Direct and Novel Regulation of cAMP-dependent Protein Kinase by Mck1p, a Yeast Glycogen Synthase Kinase-3*

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The MCK1 gene of Saccharomyces cerevisiae encodes a protein kinase homologous to metazoan glycogen synthase kinase-3. Previous studies implicated Mck1p in negative regulation of pyruvate kinase. In this study we find that purified Mck1p does not phosphorylate pyruvate kinase, suggesting that the link is indirect. We find that purified Tpk1p, a cAMP-dependent protein kinase catalytic subunit, phosphorylates purified pyruvate kinase in vitro, and that loss of the cAMP-dependent protein kinase regulatory subunit, Bcy1p, increases pyruvate kinase activity in vivo. We find that purified Mck1p inhibits purified Tpk1p in vitro, in the presence or absence of Bcy1p. Mck1p must be catalytically active to inhibit Tpk1p, but Mck1p does not phosphorylate this target. We find that abolition of Mck1p autophosphorylation on tyrosine prevents the kinase from efficiently phosphorylating exogenous substrates, but does not block its ability to inhibit Tpk1p in vitro. We find that this mutant form of Mck1p appears to retain the ability to negatively regulate cAMP-dependent protein kinase in vivo. We propose that Mck1p, in addition to phosphorylating some target proteins, also acts by a separate, novel mechanism: autophosphorylated Mck1p binds to and directly inhibits, but does not phosphorylate, the catalytic subunits of cAMP-dependent protein kinase.

The genome of Saccharomyces cerevisiae encodes four different protein kinases highly similar (greater than 40% identity) to mammalian glycogen synthase kinase-3 (GSK-3) (1). One of these kinases is encoded by MCK1 (2, 3). Like its vertebrate homologues, Mck1p displays dual specificity in vitro, in that it is capable of autophosphorylating on serine and tyrosine residues, but only phosphorylates exogenous substrates on serine and threonine (3).

The protein kinases of the glycogen synthase kinase-3 (GSK-3) sub-family have been implicated in a variety of regulatory processes. In mammals, the prototypic members, GSK-3α and GSK-3β, phosphorylate and are thought to regulate a number of metabolic enzymes (4–7). GSK-3 enzymes are intimately involved in the insulin signaling pathway and in key developmental pathways in a number of organisms (8, 9). The development of the dorsal-ventral axis in Drosophila and Xenopus embryos relies on the function of the Wingless/Wnt pathway. GSK-3 is inhibited by this pathway, allowing the expression of crucial developmental genes (9).

Yeast cells lacking Mck1p display a wide range of phenotypes, indicating that Mck1p may have multiple targets. Deletion of the MCK1 locus results in various defects in carbon metabolism, including reduced glycogen accumulation and poor growth on non-fermentable carbon sources (10, 11). Other phenotypes resulting from deletion of MCK1 include heat and cold sensitivity (10, 11), delayed sporulation (13) and sensitivity to the microtubule-destabilizing drug benomyl (12). In this work we show that mck1Δ cells are also sensitive to caffeine. Overexpression of MCK1 has been shown to suppress temperature-sensitive mutations in CBP2 and CBP5, which encode essential centromere-binding proteins. Mck1p was found to bind to and phosphorylate Cbf2p in vitro (14, 15). More recently, MCK1 and RIM11 have been implicated in the protein ubiquitination pathway (16). Finally, MCK1 has been implicated in the response to high concentrations of NaCl in growth medium (17).

Previous work has demonstrated that Mck1p down-regulates pyruvate kinase (EC 2.7.1.40), encoded by the CDC19/PYK1 gene, in vivo (11). Like mck1Δ mutants, strains overproducing pyruvate kinase fail to grow at 37 °C and do not accumulate normal amounts of glycogen. Cells overexpressing CDC19, like mck1Δ, adapt poorly to growth on non-fermentable carbon sources (11, 18, 19). Finally, diploid cells overexpressing CDC19 show markedly diminished sporulation efficiency (11), similar to that observed in mck1Δ mck1Δ diploids. Deletion of MCK1 was found to exacerbate each of these CDC19 overexpression phenotypes (11).

