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Phosphorylation of the Protein Kinase A Catalytic Subunit Is Induced by Cyclic AMP Deficiency and Physiological Stresses in the Fission Yeast, *Schizosaccharomyces pombe*

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Abstract

In the fission yeast, *Schizosaccharomyces pombe*, cyclic AMP (cAMP)-dependent protein kinase (PKA) is not essential for viability under normal culturing conditions, making this organism attractive for investigating mechanisms of PKA regulation. Here we show that *S. pombe* cells carrying a deletion in the adenylate cyclase gene, *cyr1*, express markedly higher levels of the PKA catalytic subunit, Pka1, than wild type cells. Significantly, in *cyr1Δ* cells, but not wild type cells, a substantial proportion of Pka1 protein is hyperphosphorylated. Pka1 hyperphosphorylation is strongly induced in *cyr1Δ* cells, and to varying degrees in wild type cells, by both glucose starvation and stationary phase stresses, which are associated with reduced cAMP-dependent PKA activity, and by KCl stress, the cellular adaptation to which is dependent on PKA activity. Interestingly, hyperphosphorylation of Pka1 was not detected in either *cyr1*⁺ or *cyr1Δ* *S. pombe* strains carrying a deletion in the PKA regulatory subunit gene, *cgs1*, under any of the tested conditions. Our results demonstrate the existence of a cAMP-independent mechanism of PKA catalytic subunit phosphorylation, which we propose could serve as a mechanism for inducing or maintaining specific PKA functions under conditions in which its cAMP-dependent activity is downregulated.

Introduction

Cyclic AMP (cAMP)-dependent protein kinase, also known as protein kinase A (PKA), is an evolutionarily conserved serine/threonine kinase that contributes to the regulation of diverse cell regulatory pathways in eukaryotic organisms, including signal transduction networks regulating cell growth and differentiation, cell cycle control, cytoskeletal dynamics, metabolism, and physiological stress responses [1;2;3;4]. In its inactive state, the PKA holoenzyme consists of two regulatory subunits bound with high affinity to two catalytic subunits [1]. Binding of cAMP to the PKA regulatory subunits results in their dissociation from the catalytic subunits, which are released as catalytically active monomers. In addition to its cAMP-binding regulatory subunits, PKA function has been shown to be controlled via interactions with several other regulatory factors [5;6]. In mammalian cells, interactions of PKA regulatory subunits with A-kinase anchoring proteins (AKAPs) and A-kinase interacting proteins (AKIPs) have been shown to contribute to the regulation of PKA subcellular localization [1;5;6;7]. In the budding yeast, *Saccharomyces cerevisiae*, which

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does not have AKAP or AKIP homologs, localization of the PKA regulatory subunit is regulated, in part, via interaction with the protein Zds1 [8]. PKA has also been shown to be regulated by phosphorylation and other post-translational modifications, at least in some organisms [8;9;10]. For example, a conserved threonine residue in the activation loop of the PKA catalytic subunit has been shown to be phosphorylated constitutively by phosphoinositide-dependent protein kinases (PDKs) in mammalian cells and in the fission yeast, *Schizosaccharomyces pombe* [1;10].

The PKA regulatory and catalytic subunits in *S. pombe* are encoded by the genes *cgs1* and *pka1*, respectively [11;12]. This organism also has a single adenylate cyclase encoding gene, *cyr1* (a.k.a. *git2*) [13;14]. Similar to other eukaryotes, PKA contributes to the regulation of diverse processes in *S. pombe*, including nutrient sensing, cell cycle regulation, sexual differentiation, and osmoadaptation [12;15;16;17;18]. Furthermore, the *pka1* gene has been shown to be transcriptionally induced by multiple physiological stresses, including oxidative, hyperosmotic, and heavy metal stresses [19]. Because of this, it has been classified as a core environmental stress response (CESR) gene in *S. pombe* [19]. We recently showed through analysis of functional Pka1-GFP and Cgs1-GFP fusion proteins that nuclear-cytoplasmic distribution of the PKA catalytic and regulatory subunits, respectively, is regulated in *S. pombe* in response to several physiological stresses, including hyperosmotic and nutrient stresses [20].

