A novel role for the yeast protein kinase Dbf2p in vacuolar H⁺-ATPase function and sorbic acid stress tolerance

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In *Saccharomyces cerevisiae*, the serine-threonine protein kinase activity of Dbf2p is required for tolerance to the weak organic acid sorbic acid. Here we show that Dbf2p is required for normal phosphorylation of the vacuolar H⁺-ATPase (V-ATPase) A and B subunits Vma1p and Vma2p. Loss of V-ATPase activity due to bafilomycin treatment or deletion of either *VMA1* or *VMA2* resulted in sorbic acid hypersensitivity and impaired vacuolar acidification, phenotypes also observed in both a kinase-inactive *dbf2* mutant and cells completely lacking *DBF2* (*dbf2Δ*). Crucially, *VMA2* is a multicopy suppressor of both the sorbic acid-sensitive phenotype and the impaired vacuolar-acidification defect of *dbf2Δ* cells, confirming a functional interaction between Dbf2p and Vma2p. The yeast V-ATPase is therefore involved in mediating sorbic acid stress tolerance, and we have shown a novel and unexpected role for the cell cycle-regulated protein kinase Dbf2p in promoting V-ATPase function.

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INTRODUCTION

Saccharomyces cerevisiae has evolved dedicated and sophisticated mechanisms that allow adaptation to different organic acids. Examples include the novel role of the yeast mitogen-activated protein kinase Hog1p in adaptation to citric acid stress (Lawrence *et al.*, 2004), the requirement for the membrane-bound transporter Azr1p and Aqr1p for optimal adaptation to short-chain monocarboxylic acids (Tenreiro *et al.*, 2000, 2002), and the key role played by the vacuolar H⁺-ATPase (V-ATPase) in resistance to the organic acid herbicide 2,4-dichlorophenoxyacetic acid (2,4-D; Fernandes *et al.*, 2003). The best-characterized response to acid stress in yeast is that induced by sorbic acid, a weak-acid food preservative. Optimal adaptation to sorbic acid stress requires induction of the ATP-binding cassette (ABC) efflux pump Pdr12p (Piper *et al.*, 1998), a rapid, weak acid-specific response that is essential for counter-acting intracellular anion accumulation. War1p, a novel transcription factor of the Zn₂Cys₆ family, is the stress-activated regulator of *PDR12* (Kren *et al.*, 2003). War1p

binds constitutively to the *PDR12* promoter irrespective of sorbic acid stress, which is believed to activate War1p through phosphorylation (Kren *et al.*, 2003; Schuller *et al.*, 2004). Notably, the signalling pathway that mediates War1p phosphorylation has not yet been identified.

One of the principal objectives of this study was to identify key regulatory proteins involved in the perception and signalling of weak-acid stress. Screening of the yeast haploid deletion library revealed that cells lacking the well-studied serine-threonine protein kinase Dbf2p, best known for its role in cell cycle regulation (see Yeong *et al.*, 2002), are hypersensitive to sorbic acid. However, the presence or absence of Dbf2p did not affect expression of Pdr12p, suggesting that the kinase mediates sorbic acid tolerance via a novel mechanism.

Here we show that Dbf2p is required for phosphorylation of both the V-ATPase catalytic subunit Vma1p and the non-catalytic subunit Vma2p. V-ATPases are large, complex enzymes responsible for the acidification of many internal compartments in eukaryotic cells (reviewed by Arata *et al.*, 2002; Nishi & Forgac, 2002). In yeast, the V-ATPase acidifies the vacuole by driving the translocation of protons into the lumen. This acidification is crucial for many cellular processes, including endocytosis, targeting of newly synthesized lysosomal enzymes, cytoplasmic pH homeostasis, protein processing and the coupled transport

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Abbreviations: ABC, ATP-binding cassette; CIP, calf intestinal phosphatase; 2,4-D, 2,4-dichlorophenoxyacetic acid; V-ATPase, vacuolar H⁺-ATPase.

of small molecules. Both Vma1p and Vma2p, and thus a functional V-ATPase, are required for optimal adaptation to sorbic acid. Measurement of V-ATPase-mediated vacuolar acidification revealed that like *vma1Δ* and *vma2Δ* mutants, *dbf2Δ* and kinase-inactive *dbf2* strains were also impaired in vacuolar acidification. Overexpression of *VMA2* rescued both the sorbic acid sensitivity and the impaired vacuolar acidification of *dbf2Δ* cells, implying a functional interaction between the two proteins. Our findings therefore provide strong evidence for a previously unknown role for Dbf2p in maintaining optimal V-ATPase function.

METHODS

Yeast strains and growth conditions. Basic yeast methods and growth media were as described elsewhere (Kaiser *et al.*, 1994). Yeast strains were grown at 30 °C in standard rich medium (YPD) at pH 4.5. All yeast strains are listed in Table 1, and those constructed in this study were prepared in the BY4741a background (Research Genetics). Yeast strains containing tandem affinity purification (TAP) tags were from Open Biosystems, and the *myc₁₃-DBF2* strain was prepared by PCR-mediated genomic manipulation (Longtine *et al.*, 1998). *VMA2* and *VPH1* were chromosomally tagged at the 3' end with GFP-*HIS1* cassettes using the method of Sheff & Thorn (2004) and verified by diagnostic PCR.