The findings of Brazill et al. (11) suggested that Mck1p might directly phosphorylate pyruvate kinase, thereby down-regulating the activity of this enzyme. In this report we investigate this possibility. We find that Mck1p does not in fact phosphorylate pyruvate kinase but instead acts to inhibit an intermediary kinase, which would otherwise phosphorylate the glycolytic enzyme. We identify this intermediary kinase as cAMP-dependent protein kinase (PKA). The genome of S. cerevisiae contains three genes encoding PKA catalytic subunits, TPK1, TPK2, and TPK3, and one gene, BCY1, encoding the cAMP binding and negative regulatory subunit of PKA. A purified Tpk1p-Bcy1p complex phosphorylates purified pyruvate kinase, and this phosphorylation is stimulated by cAMP. Consistent with this result, we find that loss of Bcy1p increases pyruvate kinase activity in vivo. Phosphorylation of pyruvate...
kinase by Tpk1p-Byc1p is inhibited by the addition of purified Mck1p in vitro. Mck1p also inhibits purified Tpk1p alone. This inhibition is dependent on Mck1p catalytic activity, but Mck1p does not phosphorylate Tpk1p (or indeed Byc1p) in vitro. Mck1p autophosphorylation on tyrosine-199 is required for efficient phosphorylation of exogenous substrates but not for Mck1p autophosphorylation on other residues or for inhibition of Tpk1p in vitro. Finally, autophosphorylation by Mck1p on tyrosine (and hence phosphorylation of exogenous substrates) is not required for the regulation of PKA by Mck1p in vivo. We propose that, in addition to phosphorylating some target proteins, Mck1p also directly binds to and inhibits, but does not phosphorylate, the catalytic subunits of PKA.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Materials**—Purified bovine brain myelin basic protein (MBP), CAMP, LRRASLG (Kemptide), PKI, l-lactate dehydrogenase, DEAE-Sephadex, CM-Sephadex, and AEMP-agarose were purchased from Sigma-Aldrich Co. (St. Louis, MO). S-Sepharose was purchased from Amersham Biosciences, Inc. (Piscataway, NJ). Nickel-agarose was purchased from Novagen (Madison, WI) and prepared according to the manufacturer’s instructions. Anti-His<sub>6</sub> tag antibodies were purchased from Covance Research Products (Richmond, CA). Anti-phosphotyrosine monoclonal antibody clone 4G10 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Modified Bradford protein concentration assay reagent was purchased from Bio-Rad Laboratories Inc. (Hercules, CA).

Partially purified pyruvate kinase was a gift of Tom Nowak (University of Notre Dame, Notre Dame, IN) and fully purified pyruvate kinase was generously provided by Barry Stoddard (Fred Hutchinson Cancer Research Center, Seattle, WA).

**Growth of Yeast Cells**—Yeast strains were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose) (20) where appropriate. Selection for plasmid maintenance was provided where necessary by growing strains in synthetic minimal medium supplemented with the appropriate nutrients (20). Media containing non-fermentable carbon sources (acetate, glycerol, m lactate, or ethanol) were made by adding the relevant compound to suitable medium to a final concentration of 2%. In the cases of lactate and acetate, agar plates were made using unbuffered acids at 0.2%. All other plates contained 2% glucose as the carbon source. All plates contained 2% glucose as the carbon source. All plates contained 2% glucose as the carbon source. All plates contained 2% glucose as the carbon source. All plates contained 2% glucose as the carbon source.
volumes of buffer A. A second wash was performed with six column volumes of buffer A containing 20 mM imidazole. The Mck1p protein was then eluted with three column volumes each of buffer A containing 50 and 100 mM imidazole. These final fractions were assayed for protein concentration using a Bradford-based assay (32) and checked for Mck1p containing SDS-PAGE (33) and Western blotting (34). Peak fractions were pooled and dialyzed overnight against buffer B (50 mM MES, pH 6.5, 1 mM EDTA, 25% glycerol). The protein was then further purified by running the dialysate over an S-Sepharose column (1-mL bed volume) equilibrated in buffer B. The column was washed with ten volumes of buffer B and a further five volumes of buffer B containing 100 mM NaCl. Purified Mck1p was then eluted using buffer B containing increasing concentrations of NaCl. Mck1p typically eluted in the range of 300–400 mM NaCl. Fractions were assayed by SDS-PAGE followed by silver staining or Western blotting. Western blotted membranes were probed with anti-Mck1p (2) or anti-His6 tag antibodies.

