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In Vivo Phosphorylation of Ser^{21} and Ser^{83} during Nutrient-induced Activation of the Yeast Protein Kinase A (PKA) Target Trehalase*

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Background: Activation of yeast trehalase has been a convenient read-out for nutrient signaling to PKA, but demonstration of phosphorylation *in vivo* is lacking.
Results: Nutrient activation is associated with phosphorylation, but phosphorylation is not enough for activation.
Conclusion: Nutrient activation of trehalase is a reliable read-out for nutrient activation of PKA *in vivo*.
Significance: Nutrient-sensing mechanisms can be identified using trehalase activation as a read-out.

The readdition of an essential nutrient to starved, fermenting cells of the yeast *Saccharomyces cerevisiae* triggers rapid activation of the protein kinase A (PKA) pathway. Trehalase is activated 5–10-fold within minutes and has been used as a convenient reporter for rapid activation of PKA *in vivo*. Although trehalase can be phosphorylated and activated by PKA *in vitro*, demonstration of phosphorylation during nutrient activation *in vivo* has been lacking. We now show, using phosphospecific antibodies, that glucose and nitrogen activation of trehalase *in vivo* is associated with phosphorylation of Ser^{21} and Ser^{83}. Unexpectedly, mutants with reduced PKA activity show constitutive phosphorylation despite reduced trehalase activation. The same phenotype was observed upon deletion of the catalytic subunits of yeast protein phosphatase 2A, suggesting that lower PKA activity causes reduced trehalase dephosphorylation. Hence, phosphorylation of trehalase *in vivo* is not sufficient for activation. Deletion of the inhibitor Dcs1 causes constitutive trehalase activation and phosphorylation. It also enhances binding of trehalase to the 14-3-3 proteins Bmh1 and Bmh2, suggesting that Dcs1 inhibits by preventing 14-3-3 binding. Deletion of Bmh1 and Bmh2 eliminates both trehalase activation and phosphorylation. Our results reveal that trehalase activation *in vivo* is associated with phosphorylation of typical PKA sites and thus establish the enzyme as a reliable read-out for nutrient activation of PKA *in vivo*.

Depending on the available nutrients, cells of the yeast *Saccharomyces cerevisiae* show dramatic changes in properties affected by the activity of protein kinase A (PKA) (1–5). During growth on glucose, storage carbohydrate levels are low, stress tolerance is low, cell wall composition is very sensitive to lyticase treatment, etc. During growth on respirative carbon sources, on the other hand, as well as upon starvation of glucose-fermenting cells for another essential nutrient, these properties are reversed. This has led to the concept that a complete fermentable growth medium is required to maintain high PKA activity. In addition, it has allowed the investigators to establish conditions under which glucose but also other essential nutrients, like nitrogen, phosphate and sulfate, trigger rapid activation of the PKA pathway (6).

The discovery that different essential nutrients can trigger rapid activation of the PKA pathway in appropriately starved fermenting cells has laid the basis for detailed studies on the nutrient-sensing and signaling systems involved (1, 6–11). This has required the use of reporter systems to follow rapid activation of the PKA pathway: trehalase activation; mobilization of trehalose and glycogen; loss of stress tolerance, repression of stress response (STRE-controlled) genes; induction of ribosomal protein genes; etc. The 5–10-fold increase in trehalase activity, which can be detected within 3–5 min after the addition of the agonist nutrient, has been a favorite reporter system in our studies on nutrient activation of the PKA pathway because of the rapidity and purely post-transcriptional character of the response.

Recently, we reported that the two main protein phosphatases of eukaryotic cells, PP2A[2] and PP1, are also rapidly activated within a few min after the addition of glucose to cells growing on a non-fermentable carbon source. This activation is dependent on glucose activation of the cAMP-PKA pathway and thus suggests that both phosphatases are positively regulated by PKA (12).

The yeast *S. cerevisiae* has two enzymes for trehalose hydrolysis: neutral trehalase, encoded by *NTH1* (13), and acid tre-

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[2] The abbreviations used are: PP2A, protein phosphatase 2A; PP1, protein phosphatase 1; Ab, antibody; SD, synthetic dextrose.

TABLE 1
Yeast strains used in this study

Strain	Relevant genotype	Source/Reference
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Ref. 60
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Ref. 60
BJ2168	*MAT***a** *leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1407 gal2*	Ref. 61
JT21766	BY4741 *tpk2::HIS3 tpk3::LEU2*	This study
JT21765	BY4742 *tpk1::HIS3 tpk3::LEU2*	This study
JT21721	BY4742 *tpk1::KanMX4 tpk2::KanMX4*	This study
DC90	BY4742 *tpk1::HIS3 tpk2^{w3} tpk3::LEU2*	This study
DC127	BY4742 *pph21::KanMX4 pph22::KanMX4*	Ref. 12
WS01	BY4742 *BMH1-GFP::KanMX6*	This study
WS02	BY4742 *BMH2-GFP::KanMX6*	This study
b.1986	BY4742 *dcs1::KanMX4*	Ref. 31
GV299	BY4742 *dcs1::KanMX4 BMH1-GFP::KanMX6*	This study
GV300	BY4742 *dcs1::KanMX4 BMH2-GFP::KanMX6*	This study
L6281	*MAT***a** *ura3-52 leu2::hisG*	Ref. 48
L6245	L6281 *his3::hisG bmh2::HIS3+ bmh1::HIS3+*	Ref. 48
pJ69-4A	*MAT***a** *leu2-312 ura3-52 trp1-901 his3-200 gal4Δ gal80 Δ GAL2-ADE2 lys2::GAL1-HIS3 met2::GAL7-LacZ*	Ref. 62
WS10	pJ69-4A *dcs1::KanMX4*	This study

halase, encoded by *ATH1* (14). Neutral trehalase is responsible for the rapid changes in trehalose content observed upon stimulation of the PKA pathway with glucose and other nutrients (15). Rapid glucose activation of neutral trehalase in glucose-deprived cells was first described by Van der Plaat in 1974 (16), whereas later also amino acid, phosphate, sulfate, and ammonium activation (6) were reported in appropriately starved cells. Van der Plaat (16, 17) provided evidence for the involvement of PKA, demonstrating a correlation with glucose-induced increase in cAMP and also *in vitro* activation of trehalase by incubation with cAMP and PKA. App and Holzer (18) demonstrated for the first time that *in vitro* activation of trehalase by PKA was correlated with phosphorylation. Extensive evidence indicates that rapid nutrient activation of trehalase *in vivo* is mediated by PKA. Mutants with reduced or constitutively high cAMP levels and mutants with reduced or constitutively high PKA activity show similarly reduced or constitutively elevated trehalase activity (6, 8, 19–22).

The precise phosphorylation site(s) responsible for activation of *S. cerevisiae* trehalase has remained enigmatic. The enzyme contains eight putative PKA phosphorylation sites: Ser[20], Ser[21], Ser[60], Ser[83], Ser[475], Thr[58], Thr[135], and Thr[149]. Site-directed mutagenesis of individual sites did not reveal a specific site involved in activation, and mutagenesis of multiple sites led to a gradual loss of trehalase activity, preventing proper assessment of a role in the activation process (23). Recently, mass spectrometry evidence was reported for phosphorylation of purified trehalase by PKA *in vitro* on Ser[20], Ser[21], Ser[60], and Ser[83] (24). *Schizosaccharomyces pombe* neutral trehalase has a structure similar to that of *S. cerevisiae* trehalase and is rapidly activated under similar environmental conditions (25, 26). Mutagenesis of the putative PKA phosphorylation sites resulted in inactive trehalases unresponsive to environmental stimulation. Hence, also for *S. pombe* trehalase, it remains unclear what putative PKA phosphorylation sites are relevant for activation *in vivo* (27).

The extent of trehalase activation is always lower *in vitro* compared with *in vivo*, suggesting the existence of additional regulatory mechanisms. Two such mechanisms have been identified. Dcs1, an mRNA decapping enzyme, has been shown to interact with and act as a negative regulator of trehalase activity (28, 29). The yeast 14-3-3 proteins, encoded by Bmh1 and Bmh2, have recently been demonstrated to bind to phosphorylated residues in the N terminus of trehalase. Furthermore, the addition of recombinant Bmh1 protein to *in vitro* phosphorylated trehalase stimulated activation of the enzyme up to 7-fold (24, 30). The individual phosphorylation sites sustain no activation or only poor activation of trehalase *in vivo*, suggesting that phosphorylation on all or a majority of the sites is important for full 14-3-3 binding and thus for full activation (24). The possible importance of both mechanisms for *in vivo* regulation of trehalase has remained unclear.

