The localization and concentration of the PDE2-encoded high-affinity cAMP phosphodiesterase is regulated by cAMP-dependent protein kinase A in the yeast Saccharomyces cerevisiae

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Keywords

cyclic AMP; high-affinity cAMP phosphodiesterase; Pde2; nuclear localization; protein kinase A; Saccharomyces cerevisiae.

Abstract

The genome of the yeast Saccharomyces cerevisiae encodes two cyclic AMP (cAMP) phosphodiesterases, a low-affinity one, Pde1, and a high-affinity one, Pde2. Pde1 has been ascribed a function for downregulating agonist-induced cAMP accumulation in a protein kinase A (PKA)-governed negative feedback loop, whereas Pde2 controls the basal cAMP level in the cell. Here we show that PKA regulates the localization and protein concentration of Pde2. Pde2 is accumulated in the nucleus in wild-type cells growing on glucose, or in strains with hyperactive PKA. In contrast, in derepressed wild-type cells or cells with attenuated PKA activity, Pde2 is distributed over the nucleus and cytoplasm. We also show evidence indicating that the Pde2 protein level is positively correlated with PKA activity. The increase in the Pde2 protein level in high-PKA strains and in cells growing on glucose was due to its increased half-life. These results suggest that, like its low-affinity counterpart, the high-affinity phosphodiesterase may also play an important role in the PKA-controlled feedback inhibition of intracellular cAMP.

Introduction

Throughout the eukaryotic kingdom, cyclic AMP (cAMP) signaling is known to modulate a variety of cellular functions such as carbohydrate metabolism, cell growth, differentiation, gene transcription and stress responses (Beebe, 1994; Thevelein & de Winde, 1999). The second messenger cAMP is produced by adenylate cyclase via stimulation of G protein-coupled receptors, and its main target is cAMP-dependent protein kinase A (PKA). The only way to inactivate cAMP is to degrade it through the action of cAMP phosphodiesterases (PDEs), which are thus considered to play a key regulatory role (Beavo & Brunton, 2002).

In mammalian cells, it is well recognized that cAMP signaling responses are compartmentalized. Compartmentalization allows spatially distinct pools of PKA to be differentially activated, of which the basis is that PKA isoforms are anchored at specific intracellular sites by proteins called A-kinase anchoring proteins (Wong & Scott, 2004; Langeberg & Scott, 2005; Smith & Scott, 2006; Beene & Scott, 2007). Therefore, discrete PKA populations could respond to gradients of cAMP in cells and modify localized target proteins. The basis of such gradients of cAMP depends largely on phosphodiesterases that are localized in distinct intracellular sites (Houslay, 2001; Houslay & Adams, 2003). Of the 11 families of phosphodiesterases in mammalian cells, PDE4 phosphodiesterases play a pivotal role in controlling spatially distinct pools of cAMP by virtue of their cAMP-specific activity and unique intracellular targeting (Houslay, 2001; Houslay & Adams, 2003). In addition, an upregulation of PDE4 by constitutive activation of the cAMP pathway has been observed in several types of mammalian cells (Persani et al., 2000, 2001; Georget et al., 2003). The increase in cAMP-degrading activity constitutes an intracellular feedback mechanism counteracting the phenotypic expression of the activating mutations.

The budding yeast Saccharomyces cerevisiae contains two cAMP phosphodiesterases, Pde1 and Pde2, with unrelated primary sequences (Suoranta & Londesborough, 1984; Sass et al., 1986; Nikawa et al., 1987b). Pde2 has a much lower \( K_m \) (170 nM) (Suoranta & Londesborough, 1984; Sass et al., 1986) than Pde1 (between 20 and 250 \( \mu \)M) (Londesborough & Lukkari, 1980), and shares a homologous region consisting of about 200 amino acids with phosphodiesterases in...
cAMP accumulation, and its activity is most likely regulated by PDE1 results in a highly activated cAMP-PKA pathway and the related phenotypes, whereas overexpression has the opposite effect (Ma et al., 1999; Park et al., 2005). Pde2 controls the basal cAMP level in the cell (Ma et al., 1999) and thereby protects it from detrimental changes in the extracellular environment (Wilson et al., 1993). Although PDE2 is not an essential gene in yeast, previous studies revealed synthetic genetic interactions of PDE2 with many other genes, which significantly influence stress response, nutrient utilization and the life span of the cell (Hubbard et al., 1992; Mitsuzawa, 1993; Balcìunas & Ronne, 1999; Poplinski et al., 2007).