**Purification of Tpk1p-His6** —The procedure for purifying PKA subunits from yeast was based on the work of Hixon and Krebs (35). Tpk1p, His6-tagged at its C terminus, was overproduced in strain BJ2168 using the 2-μm plasmid pTRYAT1H, which expresses His6-tagged Tpk1p under the control of the constitutive ADH1 promoter. The regulatory subunit Bcy1p was co-overproduced with His6-tagged Tpk1p using a second-2-μm plasmid, YEpBCY1, which expresses BCY1 under the control of its own promoter. Such co-overexpression has been previously found to enhance TPK1 expression (36). Cells were harvested from a 2-L culture and lysed by adding ammonium sulfate to 40% saturation. The precipitate was resuspended in 50 mM MOPS, pH 7.5, 1 mM EDTA. The lysate was fractionated by adding ammonium sulfate to 40% saturation. The precipitate was resuspended in 50 mM MOPS, pH 7.5, with 0.1% Tween 20. This fraction was loaded onto a nickel-agarose column (1-mL bed volume) equilibrated in the same buffer. The column was washed in 10 volumes of buffer before the protein was eluted with increasing concentrations of NaCl. The column was washed with 10 volumes of buffer containing 10 mM imidazole followed by a further six column volumes with 20 mM imidazole. These final fractions were assayed for protein concentration with varying concentrations of NaCl added. The column was washed with 10 volumes of buffer, and the protein was eluted with increasing concentrations of NaCl in the same MOPS buffer. Fractions were assayed by immunoblotting for the His6 tag, and peak fractions (typically in the range 100–200 mM NaCl) were pooled. The pooled fractions were dialyzed overnight in buffer B (50 mM MES, pH 6.5, 1 mM EDTA, 25% glycerol, as for the purification of Mck1p) and applied to a CM-Sephadex column (0.5-ml bed volume) equilibrated in the same MOPS buffer. Fractions were assayed by immunoblotting for the His6 tag, and peak fractions (typically in the range 100–200 mM NaCl) were pooled and dialyzed overnight against buffer B (50 mM MES, pH 6.5, 1 mM EDTA). The purity of the proteins was determined by SDS-PAGE followed by silver staining.

**RESULTS**

**Mck1p Does Not Phosphorylate Pyruvate Kinase in Vitro** —The results of Brazil et al. (11) suggested that Mck1p might down-regulate pyruvate kinase by direct phosphorylation. We set out to determine if purified Mck1p phosphorylates purified pyruvate kinase in vitro. Mck1p was purified to homogeneity (Fig. 1) using a C-terminal His6 tag, a nickel-agarose column, and cation exchange chromatography. The purified enzyme retained catalytic activity toward myelin basic protein (MBP) (Fig. 4) and had a Km for ATP of 70 μM, comparable to that previously determined for the untagged enzyme (27 μM in Dalley et al. (2); 70 μM in Lim et al. (3)). Purified Mck1p did not phosphorylate purified pyruvate kinase in vitro. These data raise the possibility that Mck1p does not regulate pyruvate kinase directly but instead acts on this key metabolic enzyme through an intermediary protein kinase.

We found that pyruvate kinase is phosphorylated by an unknown protein kinase in a partially purified preparation of the enzyme. Mck1p was absent from this pyruvate kinase sample, as determined by immunoblotting. Because PKA is known to phosphorylate and regulate pyruvate kinase in other organisms (41–43), we examined the effect of adding cAMP on phosphorylation of partially purified pyruvate kinase. We found that cAMP strongly stimulated phosphorylation of the enzyme. This phosphorylation was inhibited by the addition of purified Mck1p to the reaction. This led us to postulate that PKA, directly or indirectly, phosphorylates pyruvate kinase in partially purified preparations. Mck1p is capable of inhibiting this PKA-dependent phosphorylation.

**Tkpk1 Phosphorylates Pyruvate Kinase in Vitro** —We set out to determine if purified PKA is capable of phosphorylating purified pyruvate kinase in vitro. The PKA catalytic subunit Tpk1p was purified as a complex with its regulatory subunit Bcy1p. It has previously been found that efficient production of Tpk1p by the TPK1 gene requires the co-overexpression of the gene encoding Bcy1p (36). Strains used during this study for the overproduction of Tpk1p, therefore, used a BCY1-overexpression plasmid, YEpBCY1, to boost the expression of TPK1.
Tpk1p, tagged on its C terminus with a His\textsubscript{6} epitope, was initially purified as a complex with Bcy1p as described under “Experimental Procedures.” This protocol yielded a purified complex of Tpk1p with Bcy1p as judged by silver staining of an SDS-PAGE gel (Fig. 1).

The purified PKA complex was assayed for its ability to phosphorylate purified pyruvate kinase in vitro. As shown in Fig. 2A, we found that pyruvate kinase can indeed be phosphorylated by a purified preparation of Tpk1p-Bcy1p complex. This observation suggests that PKA is sufficient to phosphorylate pyruvate kinase in vitro. Tpk1p-Bcy1p complex is known to be catalytically inactive (44). We therefore attribute the activity observed in the above experiment to be due to a small amount of free Tpk1p in our preparation of the complex.