In this paper, we have made use of custom-made phospho-specific antibodies against two phosphorylation sites in trehalase: Ser[21] and Ser[83]. We show that glucose and nitrogen activation *in vivo* are associated with rapid phosphorylation of both sites. However, we also show that phosphorylation of these sites is not sufficient for activation of trehalase *in vivo*. Reduction of PKA activity surprisingly results in constitutive phosphorylation of trehalase. Furthermore, we show that the yeast 14-3-3 proteins are required for both activation of trehalase and phosphorylation of the two sites. Deletion of the trehalase inhibitor, Dcs1, causes constitutive activation and phosphorylation of trehalase and also results in stronger binding of 14-3-3 to trehalase, suggesting that Dcs1 inhibits by preventing 14-3-3 binding. Our results underscore the reliability of the rapid increase in trehalase catalytic activity as a valid marker for nutrient activation of the PKA pathway.

EXPERIMENTAL PROCEDURES

Yeast Strains—The strains used in this study are listed in Table 1. Strains JT21766 and JT21765 were constructed by Dr. T. Peeters (Leuven, Belgium). The *tpk3::LEU2* construct was obtained by PCR amplification from vector pRS425 and transformed into BY4742. The resulting *tpk3::LEU2* strain was crossed with either the *tpk1::KanMX4* or *tpk2::KanMX4* strains from the yeast deletion collection (31). Segregants carrying the double deletion were subsequently transformed with plasmid M4754 to swap the *KanMX4* marker for *HIS3* (32). Strain JT21721 was constructed by Dr. T. Peeters using crossing of the corresponding deletion mutants from the deletion collection followed by sporulation and segregant selection. The DC90 strain carrying the *tpk2^{w3}* allele was created by Dr. D. Castermans (Leuven, Belgium), following the procedure described by

Cameron *et al.* (33). Briefly, a *tpk1*Δ *TPK2 tpk3*Δ *bcy1*Δ strain was plated on galactose-containing medium. The enhanced PKA activity in such a strain prevents growth on media with galactose as the carbon source, and spontaneous mutations in *TPK2* arise that lower PKA activity and allow growth on galactose. Growing colonies were selected and mutations in *TPK2* were identified by Sanger sequencing. The *tpk2*^{w3} allele has a G415C substitution in the ORF, which translates into D139H replacement in the protein. The allele was isolated by PCR, cloned into YIplac33, and integrated into the genome at the *TPK2* locus. The plasmid was subsequently lost by plating the cells on 5-fluoroorotic acid medium, effectively replacing wild type *TPK2* with the *tpk2*^{w3} allele. Strain DC127 was created by Dr. D. Castermans (Leuven, Belgium) by crossing BY4741 *pph21*Δ and BY4742 *pph22*Δ (12). Strains WS01, WS02, GV299, and GV300 were constructed as described (34). The GFP-KanMX6 cassette was isolated with PCR from plasmid pFA6a-GFP(S65T)-KanMX6 and transformed into BY4742 or b.1986. Strain WS10 was obtained by transforming the KanMx4 cassette isolated from b.1986 into PJ69-4A.

Plasmid Construction—Plasmid p*TPI1-NTH1-HA-URA3* was constructed using 5′ BamHI PCR primer CGGGATCCATGAGTCAAGTTAATACAAGC and 3′ SmaI PCR primer TCCCCCCGGGTAGTCCATAGAGGTTTCTTTC. The PCR product was digested and ligated into the episomal vector pYX212, which contains the TPI promotor and the *URA3* selection gene. Plasmid p*TPI1-NTH1-HA-LEU2* was constructed by subcloning of the *NTH1-HA* allele, using BamHI and EcoRV restriction sites, into pYX242, which contains the same TPI promotor as pYX212. Plasmids p*TPI1-BMH1-HA-URA3* and p*TPI1-BMH2-HA-URA3* were constructed using 5′ EcoRI PCR primer CTACGGAATTCATGTCAACCAGTCGTGAAG or CTACGGAATTCATGTCCCAAACTCGTGAAG and 3′ BamHI PCR primer CGTAGGGATCCCTTTGGTGCTTCACCTTC or CGT-AGGGATCCTTTGGTTGGTTCACCTTGAG, respectively. PCR products were digested and ligated into pYX212. Plasmid pGEX-4T1-NTH1 used in the *in vitro* kinase assay was constructed using 5′ BamHI PCR primer CGGGATCCATGAGTCAAGTTAATACAAGC and 3′ NotI PCR primer TTTTCCTTTTGCGGCCGCCTATAGTCCATAGAGGTTTC. The PCR product was digested and ligated in frame with the GST tag into the pGEX-4T1 vector. Plasmid p*ADE-GFP-HA$_3$-TPK1*, used to express Tpk1 for the *in vitro* kinase assay, was constructed as described (35). Plasmids pGAD424-BMH1 and pGAD424-BMH2 used in the two-hybrid assay were constructed using 5′ BamHI PCR primers GAAGGATCCGGATGTCAACCAGTCGTGAAGATTC and 3′ PstI AAAACTGCAGTTACTTTGGTGCTTCACCTTC or 5′ GAAGGATCCGGATGTCCCAAACTCGTGAAGATTC and 3′ PstI AAAACTGCAGTTATTTGGTTGGTTCACCTTGAG. The PCR product was digested and ligated into the pGAD424 vector. Plasmid pGBT9-NTH1 used in the two-hybrid assay was constructed using 5′ SmaI TCCCCCGGGGATGAGTCAAGTTAATACAAGC and 3′ PstI AAAACTGCAGCTATAGTCCATAGAGGTTTC PCR primers. The PCR product was digested and ligated into the pGBT9 vector.

Growth Conditions—For the experiments involving glucose activation of trehalase, cells were grown in rich yeast peptone (YP) containing 3% glycerol and 0.1% glucose under continuous shaking until exponential phase. For the experiments involving nitrogen activation, cells were grown in SD medium with 2% glucose and appropriate amino acids into exponential phase. Exponential phase cells were subsequently harvested and transferred to nitrogen starvation medium (1.7 g/liter yeast nitrogen base without amino acids and without ammonium sulfate) with 4% glucose. Cells were starved for 6 h at 30 °C under continuous shaking.

In Vitro Kinase Assay—GST-Nth1 fusion proteins were expressed in *Escherichia coli* from a pGEX-4T1 vector. After a 3-h induction with 0.3 mM IPTG, a clarified bacterial lysate was prepared, and the fusion protein was bound to gluthatione-Sepharose (GE Healthcare) following standard procedures. Tpk1 was expressed in the partially protease-deficient yeast strain BJ2168 from plasmid p*ADE-GFP-HA$_3$-TPK1* and immunoprecipitated as described below. Bead-bound Tpk1 and GST-Nth1 were washed and resuspended in 100 μl of kinase buffer (50 mM Tris, pH 8.0, 1 mM EGTA, 1 mM DTT, 5 mM $MgCl_2$, 0.5 mM Na_3VO_4, 10 mM β-glycerol phosphate). 20 μl of bead-bound Tpk1 and GST-Nth1 were mixed, and the reactions were started by the addition of 50 μM ATP. Reactions were incubated for 45 min at 30 °C while shaking and terminated by the addition of 5× SDS sample buffer (250 mM Tris/HCl, pH 8, 50 mM 2-mercaptoethanol, 10% SDS, 0.5% bromphenol blue, and 50% glycerol). Samples were heated for 3 min at 95 °C and separated on SDS-PAGE. The gel was stained with Coomassie, and bands corresponding to Nth1 were analyzed by mass spectrometry.

