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Role of Sch9 in regulating Ras-cAMP signal pathway in *Saccharomyces cerevisiae*

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ABSTRACT

In *Saccharomyces cerevisiae* PKA plays a major role in regulating cell growth, metabolism, and stress resistance. We report that Sch9 regulates PKA directly and SCH9 deletion enhances PKA activity by showing that: (1) Bcy1 predominately localized in the nucleus in glycerol-grown sch9Δ cells; (2) large part of the catalytic subunits of PKA transferred from the nucleus to the cytoplasm in sch9Δ cells; (3) higher protein stability of Tpk2 resulted in higher protein level of Tpk2 in sch9Δ than in wild type cells. Our investigations suggest that Sch9 regulates phosphorylation of Bcy1. We also observed hyper-phosphorylation of Cdc25 in sch9Δ, in contrast to the tpk2Δ and tpk2Δsch9Δ mutants, suggesting that feedback inhibition of PKA on Cdc25 is through Tpk2.

1. Introduction

All organisms sense and respond to nutrient derived signals to adapt their physiology and adopt appropriate developmental decisions to promote survival. In the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), glucose signals activate the production of cellular cAMP. This signaling pathway is called the Ras-cAMP pathway, which plays a major role in the regulation of cell growth, metabolism and stress resistance, in particular in connection with the available nutrient conditions [1]. The level of cAMP is controlled by an elaborate pathway of which many components have been identified. cAMP is synthesized by adenylae cyclase encoded by the *CYR1/CDC35* gene [2]. The activity of adenylae cyclase is controlled by Ras proteins and Gpr1-Gpa2. The Ras protein is a small G protein that demonstrates its biological functions through a cycle of GDP/GTP exchange and GTP hydrolysis. The GTP-bound form is active while the hydrolysis of the bound GTP to GDP inactivates it. The GDP/GTP exchange on the Ras protein is catalyzed by Cdc25 [3] and the GTP hydrolysis reaction is stimulated by Ira1 and Ira2 [4]. *GPR1* encodes a glucose receptor molecule with seven transmembrane regions, and the third cytosolic loop and C-terminal end were shown to interact with Gpa2 [5]. Cellular cAMP is hydrolyzed by low-affinity (Pde1) [6] and high-affinity (Pde2) [7] cAMP phosphodiesterases through which can realize the feedback inhibition on PKA [8,9].

The downstream target of cAMP in yeast is PKA which is a tetramer consisting of two regulatory subunits (encoded by the gene *BCY1*) and two catalytic subunits (encoded by the genes *TPK1*, *TPK2* and *TPK3*) [10]. Although the three genes (*TPK1*, *TPK2*, and *TPK3*) play redundant roles during cell growth [11], distinct roles have been shown for each subunit in pseudohyphal growth. cAMP controls the localization of Tpk1, and the carbon source determines that of Bcy1 [12]. During exponential growth on glucose, both Bcy1 and Tpk2 localized in nucleus, whereas Tpk1 and Tpk3 showed a mixed pattern of nucleus-cytoplasm localization. During exponential growth on glycerol and during stationary phase, the PKA subunits showed mostly cytoplasmic localization [13]. The N-terminal domain of Bcy1 served to target it properly during logarithmic phase and stationary phase [12].

Another pathway involved in signaling the presence of glucose and nitrogen to yeast cells is the so-called Fermentable Growth Medium (FGM) induced pathway [14]. Protein kinase Sch9, an orthologue of mammalian PKB/Akt, is one of major components of this pathway. Sch9 plays an important role in nitrogen signaling and glucose signaling in the budding yeast. When Sch9 was deleted, the activity of the protein kinase A in derepressed cells was strongly elevated and this was supported both by in vivo measurements of protein kinase A targets and by direct in vitro measurements of its activity [14], but the mechanism is still unclear. Subsequent studies showed that Sch9 was an activator of translation initiation, the expression of ribosomal protein, ribosome biogenesis genes, a regulator of cell size control and transcription in response to nutrient availability and osmotic stress responses [14–18]. Sch9 also participated in a conserved signaling network

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involving upstream acting kinases: Pkh1/2, which phosphorylate Thr570 in the activation loop of Sch9, and Tor1/2, which phosphorylated six amino acids in the C terminus of Sch9 and these phosphorylation events were required for catalytic activity [19–21].