If PKA can indeed phosphorylate purified pyruvate kinase, addition of cAMP should stimulate this phosphorylation. We found that adding cAMP to our assays dramatically increased the incorporation of radiolabeled phosphate into pyruvate kinase (Fig. 2A). As an alternative means of stimulating PKA activity, we purified Tpk1p away from the inhibitory Bcy1p subunit. This was accomplished by using cAMP-conjugated agarose, which specifically binds to the Bcy1p subunit, to dissociate the purified Tpk1p-Bcy1p complex as described under “Experimental Procedures.” This step efficiently yielded a homogeneous preparation of Tpk1p, confirmed by silver staining of SDS-PAGE gels (Fig. 1) and Western blotting with anti-His\textsubscript{6} tag monoclonal antibodies. Subsequent experiments using only the purified Tpk1p subunit to phosphorylate pyruvate kinase demonstrated that Tpk1p on its own is sufficient to phosphorylate pyruvate kinase in vitro (Fig. 2B). Omission of the regulatory Bcy1p subunit from the assays abolished the stimulatory effect of cAMP on the phosphorylation reaction. These data show that activation of PKA by addition of cAMP or by depletion of the Bcy1p subunit stimulates phosphorylation of pyruvate kinase.

If PKA phosphorylates pyruvate kinase, this phosphorylation should depend on PKA activity. We examined the effect of a specific inhibitor of PKA, mammalian protein kinase inhibitor (PKI), on the phosphorylation of pyruvate kinase by the purified preparation of PKA. PKI is a known and specific inhibitor of cAMP-dependent protein kinases (45). PKI was added to assays in which purified Tpk1p-Bcy1p complex was used to phosphorylate pyruvate kinase in the presence of cAMP. Under these conditions, PKI was found to efficiently inhibit the phosphorylation of pyruvate kinase by our PKA preparation (Fig. 2A). This finding shows that the catalytic activity of PKA is required for phosphorylation of pyruvate kinase in our in vitro assays.

These results, taken together, indicate that PKA is capable of directly phosphorylating pyruvate kinase in vitro.

PKA Positively Regulates Pyruvate Kinase in Vivo—We have shown above that PKA directly phosphorylates pyruvate kinase in vitro. Given that PKA is a known regulator of this enzyme in other organisms (41–43), we investigated the relationship between PKA and pyruvate kinase activities in living yeast. We found that haploid bcy1Δ cells have higher pyruvate kinase activity than do isogenic wild-type cells. However, this difference in activity could be an artifact of the slow growth of haploid bcy1Δ mutants. To circumvent this problem, we took advantage of an accidental observation: Like homozygous bcy1Δ cells, heterozygous bcy1Δ diploid cells are unable to sporulate, a phenotype suggestive of PKA activation. Unlike haploid bcy1Δ cells, heterozygous bcy1Δ cells do not have a vegetative growth defect. These data suggest that BCY1 is haploinsufficient for sporulation. Consistent with this view, we find that introduction of BCY1 on a plasmid into the heterozygous mutant restores the ability of the strain to sporulate efficiently. We infer that a heterozygous bcy1Δ strain has higher PKA activity than wild-type, sufficient to inhibit sporulation but insufficient to slow vegetative growth. As shown in Fig. 3, we find that pyruvate kinase activity is elevated in a heterozygous bcy1Δ diploid strain. We conclude that PKA is indeed a positive regulator of pyruvate kinase activity in vivo.

Mck1p Inhibits the Kinase Activity of Tpk1p in Vitro—We have shown that Mck1p inhibits the phosphorylation of pyruvate kinase by PKA in partially purified pyruvate kinase samples. To determine if this inhibition is direct, we investigated the effect of adding purified Mck1p to in vitro assays in which purified Tpk1p-Bcy1p was used to phosphorylate purified pyruvate kinase. In each assay, PKA was activated by adding cAMP. Addition of purified Mck1p to such assays was found to substantially reduce phosphate incorporation into pyruvate kinase (Fig. 2A). We conclude that Mck1p can directly inhibit the phosphorylation of pyruvate kinase by PKA in vitro.