Mass Spectrometry Analysis—Nth1 bands were excised from the gel, reduced and alkylated with dithiotreitol and iodoacetamide, and finally digested with sequencing grade trypsin in 25 mM ammonium bicarbonate, pH 8.0. Proteolytic digests were acidified by adding 5% formic acid (final concentration) and analyzed using a nanoAcquity ultrahigh pressure liquid chromatograph (Waters, Manchester, UK) directly coupled to a Q-TOF premier (Waters). The mass spectrometer was set in a data-dependent mode. Survey scans of 1 s were acquired in positive ion centroid mode from m/z 400 to 1500, and low energy collision-induced dissociation spectra were acquired in profile mode from m/z 50 to 2000 for 3 s. Peptides were identified by MS/MS ion search by using an in-house licensed MASCOT 2.0 server (available from the Matrix Science Web site). Searches were done in the SwissProt database (UniProt_SwissProt 50.8) using the following parameters: *S. cerevisiae* as taxonomy restriction, trypsin as proteolytic enzyme with two missed cleavages allowed, peptide tolerance 25 ppm, fragment tolerance 0.05 Da, carbamidomethylcysteine as fixed and phosphorylated serine, and phosphorylated threonine and oxidized methionine as variable modifications. Phosphorylated peptides identified by mascot were validated by manual interpretation of the fragmentation spectra.

Phosphospecific Antibody Production—Antibodies against phosphorylated Ser[21] and Ser[83] were generated by Eurogentec, using keyhole limpet hemocyanin-conjugated phosphopeptides RQRRLSpSLSEFND (flanking Ser[21]) and LQQTRRGpSEDDTY (flanking Ser[83]). Phosphospecific antibodies were

purified by affinity chromatography using phosphopeptide-conjugated resin.

Trehalase Activity Assay—Appropriately nutrient-deprived cells were cooled on ice for 30 min, harvested by centrifugation, and washed with Mes/KOH buffer (25 mM, pH 6). Cells were resuspended in fresh medium (either YPglycerol or nitrogen starvation medium, depending on the deprivation condition) at a density of 25 mg of wet weight/ml. Trehalase activity was determined in crude cell extracts as described (36). The glucose liberated was assayed by the glucose oxidase/peroxidase method. Protein concentration was determined by the Lowry procedure. The specific activity of trehalase is expressed as nmol of glucose liberated/min/mg of protein.

Protein Extraction and Immunoprecipitation—~100 mg of cells were lysed mechanically in 500 μl of extraction buffer containing 1× PBS, 10% glycerol, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 1 mM EDTA, phosphatase inhibitors (10 mM NaF, 0.1 mM β-glycerol phosphate, 0.4 mM Na_3VO_4), and 10 μl/ml Sigma protease inhibitor mixture for yeast extracts. Crude cell extracts were cleared twice by centrifugation. Protein concentration was measured using the Bradford assay (Bio-Rad) or Pierce 660-nm protein assay. Samples were diluted to match the protein concentration of the sample with the lowest concentration. HA-tagged trehalase was immunoprecipitated using protein G-conjugated Dynabeads (Invitrogen) and high affinity anti-HA antibody (clone 3F10, Roche Applied Science). Dynabeads were equilibrated in lysis buffer, and immunoprecipitation was carried out at 4 °C under continuous gentle agitation. Immunoprecipitates were washed three times with lysis buffer and subsequently boiled in 2.5× SDS sample buffer containing 125 mM Tris/HCl, pH 8, 25 mM 2-mercaptoethanol, 5% SDS, 0.25% bromphenol blue, and 25% glycerol.

Western Blotting—After SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Hybond, GE Healthcare). Nonspecific antibody/reagent binding sites on the blots were blocked using 5% skimmed milk (anti-HA Ab and anti-GFP Ab) or 5% BSA (anti-phosphospecific Abs) for 1 h. Blots were incubated with antibody solutions overnight at 4 °C under continuous, gentle agitation, washed three times with 1× PBST, and incubated with secondary antibodies (anti-rabbit or anti-mouse Abs conjugated to horseradish peroxidase) (GE Healthcare). Proteins were visualized using Pierce ECL reagents and the ImageQuant LAS4000 mini CCD imaging system (Fujifilm).

λ-Phosphatase Treatment—Immunoprecipitates were obtained as described previously and washed three times with 1× PBST (0.05% Triton X-100) to remove all traces of phosphatase inhibitors. After removal of the last wash, beads were resuspended in 50 μl of 1× λ-phosphatase buffer (50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35, pH 7.5) supplemented with 1 mM $MnCl_2$ and 80 units of λ-phosphatase (New England Biolabs). Samples were incubated for 30 min at 30 °C; subsequently, supernatant was removed, and the beads were boiled in SDS sample buffer.

RESULTS

Trehalase Is a Phosphoprotein—Based on its amino acid sequence, two Pfam domains have been identified in trehalase: a small, 30-amino acid-long Ca^{2+} binding domain located near the N terminus and a much larger catalytic domain spanning residues 163–721 (Fig. 1*A*). Apart from these two domains, an N-terminal extension of about 100 amino acids is present. Over the last 5 years, several large scale phosphoproteomic studies have identified 11 phosphorylated serines or threonines in trehalase (37–41). Interestingly, all of the identified residues are located in the 100-amino acid-long N-terminal extension, which suggests that this region plays an important role in the regulation of the enzyme. In 2008, Panni *et al.* (30) mapped the binding site of the yeast 14-3-3 proteins to this region. They showed that phosphorylation at either Ser^{21} or Ser^{23} was necessary to promote binding of yeast 14-3-3 proteins to trehalase.

To identify putative PKA-specific phosphorylation sites in trehalase, we performed an *in vitro* kinase assay. Recombinant GST-tagged trehalase purified from *E. coli* was phosphorylated with HA-tagged Tpk1 immunoprecipitated from yeast in the presence of ATP, cAMP, and $MgCl_2$. The samples were sepa-

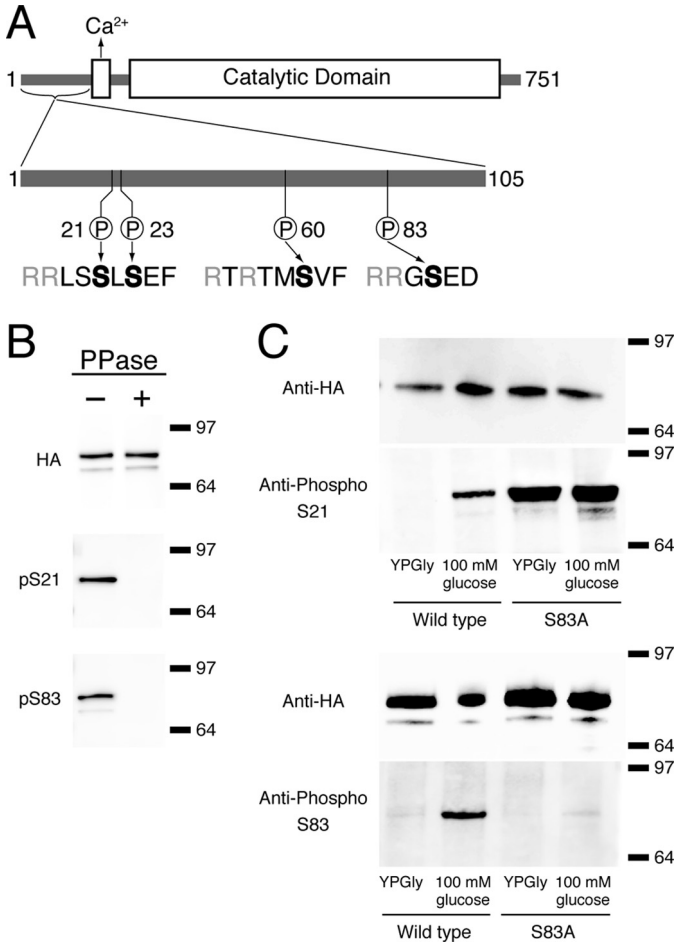

FIGURE 1. **Structure of neutral trehalase and evaluation of the specificity of the custom-made phosphospecific Abs.** *A*, trehalase consists of a catalytic domain, a Ca^{2+}-binding domain, and an N-terminal extension. Phosphorylated serines and adjacent residues in the N-terminal extension are depicted to illustrate putative PKA phosphorylation sites. *B*, immunoprecipitation of trehalase expressed in BY4742 from plasmid p*TPI1-NTH1-HA-URA3* treated with (+) or without (−) phosphatase. Equal amounts of immunoprecipitated Nth1-HA were loaded on all three Western blots. The *upper blot* was incubated with anti-HA Ab, the *middle blot* with anti-phospho-Ser^{21} Ab (*pS21*), and the *bottom blot* with anti-phospho-Ser^{83} Ab (*pS83*). *C*, phosphorylation status of Ser^{21} and Ser^{83} in trehalase after the addition of 100 mM glucose to respiring yeast cells. Strains were transformed with p*YX212-NTH1^{S21A}-HA* or p*YX212-NTH1^{S83A}-HA*.

