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The *PDE1*-encoded Low-Affinity Phosphodiesterase in the Yeast *Saccharomyces cerevisiae* Has a Specific Function in Controlling Agonist-induced cAMP Signaling

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The yeast *Saccharomyces cerevisiae* contains two genes, *PDE1* and *PDE2*, which respectively encode a low-affinity and a high-affinity cAMP phosphodiesterase. The physiological function of the low-affinity enzyme Pde1 is unclear. We show that deletion of *PDE1*, but not *PDE2*, results in a much higher cAMP accumulation upon addition of glucose or upon intracellular acidification. Overexpression of *PDE1*, but not *PDE2*, abolished the agonist-induced cAMP increases. These results indicate a specific role for Pde1 in controlling glucose and intracellular acidification-induced cAMP signaling. Elimination of a putative protein kinase A (PKA) phosphorylation site by mutagenesis of serine^{252} into alanine resulted in a Pde1^{ala252} allele that apparently had reduced activity in vivo. Its presence in a wild-type strain partially enhanced the agonist-induced cAMP increases compared with *pde1*Δ. The difference between the Pde1^{ala252} allele and wild-type Pde1 was strongly dependent on PKA activity. In a *RAS2*^{val19} *pde2*Δ background, the Pde1^{ala252} allele caused nearly the same hyperaccumulation of cAMP as *pde1*Δ, while its expression in a PKA-attenuated strain caused the same reduction in cAMP hyperaccumulation as wild-type Pde1. These results suggest that serine^{252} might be the first target site for feedback inhibition of cAMP accumulation by PKA. We show that Pde1 is rapidly phosphorylated in vivo upon addition of glucose to glycerol-grown cells, and this activation is absent in the Pde1^{ala252} mutant. Pde1 belongs to a separate class of phosphodiesterases and is the first member shown to be phosphorylated. However, in vitro the Pde1^{ala252} enzyme had the same catalytic activity as wild-type Pde1, both in crude extracts and after extensive purification. This indicates that the effects of the S252A mutation are not caused by simple inactivation of the enzyme. In vitro phosphorylation of Pde1 resulted in a modest and variable increase in activity, but only in crude extracts. This was absent in Pde1^{ala252}, and phosphate incorporation was strongly reduced. Apparently, phosphorylation of Pde1 does not change its intrinsic activity or affinity for cAMP but appears to be important in vivo for protein-protein interaction or for targeting Pde1 to a specific subcellular location. The PKA recognition site is conserved in the corresponding region of the *Schizosaccharomyces pombe* and *Candida albicans* Pde1 homologues, possibly indicating a similar control by phosphorylation.

INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* contains two cAMP phosphodiesterases, Pde1 and Pde2, that are unrelated in primary sequence (Fujimoto *et al.*, 1974; Londesborough, 1974; Suoranta and Londesborough, 1984; Sass *et al.*, 1986; Nikawa *et al.*, 1987b). The high affinity Michaelis-Menten constant ([K_m] = 170 nM) cAMP phosphodiesterase Pde2 belongs to a well studied class of phosphodiesterases of which representatives have been found in many species, including mammals (Suoranta and Londesborough, 1984; Char-

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bonneau *et al.*, 1986). Several enzymes of this class are known to be regulated by phosphorylation and are involved in control of agonist-induced cAMP responses (Conti *et al.*, 1995). On the other hand, only four homologues of *S. cerevisiae* Pde1 are currently known (Wera *et al.*, 1997): they have been identified in *Vibrio fischeri* (Dunlap and Callahan, 1993), *Dictyostelium discoideum* (Lacombe *et al.*, 1986), *Schizosaccharomyces pombe* (DeVoti *et al.*, 1991), and *Candida albicans* (Hoyer *et al.*, 1994). Up to now there is no evidence for regulation of any one of these enzymes by phosphorylation. This report provides the first evidence that a member of this family is involved in controlling agonist-induced cAMP signaling and suggests that the enzyme is regulated in vivo by phosphorylation.

Pde1 displays a low affinity for cAMP with a K_m value that varies between 20 and 250 μM, depending on the assay conditions (Fujimoto *et al.*, 1974; Londesborough and Lukkari, 1980). Londesborough and Lukkari (1980) calculated that at 10 μM cAMP (the upper limit of the cAMP level they estimated to occur in yeast), at 30°C and pH 6.4, Pde1 can degrade 27 nmol cAMP/min/g. They suggested that, in spite of its high K_m, Pde1 might contribute significantly to the degradation of the high cAMP concentration that occurs in yeast cells after addition of glucose. This proposed function for Pde1, however, has never been supported by experimental evidence.

Addition of glucose to yeast cells grown on a nonfermentable carbon source results in a rapid and transient increase in intracellular cAMP (van der Plaat, 1974; Thevelein *et al.*, 1987b). A higher and longer-lasting cAMP spike occurs after intracellular acidification induced by protonophores such as 2,4-dinitrophenol (Trevillyan and Pall, 1979; Caspani *et al.*, 1985; Thevelein *et al.*, 1987a). cAMP synthesis in yeast cells is controlled by an elaborate pathway (reviewed by Broach and Deschenes, 1990; Thevelein, 1991, 1992; and Tatchell, 1993). Adenylate cyclase activity is largely dependent on the Ras proteins, the activity of which is controlled by the guanine nucleotide exchange proteins, Cdc25 and Sdc25, and the GTPase-activating proteins, Ira1 and Ira2. Recent work has shown that intracellular acidification, but not glucose, leads to a rapid increase in the ratio of GTP/GDP bound to the Ras proteins. On the other hand, for glucose activation of cAMP synthesis, another G protein, Gpa2, is required (Colombo *et al.*, 1998).

It is known that cAMP accumulation in yeast is strongly inhibited by protein kinase A (PKA)[1], since mutants with reduced activity of the protein kinase display hyperaccumulation of cAMP, while mutants with unbridled PKA activity display a reduced cAMP level (Nikawa *et al.*, 1987a). Also, for the glucose-induced cAMP signal, a close, inverse correlation is observed between the amplitude and duration of the cAMP spike and the activity of PKA (Mbonyi *et al.*, 1990). In strains lacking the two phosphodiesterases, the basal cAMP level is only elevated two- to threefold compared with the level in a wild-type strain, indicating that most of the feedback-inhibition on cAMP accumulation is independent of the phosphodiesterases, or at least that their effect can be mimicked by other mechanisms in such a genetic background (Nikawa *et al.*, 1987b). Several targets for this feedback-inhibition mechanism have been proposed: Cdc25 (Munder and Küntzel, 1989), Ras (Resnick and Racker, 1988), Ira (Tanaka *et al.*, 1989, 1990), and adenylate cyclase itself (De Vendittis *et al.*, 1986). Recent work has shown that the feedback inhibition apparently does not act through a mechanism influencing the ratio of GTP/GDP on the Ras proteins (Colombo *et al.*, 1998).

Although the transient nature of the glucose-induced cAMP signal correlates inversely with the activity of PKA and therefore with the intensity of the feedback-inhibition mechanism, the rapid decrease in cAMP levels after the initial increase appears to be more consistent with PKA-mediated activation of phosphodiesterase activity. Previous results have also indicated that phosphodiesterase activity in yeast might be activated by PKA-mediated phosphorylation. As opposed to a *RAS2*^{val19} strain, a *RAS2*^{val19} *pde1*Δ *pde2*Δ strain displays a very high cAMP level. This indicates that in a *RAS2*^{val19} strain the phosphodiesterases are able to prevent hyperaccumulation of cAMP. However, in a strain with reduced PKA activity, the phosphodiesterases are apparently unable to prevent cAMP hyperaccumulation (Nikawa *et al.*, 1987a). This is consistent with stimulation of phosphodiesterase activity by PKA (Thevelein, 1992).

It has been unclear up to now which phosphodiesterase, Pde1 or Pde2, or both, is responsible for the degradation of the elevated cAMP levels in yeast cells after stimulation with glucose or intracellular acidification and how the activity of this phosphodiesterase is controlled. In the present article we show that Pde1 is specifically involved in control of agonist-induced cAMP signaling and that it is most likely regulated by reversible phosphorylation. Our results identify serine^{252} of Pde1 as a possible target site of the feedback-inhibition mechanism involved in control of agonist-induced cAMP signaling.

MATERIALS AND METHODS

Strains, Media, and Growth Conditions

S. cerevisiae strains used in this work are shown in Table 1. All results shown were obtained with the strains in the W303–1A background except where otherwise stated. Composition of the growth media was as follows. Rich media contained 2% bacto-peptone, 1%

[1] Abbreviation used: PKA, protein kinase A.

