suggested to be involved in the K^+ transport system that determines intracellular K^+ homeostasis and a correlation of K^+ accumulation and the *HAL1*-mediated salt tolerance was suggested (Gaxiola *et al.*, 1992). A possibility exists that the adaptation to high NaCl medium is achieved by the enhancement of the expression of *HAL1* in a PP2B-dependent manner.

Of the null mutants of the catalytic subunits of PP2B, the *cmp1 cmp2* double mutant was sensitive to high pH, while the *cmp1* or *cmp2* single mutant apparently grew normally under these conditions. This indicates that the *CMP1* and *CMP2* products share physiological functions and either one of these two gene products is sufficient to enable growth under these stress conditions. In contrast, the mutation in *CMP2*, but not in *CMP1*, caused poor growth in high salt medium, suggesting that *CMP2* has a greater contribution to the growth in high salt medium than *CMP1*. Similarly, it has been reported that *CMP2* is more important than *CMP1* in the recovery process from the growth arrest by α-factor (Foor *et al.*, 1992). The differences in the phenotypes of *CMP1* and *CMP2* mutants could result either from the functional difference of two PP2B isozymes containing the *CMP1* or *CMP2* products, or the difference in the amount of two PP2B isozymes. The significant difference (64% identity) in the deduced amino acid sequences of the *CMP1*

and *CMP2* products may suggest the functional differentiation of the PP2B isozymes (Liu *et al.*, 1991b). The growth behaviour of the *cnb1* mutant under various growth conditions was very similar to that of the *cmp1 cmp2* double mutant, indicating that the regulatory subunit is essential for the function of PP2B. A similar result has been demonstrated for recovery from α-factor arrest (Cyert and Thorner, 1992).

Materials and methods

Materials

Restriction endonucleases and DNA modification enzymes were purchased from Nippon Gene and Takara Shuzo Co. DNA labelling and nucleic acid detection kits were purchased from Boehringer Mannheim. Plasmid pGEMEX-1 was purchased from Promega Corp. FK506 was a gift from Fujisawa Pharmaceutical Co.

Strains, media and growth conditions

S.cerevisiae strains used are listed in Table I. The composition of yeast media YPD and SC was described previously (Liu *et al.*, 1991b).

PCR

The following degenerated oligonucleotide primers were used for PCR. Primer a, 5′-CTC GAG GGI GA(C/T) TA(C/T) GTI GA(C/T) (A/C)GI GG-3′ (sense strand); primer b, 5′-GGA TCC (C/T)TC (C/T)TC IC(G/T) IA(A/G) IAC I(C/G)(A/T) (A/G)(A/T)A-3′ (antisense strand); and primer c, 5′-GGA TCC CCA IGT (A/G)AA IAC (A/G)TC CAT (A/G)AA-3′ (antisense strand). The sense primer contains the restriction site for *Xho*I, followed by the sequences found to be conserved in all types of serine/threonine protein phosphatases. The antisense primers contain the restriction site for *Bam*HI, followed by the sequences specifically conserved in PP2B from various sources. These primers were synthesized by an autosynthesizer and purified by electrophoresis. DNA amplification was carried out on a Perkin-Elmer thermal cycler in a 100 μl mixture containing 10 mM Tris−HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μl dNTP, 0.001% gelatin, 1 μmol each of sense and antisense primers, 2.5 units of AmpliTaq DNA polymerase and 1 μg of genome DNA of *S.cerevisiae* strain YLL34-5d (Liu *et al.*, 1991b) as a template. Forty cycles of amplification were performed (94°C for 2 min, 42°C for 2 min and 55°C for 2 min). The PCR products were separated in a 1% agarose gel and the DNA bands were isolated. The purified products were labelled and used to screen the YCp50-based yeast genomic library.

Disruption of CMP1 and CMP2

The disruption plasmids with null alleles of *cmp1::LEU2* and *cmp2::URA3* described in our previous paper were introduced into isogenic diploid W303 and gene disruption mutants were obtained (Liu *et al.*, 1991b). Similarly, the disruption plasmid with null allele of *cmp2::HIS3* was constructed by replacing the *Xba*I−*Kpn*I fragment within the open reading frame of *CMP2* with *HIS3*. The gene disruption mutant was obtained as described above.