It is possible that Mck1p does not act directly on the catalytic subunit of PKA but rather prevents cAMP-stimulated dissociation of the inhibitory Bcy1p subunit. To investigate if the inhibition of PKA by Mck1p requires the Bcy1p subunit, we examined the effect of Mck1p on the ability of purified Tpk1p to phosphorylate pyruvate kinase. Mck1p inhibited Tpk1p-dependent phosphorylation of pyruvate kinase both in the presence and absence of the regulatory subunit Bcy1p (Fig. 2, A and
Thus Mck1p acts directly on the catalytic subunit of PKA. There are several possible ways in which Mck1p might inhibit phosphorylation of pyruvate kinase by PKA: by inhibiting the catalytic activity of PKA; by altering substrate specificity of the kinase; or by sequestering the substrate, pyruvate kinase. To address the second possibility, we set out to determine if the inhibitory effect of Mck1p was limited to just one substrate for PKA. Assays were performed in which purified Tpk1p was used to phosphorylate Kemptide, a synthetic peptide substrate of PKA, in the presence or absence of Mck1p (Fig. 5B). We observed that Mck1p is capable of inhibiting the PKA-dependent phosphorylation of Kemptide. This observation indicates that Mck1p does not alter the substrate specificity of PKA. These results also allow us to address the possibility that Mck1p might sequester PKA substrates, rendering them unavailable for phosphorylation by PKA. Kemptide was present in massive excess relative to both Mck1p and Tpk1p in the above assays. These data show that Mck1p is unlikely to act by sequestering a particular substrate of PKA. Taken together, we conclude from these results that Mck1p directly inhibits the catalytic activity of PKA in vitro.

Mck1p Requires Catalytic Activity to Inhibit PKA but Does Not Phosphorylate It—We set out to further investigate the mechanism by which Mck1p inhibits PKA. The simplest possibility is that Mck1p directly phosphorylates Tpk1p. If this mechanism is correct, then the catalytic activity of Mck1p should be required for it to inhibit Tpk1p. We generated a catalytically inactive version of Mck1p (D164A) and purified it to homogeneity as described under “Experimental Procedures.” Purified Tpk1p was used to phosphorylate pyruvate kinase or Kemptide in the presence of the purified catalytically inactive Mck1p and cAMP. Under these conditions no inhibition of Tpk1p by Mck1p (D164A) was observed (Figs. 2C and 5). This observation demonstrates that the kinase activity of Mck1p is indeed required for its ability to inhibit Tpk1p in vitro.

However, in all the protein kinase assays so far discussed, Tpk1p was not phosphorylated when catalytically active Mck1p was present, even though Tpk1p was efficiently inhibited under these conditions (Fig. 5A). This surprising observation led us to conclude that Mck1p regulates PKA by a novel mechanism: Mck1p must undergo autophosphorylation before it is capable of binding to and inhibiting Tpk1p. This direct inhibition does not involve phosphorylation of the target.

The Y199F Mutation in Mck1p Abolishes Autophosphorylation on Tyrosine and Is a Separation-of-Function Mutant—This novel mechanism of action of Mck1p points to a critical role for autophosphorylation of the kinase. Comparison of the amino acid sequence of Mck1p with that of other GSK-3 family members suggests a candidate residue upon which the postulated regulatory autophosphorylation may occur. The tyrosine residue at position 199 of Mck1p is highly conserved among GSK-3-related kinases. This residue falls within the so-called “activation loop” of the canonical protein kinase structure. Mutation of this activation-loop tyrosine to phenylalanine in other members of the GSK-3 subfamily (9, 37) compromises phosphotransferase activity with respect to exogenous substrates.

We set out to determine if tyrosine-199 is the site of tyrosine autophosphorylation on Mck1p. A mutant allele encoding Mck1p with its activation-loop tyrosine mutated to phenylalanine (Y199F) was constructed and purified. The autophosphorylation state of the mutant protein was determined in vitro. The activation-loop mutation entirely abolishes the ability of Mck1p to autophosphorylate on tyrosine (Fig. 4). We conclude from this observation that tyrosine-199 is the likely target of autophosphorylation on tyrosine in Mck1p.

To address the possibility that Mck1p can autophosphorylate on residues other than tyrosine, we determined the ability of the activation-loop mutant Mck1p to incorporate radiolabeled phosphate. We found that the activation-loop mutation fails to block Mck1p autophosphorylation entirely, despite abolishing autophosphorylation on tyrosine (Fig. 4). The phosphorylations noted in the above experiment are due to autophosphorylation, because catalytically inactive Mck1p(D164A) is not capable of incorporating radiolabeled phosphate in parallel experiments (Fig. 4). We conclude that the mutant Mck1p(Y199F) is still capable of autophosphorylating on residues other than tyrosine-199.