Table 1. *Saccharomyces cerevisiae* strains used in this work

Strain	Genetic background	Relevant genotype	Complete genotype	Source and/or reference
W303-1A	W303-1A	wild type	*MAT***a** *leu2-3, 112 ura3-1 trp1-92 his3-11, 15ade2-1 can1-100 GAL SUC mal*	Thomas and Rothstein (1989)
PM941	W303-1A	*pde1Δ*	*MAT***a** *leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100 GAL SUC mal pde1::URA3*	This work
PM942	W303-1A	*pde2Δ*	*MAT***a** *leu2-3, 112 ura3-1 trp 1-92 his3-11, 15 ade2-1 can 1-100 GAL SUC mal pde2::URA3*	This work
PM943	W303-1A	*pde1Δ pde2Δ*	*MAT***a** *leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100 GAL SUC mal pde1::TRP1 pde2::URA3*	This work
PM850	W303-1A	wild type + YEplac195	*MAT***a** *leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100 GAL SUC mal* + YEplac195 *(URA3)*	This work
PM851	W303-1A	wild type + YEp*PDE1*	*MAT***a** *leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100 GAL SUC mal* + YEp*PDE1 (URA3)*	This work
PM852	W303-1A	wild type + YEp*PDE2*	*MAT***a** *leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100 GAL SUC mal* + YEp*PDE2 (URA3)*	This work
PM581	W303-1A	$pde1^{ala252}$	*MAT***a** *leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100 GAL SUC mal* $pde1^{ala252}$	This work
PM582	W303-1A	$pde1^{ala252}$ *pde2Δ*	*MAT***a** *leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100 GAL SUC mal* $pde1^{ala252}$ *pde2::URA3*	This work
PM541	W303-1A	*pde1Δ pde2Δ* + YCp*PDE1*	*MAT***a** *leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100 GAL SUC mal pde1::TRP1 pde2::URA3* + YCpL-*PDE1*	This work
PM542	W303-1A	*pde1Δ pde2Δ* + YCp$pde1^{ala252}$	*MAT***a** *leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100 GAL SUC mal pde1::TRP1 pde2::URA3* + YCpL-$pde1^{ala252}$	This work
TK161-R2V	SP1	$RAS2^{val19}$	*MAT***a** *leu2 his3 trp1 ade8 can1 ura3* $RAS2^{val19}$	Broek *et al.* (1985)
DC124	SP1	wild type	*Matα his4 leu2 ura3 trp1 ade8 can1*	M. Wigler (Cold Spring Harbor)
PM944	SP1	$RAS2^{val19}$ *pde1Δ*	*MAT***a** *leu2 his3 trp1 ade8 can1 ura3* $RAS2^{val19}$ *pde1::URA3*	This work
PM945	SP1	$RAS2^{val19}$ *pde2Δ*	*MAT***a** *leu2 his3 trp1 ade8 can1 ura3* $RAS2^{val19}$ *pde2::URA3*	This work
PM946	SP1	$RAS2^{val19}$ *pde1Δ pde2Δ*	*MAT***a** *leu2 his3 trp1 ade8 can1 ura3* $RAS2^{val19}$ *pde1::TRP1 pde2::URA3*	This work
PM584	SP1	$RAS2^{val19}$ $pde1^{ala252}$	*MAT***a** *leu2 his3 trp1 ade8 can1 ura3* $RAS2^{val19}$ $pde1^{ala252}$	This work
PM586	SP1	$RAS2^{val19}$ $pde1^{ala252}$ *pde2Δ*	*MAT***a** *leu2 his3 trp1 ade8 can1 ura3* $RAS2^{val19}$ $pde1^{ala252}$ pde2::URA3	This work
J105	SP1	*pde1Δ*	*MAT***a** *leu2 his3 ura3 trp1 ade8 can1 pde1::LEU2*	Nikawa *et al.* (1987b)
J104	SP1	*pde2Δ*	*MAT***a** *leu2 his3 ura3 trp1 ade8 can1 pde2::HIS3*	Sass *et al.* (1986)
J106	SP1	*pde1Δ pde2Δ*	*MAT***a** *leu2 his3 ura3 trp1 ade8 can1 pde1::URA3 pde2::HIS3*	Nikawa *et al.* (1987b)
PM975	SP1	$tpk1^{w1}$ *tpk2 tpk3 bcy1* + YCplac33	*MAT***a** *his3 leu2 ura3 trp1 ade8* $tpk1^{w1}$ *tpk2::HIS3 tpk3::TRP1 bcy1::LEU2* (RS13-58A-1) + YCplac33*(URA3)*	Nikawa *et al.* (1987a) This work
PM976	SP1	$tpk1^{w1}$ *tpk2 tpk3 bcy1* + YCp*PDE1*	*MAT***a** *his3 leu2 ura3 trp1 ade8* $tpk1^{w1}$ *tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 (RS13-58A-1)* + YCpU-*PDE1*	This work
PM977	SP1	$tpk1^{w1}$ *tpk2 tpk3 bcy1* + YCp$pde1^{ala252}$	*MAT***a** *his3 leu2 ura3 trp1 ade8* $tpk1^{w1}$ *tpk2::HIS3 tpk3::TRP1 bcy1::LEU2* (RS13-58A-1) + YCpU-$pde1^{ala252}$	This work
PM978	SP1	$tpk1^{w1}$ *tpk2 tpk3 bcy1* + YCp*PDE2*	*MAT***a** *his3 leu2 ura3 trp1 ade8* $tpk1^{w1}$ *tpk2::HIS3 tpk3::TRP1 bcy1::LEU2* (RS13-58A-1) + YCp*PDE2 (URA3)*	This work
PM979	SP1	$tpk1^{w1}$ *tpk2 tpk3 bcy1* + YCp$pde1^{asp252}$	*MAT***a** *his3 leu2 ura3 trp1 ade8* $tpk1^{w1}$ *tpk2::HIS3 tpk3::TRP1 bcy1::LEU2* (RS13-58A-1) + YCpU-$pde1^{asp252}$	This work
PM545	W303-1A	*PDE1-HA*	*MAT***a** *leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100 GAL SUC mal-PDE1-HA*	This work
PM546	W303-1A	$PDE1^{S252A}$-*HA*	*MAT***a** *leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100 GAL SUC mal* $pde1^{ala252}$-HA	This work

yeast extract, and 2% glucose (YPD). Synthetic media contained 0.67% yeast nitrogen base without amino acids (Difco, Detroit, MI) and 2% glucose (SDglucose) or 3% glycerol (SDglycerol), supplemented with the appropriate auxotrophic requirements. The cells

were grown at 30°C in the appropriate medium (as specified in the figure legends).

Plasmid and Strain Constructions

The vectors YCplac33, YCplac111, and YEplac195 (Gietz and Sugino, 1988) were used for the construction of new plasmids. The plasmids pJJ242 and pJJ246 (Jones and Prakash, 1990) were used as source of the marker genes for disruption of *PDE1* and *PDE2*. Plasmids pYT20 (Nikawa *et al*., 1987b) and pYEp*PDE2*-2 (Sass *et al*., 1986) were generous gifts of Michael Wigler (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Plasmids YCpU-*PDE1*, YCpL-*PDE1*, YEp*PDE1*, and pUC*PDE1* were constructed by subcloning the *Xba*I–*Sma*I fragment of pYT20 containing *PDE1* in between the corresponding sites of YCplac33, YCplac111, YEplac195, and pUC18. Plasmid p*pde1*::URA3 was constructed by inserting the *Bam*HI--*Pvu*II fragment of pJJ242 containing the yeast *URA3* gene in between the *Bam*HI--*Bal*I sites of pUC*PDE1*. The *Xmn*I–*Sna*BI fragment of p*pde1*::URA3 was used for the disruption of the *PDE1* genomic locus by homologous recombination. p*pde1*::*TRP1* was constructed by the same method as described for p*pde1*::*URA3*, except that the *Bam*HI–*Pvu*II fragment with *TRP1* from pJJ246 was used. Plasmids YCp*PDE2*, YEp*PDE2*, and pUC*PDE2* were constructed by subcloning the *Bam*HI–*Spe*I fragment of YEp*PDE2*-2 in between the *Bam*HI–*Xba*I site of YCplac33, YEplac195, and pUC19, respectively. The *Sph*I–*Pvu*II fragment of pJJ242 containing the yeast *URA3* gene was inserted in between the *Sph*I–*Hpa*I sites of pUC*PDE2* generating p*pde2*::*URA3*. This plasmid was linearized by *Ssp*I for disruption of the *PDE2* genomic locus by homologous recombination.