The Research Genetics yeast haploid deletion library was screened for sorbic acid-sensitive genes as previously described (Lawrence *et al.*, 2004), using YPD (pH 4.5) supplemented with 1.5 mM sorbic acid. In liquid culture, growth was monitored in a 24-well microplate (Greiner Bio-One) by measuring OD₆₀₀ using a PowerWave XS Universal Microplate Spectrophotometer (BioTek Instruments), taking automated readings every 30 min. All treatments were performed in triplicate. Phenotypic testing was performed by diluting overnight cultures of yeast strains to OD₆₀₀ 0.1 and making 10-fold serial dilutions, then spotting 5 μl samples onto appropriate agar plates.

Site-directed mutagenesis and plasmid constructs. Routine recombinant DNA methodology was performed as described elsewhere (Sambrook & Russell, 2001). The pRS vector series was used for gene complementation and overexpression experiments (Sikorski & Hieter, 1989). Details of constructed vector sequences and oligonucleotides used are available on request. The Stratagene QuikChange method was applied to mutate residue 305 of Dbf2p, carried by pRS313-*DBF2*, from asparagine (AAC) to alanine (GCT). This created pRS313-*DBF2*(N305A), which encoded a kinase-inactive version of Dbf2p, as described elsewhere (Mah *et al.*, 2001).

Phosphoproteome analysis and MS. Phosphoproteome analysis was carried out as previously described (Makrantonis *et al.*, 2005). Briefly, affinity capture of phosphoproteins was performed using the PhosphoProtein Purification Kit from Qiagen. Approximately 25 mg total yeast protein was loaded onto the proprietary affinity columns, which were washed before the elution of bound phosphoproteins. Fractions containing the enriched phosphoproteins were compared by 2D-PAGE as previously described, and proteins were visualized with SYPRO Ruby protein stain and identified by peptide mass fingerprinting (Lawrence *et al.*, 2004). Only protein spots showing reproducible changes in protein abundance were catalogued. Peptide mass fingerprint data were obtained as described previously, using the Mascot search engine to search for proteins with calculated tryptic peptide masses that matched the measured experimentally derived masses with a mass accuracy of ±0.15 Da (Lawrence *et al.*, 2004).

Western blotting of Pdr12p, Vma1p, Vma2p and Dbf2p. Proteins were transferred from Criterion gels (Bio-Rad) to nitrocellulose membranes (GE Healthcare) by semi-dry blotting. Membranes were subsequently probed according to standard procedures with mAbs to Vma1p (anti-H⁺-ATPase 69 kDa subunit 8B1) and Vma2p (anti-H⁺-ATPase 60 kDa subunit 13D11) obtained from Invitrogen, and a polyclonal antibody to Pdr12p (kind gift of Karl Kuchler, University of Vienna). Comparative 2D-immunoblotting was performed essentially as previously described (Lawrence *et al.*, 2004). Immobilized pH gradient 11 cm strips, pH 4–7, were used with a Multiphor II apparatus for IEF (GE Healthcare), loading 40 μg total cell lysate. For the second dimension, 12.5 % Tris/HCl, 1.00 mm precast 11 %

Table 1. Yeast strains used in this study

Strain	Genotype	Source
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Research Genetics
BY-Deletions	<i>geneΔ:: KanMX4</i> deletions of 4847 genes	Research Genetics
Dbf2-TAP	BY4741a <i>DBF2-TAP</i>	Open Biosystems
PDY120	BY4741a <i>vma1Δ:: KanMX4</i> [pRS313:: <i>VMA1</i>]	This study
PDY121	BY4741a <i>vma1Δ:: KanMX4</i> [pRS423:: <i>VMA1</i>]	This study
PDY122	BY4741a <i>dbf2Δ:: KanMX4</i> [pRS423:: <i>VMA1</i>]	This study
PDY130	BY4741a <i>dbf2Δ:: KanMX4</i> [pRS313- <i>DBF2</i> (N305A)]	This study
PDY201	BY4741a <i>VMA2-GFP:: HIS3</i>	This study
PDY202	BY4741a <i>VPH1-GFP:: HIS3</i>	This study
PDY203	BY4741a <i>dbf2Δ:: KanMX4 VMA2-GFP:: HIS3</i>	This study
PDY204	BY4741a <i>dbf2Δ:: KanMX4 VPH1-GF:: HIS3</i>	This study
Vma2-TAP	BY4741a <i>VMA2-TAP</i>	Open Biosystems
VMY100	BY4741a <i>dbf2Δ:: KanMX4</i> [pRS313:: <i>DBF2</i>]	This study
VMY101	BY4741a <i>vma2Δ:: KanMX4</i> [pRS313:: <i>VMA2</i>]	This study
VMY107	BY4741a <i>dbf2Δ:: KanMX4</i> [pRS423:: <i>DBF2</i>]	This study
VMY108	BY4741a <i>vma2Δ:: KanMX4</i> [pRS423:: <i>VMA2</i>]	This study
VMY109	BY4741a <i>dbf2Δ-Myc13-kanMX6</i>	This study
VMY110	BY4741a <i>vma2Δ:: KanMX4</i> [pRS423:: <i>DBF2</i>]	This study
VMY111	BY4741a <i>dbf2Δ:: KanMX4</i> [pRS423:: <i>VMA2</i>]	This study

polyacrylamide gels (Bio-Rad) were run in a Criterion cell apparatus (Bio-Rad). For some experiments, 40 µg total lysate was treated with 50 U calf intestinal phosphatase (CIF; Sigma-Aldrich) for 1 h at 30 °C prior to IEF.