Because phosphorylation on the activation-loop tyrosine is known to be important for the activity of other yeast GSK-3 enzymes, such as Rim11p, toward exogenous substrates (37, 46), we tested the activity of the Mck1p activation-loop mutant protein toward the exogenous substrate MBP. We found that the activation-loop mutation severely compromises the ability of Mck1p to phosphorylate Mck1p(MBP). This finding indicates that autophosphorylation on tyrosine-199 is important for the kinase activity of Mck1p toward exogenous substrates.

In the previous section, we concluded that inhibition of...
Tpk1p by Mck1p does not involve phosphorylation of this target. If this mechanism is correct, then we might expect Mck1p(Y199F) to still be competent at inhibiting Tpk1p in vitro. We therefore assayed the ability of the activation-loop mutant Mck1p protein to inhibit phosphorylation of Kemptide or pyruvate kinase by Tpk1p in vitro. In addition, PKA is known to autophosphorylate in vitro. Therefore, we also assayed the ability of the activation-loop mutant Mck1p protein to inhibit autophosphorylation of purified Tpk1p·Beyp1 complex. Interestingly, in each case the mutant Mck1p protein was found to inhibit Tpk1p to the same extent as did wild-type Mck1p (Fig. 5). We conclude from this that Mck1p does not require autophosphorylation on tyrosine for its ability to inhibit Tpk1p, but that this autophosphorylation is required for phosphorylation of exogenous substrates. These data independently confirm that Mck1p inhibits Tpk1p by a novel mechanism that does not involve phosphorylation of Tpk1p. These results, taken together, show that autophosphorylation of Mck1p on tyrosine-199 is required for phosphorylation of exogenous substrates, but is dispensable for the ability of Mck1p to inhibit Tpk1p. Mck1p thus appears to have two different mechanisms of action: (1) an autophosphorylation-dependent mechanism involving autophosphorylation-dependent binding to PKA, and (2) an autophosphorylation-independent mechanism involving phosphorylation of exogenous substrates. The Y199F mutation specifically abolishes the ability of Mck1p to phosphorylate exogenous substrates, having no effect on its ability to inhibit PKA. If our in vitro analysis correctly reflects the situation in vivo, then we predict that the Mck1p(Y199F) should retain some functions in vivo whereas a catalytically inactive mutant should be devoid of function. We therefore determined if the activation-loop mutant mck1(Y199F) and a catalytically inactive mutant mck1(D164A) could complement an mck1Δ mutant. An mck1Δ strain was first transformed with a 2-μm plasmid encoding the activation-loop mutant under the control of its own promoter. The wild-type allele expressed from such a construct has previously been found to complement mck1Δ in vivo (3). The activation-loop mutant gene fully complemented the temperature-sensitive growth defect of mck1Δ cells (Fig. 6). However, the activation-loop mutant failed to complement the caffeine sensitivity of mck1Δ cells. We also examined the ability of the catalytically inactive allele to support growth at 37°C and on medium containing 10 mM caffeine. We found that the catalytically inactive Mck1p was unable to support growth under either condition. We conclude that Mck1p is indeed able to perform some of its functions in vivo without phosphorylating its target protein(s). The catalytic activity of Mck1p is required for its in vivo functions. Therefore, we further conclude that Mck1p acts on one or more targets in vivo by a mechanism that does not involve phosphorylation of the target protein(s) but does require autophosphorylation. Collectively, our in vivo results corroborate our in vitro findings. The above observations indicate that Mck1p does not have to phosphorylate an exogenous substrate to support growth at 37°C. Interestingly, either overexpression of the pyruvate kinase gene CDC19 or deletion of BCY1 confers temperature-sensitive growth. These in vivo observations are thus consistent with the activation-loop mutant version of Mck1p retaining the ability to inhibit PKA in vivo and to thereby down-regulate pyruvate kinase.

The Activation-loop Mutant Form of Mck1p Is Competent to Inhibit PKA Activity In Vivo—Mck1p directly inhibits the activity of PKA in vitro. This inhibition occurs by a novel mechanism involving autophosphorylation-dependent binding but does not involve phosphorylation of PKA subunits. The activation loop mutant form of Mck1p specifically retains the ability to inhibit PKA activity in vitro but is defective in phosphorylation of exogenous substrates. If Mck1p inhibits PKA activity by such a novel mechanism in living cells, then the following should be true: catalytically inactive Mck1p should be unable to inhibit PKA in vivo, but the activation-loop mutant form of Mck1p should be competent for this function.