It is known that addition of glucose to yeast cells grown on a nonfermentable carbon source results in a rapid and transient increase in intracellular cAMP (Thevelein et al., 1987), and that cAMP accumulation in yeast is strongly inhibited by PKA (Nikawa et al., 1987a). Mutants with reduced activity of PKA display hyperaccumulation of cAMP, whereas mutants with hyperactive PKA display a reduced cAMP level (Nikawa et al., 1987a). Several targets of feedback-inhibition on cAMP synthases have been proposed: Cdc25 (Munder & Kuntzel, 1989), Ras (De Vendittis et al., 1987a). In the second step, the RAS2 gene amplified from plasmid YCplac33 (Gietz & Sugino, 1988) using primer pair KRAS2-F and KRAS2-R. In the first step, the RAS2 gene in W303-1A was disrupted with the URA3 gene amplified from plasmid YCplac33 (Gietz & Sugino, 1988) using primer pair KRAS2-F and KRAS2-R, and used to transform the above strain HY222, HY224, HY225 were obtained by sporulation of HY222 and subsequent isolation of spores. Deletion of PDE1 was achieved by transformation with a pde1-Δ::ADE2 cassette, which was obtained by PCR amplification of the ADE2 gene from genomic DNA of S18-2D (Nikawa et al., 1987a) using primer pair KPDE1-F and KPDE1-R. Strains HY215, HY220, HY222 were obtained by sporulation of HY218 and subsequent isolation of spores. Deletion of PDE1 was achieved by transformation with a pde1-Δ::ADE2 cassette, which was obtained by PCR amplification of the ADE2 gene from genomic DNA of S18-2D (Nikawa et al., 1987a) using primer pair KPDE1-F and KPDE1-R. Strains HY222, HY224, HY225 were obtained by sporulation of HY222 and subsequent isolation of spores. Strain PMA203 was constructed in two steps. In the first step, the RAS2 gene in W303-1A was disrupted with the URA3 gene amplified from plasmid YCplac33 (Gietz & Sugino, 1988) using primer pair KRAS2-F and KRAS2-R, and used to transform the above strain HY222, HY224, HY225 were obtained by sporulation of HY222 and subsequent isolation of spores.

Strain PMA203 was constructed in two steps. In the first step, the RAS2 gene in W303-1A was disrupted with the URA3 gene amplified from plasmid YCplac33 (Gietz & Sugino, 1988) using primer pair KRAS2-F and KRAS2-R, and used to transform the above strain HY222, HY224, HY225 were obtained by sporulation of HY222 and subsequent isolation of spores.

This work studies PKA-regulated localization and the protein level of Pde2. We show that the intracellular localization of Pde2 is regulated by PKA, and that the protein level of Pde2 is positively correlated with the activity of PKA.

**Materials and methods**

**Strains, media and growth conditions**

*Saccharomyces cerevisiae* strains, listed in Table 1, were all derived from W303-1A (MATa ade2-1 trpl-1 can1-100 leu2-3,112 his3-11,15 ura3-1). The oligonucleotides used in the study for construction of strains and plasmids are described in Table 2. Strain HY203 was constructed by transforming W303-1A with a tpk1Δ::URA3 deletion fragment, which was obtained by PCR amplification of the URA3 gene on the plasmid YCplac33 (Gietz & Sugino, 1988) using primer pair KTPK1-F and KTPK1-R. To construct strain HY205, the tpk1w1 allele was amplified from genomic DNA of S18-2D (Nikawa et al., 1987a) with primer pair TPK1-F and TPK1-R, and used to transform strain HY203 to uracil auxotrophy. Deletion of TPK2 and TPK3 was achieved by transformation with PCR fragments generated from genomic DNA of S18-2D (Nikawa et al., 1987a) using primers flanking tpk2 or tpk3 loci. Strain HY218 was constructed by transforming the diploid strain HY217 with an bcy1Δ::LEU2 allele, which was obtained by PCR amplification of the LEU2 gene on the plasmid YEplac181 (Gietz & Sugino, 1988) using primer pair KBCY1-F and KBCY1-R. Strains HY219, HY220, HY222 were obtained by sporulation of HY218 and subsequent isolation of spores. Deletion of PDE1 was achieved by transformation with a pde1-Δ::ADE2 cassette, which was obtained by PCR amplification of the ADE2 gene from genomic DNA of S18-2D (Nikawa et al., 1987a) using primer pair KPDE1-F and KPDE1-R. Strains HY218 and HY219 were obtained by sporulation of HY218 and subsequent isolation of spores.