In *S. cerevisiae*, as well as in higher eukaryotes, highly conserved protein kinase PKA and Sch9 integrate the nutrient and other environmental cues to regulate ribosome biogenesis, stress response, cell size, autophagy, and other cellular processes [22]. In recent years, the general understanding of multiple ways of crosstalk between PKA and Sch9 have greatly advanced [23]. However, the fine mechanisms of how Sch9 regulate Ras-cAMP pathway remains need to be clarified.

Here we report that Sch9 inhibits PKA activity. We observed that Sch9 regulated the localization and phosphorylation of Bcy1, and there were more Tpk1, Tpk2, Tpk3 localized in cytoplasmic in *sch9*Δ cells than in wild type cells. Most importantly, we found that higher protein stability of Tpk2 in *sch9*Δ cells resulted in higher protein level of Tpk2 in *sch9*Δ cells. All these results suggested that deletion of *SCH9* enhanced PKA activity. Our researches also demonstrated that deletion of *SCH9* resulted in hyper-phosphorylation of Cdc25, while this hyper-phosphorylation of Cdc25 disappeared in *tpk2*Δ mutant and *sch9*Δ*tpk2*Δ mutant. These results suggested that Sch9 regulated phosphorylation of Cdc25 though Tpk2 to realize feedback inhibition on Ras-cAMP signal pathway.

2. Materials and methods

2.1. Yeast strains, growth media, growth conditions and plasmids

Yeast strains used in this study were listed in Table 1. Yeast cells were grown in YPD (1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose) or in synthetic complete (SC) medium (0.17% (w/v) nitrogen base, with adenine, uracil, histidine, leucine, tryptophan and amino acids as appropriate) but lacking essential components to select for plasmids. Yeast cells were grown into mid-exponential phase (OD_{600} 1.5) at 30 °C. Carbon source-derepressed cells used for the experiments described in this study were grown on YPG (1% (w/v) yeast extract, 2% (w/v) peptone and 3% (w/v) glycerol)) until the cultures reached an OD_{600} of 1.5. To obtain cells in stationary phase, cells were cultured in YPD for at least 2 days.

All plasmids and primers used in this study were listed in Tables 2 and 3, respectively.

2.2. Western blot analysis

Yeast cells cultures were grown at 30 °C. Cells were harvested by centrifugation at 4 °C and washed in ice-cold sterile water, and then the pellets were stored at −80 °C until use. All subsequent steps were carried out at 4 °C. Cells were resuspended in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.1% NP-40/Igepal CA-630, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, 1 mM sodium orthovanadate , 10 mM glycerol-2-phosphate, and a mixture of protease inhibitors (Roche). And then cells were disrupted by vortexing them for 30s in the presence of glass beads using Fastprep FP120 (Bio101 Thermo Savant). The resulting suspensions were spun down in a centrifuge at 18 000*g* for 5 min. After addition of equal volume of 2× sample buffer to the supernatant, samples were heated to 95 °C for 5 min before equal amount of total protein being separated by SDS–PAGE. Immunodetection of proteins were carried out using anti-hemagglutinin (HA) monoclonal antibody (mouse immunoglobulin G [IgG3], TIANGEN) or Anti-GAPDH Antibody (HRP conjugated) (mouse monoclonal antibody, TIANGEN) as loading control. The secondary antibody was anti-mouse IgG conjugated with horseradish peroxidase purchased from TIANGEN. Proteins were visualized using LumiGlo (KPL) according to the manufacturer's instructions.

Table 1
Strains used in this study.