Disruption of CNB1

A cDNA copy originally designated YCNB encoding the B subunit of PP2B was renamed as *CNB1*, as this gene turned out to be identical to that reported by Cyert and Thorner (1992). The *Eco*RI−*Xho*I cDNA fragment of *CNB1* was subcloned into plasmid pGEMEX. To create an insertion mutant allele of *CNB1*, a blunt-ended 1.7 kb DNA fragment of *HIS3* was then inserted into the *Pvu*II site which is localized in the open reading frame of *CNB1*. The resulting plasmid was isolated by the alkali−SDS lysis method and digested with restriction endonucleases *Eco*RI and *Apa*I. The reaction mixture was used to transform diploid strain W303. His^+ transformants were obtained by selecting for histidine auxotrophy. Restriction enzyme digestion with *Eco*RI and Southern blot analysis of genomic DNA from the resulting His^+ transformants confirmed that gene replacement had occurred at the *CNB1* locus. Northern analysis of total RNA isolated from wild type and *cnb1* mutant progenies indicated that the expression of *CNB1* was completely abolished by the insertion mutation. The nonessentiality of *CNB1* confirms previous results obtained by Cyert and Thorner (1991). DHT14 is one of the resulting transformants.

Disruption of FKB1

The plasmid for *fkb1::HIS3* mutation was constructed by replacing the internal 0.15 kb *Bst*EII−*Hpa*I fragment of *FKB1* with a 1.7 kb *Bam*HI fragment of *HIS3*. For construction of null mutants, the plasmid was introduced into isogenic diploid W303 and His^+ prototrophs were selected. Southern hybridization analysis confirmed that these transformants were heterozygous for the mutated allele. The diploids were induced to sporulate and the resulting tetrads were dissected. His^+ and His^- segregated 2:2 in all tetrads.

Reagent sensitivity assay

The sensitivity of the cells to NaCl, LiCl and other salts was evaluated on freshly prepared solid YPD medium containing various concentrations of the reagents. pH sensitivity was evaluated in liquid YPD medium adjusted to various pHs with 5 N HCl or 5 N KOH.

Measurement of intracellular Na^+ and K^+ concentrations

To an exponentially growing cell culture (~4×10^7 cells/ml) in 100 ml YPD, NaCl was added to a concentration of 0.85 M. Five millilitre samples were taken at various times after shifting to high NaCl medium and the cells were harvested by centrifugation at 4°C. The cells were washed three times with ice-cold water containing 1.5 M sorbitol and 20 mM $MgCl_2$, rinsed with cold deionized water and then extracted with 400 μl of deionized water by incubation at 95°C for 30 min (Gaxiola *et al.*, 1992). Na^+ and K^+ concentrations in aliquots of the clarified extract were determined by isotachophoresis (Shimadzu, model R112).

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Table I. *Saccharomyces cerevisiae* strains

Strain	Genotype	Source
DHT22-1b	*MATa trp1 leu2 ade2 ura3 his3 can1-100*	W303-1A from R.Rothstein
WHU3B	*MATa trp1 leu2 ade2 can1-100* (Ura^+His^+ transformant of DHT22-1b)	This study
DHT22-1c	*MATa trp1 ade2 ura3 his3 can1-100 cmp1::LEU2*	This study
DHT22-1d	*MATa trp1 leu2 ade2 ura3 can1-100 cmp2::HIS3*	This study
DHT22-1a	*MATa trp1 ade2 ura3 can1-100 cmp1::LEU2 cmp2::HIS3*	This study
DHT23-1b	*MATa trp1 leu2 ade2 can1-100 cmp1::URA3 cmp2::HIS3*	This study
DHT14	*MATa trp1 leu2 ade2 ura3 can1-100 cnb1::HIS3*	This study
DHF1	*MATa trp1 leu2 ade2 ura3 can1-100 fkb1::HIS3*	This study

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