rated with SDS-PAGE, and protein bands corresponding to trehalase were excised and digested with trypsin. The resulting tryptic peptides were analyzed, after TiO_2 chromatography for phosphopeptide enrichment, by nano-ultrahigh pressure liquid chromatography electrospray ionization Q-TOF mass spectrometry. Two phosphorylated residues were identified, Ser[60] and Ser[83], both previously observed in some of the phosphoproteomic studies mentioned above. To evaluate the relevance of the N-terminal phosphorylations for nutrient-induced trehalase activation, we ordered phosphospecific Abs against peptides containing phospho-Ser[21] and phospho-Ser[23] (the residues designated by Panni *et al.* (30) as required for 14-3-3 binding to trehalase) and against phospho-Ser[60] and phospho-Ser[83] (the putative PKA phosphorylation sites discovered in our *in vitro* kinase assay). Upon validating the quality of the Abs, using alkaline phosphatase treatment of immunopurified trehalase, we found that only the anti-phospho-Ser[21] and anti-phospho-Ser[83] Abs could distinguish properly between the phosphorylated and unphosphorylated versions of the whole trehalase protein (Fig. 1*B*). The specificity of the antibodies was also tested using versions of Nth1 in which either Ser[21] or Ser[83] was mutated to alanine. In the case of phospho-Ser[83], the signal in the Western blot was no longer observed, whereas the S21A mutant protein now showed a constitutive signal with the antibody (Fig. 1*C*). Apparently, mutagenesis of Ser[21] to alanine causes a structural change in the domain, which allows the antibody to detect the site in both the phosphorylated and nonphosphorylated form. Hence, we do not think that this result compromises the conclusion that this antibody is also specific for the phosphorylated native Ser[21] residue. This conclusion was supported by later results (see below). In our subsequent experiments, only these two Abs were used. It must be noted that both Ser[21] and Ser[83] fulfill the requirements of the consensus PKA phosphorylation site, RX_2(S/T) and RRX(S/T), respectively (42).

Trehalase Is Phosphorylated in Vivo in Response to a Glucose Signal—Upon the addition of glucose to derepressed yeast cells, trehalase was rapidly activated (Fig. 2*A*). To evaluate the phosphorylation status of the enzyme during the activation process, we expressed a C-terminal HA-tagged version of trehalase in a wild type strain and took samples over a 30-min time period after the addition of glucose. The trehalase enzyme was isolated by immunoprecipitation using anti-HA antibodies, the samples were subsequently separated by SDS-PAGE and transferred by Western blotting. The presence or absence of phosphorylation was then examined with our custom-made phosphospecific Abs. SDS-PAGE and Western blotting were done in triplicate; one blot was used for detection of total trehalase with the anti-HA Ab, and the other two were used for detection with the phosphospecific Abs. The result can be seen in Fig. 2*B*; glucose-induced trehalase activation correlates with phosphorylation of Ser[21] and Ser[83].

Aberrant Phosphorylation of Trehalase in Strains with Reduced PKA Activity—PKA is a holoenzyme consisting of two regulatory subunits encoded by *BCY1* and two catalytic subunits redundantly encoded by three *TPK* genes. Because a triple *TPK* deletion is synthetically lethal, we examined the involvement of PKA in the *in vivo* phosphorylation of trehalase using a collection of strains expressing a single *TPK* as the sole source of PKA catalytic subunit. Yeast strains expressing only *TPK1* or *TPK2* displayed both a wild type trehalase activation and phosphorylation pattern (Fig. 2, *A*, *C*, and *D*). However, glucose-induced trehalase activation was severely affected in a strain expressing only *TPK3* as the PKA catalytic subunit. Phosphorylation on Ser[21] and Ser[83] was constitutive in this strain and did not change upon the addition of glucose (Fig. 2*E*). A similar phenotype is observed in a yeast strain carrying an attenuated allele of *TPK2* as the sole source of PKA catalytic subunit (Fig. 2*F*). These results clearly show that phosphorylation alone is not sufficient to promote activation of the trehalase enzyme.

Inactivation of the Protein Phosphatase PP2A Also Causes Constitutive Phosphorylation of Trehalase—Recently, we have provided evidence that PKA stimulates PP2A activity (12). Hence, we evaluated whether the constitutive phosphorylation of trehalase in strains with reduced PKA activity might be due to lowered dephosphorylation by PP2A. Determination of trehalase phosphorylation in a yeast strain in which both catalytic subunits of PP2A, Pph21 and Pph22, are deleted indeed also revealed constitutive phosphorylation (Fig. 3). This suggests that the constitutive phosphorylation in strains with reduced PKA activity could be due to reduced dephosphorylation by PP2A rather than by enhanced phosphorylation by an alternative protein kinase.

The Yeast 14-3-3 Proteins Are Required for Activation of Trehalase in Vivo—Several genome-wide interactome studies have found the yeast 14-3-3 proteins, Bmh1 and Bmh2, in complex with trehalase (43–47). Panni *et al.* (30) demonstrated that recombinant Bmh1 could stimulate the activity of PKA-phosphorylated trehalase *in vitro*. To further assess the role of 14-3-3 in the regulation of trehalase, we measured *in vivo* glucose-induced trehalase activation and phosphorylation in 14-3-3 overexpression and deletion mutants. Overexpression of *BMH1* or *BMH2* did not have any effect on glucose-induced trehalase activation or phosphorylation when compared with wild type (Fig. 4). Deletion of both 14-3-3 isoforms is lethal in the S288c strain background but not in the Σ1278B strain. The Fink laboratory kindly provided us with their *bmh1*Δ *bmh2*Δ strain (48) in which we determined glucose-induced trehalase activation and phosphorylation. As can be seen in Fig. 5, the *bmh1*Δ *bmh2*Δ strain failed to show trehalase activation. Moreover, phosphorylation of the two PKA sites in trehalase was absent in this strain. We conclude that the 14-3-3 proteins are required for glucose-induced activation of trehalase and for establishment or maintenance of its phosphorylation.

The Yeast 14-3-3 Proteins Interact with Phosphorylated Trehalase in Vivo—Because the 14-3-3 proteins are well known to bind specifically to phosphorylated proteins, we reasoned that trehalase phosphorylation could promote interaction with 14-3-3 *in vivo*. To examine this hypothesis, we transformed strains expressing GFP-tagged alleles of *BMH1* and *BMH2* with the plasmid carrying the HA-tagged *NTH1* allele in order to perform co-immunoprecipitation. After immunoprecipitation of trehalase with anti-HA Abs, separation by SDS-PAGE, and transfer by Western blotting, we visualized the phosphorylation status with the phosphospecific Abs and any interacting 14-3-3 proteins with anti-GFP Ab (Fig. 6). In respirative yeast