Strains	Genotype	Source or reference
W303-1A	*MATa leu2-3, 112 ura3-1 trp1-92 his3-11,15 ade2-1 can1-100*	Wild type [22]
ZAL640	*MATa sch9Δ::URA3*	This study
ZAL103	*MATa tpk1Δ::repeat CDC25-3xHA*	This study
ZAL102	*MATa tpk2Δ::repeat CDC25-3xHA*	This study
ZAL101	*MATa tpk3Δ::repeat CDC25-3xHA*	This study
ZAL503	*MATa tpk1Δ::repeat sch9Δ::URA3 CDC25-3xHA*	This study
ZAL502	*MATa tpk2Δ::repeat sch9Δ::URA3 CDC25-3xHA*	This study
ZAL501	*MATa tpk3Δ::repeat sch9Δ::URA3 CDC25-3xHA*	This study
DJ401	*MATa CDC25-3xHA*	This study
DJ404	*MATa sch9Δ::URA3CDC25-3xHA*	This study

Table 2
Plasmids used in this study.

Plasmids	Source or reference
YCplac22	[23]
YEplac112	[23]
pUC18-RYUR	[24]
YCplac22-*GFP-BCY1*	This study
YCplac22-*BCY1-3xHA*	This study
YCplac22-*TPK1-GFP*	This study
YCplac22-*TPK1-3xHA*	This study
YCplac22-*TPK2-GFP*	This study
YCplac22-*TPK2-3xHA*	This study
YEplac112-*TPK3*-GFP	This study
YEplac112-*TPK3-3xHA*	This study

Table 3
Primers used in this study.

Primer	Oligonucleotide
5′BCY1-U	5′GGTACCAAGCTTAAGAGGAGCATACGACTTCG3′
5′BCY1-D	5′GGTACCGGATCCCATCGTTTATTCTTACTGTTGTC3′
3′BCY1-U	5′GGTACCGGATCCGTATCTTCTTTGCCCAAGGA3′
3′BCY1-D	5′GGTACCGAATTCCGGAATCTTTCTGTGTGGAT3′
ZBCY1-U	5′CCCGGGGGATCCAAGAGGAGCATACGACTTCG3′
bcy1gfp-D	5′GAGCTCCGGCCGCATGTCTTGTAGGATCATTGAGC3′
CTPK1-U	5′CCCGGGGGATCCACATACCGGTATACACCACA 3′
CTPK1-D	5′CCCGGGGCATGCGAAGTCCCGGAAAAGATCAG3′
CTPK2-U	5′CCCGGGGGATCCAGCTGGCCAGTCAATTTGGT3′
tpk2gfp-D	5′GAGCTCGCGGCCGCGAAATCTTGAAAGTATTCAGC3′
CTPK3-U	5′GTCGACCCCGGGCAGATACTCTCTGGTGCGCA3′
CTPK3-D	5′GTCGACGCATGCAAATTCTTTCATTAAATCCATA TATGGATCCTCCCC3′

Relevant restriction sites are underlined.

2.3. Quantitative RT-PCR assays

Total RNA were isolated from cells (1×10^7) using the RNA Extraction Kit (TIANDZ, China) and RNA concentrations were determined by measuring absorbance at 260 nm. From 1 μg of total RNA, first-strand cDNA were prepared with Quantscript RT Kit (TIANGEN, China) using oligio-dT_{15} primer. Relative quantification of *TPK2* and *ACT1* mRNA were determined with the RealMasterMix (SYBR Green) qPCR kit (TIANGEN, China) and the following primers for *TPK2*: For, 5′ATGGAATTCGTTGCAGAAAGG3′ and Rev, 5′ACG CCTTGCCCGTAAGTA3′. For *ACT1* the primers were: For, 5′TTATTG ATAACGGTTCTGGTATG3′ and Rev, 5′CCTTGGTGTCTTGGTCTAC3′. Real-time quantitative RT-PCR (Q-RT-PCR) assays were performed