Despite being involved in the regulation of multiple cellular processes, and unlike the situation in the evolutionarily distant yeast, *S. cerevisiae*, neither PKA nor cAMP is essential for viability of *S. pombe* under normal culturing conditions [11;12;13;14]. This characteristic of *S. pombe* makes it an attractive model organism for investigating mechanisms of PKA regulation. In this paper, we show that cAMP deficiency in *S. pombe* induces not only increased expression but also increased phosphorylation of the PKA catalytic subunit, Pka1. Additionally, we show that Pka1 phosphorylation is further induced in mutants lacking cAMP and to varying degrees in wild type cells in response to several physiological stresses, including stresses associated with reduced cAMP-dependent PKA activity.

Materials and methods

Yeast strains and media

S. pombe strains used in this study were SP870 (*h⁹⁰ ade6-210 leu1-32 ura4-D18*) (from D. Beach) and the previously described strains YMSM101 (*h⁹⁰ ade6-210 leu1-32 ura4-D18 pka1-GFP (S65T)-kanMX6*), YMSM102 (*h⁹⁰ ade6-210 leu1-32 ura4-D18 cyr1::ura4 pka1-GFP (S65T)-kanMX6*), YMSM103 (*h⁹⁰ ade6-210 leu1-32 ura4-D18 cgs1::ura4 pka1-GFP (S65T)-kanMX6*), YMSM104 (*h⁹⁰ ade6-210 leu1-32 ura4-D18 cyr1::LEU2 cgs1::ura4 pka1-GFP (S65T)-kanMX6*), YMSM105 (*h⁹⁰ ade6-210 leu1-32 ura4-D18 cgs1-GFP (S65T)-kanMX6*), YMSM106 (*h⁹⁰ ade6-210 leu1-32 ura4-D18 cyr1::ura4 cgs1-GFP (S65T)-kanMX6*) [20]. *S. pombe* cultures were grown in YES (0.5% yeast extract, 3% dextrose, adenine [250 mg/liter], uracil [250 mg/liter], leucine [250 mg/liter], lysine [250 mg/liter], histidine [250 mg/liter]), YES-G (same as YES, but containing 0.1% glucose and 3% glycerol), or synthetic minimal medium (EMM) [21].

Preparation and immunoblotting of cell lysates

For immunoblot analyses of whole cell extracts, cell lysates were prepared using a previously described method in which cells are boiled prior to being lysed in order to reduce the likelihood of protein degradation [22]. The protein concentration was quantified by BCA assay, and proteins were separated by SDS-PAGE on 7.5% polyacrylamide gels. GFP-

tagged proteins were detected using anti-GFP monoclonal antibody (Roche) and the SuperSignal West Dura chemiluminescence detection system (Pierce). Alpha-tubulin was visualized using anti- α -tubulin monoclonal antibody (Sigma).

Immunoprecipitation of Pka1-GFP and in vitro phosphatase assay

Cell lysates from *cyr1 Δ* mutants were prepared by breaking cells with glass beads in NP40 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate) and incubating 5 mg of total protein with Protein-G agarose beads that had been pre-conjugated with anti-GFP antibody. After washing with NP40 buffer, beads were treated with alkaline phosphatase (New England Biolabs) in NEB Buffer 3 (New England Biolabs) at 36°C for 1 hr. Beads were also used for negative controls, which included reaction without phosphatase enzyme and reaction with phosphatase enzyme plus phosphatase inhibitor (10 mM sodium orthovanadate). Beads were mixed with loading buffer and heated at 95°C for 5 minutes. The resulting supernatant was loaded onto a 7.5% polyacrylamide gel and immunoblotted as described above.

Results

cAMP deficiency results in increased expression and phosphorylation of the PKA catalytic subunit in *S. pombe*