**Plasmid constructions**

The vectors YCplac22, YEplac112, YIpplac211 (Gietz & Sugino, 1988) were used for construction of new plasmids. Plasmid YCpGFP was constructed by inserting the (green fluorescent protein) GFP sequence between the PstI and SplI sites and the 249-bp CYC1 terminator sequences between the SplI and HindIII sites of plasmid YCplac22.

Plasmid YCpPDE2-GFP was constructed as follows: the PDE2 coding sequence with 432 bp of 5’ noncoding sequence was amplified from yeast genomic DNA using primer pair PDE2-F (containing the restriction site for BamHI and nucleotide sequence – 432 to – 413 of the PDE2 gene) and PDE2-R (containing the restriction site for

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Table 1. Saccharomyces cerevisiae strains used in this study

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<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
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<td>Thomas &amp; Rothstein (1989)</td>
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<td>This work</td>
</tr>
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<td>This work</td>
</tr>
<tr>
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</tr>
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</tr>
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Table 2. Primers used in this study

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<td>KTP1-R</td>
<td>TTAGTTTGTTGCGGCACTCTACCTAAGAAACCATATTTATCC</td>
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<tr>
<td>KTP2-F</td>
<td>AGGAAACCAATCAGAGAGCAG</td>
</tr>
<tr>
<td>KTP2-R</td>
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</tr>
<tr>
<td>KTP3-F</td>
<td>CGGCTCTGTTAGAATCAAGAA</td>
</tr>
<tr>
<td>KTP3-R</td>
<td>GGGCTGTTAGAATCAAGAA</td>
</tr>
<tr>
<td>KBCY1-F</td>
<td>GTCATTTTCCAGACCTAATCTCAGCTACCAATCTAAGAAACCATATTTATCC</td>
</tr>
<tr>
<td>KBCY1-R</td>
<td>TTAGAAGCAATCCTAGATGATAGACAAAGGTAGTCTAAGAAACCATATTATC</td>
</tr>
<tr>
<td>KRA1-S</td>
<td>AGAATCTCTGCTAGATGAGGCCAATGACAAAGGTAGTCTAAGAAACCATATTATC</td>
</tr>
<tr>
<td>KRA2-R</td>
<td>CCGTGAAATTGTCTATATTTGAGTCTACCAATCTCAGATGATAGACAAAGGTAGTCTAAGAAACCATATTATC</td>
</tr>
<tr>
<td>RAAS-R</td>
<td>ATGGTTTTTGAAGATGGCAGAA</td>
</tr>
<tr>
<td>TP1-K</td>
<td>AACCATATAAGGCTGAG</td>
</tr>
<tr>
<td>TP1-R</td>
<td>GGGCCGGCATCTAATCTGCAAGGTAGTCTAAGAAACCATATTATC</td>
</tr>
<tr>
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<td>PDE2D-F</td>
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<td>GCCGCCGGATCTAATCTGCAAGGTAGTCTAAGAAACCATATTATC</td>
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<td>KPD1-R</td>
<td>AGAAGAAAGTGGCCTTCTAGCCGCAATAATTTGATATTTGCTGAGTATGAG</td>
</tr>
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Relevant restriction sites are underlined.