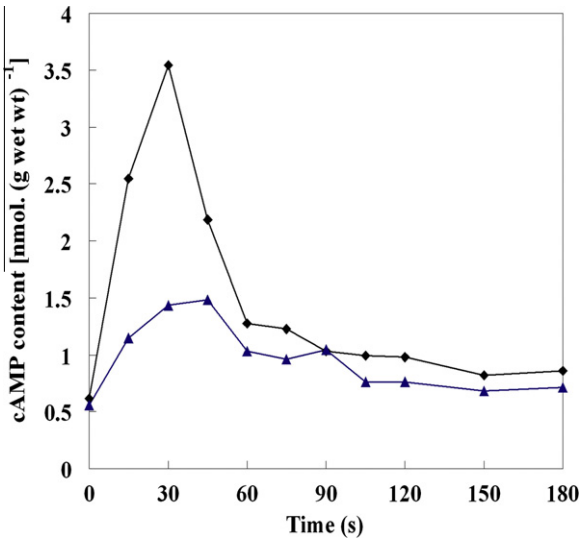

Fig. 1. Effect of Sch9 on cAMP level after addition of 100 mM glucose to glycerol-grown wild type (♦) and *sch9*Δ (▲) cells.

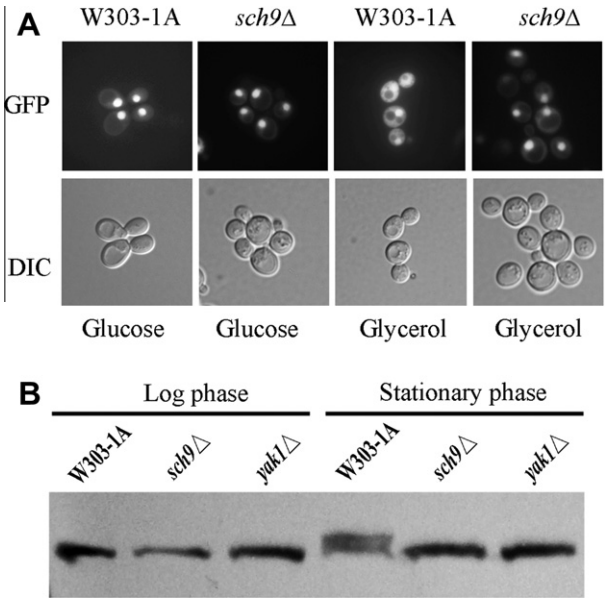

Fig. 2. Effect of Sch9 on localization and phosphorylation of Bcy1. (A) Localization of GFP-Bcy1 in exponential phase wild type cells and *sch9*Δ mutant cells. Cells carrying GFP-Bcy1 were used for fluorescence microscopy directly without fixation. (B) Expression of Bcy1 in wild type and *sch9*Δ mutant at exponential phase and stationary phase (48 h). Proteins were extracted, equal amounts of cellular protein (30 μg) were separated on a 10% SDS–PAGE gel.

using LightCycler 480 (Roche, Switzerland). For quantification, the abundance of *TPK2* was determined relative to the standard transcript of *ACT1* and the final data of relative gene expression were calculated following the $2^{-\Delta\Delta Ct}$ method, as described in [24].

2.4. Green fluorescence protein (GFP) fluorescence microscopy

Cells were used for fluorescence microscopy directly without fixation. Cells were viewed using an Olympus BX51 fluorescence microscope. Images were taken with an Olympus U-LH100HGAPO camera using SPOT (Version 4.0.2) software and then processed in Adobe Photoshop CS4.

3. Results

3.1. Sch9 regulated localization and phosphorylation of Bcy1

It was reported that addition of 100 mM glucose to glycerol-grown wild type cells caused a transient increase in cAMP level [14]. But this glucose-induced cAMP increase of *sch9*Δ cells decreased significantly than that of wild-type cells (Fig. 1), this is consistent with early research [14]. According to Crauwels, M. and his colleagues' research, when *SCH9* was deleted, the activity of the protein kinase A in derepressed cells was strongly elevated and this was supported both by in vivo measurements of protein kinase A targets and by direct in vitro measurements of its activity [14]. However, cAMP synthesis and the glucose-induced cAMP signal are to be down-regulated by high protein kinase activity. The feedback inhibition of PKA on cAMP synthesis caused the lower cAMP level in *sch9*Δ strains. But the mechanisms of Sch9 inhibit PKA activity is still unclear.