Vacuolar acidification, vacuolar staining and protein localization. Vacuolar acidification was assessed from the accumulation of fluorescent quinacrine, using published procedures in which cells were harvested in late exponential phase and transferred to uptake buffer containing 200 µM quinacrine (Morano & Klionsky, 1994; Roberts *et al.*, 1991). Vacuolar accumulation of quinacrine was monitored within 1 h of staining. Phase-contrast and fluorescence images (excitation λ =490–420 nm, emission λ =528–538 nm) were captured using an Olympus IX70 DeltaVision microscope (Applied Precision LLC). CellTracker Blue 7-amino-4-chloromethylcoumarin (CMAC; Invitrogen) was used to visualize the yeast vacuole. For staining, 1 ml of culture was harvested at OD₆₀₀ 0.8–1.0 by centrifugation for 30 s at 13 400 r.p.m. The pellet was resuspended in 100 µl 25 µM CellTracker Blue solution and incubated at 30 °C for 45 min. Cells were harvested as before, resuspended in 200 µl YPD (pH 4.5) and then incubated at 37 °C for 30 min. Stained cells (2 µl) were fixed by mixing with 2 µl 1 % low-melting-point (LMP) agarose. Phase-contrast and fluorescence images (excitation λ =340–360 nm, emission λ =450–457 nm) were captured. To study localization of GFP-tagged Vph1p and Vma2p, images were acquired as described above for quinacrine fluorescence.

RESULTS

The yeast serine-threonine protein kinase Dbf2p mediates sorbic acid stress tolerance

To identify proteins involved in sensing and signalling sorbic acid stress, we carried out a phenotypic screen of 4847 non-essential yeast gene deletion strains (Research Genetics). Deletion of genes encoding a number of regulatory proteins, including the cell cycle-regulated serine-threonine protein kinase Dbf2p, was found to confer sorbic acid hypersensitivity (Makrantonis & Coote, 2003). Compared to the parent strain, growth of the *dbf2Δ* mutant was severely impaired in the presence of increasing concentrations of sorbic acid both on YPD agar, as shown in Fig. 1(a) and in YPD medium (data not shown). Furthermore, a *dbf2* allele encoding a mutant Dbf2p (N305A) lacking kinase activity ('kinase-inactive' Dbf2p; Mah *et al.*, 2001) showed sorbic acid hypersensitivity similar to that of the *dbf2Δ* mutant, indicating that functional kinase activity was required to mediate tolerance to the weak acid. Fig. 1(b) shows that *dbf2Δ*-dependent sorbic acid hypersensitivity was rescued by complementation with *DBF2*, confirming that the effect is specific. *DBF20* encodes a functionally redundant homologue of Dbf2p to which it shows 80 % amino acid sequence identity (Toyn *et al.*, 1991). Deletion of *DBF20* had no effect on tolerance to sorbic acid stress (data not shown), consistent with the much lower kinase activity associated with Dbf20p in comparison with Dbf2p (Toyn & Johnston, 1994). Western blot analysis of a strain expressing TAP-tagged Dbf2p showed that while Dbf2p was expressed under all growth conditions used in this study, no

Fig. 1. Disruption of *DBF2* results in hypersensitivity to sorbic acid stress. (a) Overnight cultures of the parent strain (BY4741)+pRS313, *dbf2Δ*+pRS313 and *dbf2Δ*+pRS313::DBF2(N305A) grown in synthetic complete medium lacking histidine (SC-His) were serially diluted and spotted on YPD agar plates (pH 4.5) containing 0, 1.5, 1.75 and 2.0 mM (not shown) sorbic acid (SA). Plates were photographed after incubation at 30 °C for 48 h. (b) Complementation of the *dbf2Δ* sorbic acid-sensitive phenotype. Growth inhibition of BY4741+pRS313, *dbf2Δ*+pRS313 and *dbf2Δ*+pRS313::DBF2 is shown on SC-His agar (pH 4.5) in the presence or absence of sorbic acid. In each case, representative results of at least two replicate experiments are shown.

significant changes in Dbf2p level were detected under sorbic acid stress (data not shown).

The Pdr12p transporter was previously identified as the major determinant of weak organic acid stress resistance in *S. cerevisiae* (Piper *et al.*, 1998), and concurrent with this study, the transcriptional regulator War1p was shown to be required for *PDR12* expression (Kren *et al.*, 2003). To determine whether Dbf2p forms part of the pathway that transduces the sorbic acid stress signal to War1p, Western blot analysis was performed using an anti-Pdr12p polyclonal antibody. However, loss of *DBF2* affected neither the basal nor the sorbic acid-induced levels of the Pdr12p anion transporter (data not shown), indicating that the kinase is not involved in the War1p–Pdr12p pathway.

Loss of Dbf2p is associated with reduced phosphorylation of the V-ATPase

We sought to identify changes occurring in the yeast phosphoproteome due to deletion of *DBF2* that might reveal targets of the protein kinase that were relevant to its requirement in sorbic acid stress tolerance. Using our recently developed method (Makrantonis *et al.*, 2005), total yeast phosphoproteins were prepared from both the parent and the *dbf2Δ* mutant strains, grown in the presence or absence of sorbic acid. We identified Vma2p, the B subunit of the yeast V-ATPase (Liu *et al.*, 1996; Vasilyeva & Forgac, 1996), as one of a small number of proteins whose behaviour differed in phosphoprotein preparations from the two strains when analysed by 2D-PAGE. In the parent strain, two isoforms of Vma2p were identified by peptide

mass fingerprinting (Fig. 2a, isoforms 1 and 2) with 15 and 16% peptide coverage, respectively. Isoform 2 had an approximate molecular mass of 58 kDa and a pI of 5.1, while isoform 1 had a slightly higher molecular mass and a pI of ~4.9. Significantly, Fig. 2(a) shows that phosphoprotein extracts of the *dbf2Δ* mutant lacked isoform 1, although in both strains the pattern of Vma2p isoforms was not affected by the presence or absence of sorbic acid. To exclude that the loss of isoform 1 in the *dbf2Δ*

phosphoprotein extract resulted from changed Vma2p abundance, Western blot analysis was performed using an anti-Vma2p mAb and total protein extracted from the wild-type and the *dbf2Δ* mutant. Fig. 2(b) shows that the level of Vma2p was unaffected by exposure to sorbic acid or the loss of *DBF2*.