Sall and nucleotide sequence 1556–1578 of the PDE2 gene. The PCR fragment was cut with BamHI and Sall and inserted in the same enzyme pair-digested plasmid YCpGFP, creating an in-frame fusion between the PDE2 ORF and GFP. Subsequently, the BamHI–HindIII DNA fragment containing the PDE2-GFP fusion and the CYC1 terminator was subcloned in YEplac112, resulting in plasmid YEpPDE2-GFP.

Plasmid YCpHA was generated by introducing a triple copy of the anti-hemagglutinin (HA) epitope between the PstI and SphI sites of plasmid YCpGFP, replacing the GFP sequence. Then the KpnI–Sall DNA fragment of plasmid YCpPDE2-GFP containing PDE2 was subcloned correspondingly in plasmid YCpHA directly upstream of hemagglutinin epitope, resulting in plasmid YCpPDE2-HA.
Plasmid YIpPDE2-HA was constructed as follows: first, a 418-bp fragment immediately downstream of PDE2 ORF was amplified by PCR from yeast genomic DNA with primer pair PDE2D-F and PDE2D-R. This PCR fragment was then cut with SphI–HindIII and cloned in plasmid YIp lac211. Secondly, the hemagglutinin epitope was cut from plasmid YCP HA by PstI and SphI and inserted into the same enzyme pair-digested plasmid constructed as described above. Finally, the KpnI–SalI restriction fragment of plasmid YCP PDE2-GFP containing PDE2 was placed upstream of hemagglutinin, creating an in-frame fusion between the PDE2 ORF and hemagglutinin.

Epitope-tagging of Pde2

For epitope-tagging of Pde2 at the C-terminus, plasmid YIpPDE2-HA was linearized with EcoRI and transformed into strains W303-1A and HY205, respectively. The replacement of the wild-type PDE2 gene by the hemagglutinin-tagged version of PDE2 was realized through a ‘two-step gene replacement’ procedure (Dan Burke & Tim Stearns, 2000).

Fluorescence microscopy

Exponentially growing cells were used for fluorescence microscopic study directly without fixation. Nuclei were stained by addition of 5 μg/mL of 4′, 6′-diamidino-2-phenylindole to the cell suspension. Cells were viewed using an Olympus BX51 microscope with a ×100 UplanApo objective and appropriate filter cubes. Images were taken with a SPOT CCD (Diagnostic Instruments Inc.) camera and then processed in ADOBE PHOTOSHOP. Quantification of the percentage of fluorescence in the nucleus was performed using IMAGE-PRO PLUS (Media Cybernetics, Silver Spring, MD) software as follows: first, the fluorescence in the whole cell \( F_C + F_N \) and the nuclear fluorescence \( F_N \) were quantified by IMAGE-PRO PLUS as described previously (Pache et al., 2006; Wang et al., 2008); secondly, the percentage was estimated as \( F_N / (F_C + F_N) \times 100\% \).

Western blot analysis

Yeast cell cultures were grown at 30 °C. All subsequent steps were carried out at 4 °C. Cells were harvested by centrifugation and washed in sterile water, and the pellets resuspended in the extraction buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Igepal CA-630, 0.25% sodium deoxycholate, 10 mM NaF, 1 mM sodium orthovanadate, 10 mM β-glycerol phosphate, 1 mM phenylmethanesulfonyl fluoride and a mixture of protease inhibitors (Complete; Roche Molecular Biochemicals). Cells were disrupted in a FastPrep instrument (Thermo Electron) in the presence of glass beads. The resulting suspension was spun down in a microfuge at maximum speed, and the supernatant was analyzed by the Bio-Rad protein assay kit to determine protein concentration. Extracts were eluted with 2 × sodium dodecyl sulfate (SDS)-sample buffer (100 mM Tris-HCl, pH 6.8, 2% w/v β-mercaptoethanol, 4% w/v SDS, 0.2% w/v bromophenol blue, 20% w/v glycerol), and proteins (20 μg) were resolved on SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membrane. Immunodetection of proteins was carried out using 12CA5 hemagglutinin monoclonal antibody (Roche Molecular Biochemicals) and anti-Pgk1 antiserum (as a loading control). Proteins were visualized using Lumiglo (KPL) according to the manufacturer’s instructions.