Construction of the $Pde1^{ala252}$, $Pde1^{asp252}$ Alleles and the Corresponding Yeast Strains

The $Pde1^{ala252}$ and $Pde1^{asp252}$ alleles were constructed by Megaprimer PCR-mediated site-directed mutagenesis (Sarkar and Sommer, 1990) using the outer primers, 5′-GTTCATCATGGGATAGGC-3′ and 5′-CGAGTATGGTTAGTCTTGG-3′, and the following mutagenic primers, respectively (mutation in bold), 5′-GATTCTTCAG**C**TTCTCT-GCG-3′ and 5′-GATTCTTCA**TC**TTCTCTGCG-3′. The resulting PCR products were digested by *Mfe*I and *Bss*HII and cloned in between the corresponding sites of YCpU-PDE1, YCpL-PDE1, and YEpPDE1 creating the YCpU-$pde1^{ala252}$, YCpL-$pde1^{ala252}$, YEp$pde1^{ala252}$ and YCpU-$pde1^{asp252}$, YCpL-$pde1^{asp252}$, YEp$pde1^{asp252}$ alleles. The entire cloned PCR fragments were sequenced confirming the nucleotide changes causing the ser252ala and ser252asp mutations, respectively, as the only nucleotide change. The *Hin*dIII–*Hin*cII fragments of YCp$pde1^{ala252}$ and YCp$pde1^{asp252}$ were inserted in between the corresponding sites of pUC19. The resulting constructs were digested with *Bam*HI and *Hin*cII and ligated with the *Bam*HI-*Sma*I fragment of pJJ242 containing the yeast *URA3* gene. These constructs were then cut by *Eco*RI and *Sma*I, and the *Eco*RI-*Bal*I fragments of YCp$pde1^{ala252}$ and YCp$pde1^{asp252}$ were inserted, creating plasmids pPAI3 and pPAS3, respectively. Yeast strains were transformed to ura^+ with the *Sma*I–*Hae*II fragment of pPAI3 or pPAS3 to replace the wild-type *PDE1* allele, which was confirmed by Southern hybridization. The ura^+ transformants were grown on rich medium (YPD) until stationary phase (2 d) and then plated on 5′-FOA plates to select for ura^- colonies in which the *URA3* gene was lost again by homologous recombination of the overlapping *pde1*^{ala252} or *pde1*^{asp252} sequences flanking the *URA3* gene (Boeke *et al*., 1984). Genomic DNA was isolated from the resulting strains for Southern hybridization, PCR, and sequence analysis to confirm that they carried the proper *pde1* mutant allele.

Epitope-Tagging of the Pde1 and $Pde1^{ala252}$ Alleles

For epitope tagging of the Pde1 and $Pde1^{ala252}$ alleles at the C terminus, the sequence from +643 to +1107 (ATG start codon = +1) was amplified by PCR using the following primers: 5′-gaattcATAG-GCGTCAAGACTGGCGCG-3′ and 5′-cccgggTAGAAACAAAGT-GTGGCCTTC-3′. The resulting PCR product was digested with *Eco*RI and *Sma*I and cloned in the corresponding sites of PYX012 (RD Systems, Minneapolis, MN) containing an hemagglutinin (HA)-epitope tag following the *Sma*I site. The plasmid was digested with *Mfe*I and integrated at the *PDE1* locus of a wild-type strain and a strain carrying a $Pde1^{ala252}$ allele.

Determination of cAMP and Phosphodiesterase Activity

For determination of the cAMP responses, exponentially growing cells (OD_{600} = 1.5) were harvested by centrifugation at 4°C, washed with ice-cold SD-complete medium without carbon source, and resuspended in the same medium. This cell suspension was preincubated at 30°C for 10 min. Subsequently, 100 mM glucose or 2 mM 2,4-dinitrophenol (from a stock solution of 80 mM in ethanol) was added as indicated. Samples containing 75 mg cells were used for determination of cAMP as described previously (Thevelein *et al*., 1987a). The activity of Pde1 was measured as described by Wera *et al*. (1997) by following the time-dependent degradation of cAMP. Samples and controls were incubated in 50 mM Tris-HCl (pH 8), 0.1 mM EDTA, and 500 μM cAMP at 30°C. The reaction was stopped by heating, and cAMP was measured using the cAMP [^{3}H] assay system (Amersham, Arlington Heights, IL).

Determination of Heat Shock Resistance

Yeast strains were pregrown either in rich medium (YPD) or in SDglucose-uracil medium (for plasmid maintenance) until OD_{600} = 2.0–2.5. The density of the cultures was adjusted to OD_{600} = 2 with the same medium before the heat shock was performed. Heat shocks were done for the indicated periods of time in a water bath at 50°C. A series of dilutions of treated and untreated cells was spotted on YPD plates, incubated for 2 d at 30°C, and then scored for growth and photographed.

Phosphodiesterase Pde1 Purification, Gel Electrophoresis, Western Blotting, and Protein Determination

Pde1 was purified from *pde1 pde2* cells overexpressing Pde1 or $Pde1^{ala252}$. Cells in the exponential phase of growth were lysed in buffer A (50 mM Tris-HCl, pH 8, 0.1 mM EDTA, 0.3 mM PMSF). After a high-speed centrifugation the extract was loaded on a mono Q (Pharmacia, Piscataway, NJ) ion-exchange column and eluted with a linear gradient from 0 to 500 mM NaCl in buffer A. An equal volume of 4 M $(NH_4)_2SO_4$ in buffer A was added to the Pde1-containing samples, and the mixture was immediately loaded on a Phenyl Resource column (Pharmacia) that was eluted with a linear gradient from 2 to 0 M $(NH_4)_2SO_4$ in buffer A. Pde1-containing samples were concentrated using a Vivaspin 10000 concentrator (Vivascience, Binbrook Lincoln, United Kingdom) and loaded on a Pharmacia Superdex75 column equilibrated in buffer A containing 100 mM NaCl. Purified Pde1 was concentrated as before and stored at −20°C. The final preparation displayed two bands after denaturing gel electrophoresis and silver staining: a 42.6-kDa band corresponding to Pde1 (as confirmed by Western blotting with a specific antibody: see below) and a 70-kDa band. Specific activities typically amounted to 13.8 nmol/min/mg, 140 nmol/min/mg, 620 nmol/min/mg, and 9900 nmol/min/mg in the crude extract and after mono Q, Phenyl Resource, and Superdex chromatography, respectively.

Western blotting was performed using an antibody raised against the synthetic peptide ‘CKSTPAKRDPRLTILE’ (Eurogentec, Liège, Belgium), corresponding to residues 328–342 of Pde1 plus an additional N-terminal cysteine. Specificity of the antibody was confirmed by Western blotting of extracts from *pde1*Δ- and Pde1-over-