The presence of multiple isoforms of a protein with increasingly acidic pI but similar molecular mass can

Fig. 2. Dbf2p-dependent phosphorylation of Vma1p and Vma2p. (a) Portions from 2D-PAGE gels of phosphoprotein fractions from parent (BY4741) and *dbf2Δ* cells, grown in the presence or absence of 1.5 mM sorbic acid (SA). A representative result of two replicate experiments is shown, indicating two isoforms (1, 2) of Vma2p identified by mass fingerprint analysis. (b) Parent (BY4741) and *dbf2Δ* mutant cells were grown in YPD (pH 4.5) at 30 °C, then divided and grown for an additional 2 h in the presence (+) or absence (-) of 1.5 mM sorbic acid. Cell lysates were subjected to immunoblotting with an anti-Vma2p antibody. To check that equal amounts of protein were loaded, the blot was stripped and reprobed for constitutively expressed Act1p. A representative result of three replicate experiments is shown. (c, d) Vma1p and Vma2p were detected in phosphoprotein fractions prepared from parent (BY4741) and *dbf2Δ* cells grown in the absence (panels 1 and 3) or presence (panels 2 and 4) of 1.5 mM sorbic acid (SA), by immunoblotting with anti-H⁺-ATPase 60 kDa subunit 13D11 (c, Vma2p) or anti-H⁺-ATPase 69 kDa subunit 8B1 (d, Vma1p) mAbs. Proteins were separated by IEF over the pH range 4.0 (+) to 7.0 (-), as indicated, prior to second-dimension SDS-PAGE; representative results of at least three independent experiments are shown. A shift in pI of Vma1p and Vma2p from the acidic (-) toward the neutral (+) sections of the gel was observed if the phosphoprotein extracts were pre-incubated with CIP prior to immunoblotting (c, d, panels 5). (e) Protein extracts from BY4741 or *dbf2Δ* cells grown either without (-) or with 1.5 mM sorbic acid stress (+) were applied to separate phosphoprotein affinity columns, and the flow-through (F) and phosphoprotein-enriched (E) eluate fractions collected. Samples (20 μg) of each fraction were run on 1D-PAGE and immunoblotted with mAbs to detect the presence of Vma1p and Vma2p, as above. The immunoblot reveals the distribution of Vma1p and Vma2p between the eluate (phosphoprotein enriched) and flow-through fractions. Representative results are shown.

indicate differential levels of phosphorylation (Guy *et al.*, 1994). The absence of a more acidic Vma2p isoform in *dbf2Δ* phosphoprotein extracts is therefore consistent with loss of a more phosphorylated Vma2p isoform. To study the potential modification of Vma2p in more detail, Vma2p was detected by immunoblotting following 2D-PAGE separation of phosphoprotein preparations, as shown in Fig. 2(c). The preparation from the parent strain revealed two isoforms of Vma2p with slightly different pI (panel 1). The apparent pI of these isoforms shifted dramatically away from the acidic end of the IEF strip following pre-treatment with calf intestinal phosphatase (CIP), confirming that Vma2p is phosphorylated (panel 5). In phosphoprotein preparations from the *dbf2Δ* strain, all of the Vma2p isoforms were shifted towards the neutral end of the IEF gel strip relative to the isoforms present in the control samples. However, growth in the presence of sorbic acid did not significantly alter the pI of Vma2p isoforms recovered from either strain. These observations are therefore in accordance with the phosphoproteome analysis of Fig. 2(a) and support the notion that Vma2p shows Dbf2p-dependent phosphorylation that is independent of sorbic acid stress.

We performed similar experiments using a mAb to the catalytic subunit of the V-ATPase (Vma1p), which is known to interact with Vma2p. Using the parental strain, a cluster of four protein spots of similar molecular mass but slightly different pI was observed (Fig. 2d, panel 1), which shifted markedly towards the neutral end of the IEF strip following CIP pre-treatment (panel 5), again indicative of phosphorylation. In the *dbf2Δ* strain, all these Vma1p isoforms shifted toward the neutral end of the IEF gel strip, indicative of Dbf2p-dependent phosphorylation. As with Vma2p, growth in the presence of sorbic acid did not significantly alter the pI of the Vma1p isoforms in either the parent or the *dbf2Δ* strain. Western blot analysis of Vma1p showed that neither loss of *DBF2* nor exposure to sorbic acid affected the overall Vma1p level in the cell (data not shown). In summary, our findings show that both Vma1p and Vma2p are phosphorylated and that phosphorylation requires Dbf2p.