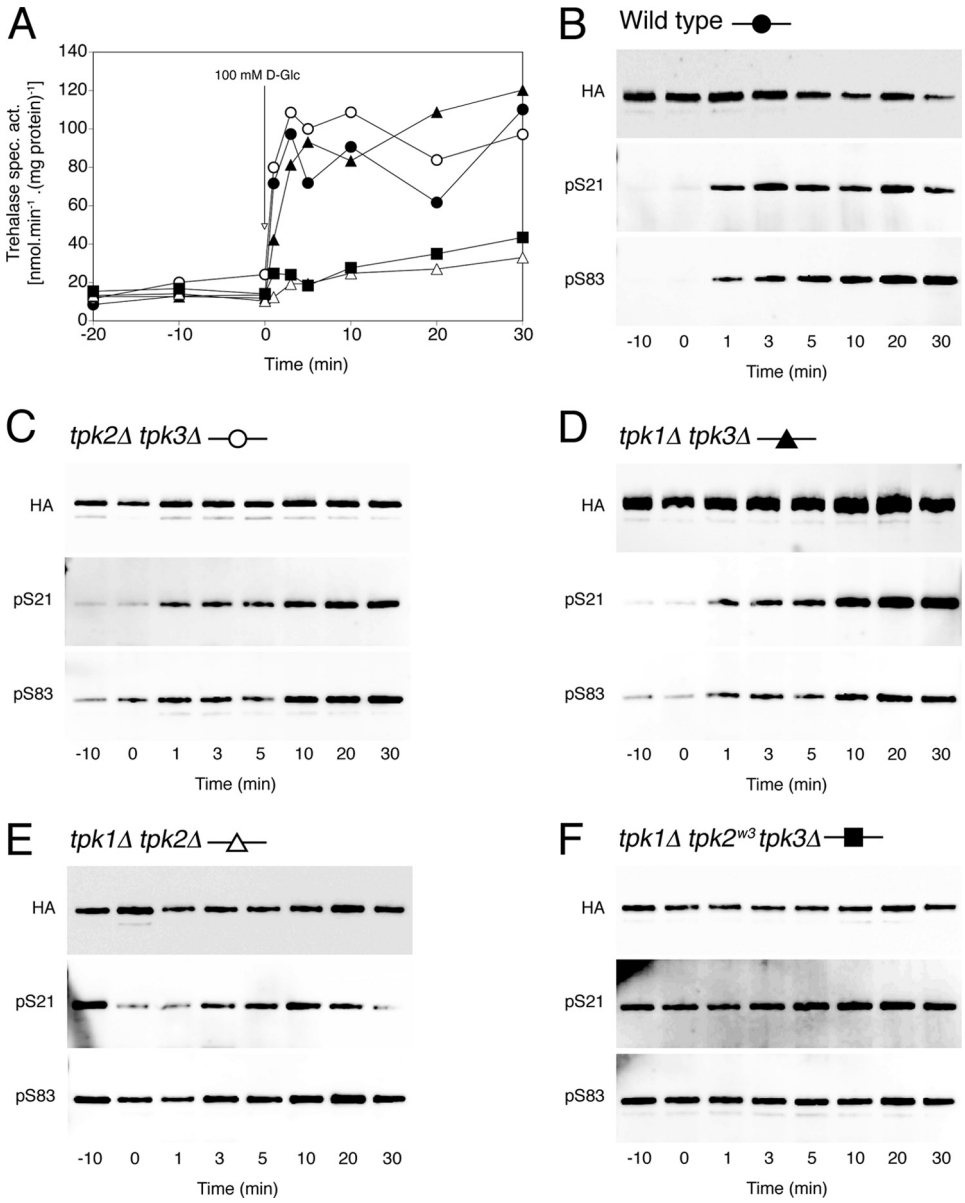

FIGURE 2. **Glucose-induced trehalase activation and phosphorylation in strains with reduced PKA activity.** Trehalase activity (*A*) and phosphorylation status of Ser[21] and Ser[83] in trehalase (*B–F*) after the addition of 100 mM glucose to respiring yeast cells. Strains were transformed with p*TPI1-NTH1-HA-URA3*. Shown are wild type (*B*, ●), *tpk2*Δ *tpk3*Δ (*C*, ○), *tpk1*Δ *tpk3*Δ (*D*, ▲), *tpk1*Δ *tpk2*Δ (*E*, △), and *tpk1*Δ *tpk2*^{w3} *tpk3*Δ (*F*, ■). *B–F*, top Western blot, detection of total Nth1-HA with anti-HA Ab; *middle Western blot*, detection with anti-phospho-Ser[21] Ab (*pS21*); *bottom Western blot*, detection with anti-phospho-Ser[83] Ab (*pS83*).

cells, only small amounts of both Bmh1 and Bmh2 can be co-immunoprecipitated with trehalase. However, upon activation of trehalase and phosphorylation of its Ser[21] and Ser[83] residues after the addition of glucose, significantly more 14-3-3 protein becomes bound to trehalase, suggesting that phosphorylation of trehalase enhances interaction with 14-3-3. This is in agreement with a recent report in which enhanced binding of Bmh1 and Bmh2 to phosphorylated trehalase was demonstrated *in vitro* (24).

Trehalase Is Phosphorylated in Vivo in Response to a Nitrogen Signal—Like respirative cells, fermenting cells deprived of nitrogen are characterized by low trehalase activity. Upon the addition of a good nitrogen source, like asparagine, trehalase is rapidly activated (5–10 min). Using the phosphospecific Abs, we show that nitrogen-induced trehalase activation is also correlated with an increase in phosphorylation on Ser[21] and Ser[83], pointing toward a similar mechanism for both glucose- and nitrogen-induced trehalase activation (Fig. 7, *A*, *B*, and *D*). Hence, this result supports the previous conclusion that PKA is mediating nitrogen-induced activation of trehalase despite the absence of cAMP signaling after the addition of a nitrogen source (6, 8, 19, 22).

Dcs1 Is an Inhibitor of Trehalase Activity—Although originally described as the yeast orthologue of human DcpS, a scavenger mRNA decapping enzyme (29), the unnamed ORF YLR270w had already been implicated in trehalase regulation, before it was named Dcs1. A genome-wide yeast two-hybrid study had reported interaction between trehalase and YLR270w (49). Additional research by the Panek laboratory (28, 50) confirmed the role of Dcs1 as an inhibitor of trehalase activ-

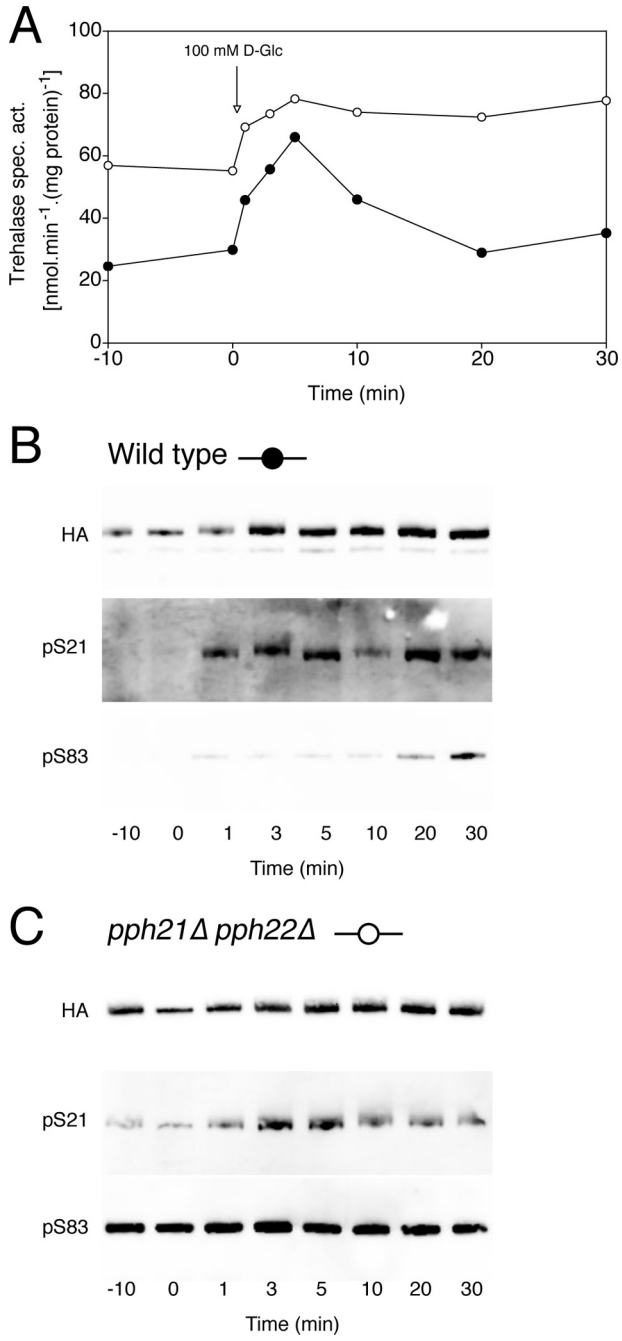

FIGURE 3. **Glucose-induced trehalase activation and phosphorylation in PP2A deletion strains.** Trehalase activity (*A*) and phosphorylation status of Ser[21] and Ser[83] in trehalase (*B* and *C*) after the addition of 100 mM glucose to respiring yeast cells. Strains were transformed with p*TPI1-NTH1-HA-URA3*. Shown are wild type (*B*, ●) and *pph21*Δ *pph22*Δ (*C*, ○). *B* and *C*, *top Western blot*, detection of total Nth1-HA with anti-HA Ab; *middle Western blot*, detection with anti-phospho-Ser[21] Ab (*pS21*); *bottom Western blot*, detection with anti-phospho-Ser[83] Ab (*pS83*).