To analyze whether Sch9 inhibit PKA directly, we investigated whether Sch9 regulated the expression and localization of Bcy1. As shown in Fig. 2A, Bcy1 predominantly localized in nucleus in rapidly glucose-grown wild type cells and *sch9*Δ cells. A large part of Bcy1 transferred from nucleus to cytoplasm in glycerol-grown wild type cells, while Bcy1 remained nucleus in glycerol-grown *sch9*Δ cells. These results suggested that Sch9 regulated localization of Bcy1.

It was reported that Bcy1 was phosphorylated in cells grown on nonfermentable carbon source or in stationary phase cells [25]. Phosphorylation of its N-terminal domain directs Bcy1 to cytoplasm. As shown in Fig. 2B, faster-migrating iso-form of Bcy1-HA was detected in exponential phase wild type cells, while a predominant slower-migrating iso-form of Bcy1-HA was detected in stationary phase wild type cells. But faster-migrating iso-form of Bcy1-HA was detected in *sch9*Δ mutant cells and *yak1*Δ mutant cells-either in exponential phase or in stationary phase. These indicated that Sch9 might regulate phosphorylation of Bcy1.

3.2. Sch9 regulated the activity and localization of catalytic subunit of PKA

To further examine how Sch9 regulates PKA, we began to investigate whether Sch9 affected protein levels and the localization of catalytic subunits (encoded by the genes *TPK1*, *TPK2*, and *TPK3*) of PKA. Firstly we determined Tpks protein levels of wild type cells and *sch9*Δ mutant cells. As shown in Fig. 3, protein levels of Tpk1 and Tpk2 in glycerol-grown wild type cells were higher than in glucose-grown wild type cells (Fig. 3A and B), while protein level of Tpk3 in glycerol-grown wild type cells were lower than in glucose-grown wild type cells (Fig. 3C). As indicated in Fig. 3A, Tpk1 protein levels of glucose-grown *sch9*Δ cells decreased slightly compared with that of wild type cells, while Tpk1 protein levels of glycerol-grown *sch9*Δ cells did not have great change compared with that of wild type cells. Fig. 3B suggested that protein levels of Tpk2 in *sch9*Δ cells enhanced markedly than in wild type cells, especially in glycerol-grown cells. Protein levels of Tpk3 in *sch9*Δ cells decreased slightly compared to wild type cells grown either on glucose or glycerol (Fig. 3C). All these results suggested that deletion of *SCH9* enhanced PKA activity by increasing protein level of Tpk2.

To clarify whether Sch9 regulated Tpk2 at transcription level, mRNA levels of *TPK2* of wild type cells and *sch9*Δ cells were tested using quantitative RT-PCR assays (Q-RT-PCR). mRNA levels of *TPK2* in glycerol-grown cells was about two to three times than in glucose-grown cells. But mRNA levels of *TPK2* in *sch9*Δ cells did not have great change compared with wild type cells (data not shown). These results indicated that Sch9 did not inhibit Tpk2 activity by regulating transcription of Tpk2.