Band intensities were quantified using SCION IMAGE software (version 4.03, Scion Corporation). Final intensities were obtained by background subtraction. Data shown in the quantification graphs were the average of three independent experiments, and are presented as normalized density ± SD.

Quantitative real-time PCR

Samples for total RNA extraction were cooled immediately by addition of ice-cold water. The cells were collected by centrifugation at 4 °C. The cell sediment was washed once with ice-cold water and stored at −70 °C. Total RNA was isolated by phenol extraction (Dan Burke & Tim Stearns, 2000). From 5 μg of total RNA, first-strand cDNA was prepared with SuperScript III reverse transcriptase (Invitrogen) using oligo(dt) primer. Relative quantification of PDE2 and ACT1 mRNA was determined using the Platinum SYBR Green qPCR kit (Invitrogen) and the following primers for PDE2: For, 5’-AGCCACATGCGGATTGTGTTA-3’, and Rev, 5’-TTGAGCAACCTCCTGATCCAGT-3’. For ACT1 the primers were: For, 5’-TGGATTCCGGTGATGGTGTTA-3’, and Rev, 5’-AAGTCCAAGCCAGCTAACAT-3’.

Phosphodiesterase assay

Phosphodiesterase activity was assayed at 30 °C by measuring the time-dependent degradation of cAMP in a mixture containing buffer A (0.1 M Tris-HCl, pH 8.0, 5 mM MgCl2), 2.5 μg albumin mL−1, 1 mM cAMP and the indicated amounts of protein (Londesborough, 1977). The reaction was stopped immediately by addition of 10 mM EDTA and then heat denaturation. cAMP was assayed subsequently, using the cAMP [3H] assay system (Amersham).

Yeast cells were harvested during the exponential phase of growth by centrifugation, washed once in cold buffer A and recentrifuged. Yeast extracts were prepared by breaking the yeast cells with glass beads in buffer A containing 1 mM β-mercaptoethanol, 0.2 mM phenylmethanesulfonyl fluoride and a mixture of protease inhibitors (Complete; Roche Molecular Biochemicals), followed by centrifugation of the
homogenates. Protein concentration was determined using the Bio-Rad protein assay kit.

**Results**

**The localization of Pde2-GFP is affected by the carbon source used**

To study the localization of Pde2 in living cells, we fused GFP to the C-termini of the Pde2 protein. The resulting chimeric protein, which was under the control of the PDE2 promoter, was found to be functional, as Pde2-GFP produced in a pde2Δ strain suppressed heat-shock sensitivity of this mutant (data not shown). We examined the localization of Pde2-GFP in wild-type cells (W303-1A) growing on glucose, glycerol, ethanol or acetate. Pde2-GFP was found to be predominantly nuclear in cells grown on glucose. However, in cells grown on glycerol (or on various other nonfermentable carbon sources) a significant portion of the fusion protein was found to be extranuclear (Fig. 1a; data obtained for the other carbon sources are not shown).

We also observed fluorescence after addition of glucose to carbon source-derepressed cells. Wild-type cells were grown on glycerol to an A600 nm of 1, and then glucose was added to a final concentration of 2% (w/v). An increased nuclear accumulation of Pde2-GFP was observed within 10 min after addition of glucose; fluorescence was found preferentially in the nucleus over a time period of 20 min (Fig. 1b). De novo protein synthesis was not required for nuclear import of Pde2, as preincubation with 100 μg cycloheximide mL⁻¹ did not prevent translocation (data not shown).

The results summarized in Fig. 1 showed a dynamic localization of Pde2, which seemed to be determined by the carbon source available. Under the experimental conditions used here, Pde2 was concentrated in the nucleus in glucose-grown cells, whereas it was distributed over the nucleus and cytoplasm in derepressed cells.

**PKA regulates the localization of Pde2**

The cAMP-dependent PKA regulates numerous cellular processes in response to glucose signals. Previous studies on phosphodiesterases in mammalian cells revealed that PDE4D3 and PKA exist in a complex targeting at specific intracellular compartments (Dodge et al., 2001; Tasken et al., 2001). We expected that the Pde2 localization would be relevant to PKA in budding yeast. Notably, the localization of the regulatory subunit of PKA, Bcy1, is determined by the carbon source (Griffioen et al., 2000, 2001).