ity. To gain more insight into the role of Dcs1 in trehalase regulation, we assessed nutrient-induced trehalase activation under a variety of conditions in a *dcs1*Δ strain. Because a *dcs1*Δ strain is unable to grow on non-fermentable carbon sources (51, 52), we tried several other methods of glucose deprivation but failed to obtain consistent, reproducible results for both activation and phosphorylation of trehalase (data not shown). Therefore, we focused on nitrogen-induced trehalase activation in nitrogen-deprived cells. The results showed that in a *dcs1*Δ strain, trehalase activity is constitutively high under nitrogen deprivation and fails to increase further upon the addition of asparagine. The phosphorylation pattern correlates well with enzyme activity: constitutive phosphorylation on both Ser[21] and Ser[83] under nitrogen deprivation and no further change after the addition of asparagine (Fig. 7, *C* and *E*). Moreover, trehalase isolated from the *dcs1*Δ strain was much more stable, suggesting that phosphorylation and/or activation of the enzyme increases its stability. We also show increased interaction between Nth1 and the 14-3-3 proteins as a function of time after the addition of nutrient and coinciding with the activation of trehalase. Deletion of *DCS1* results in constitutive interaction (Fig. 7, *C* and *E*). Using the yeast two-hybrid system, we confirmed that there is stronger interaction between trehalase and the 14-3-3 proteins in a *dcs1*Δ strain (Fig. 7*F*). This suggests that Dcs1 may inhibit trehalase activity by prevention of 14-3-3 binding.

DISCUSSION

Nutrient Activation of Trehalase in Vivo Is Associated with Phosphorylation on Putative PKA Recognition Sites—Our results have shown for the first time that rapid nutrient activation of trehalase *in vivo* is associated with rapid phosphorylation of the enzyme on two putative PKA phosphorylation sites, Ser[21] and Ser[83], in its N terminus. Other sites (*e.g.* Ser[23] and Ser[60]) may also become phosphorylated, but we were not able to obtain phosphospecific antibodies against these sites. The rapid phosphorylation *in vivo* is consistent with the view obtained from *in vitro* studies on trehalase activation (16–18) and from *in vivo* studies with mutants in the cAMP-PKA pathway (6, 8, 19–22) that PKA-mediated phosphorylation is an important mechanism underlying the rapid changes in trehalase activity observed *in vivo*. Nutrient stimulation of trehalase has not only been observed *in vivo* in the yeast *S. cerevisiae* but also in several other yeast and fungal species (26, 53, 54). Given the sequence conservation and similar predicted domains of the Nth1 neutral trehalase, this conclusion is probably valid also for neutral trehalases in other species.

Trehalase Activation as a Read-out for Activation of PKA in Vivo—Because of the regulation of PKA by allosteric cAMP-induced dissociation of the regulatory subunits from the catalytic subunits, the activity of PKA measured in cell extracts, either in the absence or in the presence of cAMP, does not reflect the actual activity *in vivo*. It only reflects the basal and maximal activity. Therefore, estimation of *in vivo* PKA activity is inferred from its effect on well established targets of the enzyme. Hence, the phosphorylation status of protein kinase substrates has been a popular read-out for inferring their *in vivo* activity. Also for PKA, phosphorylation of specific substrates has been proposed as a direct read-out for *in vivo* activity.

Although mutants with reduced activity of the PKA catalytic subunits always display reduced nutrient activation of trehalase *in vivo* (6, 8, 19–22) (Fig. 2), our current results unexpectedly showed that this reduced activation was not associated with reduced phosphorylation on the PKA consensus sites (Fig. 2). On the contrary, the Ser[21] and Ser[83] sites were constitutively phosphorylated. This allows several conclusions. First, it shows

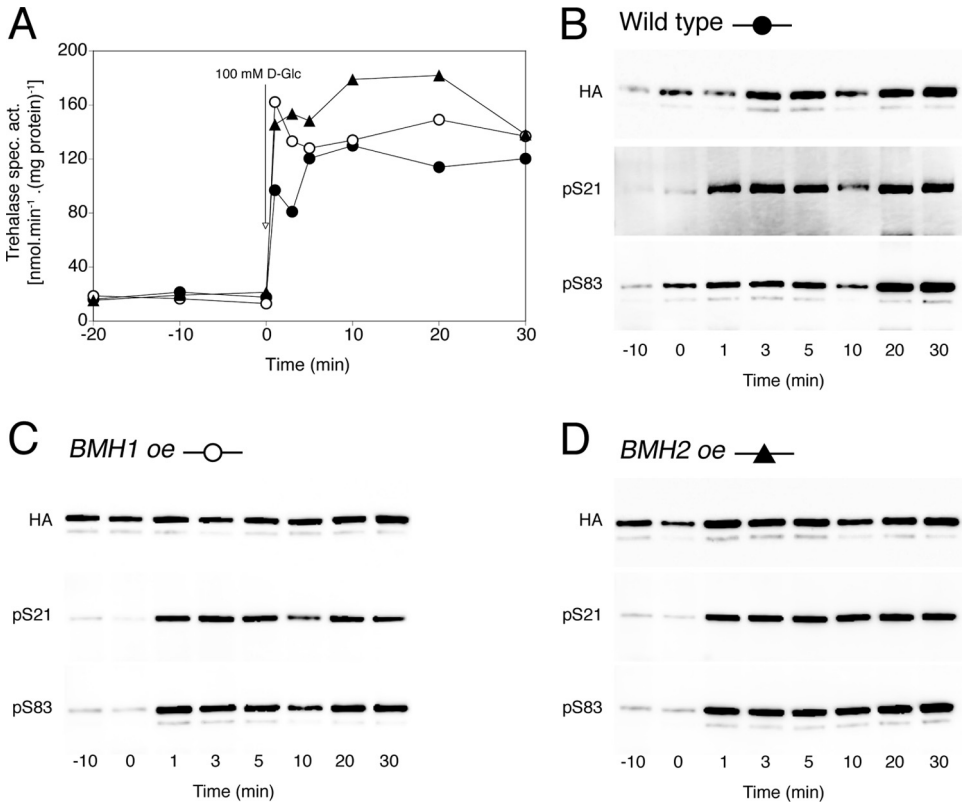

FIGURE 4. **Glucose-induced trehalase activation and phosphorylation in *BMH1* and *BMH2* overexpression strains.** Trehalase activity (*A*) and phosphorylation status of Ser[21] and Ser[83] in trehalase (*B–D*) after the addition of 100 mM glucose to respiring yeast cells. Strains shown are as follows: wild type (*B*, ●), *BMH1* overexpression (*C*, ○), and *BMH2* overexpression (*D*, ▲). BY4742 was transformed with p*TPI1-HA-URA3* (empty plasmid) and p*TPI1-NTH1-HA-LEU2* for wild type; with p*TPI1-BMH1-HA-URA3* and p*TPI1-NTH1-HA-LEU2* for *BMH1* overexpression; and with p*TPI1-BMH1-HA-URA3* and p*TPI1-NTH1-HA-LEU2* for *BMH2* overexpression.

that trehalase activity is a more reliable indicator for *in vivo* PKA activity than trehalase phosphorylation and probably also phosphorylation of PKA sites in other target proteins. Our results question the use of specific phosphorylation sites in substrate proteins as read-out for the activity of protein kinases *in vivo*. Second, it shows that phosphorylation of trehalase *in vivo* is not sufficient for its activation. It also raises the question as to what system is responsible for the constitutive phosphorylation. Reduction of PKA activity could have caused up-regulation of one or more (related) protein kinases able to phosphorylate trehalase on the same PKA recognition sites. However, for technical reasons (*i.e.* instability of the Nth1 protein), we were unable to explore the identity of possible alternative kinases (Sch9 and Yak1) responsible for the constitutive phosphorylation of trehalase. On the other hand, a yeast strain lacking the catalytic subunits of PP2A also showed constitutive trehalase phosphorylation, in agreement with previous results suggesting that PKA stimulates PP2A activity (12). Hence, reduced dephosphorylation by PP2A could explain the constitutive phosphorylation of trehalase in mutants with reduced PKA activity but does not exclude the involvement of alternative protein kinases.