We have shown above that pyruvate kinase serves as an in vivo reporter of PKA activity (Fig. 3). Cells carrying the mck1Δ allele were transformed with 2-μm plasmids encoding either wild-type Mck1p, the catalytically inactive Mck1p, or the activation-loop mutant form of Mck1p. Cell extracts from these strains, and a control strain carrying only the empty vector, were assayed for pyruvate kinase activity. We found that pyruvate kinase activity in the strain expressing wild-type Mck1p is distinctly lower than that found in mck1Δ cells (Fig. 7A), as had
have uncovered by which Mck1p inhibits PKA activity in vitro indeed pertains in vivo.

**DISCUSSION**

We have found that purified, catalytically active, Mck1p cannot phosphorylate purified pyruvate kinase in vitro. Given that Mck1p does regulate pyruvate kinase activity in vivo, the mechanism must be indirect. Here we find that another protein kinase, PKA, mediates the regulation of pyruvate kinase by Mck1p.

Multiple lines of evidence indicate that PKA directly phosphorylates pyruvate kinase. First, purified PKA (Tpk1p-Bcy1p) phosphorylates purified pyruvate kinase in vitro. This phosphorylation is stimulated by addition of cAMP or removal of the Bcy1p inhibitory subunit and is inhibited by PKI, a specific inhibitor of PKA kinase activity. In addition, PKA stimulates phosphorylation of pyruvate kinase in a partially purified preparation of the latter enzyme, indicating some specificity to the reaction. Finally, we found that purified Tpk1p alone phosphorylates purified pyruvate kinase in vitro. Encouragingly, Cytrynska et al. (50) recently and independently found that PKA can phosphorylate pyruvate kinase in partially purified preparations.

We believe that PKA is a positive regulator of pyruvate kinase activity in vivo. We have shown that partial activation of PKA, by deletion of one copy of Bcy1 in diploid cells, results in increased pyruvate kinase activity in vivo. We found that deletion of Bcy1 in haploid cells also results in increased activity of pyruvate kinase. Finally, the ability of wild-type and mutant forms of Mck1p to directly inhibit PKA activity in vitro correlates with their ability to down-regulate pyruvate kinase activity in vivo. Our findings are consistent with data from multiple other organisms demonstrating that PKA directly phosphorylates and regulates pyruvate kinase (41–43).

In addition, Cytrynska et al. (50) have recently reported that pyruvate kinase co-purifies with PKA in yeast. Our data indicate that Mck1p regulates pyruvate kinase by directly inhibiting PKA in vitro and in vivo. Purified Mck1p inhibits the phosphorylation of purified pyruvate kinase by purified PKA in vitro. Therefore, Mck1p can directly inhibit PKA activity. The capacity of mutant versions of Mck1p to down-regulate pyruvate kinase activity in vivo correlates with their capacity to directly inhibit PKA catalytic activity in vitro. Finally, mck1Δ mutants share many phenotypes with bcy1Δ mutants lacking the cAMP-binding and inhibitory subunit of PKA. Both mutants are temperature-sensitive for growth, both grow poorly on non-fermentable carbon sources, both display constitutive invasive growth, and both display high pyruvate kinase activity. Thus Mck1p, like Bcy1p, behaves genetically and biochemically as an inhibitor of PKA.

Although we have only examined the regulation of Tpk1p by Mck1p in vitro, it is likely that Mck1p modulates the activity of all three catalytic subunits of PKA in vivo. Tpk1p, Tpk2p, and Tpk3p display a high degree of amino acid sequence identity and functional redundancy in vivo (14, 15). Our results in combination with those of Cytrynska et al. (50) indicate that both Tpk1p and Tpk2p can phosphorylate pyruvate kinase. Deletion of MCK1 mimics deletion of Bcy1 in preventing growth at high temperature and on non-fermentable carbon sources; i.e., phenotypes that are attributed to activation of all three catalytic subunits of PKA. Finally, deletion of BCY1 or MCK1 promotes invasive growth, a phenotype caused specifically by activation of Tpk2p.

Mck1p acts directly on the PKA catalytic subunit, because purified Mck1p can inhibit purified Tpk1p alone. Mck1p does not act by grossly altering the substrate specificity of PKA or by sequestration of its peptide/protein substrates because Mck1p
inhibits the capacity of PKA to phosphorylate both pyruvate kinase (a globular protein) and Kemptide (a peptide) and these inhibitions occur even when the substrates are present in massive excess over the kinases.