To provide further evidence that Vma1p and Vma2p are phosphoproteins, total soluble protein was prepared from cells grown in the presence or absence of sorbic acid, and phosphoproteins were recovered by phosphoprotein affinity chromatography. Fig. 2(e) shows immunoblots of both the flow-through (F; non-phosphorylated proteins) and the eluate (E; phosphorylated proteins) fractions using anti-Vma1p and -Vma2p mAbs. In the parent strain, both Vma1p and Vma2p were detected in the eluate fraction, providing further evidence that both proteins are phosphorylated. However, Vma1p and Vma2p prepared from *dbf2Δ* cells were predominantly detected in the flow-through fraction, confirming that the proteins show reduced levels of phosphorylation in the absence of functional Dbf2p.

In a previous study, neither Vma1p nor Vma2p was identified as a direct substrate for Dbf2p (Mah *et al.*, 2005). We re-examined this by attempting to phosphorylate

Vma2p *in vitro*, as described elsewhere (Toyn & Johnston, 1994), using Dbf2p purified from yeast as either a TAP-tag or *myc*₁₃-tagged fusion protein, together with TAP-Vma2p isolated from yeast as substrate. While both Dbf2p preparations efficiently phosphorylated calf thymus histone H1, neither was able to promote direct phosphorylation of Vma2p (data not shown). Thus, Dbf2p-dependent phosphorylation of Vma2p may not be direct.

V-ATPase function is required for sorbic acid stress tolerance and is dependent on functional Dbf2p kinase

Since loss of Dbf2p function confers sorbic acid hypersensitivity, and Vma1p and Vma2p both show Dbf2p-dependent phosphorylation, we next examined whether the V-ATPase is involved in sorbic acid stress tolerance. Compared to the parent strain, growth of *vma1Δ* and *vma2Δ* mutant strains was significantly impaired at pH 4.5 in the presence of 1.5 mM sorbic acid both on YPD agar as shown in Fig. 3(a) and in YPD medium (data not shown). In each case, the defect was rescued by complementation with the corresponding gene; see Fig. 3(b). The V-ATPase is inhibited with high specificity by bafilomycin A₁, both *in vitro* and *in vivo* (Bowman *et al.*, 2004; Droese & Altendorf, 1997). Fig. 3(c) shows that treatment with 10 μM bafilomycin A₁ resulted in slight growth inhibition compared to the untreated control, while 1 mM sorbic acid at pH 4.5 resulted in marked growth inhibition. However, the combination of 10 μM bafilomycin together with 1 mM sorbic acid resulted in greater growth inhibition than that induced by exposure to sorbic acid alone, implying that a fully functional V-ATPase is required for optimal adaptation to sorbic acid stress.

To determine whether Dbf2p influences V-ATPase function, vacuolar acidification (monitored by quinacrine dye accumulation) was compared between the parent strain and the *vma1Δ*, *vma2Δ*, *dbf2Δ* and kinase-inactive *dbf2* mutants grown in the presence or absence of sorbic acid. Quinacrine is a weakly basic dye that accumulates in acidic compartments in response to proton gradients and is routinely used to assess the state of vacuolar acidification in yeast (Umemoto *et al.*, 1990; Weisman *et al.*, 1987). Earlier studies have shown that deletion of *VMA1*, encoding the V-ATPase catalytic subunit, causes inactivation of the V-ATPase and loss of vacuolar acidification (Morano & Klionsky, 1994). Cells of the parent strain displayed strongly localized vacuolar fluorescence indicative of a normal acidified vacuole, whereas quinacrine accumulation was entirely absent in the *vma1Δ* strain, indicating that the vacuole was not acidified (Fig. 4). Similarly, vacuolar fluorescence was almost undetectable in *vma2Δ* cells. Significantly, *dbf2Δ* cells showed a dramatic reduction of quinacrine fluorescence (Fig. 4), although slight residual fluorescence indicated that the *dbf2Δ* vacuolar-acidification defect, although profound, was not as complete as that in *vma1Δ* cells. The kinase-inactive *dbf2*(N305A) mutant

Fig. 3. A fully functional V-ATPase is required for optimal sorbic acid stress adaptation. (a) Overnight cultures of the parent strain (BY4741), and *vma1Δ* and *vma2Δ* mutants were serially diluted and spotted on YPD agar plates (pH 4.5) containing 0, 0.5, 1.0 and 1.5 mM sorbic acid (SA). Plates were photographed after incubation at 30 °C for 48 h. (b) Complementation of the *vma1Δ* and *vma2Δ* sorbic acid-sensitive phenotype. Growth inhibition of BY4741 + pRS313, *vma1Δ* + pRS313, *vma2Δ* + pRS313, *vma1Δ* + pRS313::VMA1 and *vma2Δ* + pRS313::VMA2 is shown on selective yeast dropout medium lacking histidine (SD-His) (pH 4.5) in the presence or absence of sorbic acid. Data shown are representative of three independent experiments. (c) Growth of BY4741 cells was followed at 30 °C in 24-well microtitre plates containing YPD medium (pH 4.5) without sorbic acid (■), with 1 mM sorbic acid (□), with 10 mM bafilomycin A1 (●) and with a combination of 1 mM sorbic acid and 10 μM bafilomycin A1 (○).

displayed a similar vacuolar-acidification defect (Fig. 4), but growth in the presence of sorbic acid did not affect vacuolar acidification in any of the strains tested. These findings indicate that the kinase activity of Dbf2p is required for optimal vacuolar acidification and therefore proper V-ATPase function, demonstrate that a fully functional V-ATPase is required for adaptation to sorbic acid stress, and imply that Dbf2p-dependent phosphorylation of the V-ATPase might be the mechanism by which Dbf2p promotes sorbic acid stress tolerance.