Phosphorylation of Trehalase Is Not Enough for Activation—The constitutive phosphorylation of the Ser[21] and Ser[83] sites in mutant strains with reduced PKA activity or lacking PP2A activity shows that trehalase phosphorylation is not enough for its activation. This discrepancy can be explained in at least two ways. First, Panni *et al.* (30) observed that, although Bmh1 and Bmh2 can bind to trehalase when Ser[21] or Ser[23] is phosphorylated, they are unable to bind if both residues are phosphorylated. Hence, it could be that the mutant strains have an aberrant trehalase phosphorylation pattern, which prevents proper binding of the 14-3-3 proteins and thus also activation of the enzyme. Second, more recently, Wang *et al.* (55) demonstrated that the yeast 14-3-3 proteins, like their mammalian counterparts, are phosphoproteins themselves. They showed that Tpk1 could phosphorylate Bmh1 *in vitro* on Ser[238]. If this phosphorylation were required to promote binding of 14-3-3 to phosphorylated substrate proteins like trehalase, this might explain why activation of trehalase is impaired in a strain with reduced PKA activity despite the phosphorylation of the PKA sites.

The Role of 14-3-3 Proteins in Trehalase Activation—The molecular anvil hypothesis, proposed by Yaffe (56) to explain the modus operandi of the 14-3-3 proteins, can be applied to the trehalase activation mechanism. The structural rigidity of the 14-3-3 dimer forces trehalase to alter its conformation upon recruitment of the 14-3-3 dimer to its phosphorylated sites. Currently, it is unclear which kinetic property, either substrate affinity or V_{max}, of the trehalase enzyme is changed *in vivo* by the conformational change. Ortiz *et al.* (57) demonstrated that for *in vitro* activated trehalase, only V_{max} is altered.

The absence of phosphorylation on the two sites of trehalase in the 14-3-3 null strain can be explained in different ways. A simple explanation is that the 14-3-3 proteins protect the phos-

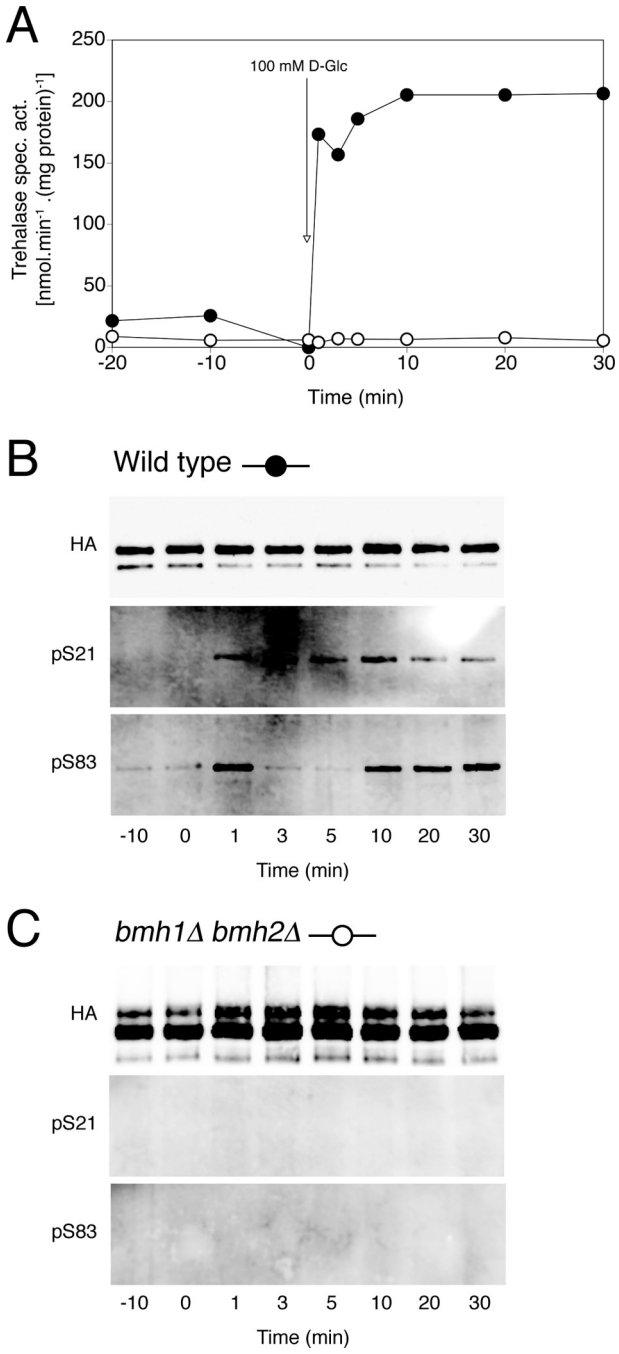

FIGURE 5. **Glucose-induced trehalase activation and phosphorylation in the *bmh1,2*Δ strain.** Trehalase activity (*A*) and phosphorylation status of Ser[21] and Ser[83] in trehalase (*B* and *C*) after the addition of 100 mM glucose to respiring yeast cells. Strains shown are as follows: wild type (*B*, ●) and *bmh1*Δ *bmh2*Δ strain (*C*, ○). Wild type and *bmh1*Δ *bmh2*Δ strains were transformed with p*TPI1-NTH1-HA-URA3*.

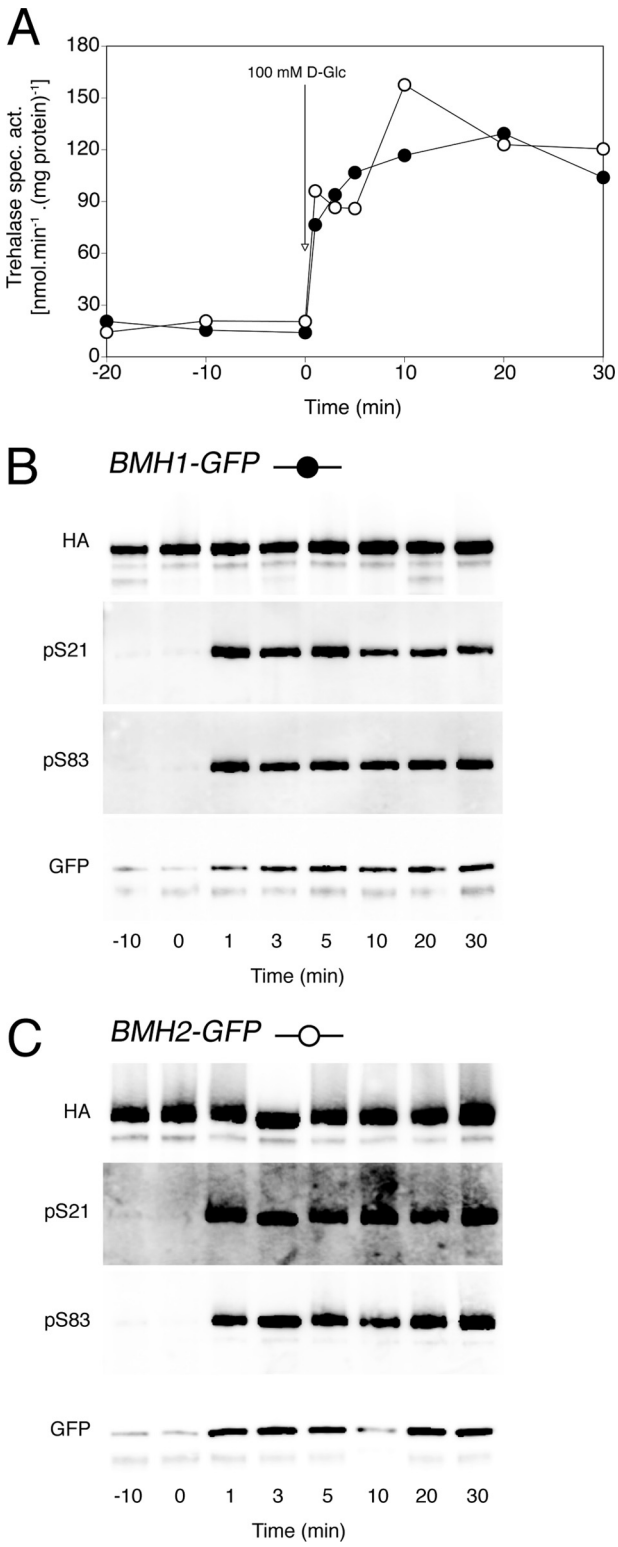

FIGURE 6. **Glucose-induced trehalase activation, phosphorylation and interaction with the Bmh1 and Bmh2 proteins.** Trehalase activity (*A*), phosphorylation status of Ser[21] and Ser[83] in trehalase (*B* and *C*), and interaction with Bmh1 (*B*, *bottom panel*) and Bmh2 (*C*, *bottom panel*) after the addition of 100 mM glucose to respiring yeast cells. The strains were transformed with plasmid p*TPI1-NTH1-HA-URA3* and were expressing in addition to Nth1-HA either *BMH1-GFP* (*B*, ●) or *BMH2-GFP* (*C*, ○). Trehalase was immunoprecipitated, and phosphorylation of Ser[21] and Ser[83] was visualized with phospho-specific Abs (*pS21* and *pS83*). In addition, anti-GFP Ab was used to visualize any interacting Bmh proteins (*GFP*).