We previously described the construction and characterization of *S. pombe* strains in which the coding sequence for green fluorescent protein (GFP) is fused to the C-terminal coding end of the endogenous *pka1* gene (*pka1-GFP*) [20]. Phenotypic characterization of these strains demonstrated that Pka1-GFP retains functions of its wild type counterpart, thus validating its use for studies on Pka1 localization, regulation, and function. Consistent with results reported by Stiefel and coworkers that *S. pombe* cells lacking adenylate cyclase express higher levels of *pka1* mRNA than wild type cells [23], we have noted that GFP fluorescence appears greater in *pka1-GFP* cells lacking the adenylate cyclase gene, *cyr1*, than in wild type (*cyr1*⁺) *S. pombe* cells (Fig. 1A). To confirm that *cyr1 Δ* cells express higher levels of Pka1 protein than wild type *S. pombe* cells, we carried out GFP immunoblot analyses of whole cell lysates prepared from *cyr1*⁺ *pka1-GFP* and *cyr1 Δ* *pka1-GFP* strains. We found that *cyr1 Δ* *pka1-GFP* cells do indeed express significantly higher levels of Pka1-GFP protein than *cyr1*⁺ *pka1-GFP* cells. However, whereas only a single band of Pka1-GFP was detected in *cyr1*⁺ *pka1-GFP* cells, two distinct Pka1-GFP bands were detected in *cyr1 Δ* *pka1-GFP* cells, one with an observed molecular mass and relative abundance similar to that of the protein detected in *cyr1*⁺ *pka1-GFP* cells, and the other exhibiting a higher observed mass, which was expressed at a level similar to that of the lower molecular mass form detected in both wild type and *cyr1 Δ* cells (Fig. 1B).

To confirm that changes in Pka1 expression detected in the *cyr1 Δ* *pka1-GFP* mutant are induced by cAMP deficiency, we cultured the strain in medium supplemented with cAMP, prepared cell lysates at various intervals post-cAMP exposure, and subjected the lysates to anti-GFP immunoblot analysis. As shown in Fig. 1C, the higher molecular mass form of Pka1-GFP was barely detectable in *cyr1 Δ* *pka1-GFP* cells after only 10 min of cAMP treatment and after 30 min of cAMP treatment was not detectable at all. After 90 min of cAMP treatment, Pka1-GFP protein detected in *cyr1 Δ* cells was also reduced to a level nearer to but still slightly higher than that detected in wild type *S. pombe* cells (Fig. 1C).

We next determined whether the higher molecular mass form of Pka1 detected in *cyr1 Δ* *pka1-GFP* cells is a consequence of increased phosphorylation, or hyperphosphorylation, of the Pka1 protein, which like mammalian PKA catalytic subunits, is phosphorylated constitutively on a conserved threonine (Thr³⁵⁶) located in the activation loop of the kinase

[10]. To do this, Pka1-GFP was immunoprecipitated from *cyr1Δ pka1-GFP* cell lysates and the immune complexes were treated with alkaline phosphatase in the presence or absence of phosphatase inhibitor. As shown in Fig. 1D, in Pka1-GFP immune complexes treated with phosphatase, but not those that were untreated or treated with both phosphatase and phosphatase inhibitor, the higher molecular mass form of Pka1-GFP was converted to a single band similar in apparent mass to the faster mobility form detected in both *cyr1Δ* cells and wild type cells. It should be noted that the phosphatase treatment used for these experiments did not result in a downward mobility shift of Pka1-GFP below that of the lower molecular mass form detected in both wild type and *cyr1Δ* cells. However, this would be expected under the conditions used for these assays, as the constitutively phosphorylated Thr³⁵⁶ residue of Pka1 is predicted to be highly resistant to phosphatase treatment, as has been shown to be the case for the corresponding phosphoamino acid residue in mammalian PKA catalytic subunits [24].

Taken together, the above findings demonstrate that Pka1 expression and phosphorylation are both upregulated in response to cAMP deficiency in *S. pombe*.

The PKA regulatory subunit, Cgs1, is required for upregulation of Pka1 phosphorylation and expression in response to cAMP deficiency

We next carried out experiments to explore the possibility that the upregulation of Pka1 expression and phosphorylation detected in *S. pombe* cells lacking adenylate cyclase might serve as mechanisms to promote or maintain certain PKA functions in the absence of cAMP stimulation. We reasoned that if this were the case, then increased Pka1 expression and phosphorylation might not be detected in adenylate cyclase mutants lacking the PKA regulatory subunit, Cgs1. Indeed, in both *cyr1⁺ cgs1Δ pka1-GFP* and *cyr1Δ cgs1Δ pka1-GFP* cells, we detected only a single band of Pka1-GFP, the relative expression and mobility of which was similar to that detected in wild type cells (Fig. 2). These results demonstrate that upregulation of Pka1 expression and phosphorylation are not induced in adenylate cyclase mutants in which PKA is rendered constitutively active as a result of deletion of the gene encoding its regulatory subunit.