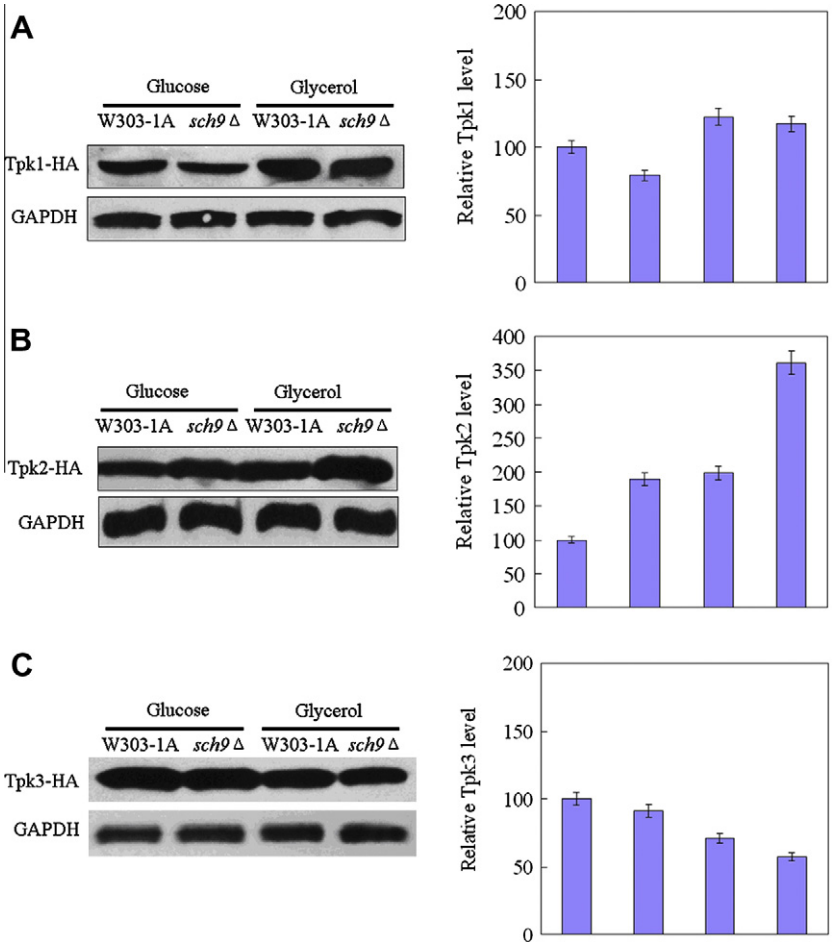

Fig. 3. Protein levels of Tpk1, Tpk2 and Tpk3 in glucose-grown and glycerol-grown W303-1A and *sch9*Δ cells. Samples were taken from exponential phase (OD_{600nm} 2.0) cells carrying 3xHA-tagged Tpk1, Tpk2 and Tpk3. Proteins were separated on 10% SDS–PAGE gels. Protein levels of GAPDH were examined as loading control. For quantitative analysis, band intensities were measured using SCION IMAGE. Normalized intensities (Tpk/GAPDH∗100) are presented as means ± S.D. of three independent experiments. (A) Comparison of Tpk1 protein levels in glucose-grown or glycerol-grown W303-1A and *sch9*Δ cells carrying single-copy plasmid YCplac22-*TPK1*-3×*HA*. Equal amounts of cellular protein (50 μg) were separated on SDS–PAGE. (B) Comparison of Tpk2 protein levels in glucose-grown or glycerol-grown W303-1A and *sch9*Δ cells carrying single-copy plasmid YCplac22-*TPK2*-3×*HA*. Equal amounts of cellular protein (50 μg) were separated on SDS–PAGE. (C) Comparison of Tpk3 protein levels in glucose-grown or glycerol-grown W303-1A and *sch9*Δ cells carrying multi-copy plasmid YEplac112-*TPK1*-3×*HA*. Equal amounts of cellular protein (100 μg) were separated on SDS–PAGE.

Then we examined whether the higher Tpk2 protein levels of *sch9*Δ cells were due to protein stability difference. We determined Tpk2 protein levels in cells treated with protein synthesis inhibitor cycloheximide (50 μg/ml). As shown in Fig. 4A, Tpk2 protein levels of glucose-grown wild type cells decreased visibly after adding protein synthesis inhibitor cycloheximide two hours. While Tpk2 protein levels of cycloheximide-treated-glucose-grown *sch9*Δ cells did not have visible change. These suggested that Tpk2 protein stability of glucose-grown *sch9*Δ celld were higher than that of wild type cells. According to Fig. 4B, Tpk2 protein levels of glycerol-grown wild type cells decreased visibly after adding protein synthesis inhibitor cycloheximide two hours. But Tpk2 protein levels of glycerol-grown *sch9*Δ cells did not change visibly. All these indicate that the higher protein levels of Tpk2 in *sch9*Δ cells were due to higher protein stability.