Loss of Dbf2p does not affect localization of the V-ATPase at the vacuolar membrane

The V-ATPase consists of two major domains: a peripheral, cytoplasmic domain (termed V₁) and an integral membrane

domain (termed V₀). To determine whether the reduced vacuolar acidification shown by *dbf2Δ* cells was due to incorrect localization of the V-ATPase, we constructed control and *dbf2Δ* strains expressing either VMA2–GFP (as a V₁ domain marker) or VPH1–GFP (as a V₀ domain marker). Using the blue fluorescent CellTracker dye to visualize the vacuoles, in both the parent and *dbf2Δ* mutant cells, Vma2–GFP and Vph1–GFP were both clearly localized normally to the vacuolar membrane in cells grown in the presence (data not shown) or absence of sorbic acid (Fig. 5). Since assembly of the V₁ subcomplex and its attachment to the V₀ subcomplex require all of the V₁ polypeptides (except Vma13p) and all of the V₀ polypeptides (Forgac, 1999; Kane *et al.*, 1992; Tomashek *et al.*, 1997), we conclude that loss of Dbf2p kinase is unlikely to affect the localization or assembly of either V-ATPase subcomplex.

Functional interaction between Dbf2p and Vma2p regulates sorbic acid stress tolerance

Previous studies have suggested a direct interaction between Dbf2p and Vma1p, the A subunit of the V-ATPase (Ho *et al.*, 2002). We therefore explored the functional relationship between Dbf2p and the V-ATPase by examining the effect of multiple extra copies of VMA1, VMA2 and DBF2 on sorbic acid sensitivity in wild-type and *dbf2Δ* strains. As expected, high-copy VMA1, VMA2 and DBF2 rescued the sorbic acid hypersensitivity of the respective *vma1Δ*, *vma2Δ* and *dbf2Δ* mutant strains, although enhanced sorbic acid tolerance was not induced (data not shown). While the sorbic acid hypersensitivity of the *dbf2Δ* mutant was unaffected by multicopy VMA1, multicopy VMA2 rescued both the sorbic acid hypersensitivity and the vacuolar-acidification defect of the *dbf2Δ* strain in the presence of sorbic acid (Fig. 6a, b, respectively). In the reciprocal experiments, high-copy DBF2 did not rescue the sorbic acid hypersensitivity of either *vma1Δ* or *vma2Δ* cells (data not shown). These results provide further evidence for a functional interaction between the Dbf2p kinase and the V-ATPase that mediates resistance to sorbic acid stress, and suggest that high-copy VMA2 may lead to enhanced V-ATPase function in the presence of sorbic acid in the *dbf2Δ* mutant.

Loss of DBF2 does not confer a typical Vma⁻-like phenotype, despite resulting in reduced vacuolar acidification

With the exception of the *vph1Δ* and *stv1Δ* mutants, which possess functionally redundant polypeptides (Manolson *et al.*, 1994), V-ATPase mutants fail to grow on YPD media buffered to pH 7.5 or supplemented with 100 mM CaCl₂ or 4 mM ZnCl₂, or on YEP medium with glycerol as the sole carbon source (reviewed by Anraku *et al.*, 1989). Despite displaying reduced vacuolar acidification, *dbf2Δ* cells grew normally under all these conditions (Fig. 7). This phenotype is similar to that displayed by the *vph1Δ* mutant, and implies that there is some residual

Fig. 4. Loss of functional Dbf2p protein kinase results in defective vacuolar acidification. V-ATPase function was studied by monitoring vacuolar acidification by vacuolar uptake and accumulation of the fluorescent dye quinacrine in the parent (BY4741) strain and in *vma1Δ*, *vma2Δ*, *dbf2Δ* and kinase-inactive *dbf2*(N305A) mutants, grown in the presence (+) and absence (–) of sorbic acid (SA) stress. Cells were grown in YPD (pH 4.5) at 30 °C to late exponential phase, transferred to uptake buffer and stained with 200 μM quinacrine. A phase-contrast image of whole cells (left panel) and the corresponding quinacrine staining of the same cells (right panel) is shown. For each strain under each condition tested, the images presented are representative, and the same result was observed in essentially all cells examined in at least two independent experiments.

V-ATPase function present in *dbf2Δ* cells, a conclusion supported by our observation that quinacrine accumulation, and thus vacuolar acidification mediated by the V-ATPase, is reduced but not completely abolished in *dbf2Δ* cells (Fig. 4).

Fig. 5. Subcellular localization of Vma2p and Vph1p. Intracellular localization of Vma2p and Vph1p was studied by observing the localization of Vma2p–GFP and Vph1p–GFP fusion proteins in the parent (BY4741) and *dbf2Δ* mutant strains by fluorescence microscopy using an Olympus IX70 DeltaVision microscope. The vacuole was visualized by the CellTracker stain (Molecular Probes), shown in blue, and representative composite images in which GFP fluorescence is merged with the CellTracker image are presented.

DISCUSSION

Through this work we have demonstrated roles for both Dbf2 protein kinase and the V-ATPase in sorbic acid stress tolerance in yeast: mutations that inactivate either the kinase or the V-ATPase confer sorbic acid hypersensitivity on yeast cells. Furthermore, we have provided several lines of evidence in support of the notion that these two observations are functionally related. Firstly, like V-ATPase mutants themselves, *dbf2* mutants show a profound defect in vacuolar acidification, a process that is dependent on a functional V-ATPase. Secondly, extra copies of *VMA2* (encoding the V-ATPase B subunit) not only suppress the sorbic acid hypersensitivity but also restore the vacuolar acidification in *dbf2* mutant cells when sorbic acid is present. Finally, both the Vma1p and the Vma2p V-ATPase subunits become hypophosphorylated when Dbf2p function is lost, providing evidence that Dbf2p-dependent phosphorylation might regulate V-ATPase function.