To assess the possibility that PKA regulates Pde2 localization, we introduced Pde2-GFP into a PKA-attenuated strain (tpk1Δ tpk2Δ tpk3Δ). In such a strain Pde2-GFP was found to be evenly distributed over the nucleus and cytoplasm not only in cells grown on nonfermentable carbon sources, but also in glucose-grown cells. Addition of glucose to glycerol-grown cells did not cause nuclear accumulation of Pde2-GFP (Fig. 2a and data not shown). These findings indicate that PKA activity is critical for the observed carbon source-dependent nuclear accumulation of Pde2.

We have also examined strains with elevated and constitutive PKA activity. Strain PMA203 carries the RAS2val19 allele, which has strongly reduced GTPase activity and is insensitive to the Ira proteins, which renders the protein a constitutive activity and, as a result, causes unregulated activity of adenylate cyclase (Toda et al., 1985; Tanaka et al., 1990, 1992). In strains of HY219, HY220 and HY221 the regulatory subunit and two of the three catalytic subunits of

![Fig. 1. Subcellular localization of Pde2-GFP in glucose-growing cells or derepressed cells. (a) W303-1A cells transformed with plasmid 112pPDE2-GFP were grown to logarithmic phase in either YPD or YPG and viewed with a fluorescence microscope. (b) Cells were grown in YPG to logarithmic phase and Pde2-GFP was observed before and after addition of 2% glucose (w/v). The numbers indicate the percentage of fluorescence in the nucleus. DAPI, 4',6'-diamidino-2-phenylindole; DIC, differential interference contrast microscopy.](https://academic.oup.com/femsyr/article-abstract/10/2/177/567883)
PKA were deleted. An increased nuclear localization of Pde2 was observed in all these strains compared with wild-type strain (Fig. 2b). The different extent of nuclear accumulation in the three bcyl mutant strains might be due to the difference in activity of the three catalytic subunits. The results also indicated that the activity of any catalytic subunit of PKA is sufficient for nuclear accumulation of Pde2. To further confirm that the nuclear accumulation of Pde2 was controlled by PKA, and that glucose affected the localization of Pde2 through activation of PKA, we shifted glucose-grown cells to medium without glucose and analyzed the Pde2-GFP localization quantitatively. As shown in Fig. 2c, in four mutant strains the nuclear accumulation of Pde2 was not affected, whereas in the wild-type strain a significant portion of Pde2-GFP was cytoplasmic.

Altogether, the data presented here show that PKA positively regulates nuclear accumulation of Pde2. Elevated PKA activity resulted in an increased nuclear concentration of Pde2, and any catalytic subunit of PKA was sufficient for accumulation of Pde2 in the nucleus, whereas in a PKA-attenuated strain, Pde2 failed to concentrate in the nucleus of cells grown on glucose.

**Addition of glucose results in the increase of Pde2 protein levels in a PKA-dependent manner**

Strain HYHA1 was constructed by replacing the original PDE2 of the W303 strain with a hemagglutinin-tagged version of the gene (see Materials and methods), and was used to determine the Pde2 level following the addition of glucose to cells grown in YPG. Western analysis with hemagglutinin antibody showed an increase in the Pde2 protein level for 2 h after the addition of glucose (Fig. 3a).
As study of the protein localization indicated a possible role of PKA in regulation of Pde2 localization as described above, we expected that a higher activity of PKA might be responsible for the increased levels of Pde2 protein upon addition of glucose. To address this, we examined strain HYHA2, containing attenuated-PKA (tpk1\textsuperscript{w1} tpk2\textsuperscript{D} tpk3\textsuperscript{D}) and a chromosome-integrated hemagglutinin-tagged version of \textit{PDE2}. Because of slow growth of this strain, samples were taken for a longer period of time. As shown in Fig. 3b, Pde2 protein concentration remained constant for 6 h after glucose addition.