Effects of glucose starvation/derepression and KCl stress on Pka1 phosphorylation

Since transcription of the *pka1* gene is upregulated in response to a variety of physiological stresses in *S. pombe* [19], it would be predicted that *S. pombe* cells lacking adenylate cyclase might be exposed to higher levels of basal stress than wild type cells. Indeed, we have recently shown that *S. pombe pka1Δ* cells have significantly higher levels of reactive oxygen species than wild type cells [25]. We reasoned, therefore, that the hyperphosphorylation of Pka1 protein detected in cells lacking adenylate cyclase activity might be indicative of a mechanism that maintains or promotes certain PKA functions under conditions in which its cAMP-dependent activities are downregulated. To test this possibility, we compared the effects of physiological stresses associated with reduced or sustained cAMP-dependent PKA activity on Pka1 phosphorylation and expression in wild type (*cyr1⁺*) and *cyr1Δ S. pombe* strains. One stress associated with downregulation of cAMP-dependent PKA activity is glucose starvation. PKA functions to repress sexual differentiation and gluconeogenesis when *S. pombe* cells are grown in glucose-enriched media and these cAMP-dependent functions of the kinase are downregulated when cells are cultured in glucose-limited media [16;26]. As shown in Fig. 3A, we detected a dramatic increase in the ratio of hyperphosphorylated Pka1 to basally phosphorylated Pka1 in *cyr1Δ pka1-GFP* cells cultured for 8 hr in glucose-limited conditions. In contrast, glucose starvation did not induce increased Pka1 phosphorylation in wild type, *cgs1Δ*, or *cyr1Δ cgs1Δ* cells (Fig. 3A, right panels). These results indicate that glucose starvation strongly induces Pka1 phosphorylation in *S. pombe* cells with low basal PKA activity.

In contrast to the situation in glucose-limited media, PKA activity is essential for growth of *S. pombe* cells cultured in media containing high concentrations of KCl [17], indicating that PKA activity must either be maintained or further stimulated under these conditions. As shown in Fig. 3B, *cyr1Δ* *pka1-GFP* cells cultured in media containing 1.2 M KCl for 8 hr exhibited dramatic upregulation of both Pka1 phosphorylation and expression in comparison to the same cells cultured under non-stressed conditions. In addition, a small proportion of Pka1 protein appeared to be hyperphosphorylated even in wild type *S. pombe* cells after 8 hr of KCl stress (Fig. 3B).

Taken together, the above results led us to hypothesize that Pka1 phosphorylation might play a more prominent role in PKA regulation under conditions in which *S. pombe* cells are subjected simultaneously to a variety of stresses, some requiring that specific PKA activities be maintained or stimulated for proper cellular response and others requiring that certain PKA functions be repressed. To explore this possibility, we determined the consequences of subjecting wild type (*cyr1⁺*) *pka1-GFP* cells simultaneously to KCl stress, the cellular response to which is dependent on PKA activity, and glucose starvation, the response to which requires downregulation of cAMP-dependent PKA activity. Strikingly, and consistent with our hypothesis, we observed that Pka1 phosphorylation was strongly induced in wild type *S. pombe* cells subjected simultaneously to both stresses (Fig. 3C). Furthermore, in *cgs1Δ* cells in which PKA is constitutively active, the combination of KCl stress and glucose starvation did not lead to increased Pka1 phosphorylation and actually triggered a discernable decrease in the abundance of Pka1 protein (Fig. 3C).

Stationary phase stress strongly induces Pka1 phosphorylation in *S. pombe* cells

Previous studies have shown that *S. pombe* *pka1Δ* mutants exhibit increased stationary phase viability relative to wild type cells, whereas *cgs1Δ* cells in which PKA is constitutively active exhibit reduced viability in stationary phase [11;15;27]. In light of these previous findings, we investigated the effects of stationary phase stress on Pka1 expression and phosphorylation in *S. pombe* cells. Interestingly, increased Pka1 expression was detected in early stationary phase cultures of both wild type and *cyr1Δ* cells (Fig. 4A). By contrast, in both *cgs1Δ* and *cyr1Δ* *cgs1Δ* strains, the level of Pka1 protein appeared similar in log phase and early stationary phase cells (Fig. 4B). Strikingly, in both wild type and *cyr1Δ* cells incubated at stationary phase density for 24 hr, the only detectable Pka1 protein was the hyperphosphorylated form (Fig. 4A). In contrast, in both *cgs1Δ* and *cyr1Δ* *cgs1Δ* cells, Pka1 levels were greatly reduced relative to those detected in log phase and early stationary phase cells and a hyperphosphorylated form of the protein was not detected in either strain (Fig. 4B).