Role of the V-ATPase in sorbic acid stress tolerance

We found that loss of *VMA1* or *VMA2*, or exposure to the specific V-ATPase inhibitor bafilomycin A₁, induced hypersensitivity to sorbic acid. These observations are supported by identification of other V-ATPase genes (*VMA4*, *VMA5*, *VMA7*, *VMA8*, *VMA13* and *VMA22*) as mediators of sorbic acid tolerance in screens of the yeast deletion mutant collection (Mollapour *et al.*, 2004; Schuller *et al.*, 2004), several of which we independently identified in our own, similar screen (data not shown). A functional

Fig. 6. *VMA2* is a multicopy suppressor of both the sorbic acid-sensitive phenotype and the vacuolar-acidification defect of *dbf2Δ* cells. (a) Cultures of wild-type (WT; BY4741) and *dbf2Δ* cells containing either the empty multicopy plasmid pRS423 or pRS423 carrying *VMA2* were serially diluted and spotted on SC-His, pH 4.5, containing 0, 1.0 or 1.5 mM sorbic acid (SA), and incubated for 48 h at 30 °C. (b) Vacuolar uptake of quinacrine in the presence (+) and absence (-) of sorbic acid (SA) stress was monitored by vacuolar accumulation of quinacrine fluorescence in cells of BY4741 + pRS423, *dbf2Δ* + pRS423 and *dbf2Δ* + pRS423::VMA2. A phase-contrast image (left panel) and the corresponding quinacrine fluorescence image of the same cells (right panel) are shown. For each strain under each condition tested, the images presented are representative, and the same result was observed in essentially all cells examined in at least two independent experiments.

V-ATPase is therefore required for optimal adaptation to sorbic acid stress, and available evidence supports the hypothesis that V-ATPase function is required to mediate tolerance to a range of growth-inhibitory compounds in yeast. For example, the yeast V-ATPase plays a crucial role in mediating tolerance to the weak-acid herbicide 2,4-D (Fernandes *et al.*, 2003), and yeast mutants lacking a functional V-ATPase show multidrug sensitivity (Parsons *et al.*, 2004). When sorbic acid diffuses into the cell it dissociates into H^+ and sorbate, leading to intracellular acidification. A functional V-ATPase may therefore assist the plasma membrane ATPase Pma1p in removing protons from the cytoplasm to balance the extrusion of sorbate anions via the Pdr1p ABC transporter. In fact, loss of V-ATPase function is also known to cause reduced Pma1p activity (Fernandes *et al.*, 2003), probably by reduced

targeting of the ABC transporter to the plasma membrane (Perzov *et al.*, 2000), and so changes in V-ATPase activity may also impact on Pma1p function. Alternatively, if sorbic acid led to dissipation of the vacuolar membrane H^+ gradient this might be counteracted by V-ATPase activity. Loss of the vacuolar H^+ gradient would disrupt the ability of the vacuole to maintain the homeostasis of many essential nutrients and ions, thus inhibiting growth.

Dbf2p kinase and sorbic acid stress

Dbf2p is a multifunctional, cell cycle-regulated protein kinase that regulates cell division, transcription and the stress response (Lee *et al.*, 1999; Liu *et al.*, 1997a; Toyn & Johnston, 1994). As part of the mitotic exit network (MEN), Dbf2p plays a crucial role in exit from mitosis (reviewed by Yeong *et al.*, 2002). Within the MEN, Dbf2p is activated following phosphorylation by Cdc15p and its activity is dependent on the Dbf2p-associated protein Mob1p (Mah *et al.*, 2001). Dbf2p is also believed to influence gene expression via interaction with the CCR4-NOT complex (Liu *et al.*, 1997a), a general transcriptional regulator (reviewed by Collart, 2003) that affects the expression of genes including those that encode the stress-activated transcription factors Msn2p and Msn4p (Lenssen *et al.*, 2002). Outside its role within the MEN, the mechanisms by which Dbf2p is regulated remain undefined. In contrast to *dbf2Δ* strains, temperature-sensitive *cdc15* mutants do not show sorbic acid hypersensitivity even when grown very close to their restrictive temperature (data not shown), so we consider it likely that the classic cell-cycle regulators of Dbf2p activity are not

Fig. 7. Loss of *DBF2* does not confer a typical *Vma*⁻ phenotype. Overnight cultures of the parent strain (BY4741), and *dbf2Δ*, *vma1Δ* and *vma2Δ* mutants, were serially diluted and spotted on YPD (pH 4.5), YPD (pH 4.5) plus 100 mM $CaCl_2$, YPD plus 50 mM MES-Tris (pH 7.5), YPD (pH 4.5) plus 4 mM $ZnCl_2$, and YEP glycerol (YPG). Plates were photographed after incubation at 30 °C for 48 h.

required for this function of the kinase. Deletion of *DBF2* results in other stress-sensitive phenotypes (Liu *et al.*, 1997b), and although *DBF2* expression is induced in response to stresses such as salt and heat, we observed no significant change in the level of Dbf2p during growth in the presence of sorbic acid. This conclusion is supported by two independent transcriptional profiling studies of yeast exposed to sorbic acid stress, neither of which detected upregulation of *DBF2* (de Nobel *et al.*, 2001; Schuller *et al.*, 2004). Loss of *DBF2* also did not affect the increased expression of the ABC-transporter Pdr12p observed during sorbic acid stress (Piper *et al.*, 1998). Thus, although we cannot exclude the possibility that Dbf2p regulates Pdr12p activity, Dbf2p-dependent sorbic acid tolerance is probably independent of Pdr12p-mediated sorbate anion efflux (Holyoak *et al.*, 1999).