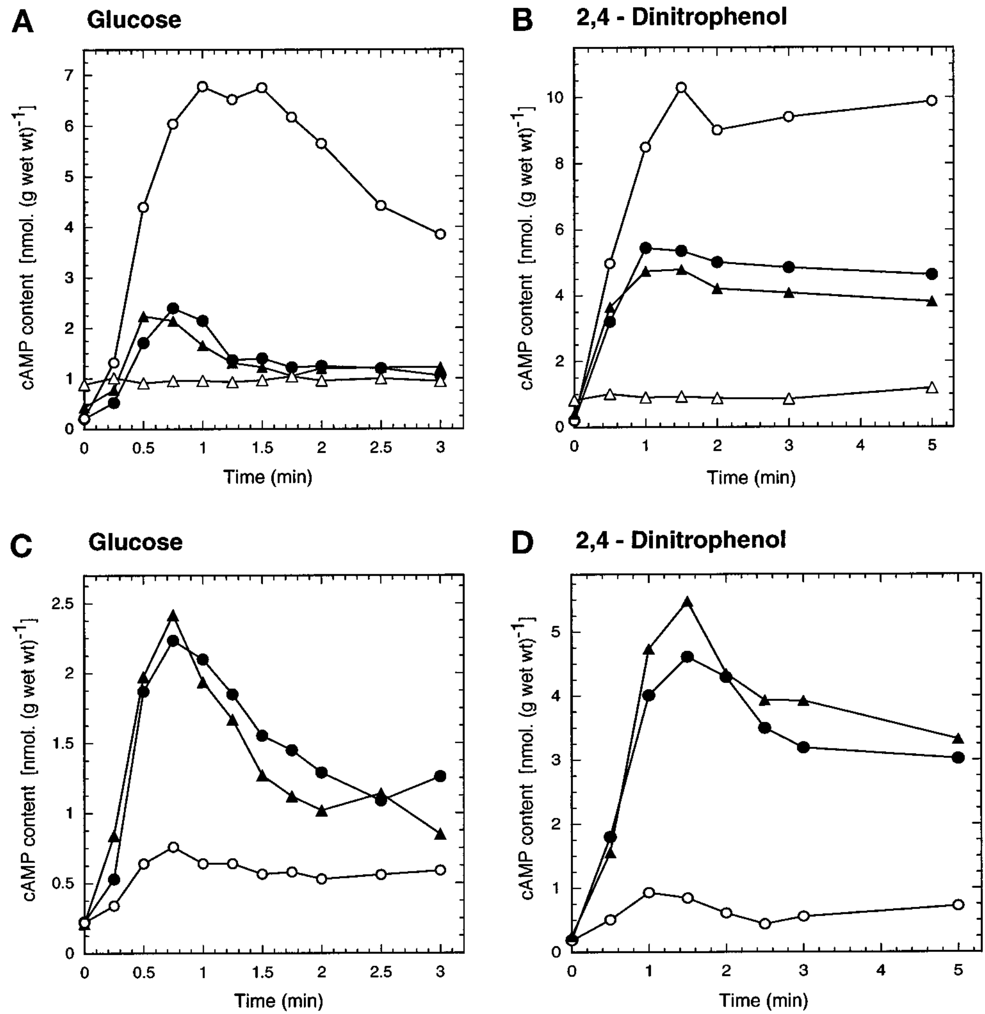

Figure 1. Intracellular cAMP level as a function of time after addition of 100 mM glucose (A and C) or 2 mM 2,4-dinitrophenol (B and D). (A and B) Phosphodiesterase-deficient strains: wild-type strain (W303–1A) (●); *pde1*Δ strain (PM941) (○); *pde2*Δ strain (PM942) (▲); and *pde1*Δ *pde2*Δ strain (PM943) (△). (C and D) Strains with overexpression of *PDE1* or *PDE2*: wild-type strain + YEplac195 (PM850) (●); wild-type strain + YEp*PDE1* (PM851) (○); wild-type strain + YEp*PDE2* (PM852) (▲).

expressing cells with preimmune and immune sera. Protein was determined using the Lowry method (Lowry *et al.*, 1951).

Phosphorylation of Pde1

***In Vitro*.** For phosphorylation of Pde1 in vitro, samples were incubated at 30°C for 30 min in the presence of 2 mM magnesium acetate, 0.1 mM ATP, and the catalytic subunit of bovine PKA (Sigma Chemical, St. Louis, MO). For labeling experiments 0.3 μCi/ml [γ^{32}P]-labeled ATP (Amersham) was included. For determination of the stoichiometry of phosphate incorporation, the purity of Pde1 was estimated at 50%.

***In Vivo*.** Cells, grown overnight to exponential phase in YP medium containing 2% glycerol, were harvested by centrifugation, washed once in water, and resuspended to OD_{600} = 5 in low-phosphate medium (Bio-101) containing 0.1% glucose and 2% glycerol. ^{32}P was added to a final concentration of 50–150 μCi/ml, and incubation was continued for another hour. ^{32}P incorporation was measured and was typically higher than 99%. Aliquots of 5 ml of cell suspension were prepared, and 2% glucose was added where appropriate. After 3 min, cells were harvested and extracts were prepared and immunoprecipitated with anti-HA antibodies (Boehringer Mannheim, Indianapolis, IN) and protein A Sepharose (Sigma) as described.

RESULTS

Pde1 Plays a Specific Role in Agonist-induced cAMP Signaling

When glucose is added to yeast cells grown on a nonfermentable carbon source, such as glycerol, a transient spike in the cAMP level is observed within ~1–2 min (Figure 1A). In a *pde1*Δ mutant, lacking the low-affinity cAMP phosphodiesterase, this cAMP signal was much higher (approximately threefold) and also longer-lived (Figure 1A). In the *pde2*Δ mutant, which lacks the high-affinity cAMP phosphodiesterase, the cAMP signal was not significantly changed (Figure 1A) or partially reduced (in the SP1 background, our unpublished results). In the *pde1*Δ *pde2*Δ strain the cAMP signal was virtually absent (Figure 1A). The latter strain always displayed a significantly higher (approximately twofold) basal cAMP level, as has been reported previously (Nikawa *et al.*, 1987b). Similar results were obtained with *pde*Δ mutants in the

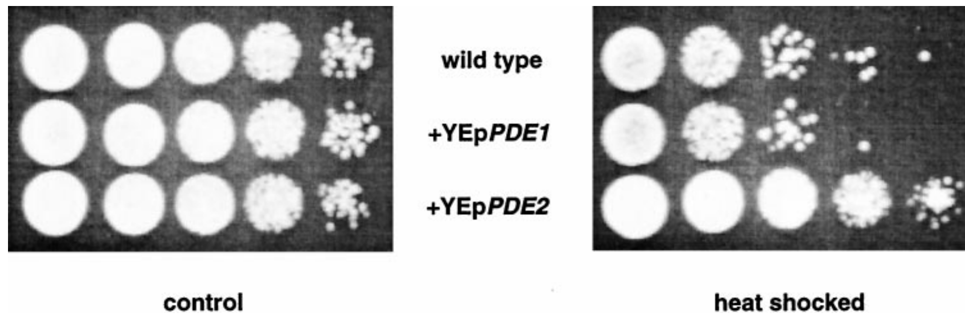

Figure 2. Cells of a wild-type strain (W303–1A) transformed with either YEp*PDE1* (PM851), YEp*PDE2* (PM852), or the empty vector YEplac195 (PM850) were grown in SD glucose-uracil medium until OD_{600} = 2.0–2.5, after which the OD was adjusted to 2 with the same medium. The cell suspension was heat shocked for 30 min at 50°C in a water bath. Treated and untreated cell suspensions were spotted on YPD plates and incubated at 30°C for 2 d. Serial dilutions were made with a factor of 10.

SP1 background (our unpublished results). The reduction or disappearance of the cAMP signal in the *pde2*Δ and *pde1*Δ *pde2*Δ strains seems at first sight contradictory, but can be explained by enhanced feedback inhibition of PKA on cAMP synthesis (see DISCUSSION).

Intracellular acidification triggered by addition of the protonophore 2,4-dinitrophenol at an extracellular pH of 6 to wild-type cells causes a higher and longer-lived increase in the cAMP level than glucose addition (Figure 1B). This intracellular acidification-induced cAMP increase was enhanced (approximately twofold) in the *pde1*Δ strain compared with the wild-type strain (Figure 1B). It was reduced to a variable extent in the *pde2*Δ strain and eliminated in the *pde1*Δ *pde2*Δ strain (Figure 1B). Similar results were obtained with *pde*Δ mutants in the SP1 background. The effects of the single *pde1*Δ and *pde2*Δ mutations were even somewhat more pronounced (our unpublished results).

Since these results pointed to a possible specific role of the Pde1 low-affinity phosphodiesterase in controlling agonist-induced cAMP signaling, we investigated glucose- and 2,4-dinitrophenol–induced cAMP stimulation in strains overexpressing Pde1 or Pde2. The overexpression of *PDE1* and *PDE2* was confirmed by Northern blotting. At least a tenfold higher level of *PDE1* or *PDE2* transcripts was detected (our unpublshed results). Figure 1C shows that the glucose-induced cAMP signal was not affected by overexpression of Pde2, but it was largely eliminated by overexpression of Pde1. Figure 1D shows that the same is true for the cAMP increase triggered by intracellular acidification.

To check whether overexpression of *PDE1* or *PDE2* affects the basal cAMP level significantly in vivo, we compared the heat resistance of the overexpression strains with that of the wild-type strain. This is a more sensitive in vivo assay than determination of the basal cAMP level, since the latter only changes slightly upon modification of the *PDE* genes separately (Nikawa *et al.*, 1987b). It is known that yeast strains with reduced activity of the cAMP pathway show enhanced heat resistance (Iida and Yahara, 1984; Shin *et al.*, 1987). Figure 2 shows that the strain with the multicopy *PDE2* plasmid displayed an enhanced heat resistance compared with the control strain (W303–1A). This most likely indicates that overexpression of *PDE2* reduces the basal cAMP level in vivo and also confirms that the Pde2–overexpression construct is functional. Interestingly, the strain with overexpression of the Pde1 enzyme did not show a significant change in heat resistance (Figure 2), indicating, most likely, that overexpression of this enzyme does not significantly affect the basal cAMP level during growth. These results show that Pde2, rather than Pde1, controls the basal cAMP level during growth. Similar results were obtained with yeast strains of the SP1 and M5 background expressing either Pde1 or Pde2 from the same plasmid.