Discussion

We have shown here that cAMP deficiency results in upregulation of both expression and phosphorylation of the PKA catalytic subunit, Pka1, in the fission yeast, *S. pombe*. Interestingly, increased expression and phosphorylation of Pka1 were not detected in *S. pombe* cells lacking both adenylate cyclase and the PKA regulatory subunit, Cgs1. This finding suggests that the Pka1 phosphorylation induced by cAMP deficiency might represent a physiological response to low PKA activity and, therefore, be stimulatory with respect to PKA function. The dependence on Cgs1 for any detectable phosphorylation of Pka1 in both *cyr1Δ* and *cyr1⁺* wild type *S. pombe* strains under both normal and physiologically stressed culturing conditions suggests a possible role for Cgs1 in promoting interaction between Pka1 and the protein kinase that phosphorylates it. In this regard, it is noteworthy that addition of cAMP to cultures of *S. pombe* cells lacking adenylate cyclase induces rapid dephosphorylation of Pka1. This finding suggests that the protein phosphatase(s) responsible

for dephosphorylating Pka1 under normal growth conditions might be physically associated with the PKA complex in *S. pombe*.

Interestingly, we found that in *S. pombe* cells lacking adenylate cyclase, Pka1 phosphorylation was strongly induced in response to both glucose starvation, which is associated with downregulation of cAMP-dependent PKA activity [16], and by KCl stress, the cellular response to which is dependent on PKA function [17]. Pka1 phosphorylation was also strongly induced by stationary phase stress in both wild type and *cyr1Δ S. pombe* strains. These findings demonstrate what is to our knowledge the first example in any eukaryotic organism of *in vivo* phosphorylation of a PKA catalytic subunit induced by a mechanism independent of cAMP stimulation. We hypothesize that the cAMP-independent phosphorylation of Pka1 detected in our experiments might serve as a mechanism to maintain or promote specific PKA functions under conditions in which its cAMP-dependent activities are downregulated. cAMP-independent PKA stimulatory mechanisms might play particularly important roles in *S. pombe* cells in their natural environments (e.g., plant surfaces), where they would frequently be subjected to multiple physiological stresses, some of which might require that specific PKA functions be sustained or upregulated and others requiring downregulation of specific PKA functions. A simple example of such a situation is that of *S. pombe* cells cultured in a glucose-limited, hypertonic growth medium in which cAMP-dependent gluconeogenic inhibitory functions of PKA are downregulated, while PKA functions required for growth in hypertonic conditions would presumably need to be maintained or upregulated. We found that under this culturing condition, as our hypothesis would predict, Pka1 phosphorylation was strongly induced in wild type *S. pombe* cells but not in *cgs1Δ* cells in which PKA is constitutively active. Given the diverse roles played by PKA in eukaryotic organisms, we consider it likely that stress-induced phosphorylation of PKA catalytic subunits represents an underappreciated mechanism for regulating PKA function independently of or in concert with cAMP.

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Fig. 1. cAMP deficiency results in increased expression and phosphorylation of the PKA catalytic subunit in *S. pombe*