**Protein level of Pde2 is positively correlated with PKA activity**

To gain further evidence for the role of PKA in regulating Pde2 protein level, we introduced plasmid YCp\textit{PDE2-HA}, containing \textit{PDE2-HA} under the control of \textit{PDE2} promoter, into wild-type strain and strains with altered PKA activity. Western analysis (Fig. 4a) showed that Pde2 protein levels were higher in mutants with hyperactive PKA than in wild-type strain. To further confirm that the upregulation of Pde2 protein level especially resulted from elevated PKA activity, we shifted glucose-grown cells to medium without glucose for 2 h and then collected cells for Western analysis. A similar result was obtained as when cells were grown on glucose (data not shown). We have also detected the PKA-attenuated strain (tpk1\textsuperscript{w1} tpk2\textsuperscript{D} tpk3\textsuperscript{D}). Figure 4b indicated a downregulated protein level in the tpk1\textsuperscript{w1} tpk2\textsuperscript{D} tpk3\textsuperscript{D} strain compared with the wild-type strain.

To determine the phosphodiesterase activity of Pde2, we measured the time and dose-dependent degradation of exogenous cAMP added to cell extracts from \textit{pde1} mutants. Figure 4c showed increased cAMP-degrading activity in the extracts of mutants with constitutively activated PKA and decreased cAMP-degrading activity in the extracts from the PKA-attenuated strain. Although we could not exclude other mechanisms potentially responsible for the observed increase in Pde2 activity (such as phosphorylation), the...
increased Pde2 activity resulted, at least in part, from upregulation of the Pde2 protein level.

Data presented here indicated a positive correlation between the protein level of Pde2 and the activity of PKA. We suspected that the increase of Pde2 concentration in strains with elevated PKA activity was a result of upregulation of the PDE2 mRNA, as many PKA-mediated effects can be accounted for by changes in transcription (Roos et al., 2005). We investigated the transcription of PDE2 in the four PKA-elevated strains and the wild-type strain by quantitative real-time PCR. Unexpectedly, no increase in the PDE2 mRNA level was detected in any of the four mutants, despite their elevated PKA activity (data not shown). Therefore, the increased protein levels of Pde2 in mutants with hyperactive PKA were not due to upregulation of the mRNA level of the gene by PKA.

The elevated levels of Pde2 protein in strains with hyperactive PKA are due to the increased protein stability of Pde2

To explore the nature of the enhanced Pde2 protein levels in glucose-growing cells and in strains with hyperactive PKA, we conducted protein stability analysis by Western blotting in the presence of cycloheximide, the protein synthesis inhibitor. For protein stability analysis, cells were grown in appropriate medium to exponential phase. The culture was then split into two subcultures. Cycloheximide was added to a final concentration of 100 μg mL⁻¹ to one subculture. Both subcultures were kept synchronically and samples were taken before and after addition of cycloheximide for Western analysis. As shown in Fig. 5a, the basal Pde2 abundance remained constant for 1 h after the addition of cycloheximide in glucose-grown cells. However, in glycerol-grown cells, Pde2 concentration decreased to 50% of its initial level. To address the correlation of Pde2 protein stability with PKA, the same experiment was performed on glucose-growing cells with hyperactive or attenuated PKA. Data in Fig. 5b show that even more stable Pde2 protein was detected in cells with hyperactive PKA. In strain RAS2Δ and bcy1Δ tpk2Δ tpk3Δ, Pde2 level remained unchanged for 2 h after the addition of cycloheximide. However, the concentration of Pde2 protein decreased by about 30% in strain W303 in the same period of time (Fig. 5b). In contrast, the stability of Pde2 decreased in the PKA-attenuated strain (Fig. 5c). Therefore, the increased stability of the Pde2 protein was apparently the result of high PKA activity rather than the presence of glucose.