Serine^{252}, a Putative PKA Phosphorylation Site, Is Important for Pde1 Activity In Vivo

Since previous data in the literature indicated a possible role of PKA-regulated phosphodiesterase activity in the control of cAMP levels in yeast (see INTRODUCTION), we have scanned the Pde1 sequence for putative PKA phosphorylation sites. At amino acid positions 249–252, an RRXS sequence was found, which is an ideal consensus site for phosphorylation by PKA. We have changed serine^{252} by site-directed mutagenesis into alanine (see MATERIALS AND METHODS). A strain with the wild-type Pde1 allele replaced by the mutant Pde1^{ala252} allele displayed an enhanced (approximately twofold) and also longer-lived glucose-induced cAMP signal compared with the wild-type strain (Figure 3A). However, the increase was not as high as in the *pde1*Δ strain. This seems to indicate that the *pde1*^{ala252} allele apparently displays a partial activity in vivo with respect to the control of glucose-induced cAMP accumulation. In a *pde1*^{ala252} *pde2*Δ strain, the glucose-induced cAMP signal was eliminated as was seen in the *pde1*Δ *pde2*Δ strain. Deletion of Pde2 combined with partial inactivation of Pde1 (at least as judged from the effect in vivo on cAMP accumulation) causes the same elimination of the cAMP signal as double deletion of Pde1 and Pde2.

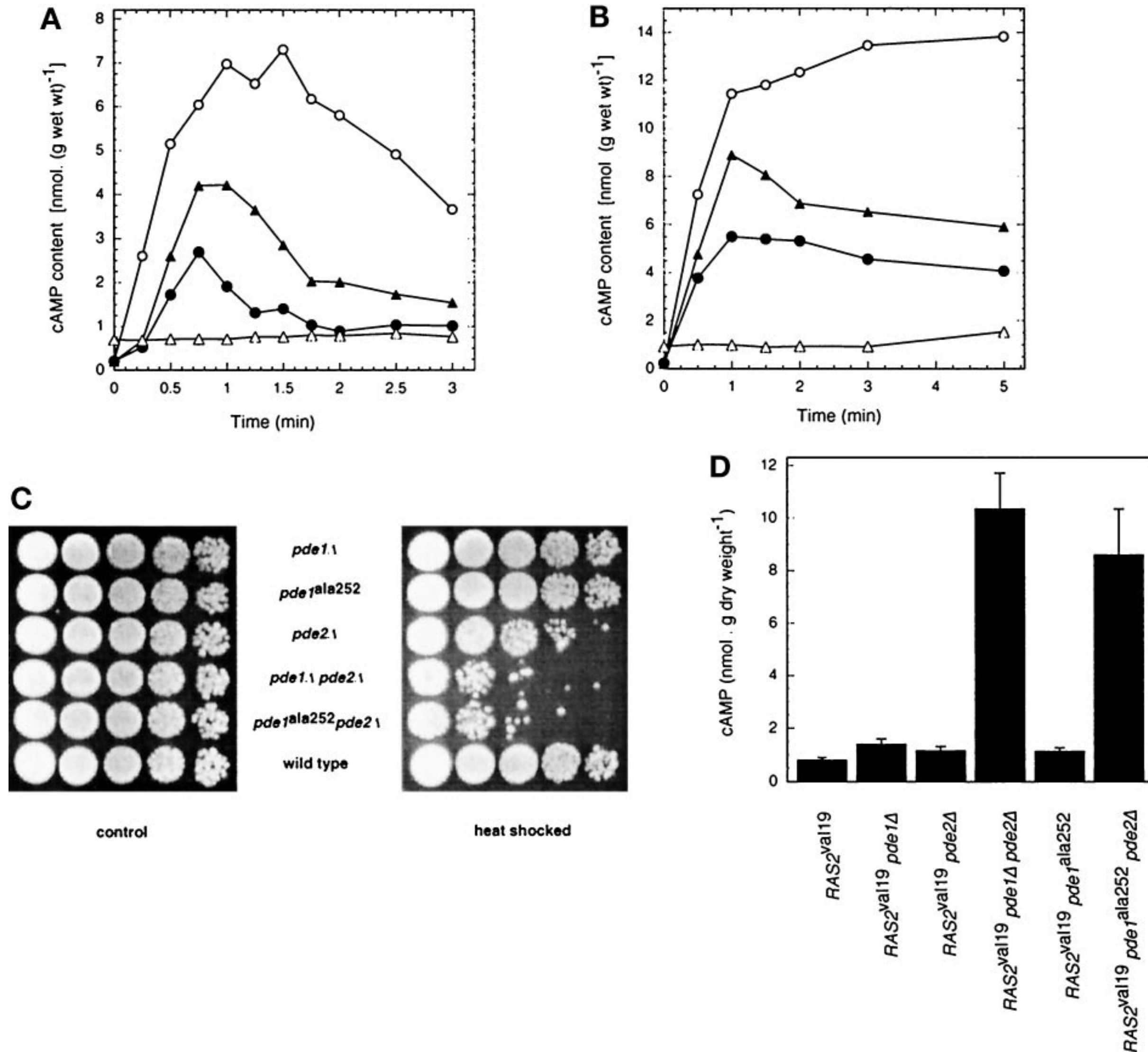

Figure 3. Intracellular cAMP level as a function of time after addition of 100 mM glucose (A) or 2 mM 2,4-dinitrophenol (B) in strains in which *PDE1* has been replaced by *pde1*^{ala252}: wild-type strain (W303–1A) (●); *pde1*Δ strain (PM941) (○); *pde1*^{ala252} strain (PM581) (▲); and *pde1*^{ala252} *pde2*Δ strain (PM582) (△). (C) Heat shock resistance of strains carrying different alleles of the *PDE* genes. Cells were grown in YPD medium until OD_{600} = 2.0–2.5, after which the OD was adjusted to 2 with the same medium. The cell suspension was heat shocked for 20 min at 50°C in a water bath. Treated and untreated cell suspensions were spotted on YPD plates and incubated at 30°C for 2 d. Serial dilutions were made with a factor of 10. (D) Basal cAMP level during exponential growth on YPD medium in *RAS2*^{val19} strains with deletion and/or modification of the phosphodiesterase genes.

The results obtained for the acidification-induced cAMP increase in the strains with the Pde1^{ala252} mutant allele were very similar to those obtained for the glucose-induced cAMP signal. The strain in which the wild-type Pde1 allele was replaced by the mutant Pde1^{ala252} allele showed a higher 2,4-dinitrophenol–induced cAMP increase, while additional deletion of *PDE2* in this strain practically eliminated the cAMP increase (Figure 3B). Reintroduction on a single-copy plasmid of the wild-type Pde1 allele, but not of the mutant Pde1^{ala252} allele, in the *pde1*Δ *pde2*Δ strain reduced the basal cAMP level and restored the glucose- and acidification-induced cAMP increases practically up to the level observed in the wild-type strain (our unpublished results).

We have also investigated whether substitution of serine^{252} by an aspartate residue might result in a phenotype indicating a constitutively activated Pde1 enzyme. We constructed a strain in which the wild-type Pde1 allele was replaced by the Pde1^{asp252} allele. The glucose- and acidification-induced cAMP increases were enhanced in a similar way in this strain

as in a strain where Pde1 was replaced by Pde1^{ala252} (our unpublished results). This shows that Pde1^{asp252} does not display higher catalytic activity and that apparently its activity in vivo is reduced to a similar extent as in Pde1^{ala252} because of the loss of the putative phosphorylation site.

All previous experiments have been performed with cells grown in the absence of glucose. In glucose-repressed cells, glucose is unable to trigger rapid cAMP accumulation. However, intracellular acidification produces a similar increase in the cAMP level in glucose-repressed and -derepressed wild- type cells (Beullens *et al.*, 1988; Argüelles *et al.*, 1990). In glucose-repressed cells we observed the same effects of the *PDE1* mutations as in derepressed cells. This means that in glucose-repressed cells of both the *pde1*Δ strain and the strain in which the wild-type *PDE1* gene has been replaced by the *pde1*^{ala252} allele, similar enhancements of the cAMP level upon intracellular acidification were observed compared with the level in the wild- type strain as were observed in glucose-derepressed cells (our unpublished results).

We have also investigated the effect of a *pde1* null allele and *pde1*^{ala252} on the heat shock resistance of the cells. Figure 3C shows that *pde1*^{ala252} in combination with *pde2*Δ has the same effect on heat shock resistance as *pde1*Δ combined with *pde2*Δ. In the presence of a wild-type *PDE2* allele, however, there is no significant difference in heat shock resistance between a strain carrying *PDE1*, *pde1*Δ, or *pde1*^{ala252} (Figure 3C). This indicates again that Pde1 itself has only little control over the basal cAMP level of the cells. Only in the absence of *PDE2* is there a clear effect of deletion of *PDE1*, and in this background the *pde1*^{ala252} allele behaves as an inactive allele in vivo.