DBF2 and V-ATPase function

Our discovery of Dbf2p-dependent phosphorylation of the V-ATPase subunits Vma1p and Vma2p prompted us to ask whether the Dbf2p kinase influences V-ATPase function. Normal V-ATPase activity results in ATP hydrolysis-dependent vacuolar acidification that is lost in V-ATPase mutants (reviewed by Anraku *et al.*, 1992) and that can be monitored by vacuolar accumulation of quinacrine (Umemoto *et al.*, 1990; Weisman *et al.*, 1987). Since both *dbf2Δ* and 'kinase-inactive' *dbf2* mutants showed a drastic reduction in vacuolar quinacrine accumulation (and thus vacuolar acidification), our results indicate that Dbf2p kinase activity is required for proper V-ATPase function. Compelling support for this hypothesis comes from our finding of *VMA2* as a multicopy suppressor of both the sorbic acid-sensitive phenotype and the vacuolar-acidification defect of *dbf2Δ* cells. Since *dbf2Δ* cells show apparently normal assembly and localization of the V-ATPase, it is possible that elevated expression of Vma2p partially restores the level of phosphorylated Vma2p generated by a Dbf2p-related kinase (Dbf20p, for example). *dbf2Δ* and V-ATPase mutants both show glycogen hyperaccumulation (Wilson *et al.*, 2002), providing an additional link between Dbf2p and V-ATPase function.

The *S. cerevisiae* V-ATPase is composed of 13 subunits organized into two functional domains, a peripheral V_1 domain responsible for ATP hydrolysis and an integral V_0 domain responsible for proton translocation (Arata *et al.*, 2002). Since deletion of any of the V-ATPase subunit genes leads to complete loss of enzyme assembly and activity (see Forgac, 1999), the seemingly normal localization in the vacuolar membrane of key V_1 and V_0 domain proteins in the *dbf2Δ* mutant implies that the apparent loss of V-ATPase function is not due to disrupted V-ATPase assembly. Thus, Dbf2p is more likely to be a regulator of V-ATPase activity than of synthesis or assembly. Since we did not observe the classic Vma^- phenotype in *dbf2Δ* cells, despite their vacuolar-acidification defect, *dbf2Δ* cells may have residual V-ATPase activity. This situation is

reminiscent of the *vph1Δ* V-ATPase mutant, in which the presence of a functional homologue (Stv1p; Manolson *et al.*, 1994) presumably confers residual function, and is consistent with slight residual vacuolar accumulation of quinacrine in Dbf2p-deficient cells. It is also possible that Dbf20p, which is redundant with Dbf2p for at least some functions (Toyn & Johnston, 1994), supports a low level of V-ATPase function in *dbf2Δ* mutant cells.

Since the phosphorylation state of Vma1p or Vma2p in either wild-type or Dbf2p-deficient cells was independent of sorbic acid stress, Dbf2p-dependent V-ATPase activity may not be regulated by sorbic acid. If Dbf2p is not part of a sorbic acid signalling pathway then Dbf2p may simply promote V-ATPase activity in a constitutive manner such that when sorbic acid is present, V-ATPase function is already optimal. Given that earlier studies have failed to identify Vma1p and Vma2p as Dbf2p substrates (Mah *et al.*, 2005), and that we were unable to detect direct phosphorylation of Vma2p by Dbf2p *in vitro*, other as-yet unidentified protein(s) acting downstream of Dbf2p may carry out this function. A number of protein kinases have been identified amongst the potential substrates of Dbf2p (Mah *et al.*, 2005; Ptacek *et al.*, 2005), although none of these has yet been associated with V-ATPase function. However, we cannot exclude the possibility that Dbf2p phosphorylates either Vma1p or Vma2p *in vivo*: Dbf2p, Vma1p and Vma2p exist as components of larger complexes and their interacting proteins may be required to aid phosphorylation. Further work is required to identify the actual protein kinase(s) that directly phosphorylate Vma1p and Vma2p, and to demonstrate the significance of this phosphorylation for V-ATPase function. Other potential Dbf2p substrates include two vacuolar amino acid transporters (Avt3p and Avt4p; Ptacek *et al.*, 2005) along with additional proteins involved in vacuolar function (Mah *et al.*, 2005), providing further evidence for a functional link between Dbf2p and the yeast vacuole.

In summary, we have shown that both Dbf2p kinase and the V-ATPase are required for sorbic acid stress tolerance and have provided evidence that Dbf2p is required for proper V-ATPase function. *VATB1* (encoding the human kidney isoform of the V-ATPase B subunit) shares 78% identity with its yeast homologue Vma2p, and mutations in *VATB1* cause distal renal tubular acidosis, a condition characterized by impaired renal acid secretion resulting in metabolic acidosis and sensorineural hearing loss (Karet *et al.*, 1999). It will be interesting to determine whether, as we have shown in yeast, the human homologue of yeast Dbf2p (NDR1) influences the function of human V-ATPases.

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