(A) Fluorescence photomicrographs of *cyr1⁺ pka1-GFP* (Wild Type) and *cyr1Δ pka1-GFP* (*cyr1Δ*) cells cultured to mid-log phase in YES medium showing relative intensity of Pka1-GFP fluorescence in the two strains. (B) GFP immunoblot analysis of Pka1-GFP fusion proteins in cell lysates prepared from *cyr1⁺ pka1-GFP* (WT) and *cyr1Δ pka1-GFP* (*cyr1Δ*) strains cultured to mid-log phase in YES medium. *S. pombe* strain SP870 was used as a control. Lower panel shows α -tubulin immunostaining of the same blot to show relative loading of the protein extracts. White arrow points to lower molecular mass form of Pka1-GFP detected in both *cyr1⁺* and *cyr1Δ* cells. Black arrow points to higher molecular mass form of Pka1-GFP detected only in *cyr1Δ* cells. (C) GFP immunoblot analysis of Pka1-GFP fusion proteins in cell lysates prepared from *cyr1⁺ pka1-GFP* (WT) cells and *cyr1Δ pka1-GFP* (*cyr1Δ*) cells treated with 5 mM cAMP for the indicated times. (D) Pka1-GFP protein was immunoprecipitated from *cyr1Δ pka1-GFP* cells and treated, or not, with alkaline phosphatase alone or alkaline phosphatase and the phosphatase inhibitor sodium orthovanadate (2.5 mM) as indicated.

Fig. 2. The PKA regulatory subunit, Cgs1, is required for upregulation of Pka1 expression and phosphorylation in response to cAMP deficiency in *S. pombe*

GFP immunoblot analysis of Pka1-GFP fusion proteins in cell lysates prepared from *cyr1*⁺ *pka1*-GFP (WT), *cyr1Δ pka1*-GFP (*cyr1Δ*), *cgs1Δ pka1*-GFP (*cgs1Δ*), and *cyr1Δ cgs1Δ pka1*-GFP (*cyr1Δ cgs1Δ*) strains cultured to mid-log phase in YES medium.

Fig. 3. Effects of glucose starvation and KCl stress on Pka1 expression and phosphorylation in wild type, *cyr1Δ*, *cgs1Δ*, and *cgs1Δ cyr1Δ* *S. pombe* strains

(A) Effects of glucose starvation on Pka1 expression and phosphorylation. GFP immunoblot analysis of Pka1-GFP fusion proteins in cell lysates prepared from mid-log phase cultures of *cyr1⁺ pka1-GFP* (WT), *cyr1Δ pka1-GFP* (*cyr1Δ*), *cgs1Δ pka1-GFP* (*cgs1Δ*), and *cyr1Δ cgs1Δ pka1-GFP* (*cyr1Δ cgs1Δ*) strains incubated for 8 hr in YES medium (–) or in glucose-limited YES-G medium (+). (B) Effects of KCl stress on Pka1 expression and phosphorylation. Strains in panel A were cultured to mid-log phase in YES medium, diluted with equal volumes of YES or YES containing 2.4 M KCl, and incubated for 8 hr prior to preparation of cell lysates and GFP immunoblot analysis to detect Pka1-GFP fusion proteins. (C) Effects of combined KCl and glucose starvation stresses on Pka1 expression and phosphorylation. *cyr1⁺ pka1-GFP* (WT) and *cyr1⁺ cgs1Δ pka1-GFP* (*cgs1Δ*) strains were cultured overnight to mid-log phase in YES containing 1.2 M KCl, washed 3x with YES-G containing 1.2 M KCl (YES-G + KCl), resuspended at 5×10^6 cells/ml in YES-G + KCl, and incubated at 30°C for 8 hr prior to preparation of cell lysates and GFP immunoblot analysis to detect Pka1-GFP fusion proteins. –, non-stressed control cultures grown in YES; +, cultures subjected to both KCl and glucose starvation stresses.

Fig. 4. Effects of stationary phase stress on expression and phosphorylation of Pka1 in *S. pombe* *cyr1⁺ pkal-GFP* (WT)(A, left panels), *cyr1Δ pkal-GFP* (*cyr1Δ*)(A, right panels), *cgs1Δ pkal-GFP* (*cgs1Δ*)(B, left panels), and *cyr1Δ cgs1Δ pkal-GFP* (*cyr1Δ cgs1Δ*)(B, right panels) strains were cultured to mid-log phase density (log) or early stationary phase density (0 h) or incubated at stationary phase density for 24 hr (24 h), as indicated, prior to preparation of cell lysates and GFP immunoblot analysis to detect Pka1-GFP fusion proteins. Middle panels of (B) are longer exposures of the GFP immunoblot to show low levels of Pka1 protein detectable in *cgs1Δ* and *cyr1Δ cgs1Δ* strains after incubation at stationary phase density for 24 hr.