To gain further evidence for the importance of the putative serine^{252} phosphorylation site in controlling Pde1 activity in vivo, we have also introduced the *pde1*^{ala252} allele in *RAS2*^{val19} strains. A *RAS2*^{val19} strain displays only a 2- to 3-fold higher cAMP level during growth on YPD medium compared with the wild-type strain (Broek *et al.*, 1985; Toda *et al.*, 1985). However, when the two *PDE* genes are deleted in a *RAS2*^{val19} strain, the cAMP level increases to very high values, similar to those observed in PKA-attenuated strains (Nikawa *et al.*, 1987a) (Figure 3D). The basal cAMP level during growth on YPD medium was only slightly higher (approximately twofold) in a *RAS2*^{val19} strain in which either *PDE1* or *PDE2* has been deleted compared with the level in the *RAS2*^{val19} strain (Figure 3D). This indicates that both phosphodiesterases are able to hydrolyze efficiently the very high cAMP level that accumulates in their absence in the *RAS2*^{val19} *pde1*Δ *pde2*Δ strain (Figure 3D). However, when the *pde1*^{ala252} allele was present in a *RAS2*^{val19} *pde2*Δ strain instead of the wild-type

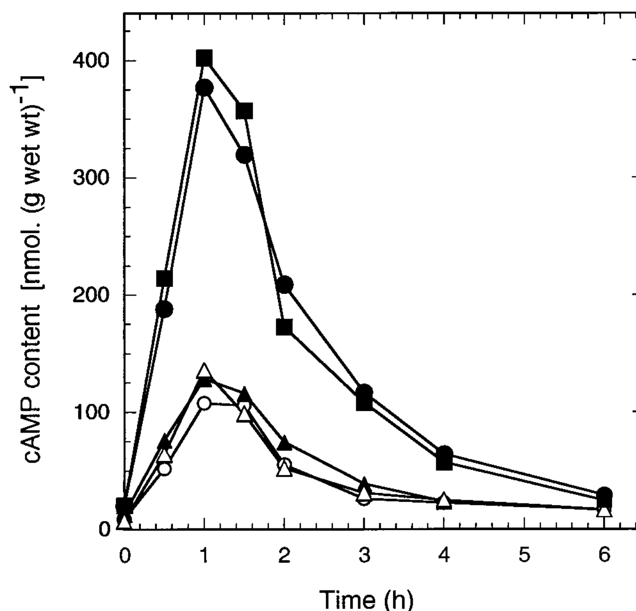

Figure 4. Intracellular cAMP level as a function of time after addition of 100 mM glucose to cells of a PKA-attenuated strain (*tpk1*^{w1} *tpk2*Δ *tpk3*Δ *bcy1*Δ) expressing an additional copy of distinct Pde1 alleles or Pde2 from the centromeric plasmid YCplac33. The cells were pregrown into exponential phase on SDglucose medium and resuspended after washing with water in the same medium without glucose. Control strain: empty YCplac33 (PM975) (●); YCplac33 + *PDE1* (PM976) (○); YCplac33 + *pde1*^{ala252} (PM977) (▲); YCplac33 + *pde1*^{asp252} (PM 979) (△); YCplac33 + *PDE2* (PM978) (■).

PDE1 allele, a very high cAMP level was observed, barely lower compared with the level in the *RAS2*^{val19} *pde1*Δ *pde2*Δ strain (Figure 3D). This indicates that the putative serine^{252} phosphorylation site in Pde1 is in some way essential for efficient hydrolysis of the very high cAMP level in the *RAS2*^{val19} *pde1*Δ *pde2*Δ strain.

We have also expressed the wild-type Pde1 allele and the Pde1^{ala252} and Pde1^{asp252} mutant alleles from the centromeric plasmid YCplac33 in a PKA-attenuated strain (*tpk1*^{w1} *tpk2*Δ *tpk3*Δ *bcy1*Δ) that displays an elevated basal cAMP level and a very high cAMP increase after addition of glucose (Nikawa *et al.*, 1987a; Mbonyi *et al.*, 1990). Figure 4 shows that expression of the wild-type Pde1 allele and the Pde1^{ala252} and Pde1^{asp252} mutant alleles resulted in the same reduction of the glucose-induced cAMP spike in the PKA-attenuated strain. This confirms that the Pde1^{asp252} allele does not display more activity than the other two alleles. Interestingly, in this strain the wild-type and Pde1^{ala252} displayed the same reduction in the cAMP spike, further supporting the idea that lack of PKA-mediated phosphorylation of the serine^{252} site in Pde1 lowers its activity in vivo to the same extent as substitution of serine^{252} by alanine. A control

experiment in which Pde2 was expressed from the same plasmid in this PKA-attenuated strain further confirmed that only Pde1 is able to down-regulate agonist-induced cAMP signaling (Figure 4).

The Serine^{252} Site Is Not Important for Pde1 Activity In Vitro

The results mentioned above demonstrate that Pde1 is involved in the feedback inhibition of cAMP accumulation and that serine^{252} of Pde1 is crucial for this phenomenon. A likely possibility to explain our results would hence be an activation of Pde1 through direct phosphorylation of serine^{252} by PKA. Serine^{252} is localized in a perfect PKA recognition site (Kennelly and Krebs, 1991). However, additional potential PKA phosphorylation sites are present in the Pde1 sequence (such as threonine^{154}). On the other hand, an alternative explanation for our results is that the serine^{252} residue is essential for catalytic activity of Pde1 and that its replacement by an alanine residue simply generates a completely or partially inactive enzyme.

To distinguish between these possibilities, we have purified wild-type Pde1 enzyme and the Pde1^{ala252} mutant enzyme from cells overexpressing one of the two types to near homogeneity using ion-exchange chromatography, hydrophobic-interaction chromatography, and gel filtration as described in the MATERIALS AND METHODS. A purified preparation separated with SDS-PAGE and visualized with silver staining is shown in Figure 5A. Both wild-type and mutant preparations behaved identically on denaturing gel electrophoresis (our unpublished results). The 42.6-kDa band could be identified as Pde1 on the basis of its recognition by specific Pde1 antibodies (see MATERIALS AND METHODS).

Purified preparations of the wild-type Pde1 enzyme and the Pde1^{ala252} mutant form displayed a very similar specific phosphodiesterase activity (7 $\pm$ 1.8 μmol cAMP/min/mg for wild-type Pde1 and 7 $\pm$ 0.2 μmol cAMP/min/mg for Pde1^{ala252}). This implies that serine^{252} is not essential for catalytic activity of Pde1 and that our results cannot be explained simply by loss of Pde1 catalytic activity.

Hence, it is most likely that serine^{252} is important because it is a phosphorylation site of Pde1 and that its phosphorylation, possibly by PKA, is crucial for feedback inhibition. To check whether Pde1 is a substrate of PKA, we incubated a purified preparation (cf. Figure 5A) of wild-type Pde1 with a commercial preparation of bovine heart PKA and [γ^{32}P]-labeled ATP. As shown in Figure 5B, this led to incorporation of radioactive label in a band corresponding to the position of Pde1. Incubation of Pde1 and labeled ATP alone or PKA and labeled ATP alone did not lead to incorporation of label in the 42.6-kDa band (Figure 5B). We can thus conclude that Pde1 is indeed a substrate for PKA. This phosphorylation had, however, no effect on the phosphodiesterase activity of the purified preparation as shown in Figure 5C. On average, the activity of phosphorylated Pde1 was 100.2 $\pm$ 6.1% of the control activity (n = 3). Since this Pde1 assay is performed at a high (500 μM) cAMP concentration, we also checked whether phosphorylation had any effect on the phosphodiesterase activity at the much lower concentration of 10 μM. Under these more physiological conditions, however, phosphorylation of purified Pde1 also remained without significant effect on the activity (our unpublished results).

Incubation of the mutant form Pde1^{ala252} with bovine heart PKA and [γ^{32}P]-labeled ATP still resulted in incorporation of label (Figure 5B). This shows that Pde1 has at least one other in vitro phosphorylation site in addition to serine^{252}. However, determination of the stoichiometry of phosphate incorporation (see MATERIALS AND METHODS) showed that it was reduced from 1.25 mol/mol in the wild-type strain to 0.4 mol/mol for the Pde1^{ala252} allele. This indicates that mutation of the serine^{252} residue eliminates a major part of the phosphorylation of Pde1.