**Discussion**

**The physiological role of dynamic Pde2 localization**

Our results reveal a dynamic localization pattern of Pde2 that is regulated by PKA. To explore the physiological role of PKA-regulated Pde2 localization, we investigated the correlation of localization pattern of Pde2 with that of PKA. Previous study has revealed that nutritional signals control the localization of both types of yeast PKA subunits by at least two different molecular mechanisms (Griffioen et al., 2000): (1) the localization of Tpk1 is affected by CAMP level, in that deprivation of CAMP in rapidly growing yeast cells resulted in a pronounced nuclear localization of Tpk1, and the subsequent addition of 3 mM CAMP to such cells led to rapid entry of Tpk1 into the cytoplasm, whereas Bcy1 remained nuclear; and (2) the localization of Bcy1 is determined by the carbon source used. Bcy1 is predominantly accumulated in the nucleus during growth on glucose and partly cytoplasmic on a nonfermentable carbon source (Griffioen et al., 2000). Therefore, Pde2 localization in the
same compartment with Bcy1 may downregulate cAMP signaling more efficiently by differential degradation of cAMP. Moreover, it was revealed that accumulation of Tpk1 in the nucleus is dependent on interaction with Bcy1 (Griffioen et al., 2000). During growth on glucose, especially degradation of cAMP in the nucleus, the conditions are presumably in favor of recombination of the regulatory and catalytic subunits, which downregulates PKA activity.

Zds1 was found to be required for cytoplasmic localization of Bcy1 in carbon source-derepressed cells (Griffioen et al., 2001). Bcy1 is largely absent from the cytoplasm in ethanol-grown zds1 mutant cells. As the movement of Pde2 in response to the carbon source is similar to that of Bcy1, we expected a role of Zds1 in the regulation of Pde2 localization. To this end, we performed fluorescence microscopy of Pde2 in zds1 mutant cells. However, the localization pattern of Pde2 was not affected by the absence of Zds1, either in glucose-grown cells or in derepressed cells (data not shown).

**Localization of Pde2 differs from that of the type 4 phosphodiesterases in mammalian cells**

Intracellular localization of Pde2 in budding yeast differs from the localization of type 4 phosphodiesterases in that: (1) PDE4 phosphodiesterases are found in discrete compartments in mammalian cell, including plasma membrane, Golgi region, cytoplasm and perinuclear region; (2) there are several isoforms of PDE4; (3) the upstream conserved regions through which PDE4s interact with anchoring proteins targeting at specific compartment, are not contained in Pde2. In spite of these differences, the region where Pde2 functions remains unclear. There might be specific region(s) for Pde2-localized function through interactions with a binding partner or for its recognition by a protein degradation system.

**High PKA activity stabilizes Pde2 protein**

The results of quantitative real-time PCR (data not shown) did not support our first hypothesis that an upregulation of PDE2 mRNA level might occur in strains with elevated PKA activity. Further analysis demonstrated that elevated activity of PKA resulted in a more stable Pde2 protein, which was responsible for the observed increase in protein level of Pde2 in strains with high PKA activity. This finding may have revealed for the first time a new mechanism for down-regulation of cAMP signaling in budding yeast. The effect of PKA on the stability of Pde2 protein might result from direct modification of the Pde2 protein by PKA, generating a more stable protein, or from modification of a protease(s), which is responsible for the degradation of Pde2 protein and makes it less effective with respect to Pde2 degradation.

**Feedback inhibition of the cAMP signaling pathway through cAMP degradation**

Previous studies on feedback control of cAMP level showed that deletion of the two phosphodiesterase genes in an RAS2<sup>val19</sup> strain caused a significant increase in the cAMP level, but in a strain with attenuated PKA activity there was a similar 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). Studies on the low-affinity cAMP phosphodiesterase Pde1 have revealed its specific function in controlling agonist-induced cAMP signaling (Ma et al., 1999). Deletion of Pde1 results in much higher glucose- and acidification-induced cAMP accumulation, and Pde1 activity is most likely controlled by PKA-regulated phosphorylation. Here we present evidence that the intracellular localization of Pde2 is regulated by PKA, and that the protein level of Pde2 is correlated positively with PKA activity, suggesting that, like its low-affinity counterpart, the high-affinity cAMP phosphodiesterase may also play an important role in the PKA-governed feedback inhibition of intracellular cAMP level. To prove this, direct evidence for Pde2 phosphorylation by PKA needs to be obtained.

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**References**