The simplest mechanism by which Mck1p might inhibit PKA activity is by phosphorylating PKA. Indeed, the catalytic activity of Mck1p is required for its capacity to inhibit purified Tpk1p. However, we have found that PKA subunits are not phosphorylated by Mck1p even though PKA activity is inhibited by this kinase in vitro. In agreement with this somewhat surprising observation, failure of Mck1p to autophosphorylate on tyrosine compromises its ability to phosphorylate exogenous substrates but does not compromise the capacity of Mck1p to inhibit PKA in vitro or in vivo. Mck1p evidently acts on PKA by a mechanism unusual for protein kinases. Autophosphorylated Mck1p most likely binds to and directly inhibits the catalytic subunits of PKA without intermolecular transfer of phosphate. We have attempted to detect direct physical interaction between Mck1p and PKA catalytic subunits in vitro but have been unsuccessful to date. The interaction may be too weak to survive our manipulations. Despite this failure to detect a direct biochemical interaction between Mck1p and Tpk1p, the interaction almost certainly occurs. Purified Mck1p inhibits purified Tpk1p in vitro demonstrating that the inhibition is direct. The inhibition does not involve sequestration of the peptide/protein substrate. Finally, the inhibition is not due to sequestration of ATP, because ATP is present in massive excess in our experiments (see “Experimental Procedures”).

Efficient inhibition of purified Tpk1p by purified Mck1p in our experiments requires an excess of Mck1p (34 to 1 ratio). We have detected some inhibition of Tpk1p when Mck1p is present at 10-fold excess. The failure to detect inhibition at lower ratios may reflect an inherently weak interaction between the proteins. Another possibility is that only a small fraction of Mck1p is autophosphorylated in our purified preparation and is thus competent to bind to and inhibit Tpk1p. Although it is possible that Mck1p inhibits Tpk1p by an as yet unknown additional enzymatic activity, this possibility is highly unlikely. Mck1p contains no domains other than those predicted to constitute a protein kinase, and the protein kinase activity of Mck1p is required for its ability to inhibit Tpk1p. Finally, it is possible that the efficient interaction between Mck1p and Tpk1p in vitro is strengthened by another, as yet unknown, co-factor. Although the requirement for an excess of Mck1p over Tpk1p in our experiments points to some complications, it is not of itself sufficient to challenge our view that Mck1p directly and non-covalently binds to, and inhibits, the catalytic subunits of PKA in vitro and in vivo. Interestingly, GSK-3 activity is required for its effective binding to Axin in mammalian cells (51). Thus, it is possible that autophosphorylation-dependent binding of GSK-3 to its targets is a common mechanism by which GSK-3 enzymes act on their substrates.

Our in vitro observations on the mechanism by which Mck1p inhibits PKA are relevant in vivo. A mutant form of Mck1p that is unable to autophosphorylate on tyrosine, Mck1p(Y199F), and cannot phosphorylate exogenous substrates, is competent to inhibit PKA in vitro and in vivo. Mck1p(Y199F) does not, however, complement all the phenotypes of mck1Δ cells. Mck1p evidently acts by two distinct and genetically separable mechanisms: phosphorylation of some target proteins and autophosphorylation-dependent binding to PKA without intermolecular transfer of phosphate.

PKA is the canonical protein kinase. It was the first protein kinase to have its structure solved, and decades of research have illuminated its mode of action and regulation. To date, PKA has only been known to be regulated by cAMP binding to Bcy1p causing that subunit to dissociate from the catalytic subunits and thereby liberating their catalytic activity. Here we report a second, novel mode of regulation of PKA. Mck1p acts as a direct inhibitor of the liberated catalytic subunits. Thus, in the presence of cAMP, Mck1p acts independently of PKA and Bcy1p to regulate the activity of the catalytic activity of PKA. To date, variation in the cytoplasmic concentration of cAMP has been taken as reflecting the activity state of PKA. Our data suggest that PKA may also be modulated independently of changes in cAMP concentration.

What is the in vivo role of Mck1p? In vegetatively growing cells, Mck1p evidently acts as a partial inhibitor of PKA. Mck1p also appears to be required for proper function of the morphogenetic checkpoint (14, 15) and for centromere function, neither of which is known to involve PKA (14, 15). The existence of multiple targets for the kinase in the cell and the diverse range of functions it affects indicate either a global role for Mck1p or a variety of independently regulated pathways. Our understanding of the exact role of Mck1p will be improved by further study of conditions and factors that regulate Mck1p autophosphorylation state and activity.

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