We then investigated whether Pde1 was phosphorylated in vivo upon addition of glucose, a physiological condition known to stimulate PKA activity. To do so we labeled wild-type yeast cells, in which the original *PDE1* gene was replaced with either an HA-tagged version of this gene or with an HA-tagged version of *pde1*^{ala252}, with ^{32}phosphate in YP medium containing glycerol as carbon source. Cell extracts were prepared before and 2 min after addition of 2% glucose. Pde1 was subsequently isolated using anti-HA antibodies and analyzed by electrophoresis, blotting, and autoradiography. Addition of glucose resulted in the incorporation of radioactive label in wild-type Pde1, but not in the Pde1^{ala252} allele (Figure 5D). Further characterization of the labeled residue was hampered by the very low concentration of Pde1 in yeast cells. Based on the purification data reported by Fujimoto *et al.* (1974) and Londesborough (1974), we calculated that 10 g of yeast cells contains only 11–13 μg ($\pm$ 0.55 nmol) of Pde1. The in vivo labeling experiments could only be performed with a strain without overexpression of Pde1, since overexpression of this enzyme abolishes the cAMP signal (see above) necessary for PKA activation. Nevertheless, the observation that wild-type Pde1, but not Pde1^{ala252}, was phosphorylated in vivo strongly supports that serine^{252} represents an in vivo phosphorylation site.

In view of the importance of serine^{252} for feedback inhibition of cAMP accumulation, we are bound to conclude that, although the phosphorylation of Pde1 by PKA has no direct effect on phosphodiesterase activity of the purified preparation in vitro, it should have an indirect effect in vivo. This could be exerted,

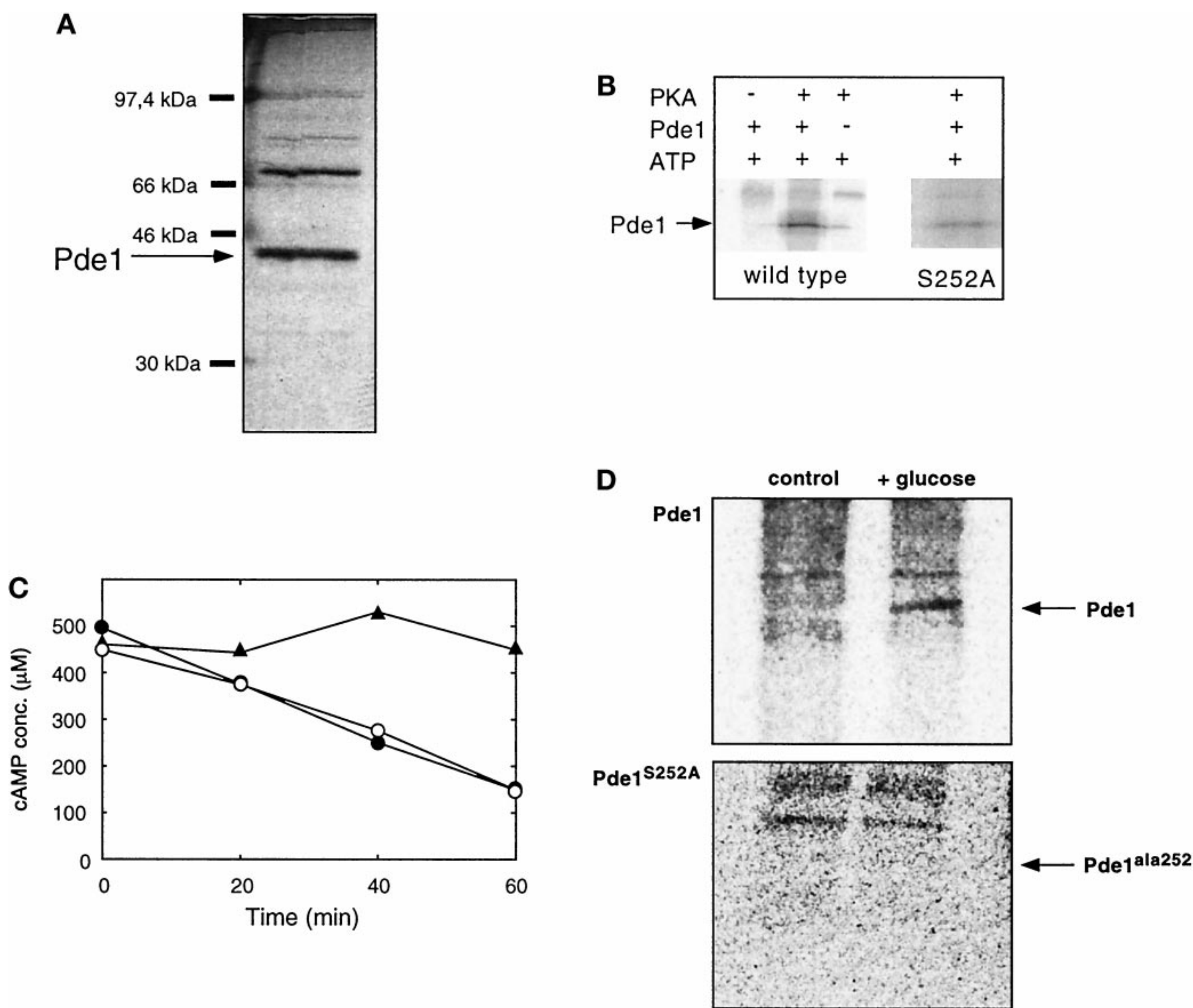

Figure 5. Purification and phosphorylation of Pde1. (A) SDS-PAGE and protein silver staining of highly purified Pde1 preparation. Pde1 has a molecular mass of 42.6 kDa. (B) Phosphorylation of wild-type Pde1 (250 ng) with PKA and [γ^{32}P]-labeled ATP results in incorporation of label into a band with the same molecular mass as Pde1. In control incubations, either the kinase or Pde1 was omitted. Samples were analyzed by SDS-PAGE and autoradiography. The arrow denotes the migration position of Pde1 (42.6 kDa). The right panel shows phosphorylation of Pde1^{ala252}, which results in much weaker incorporation of label at the same position (see RESULTS). This panel was exposed about 2.5 times longer than the panel of the wild-type strain. (C) A purified preparation of Pde1 was incubated with ATPMg and PKA as described in MATERIALS AND METHODS. Both samples and a negative control (buffer) were subsequently incubated in 100 μl buffer A containing 50 nmol (500 μM) cAMP. At the indicated time points aliquots were taken and assayed for cAMP. Purified Pde1 not treated with PKA (●) or treated with PKA (○); negative control, absence of purified Pde1 (▲). A typical result is shown. (D) HA-tagged Pde1 was immunoprecipitated from glycerol-grown ^{32}P-labeled cells of a wild-type strain (W303–1A) and a Pde1^{ala252} strain, before or after addition of 2% glucose, and analyzed by electrophoresis and autoradiography. The arrow denotes the migration position of Pde1 or Pde1^{ala252}; background signals correspond to unspecifically labeled protein A.

for instance, by targeting the phosphorylated form of Pde1 to a specific subcellular location where cAMP degradation preferentially takes place, or by interaction of phosphorylated Pde1 with an activator of phosphodiesterase activity. With respect to the latter possibility, it is noteworthy that incubation of crude extracts from yeast cells overexpressing wild-type Pde1 with MgATP and PKA led to a moderate and highly variable (154 ± 15% of unphosphorylated control, n = 6) increase in phosphodiesterase activity (Figure 6). This increase was not observed after incubation of crude extracts from yeast cells overexpress-

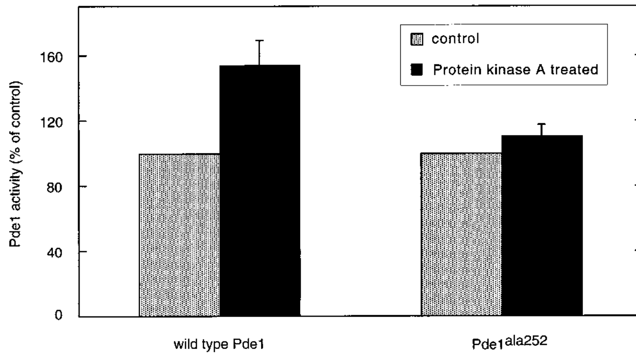

Figure 6. Effect of PKA treatment on Pde1 activity in total crude cell extracts from a wild-type strain (W303–1A) and a strain in which *PDE1* has been replaced by *pde1*^{ala252} (PM581).

ing the mutant Pde1^{ala252} form (110 ± 8% of unphosphorylated control, n = 3) (Figure 6), indicating the importance of serine^{252} for this activation. The activation of wild-type Pde1 by PKA treatment was no longer observed after the first purification step (mono Q chromatography), suggesting that an interacting protein important for the activation was lost during this step.