phorylated sites in trehalase against dephosphorylation by protein phosphatases, like PP2A. On the other hand, the absence of the 14-3-3 proteins may have a more dramatic effect on the cell. Over 200 binding partners have been described for the yeast 14-3-3 proteins, and they have been implicated in many different cellular processes, including retrograde signaling, transcriptional regulation, nucleocytoplasmic shuttling, and enzyme activation (see Ref. 58 for a recent review). Hence, the possibility cannot be excluded that PKA itself or a component

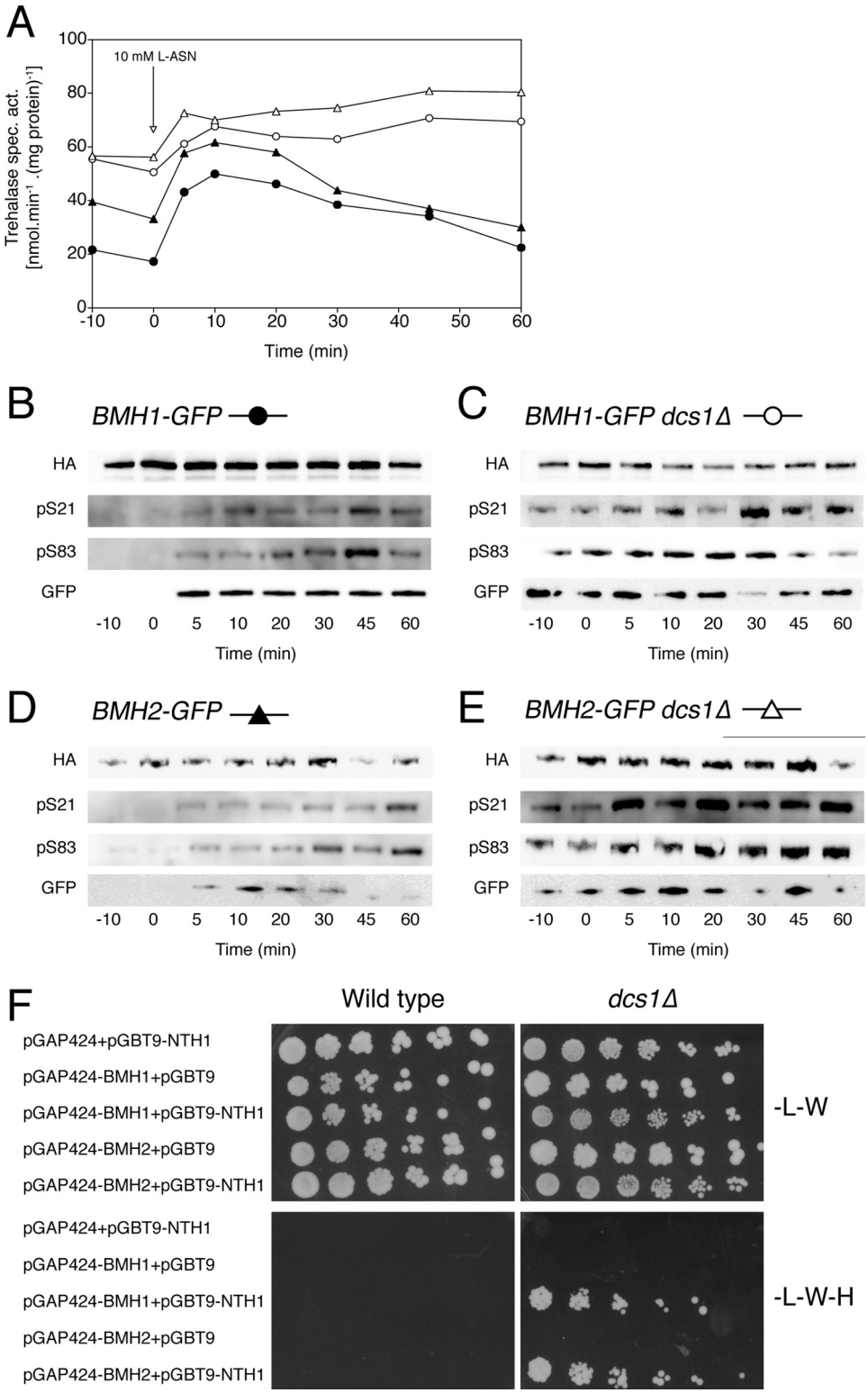

FIGURE 7. **Nitrogen-induced trehalase activation and phosphorylation in the *dcs1*Δ strain.** Trehalase activity (*A*) and phosphorylation status of Ser[21] (*pS21*) and Ser[83] (*pS83*) in trehalase (*B–E*) after the addition of 10 mM L-asparagine to fermenting, nitrogen-starved yeast cells. Wild type and *dcs1*Δ cells, expressing Bmh1-GFP or Bmh2-GFP, were transformed with p*TPI1-NTH1-HA-LEU2*. Trehalase was immunoprecipitated, and phosphorylation of Ser[21] and Ser[83] was visualized with phosphospecific Abs (pS21 and pS83). In addition, anti-GFP Ab was used to visualize any interacting Bmh proteins (*GFP*). *F*, two-hybrid analysis of direct physical interaction between Nth1 and the 14-3-3 proteins, Bmh1 and Bmh2. Two-hybrid reporter strains PJ69-4A wild type and PJ69-4A *dcs1*Δ, transformed with the indicated plasmids, expressing Nth1, Bmh1, and/or Bmh2, were grown overnight in liquid selective medium (SD−Leu−Trp) and spotted on selective SD−Leu−Trp (−*L*−*W*) and SD−Leu−Trp−His (−*L*−*W*−*H*) media.

of the signaling pathway involved in nutrient-induced activation of PKA is negatively affected by the absence of the 14-3-3 proteins. Certain phenotypic features of the 14-3-3 null strain suggest that PKA activity may be affected in such a strain. The cells flocculate spontaneously on YPD medium, and under nitrogen starvation conditions, pseudohyphal growth is enhanced.[3] Both of these properties are (partially) controlled by PKA. Moreover Gelperin *et al.* (59) described that *BMH1* overexpression could partially suppress the temperature sensitivity of the *cdc25-1* mutant, and *TPK1* overexpression could rescue a 14-3-3 null strain, indicating genetic interaction between the PKA pathway and the yeast 14-3-3 proteins.

Dcs1 Inhibits Trehalase by Prevention of 14-3-3 Binding—The action mechanism of the Dcs1 inhibitor of trehalase has remained enigmatic. Our results now show that deletion of Dcs1 enhances binding of the 14-3-3 proteins, which provides a logical explanation for its inhibitory effect. Dcs1 and 14-3-3 may function as competitive modulators, with Dcs1 having a preference for the unphosphorylated and 14-3-3 for the phosphorylated N terminus. In itself, this does not explain the enhanced phosphorylation of trehalase before the addition of nutrient (Fig. 7, *C* and *E*). However, lack of competition with Dcs1 may allow enhanced stabilization and protection of phosphorylated trehalase against dephosphorylation by the 14-3-3 proteins, shifting the equilibrium between unphosphorylated and phosphorylated forms toward the latter. This interpretation is supported by the enhanced stability of trehalase and by the correlation between enhanced activity and phosphorylation in the *dcs1Δ* strain.

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