DISCUSSION

A Specific Function for Pde1 in Controlling Agonist-induced cAMP Signaling

All previous studies on the two yeast phosphodiesterases, Pde1 and Pde2, indicated a much more prominent role for the high-affinity cAMP phosphodiesterase, Pde2, compared with the low-affinity cAMP phosphodiesterase, Pde1. For all phenotypic characteristics controlled by PKA investigated, deletion of *PDE2* always caused strong effects while deletion of *PDE1* had small-to-negligible effects (Sass *et al.*, 1986; Nikawa *et al.*, 1987b). The function of Pde1 has always been enigmatic because of its very low affinity for cAMP, at least as measured in vitro. The K_m of Pde1 in vitro is one order of magnitude higher than the estimated basal cAMP concentration in vivo (Londesborough, 1974; Londesborough and Lukkari, 1980). However, it was proposed that this low-affinity phosphodiesterase could be involved in degrading the high cAMP levels that transiently occur in yeast cells after stimulation with glucose (Londesborough and Lukkari, 1980). We have now obtained experimental evidence for a specific role of Pde1, as opposed to Pde2, in controlling glucose- and also acidification-induced stimulation of cAMP accumulation. The following results are indicative for such a function: 1) deletion of Pde1, but not of Pde2, results in much higher glucose- and acidification-induced cAMP accumulation (Figure 1, A and B); 2) overexpression of Pde1, but not Pde2, abolishes glucose- and acidification-induced cAMP accumulation (Figure 1, C and D); and 3) overexpression of Pde2, but not of Pde1, enhanced the basal heat resistance of the cells (Figure 2), which is indicative of a lower basal cAMP level (Iida and Yahara, 1984; Shin *et al.*, 1987). Hence, Pde1 appears to have a specific role in down-regulating agonist-induced cAMP increases (in transient, adaptation conditions), while Pde2 specifically controls the basal cAMP level in the cell (in stable conditions, i.e., during growth and in stationary phase).

Pde1 Activity Is Most Likely Controlled by Phosphorylation In Vivo

In addition to providing experimental evidence that Pde1 is specifically involved in controlling agonist-induced cAMP accumulation, our data suggest that its activity is controlled by phosphorylation. Previous results have pointed to the possibility that phosphodiesterase activity in yeast might be controlled by PKA-mediated phosphorylation. Deletion of the two phosphodiesterase genes in a *RAS2*^{val19} strain caused a very high increase in the cAMP level, but in a strain with attenuated PKA activity there is a similar very high cAMP level in spite of the presence of the phosphodiesterases (Nikawa *et al.*, 1987a). This indicates that high PKA activity in some way is required for efficient breakdown of cAMP by the phosphodiesterases (Thevelein, 1992). In the present article we show that this is true for both Pde1 and Pde2 since the presence of either Pde1 or Pde2 in a *RAS2*^{val19} strain is sufficient to lower the cAMP level to about the wild-type level (Figure 3D). We have also demonstrated previously that the feedback inhibition on cAMP accumulation plays a role in down-regulating the glucose-induced cAMP signal. In yeast strains with a different activity level of PKA, the glucose-induced cAMP signal was inversely correlated with PKA activity (Mbonyi *et al.*, 1990). This suggests that agonist-induced cAMP accumulation is down-regulated by cAMP itself through PKA-mediated stimulation of phosphodiesterase activity.

In the present article we have provided several arguments for regulation of Pde1 by PKA-mediated phosphorylation. 1) Mutagenesis of a putative PKA phosphorylation site (serine^{252}) in Pde1 causes dramatic effects on cAMP accumulation in vivo (Figure 3). The most striking quantitative difference between wild-type Pde1 and the Pde1^{ala252} allele was observed in a *RAS2*^{val19} *pde2*Δ background. The presence of Pde1 in such a background resulted in about the same cAMP level as in wild-type or *RAS2*^{val19} strains, whereas the presence of Pde1^{ala252} in the same background resulted in a similar very high cAMP level as observed in the *RAS2*^{val19} *pde1*Δ *pde2*Δ strain (Figure 3D). This result is in agreement with the conclusion

that phosphorylation of the serine^{252} site influences the in vivo cAMP phosphodiesterase activity of Pde1 to such an extent that it is able to hydrolyze very efficiently the huge cAMP levels that acumulate in a *RAS2*^{val19} strain. 2) In a PKA-attenuated strain (*tpk1*^{w1} *tpk2*Δ *tpk3*Δ *bcy1*Δ) there was no difference between wild-type Pde1 and the Pde1^{ala252} allele in their capacity to reduce the cAMP level (Figure 4). This corroborates that PKA-mediated phosphorylation of the serine^{252} site is required for the difference in activity in vivo between the wild-type Pde1 and mutant Pde1^{ala252} allele. 3) The Pde1 enzyme can be phosphorylated in vitro with PKA (Figure 5B) and in vivo by addition of glucose to derepressed cells (Figure 5D). Phosphorylation of the Pde1^{ala252} allele was strongly reduced in vitro (Figure 5B) while it was not phosphorylated at all in vivo under the same experimental conditions as the wild-type allele (Figure 5D). The activity of Pde1 in vitro was not affected by site-directed mutagenesis of serine^{252} nor did PKA treatment of purified Pde1 affect its activity. The value of 1.25 mol/mol obtained for the stoichiometry of phosphate incorporation in vitro into the wild-type Pde1 allele indicates that insufficient phosphate incorporation cannot be the cause of the absence of effect of phosphorylation on the enzymatic activity. Moreover, in the Pde1^{ala252} allele the stoichiometry was reduced to 0.4 mol/mol indicating a major effect of serine^{252} mutagenesis on the phosphorylation of the enzyme. The fact that the wild-type Pde1 and mutant Pde1^{ala252} alleles displayed the same enzymatic activity in vitro is interesting because it clearly demonstrates that site-directed mutagenesis of serine^{252} does not simply abolish or reduce the activity of the enzyme, which would have made this manipulation similar to a deletion or partial inactivation. It shows that serine^{252} is important for another reason, presumably as a phosphorylation site. The absence of in vivo phosphorylation of the Pde1^{ala252} allele is consistent with the latter. In crude extracts a modest increase in activity of Pde1 could be observed upon PKA treatment, suggesting that phosphorylation of Pde1 could lead to interaction with an activating protein (Figure 6). Taken together, our results indicate that serine^{252} of Pde1 is not essential for catalytic activity, but most likely represents a PKA phosphorylation site of which the phosphorylation promotes interaction of Pde1 with protein(s) that in some way enhances its cAMP phosphodiesterase activity or at least renders it much more efficient in hydrolyzing cAMP in vivo. Interaction with such an activating protein in vivo might explain why Pde1, in spite of its high, supraphysiological K_m *in vitro*, exerts an important effect on agonist-induced cAMP signaling. At present we cannot exclude, however, that in vivo a PKA-induced kinase (rather than PKA itself) phosphorylates serine^{252}. Such an indirect effect of PKA would offer an alternative explanation why in vitro phosphorylation of Pde1 with PKA remains without effect.

Interference with Feedback Inhibition of cAMP Synthesis

Our results on the effect of *PDE* deletion on agonist-induced cAMP signaling illustrate that interpretation of the effects observed is not straightforward. Normally one would expect reduction of phosphodiesterase activity to result in higher cAMP increases. However, this is only observed for *PDE1* deletion (Figure 1, A and B). Deletion of *PDE2* causes either a slight decrease or no significant effect, while more strikingly double deletion of *PDE1* and *PDE2* causes a complete elimination of the agonist-induced cAMP increases (Figure 1, A and B). The basal cAMP level was clearly enhanced in the *pde1*Δ *pde2*Δ strain and in the *pde1*^{ala252} *pde2*Δ strain (Figures 1 and 3), and *pde1*Δ *pde2*Δ strains are well known to display a phenotype indicative of elevated PKA activity (Sass *et al.*, 1986; Nikawa *et al.*, 1987b). Therefore, a likely explanation for the absence of the increases of cAMP in these strains is that the elevated PKA activity causes constitutively high feedback inhibition of cAMP synthesis. As a result, agonist-induced cAMP signaling is constitutively down-regulated. A similar elimination of the agonist-induced cAMP increases has been observed in *bcy1*Δ strains, which also display constitutively high PKA activity (Colombo *et al.*, 1998).

The Pde1 Class of Phosphodiesterases

At present, only four phosphodiesterases with homology to Pde1 have been identified (Wera *et al.*, 1997). The enzymes of this class seem to have rather versatile functions, but interestingly both the *C. albicans* (Hoyer *et al.*, 1994) and *S. pombe* (DeVoti *et al.*, 1991) Pde1 homologues contain a PKA recognition site at a similar location C-terminal of the conserved catalytic domain, serine^{238} and serine^{251}, respectively. This part of the Pde1 homologues is otherwise not well conserved. The presence of the conserved PKA recognition site might indicate that the *C. albicans* and *S. pombe* Pde1 enzymes are also involved in controlling agonist-induced cAMP signaling. The existence of a specific cAMP phosphodiesterase for control of this process in *S. cerevisiae* underscores the physiological importance of rapid cAMP signaling. A proper, precisely modulated response to sudden changes in the nutrient supply might provide a selective advantage, not only in yeasts but also in other microorganisms.

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