We next tested whether Sch9 had effects on localization of Tpks. As shown in Fig. 5A, Tpk1-GFP mainly localized in nucleus of exponential phase glucose-grown wild type cells, only a little part of Tpk1-GFP mainly localized in cytoplasm. Cytoplasmic localization of Tpk1-GFP in glycerol-grown wild type cells increased than in glucose-grown cells. While cytoplasmic localization of Tpk1 in glucose-grown and glycerol-grown *sch9*Δ cells increased markedly than in wild type cells. According to Fig. 5B, Tpk2-GFP predominantly localized in nucleus of exponential phase glucose-grown wild type cells, while some part of Tpk2 transferred from nucleus into cytoplasm in exponential phase glycerol-grown wild type cells. But Tpk2 predominantly localized in cytoplasm of *sch9*Δ cells either grown on glucose or glycerol. Tpk3-GFP predominantly localized in nucleus of exponential phase glucose-grown wild type cells, while part of Tpk3 transferred from nucleus into cytoplasm in exponential phase glycerol-grown wild type cells. Cytoplasmic localization of Tpk3 in *sch9*Δ cells either grown on glucose or glycerol increased slightly than in wild type cells (Fig. 5C). Together, these results demonstrated that deletion of *SCH9* caused transferring of Tpks into cytoplasm.

3.3. SCH9 deletion resulted in hyper-phosphorylation of Cdc25

It was reported recently that Cdc25 was hyper-phosphorylated in strains with higher PKA activity, and that Cdc25 was in lower phosphorylation level in lower PKA activity strains, while hyper-phosphorylated Cdc25 have lower GEF (Guanine Nucleotide Exchange Factor) activity on Ras in vitro [26]. These results suggest that phosphorylation of Cdc25 by activated PKA is a feedback inhibition mechanism on cAMP signaling. Our results indicate that deletion of *SCH9* caused high PKA activity. In order to investigate

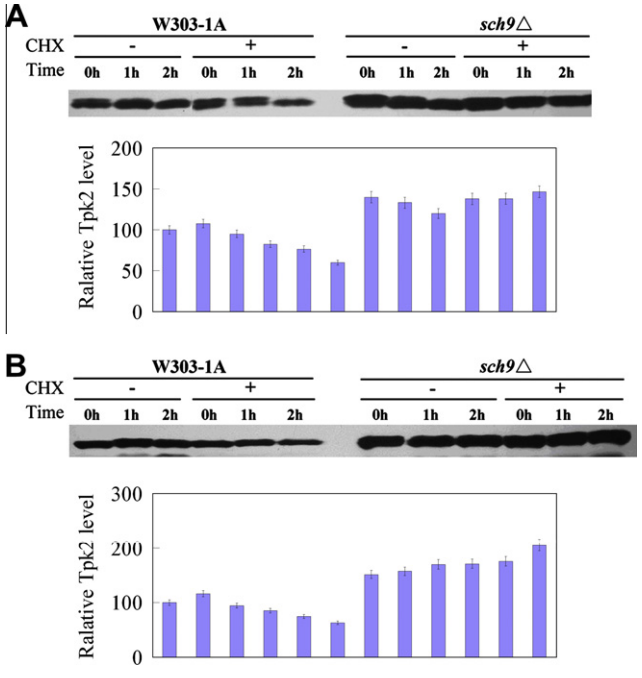

Fig. 4. Analysis of Tpk2 protein stability. Proteins were extracted form W303-1A and *sch9*Δ cells carrying YCplac22-*TPK2*-3×*HA* grown in YPD or YPG in the presence or absence of cycloheximide(CHX) (50 μg/ml). Typical results were shown. Band intensities were quantified with SCION IMAGE, and the intensity of W303-1A (YPD or YPG, 0 min) was set to 100, and data are presented as means ± S.D. (A) Analysis of Tpk2 protein stability in glucose-grown cells. (B) Analysis of Tpk2 protein stability in glycerol-grown cells.

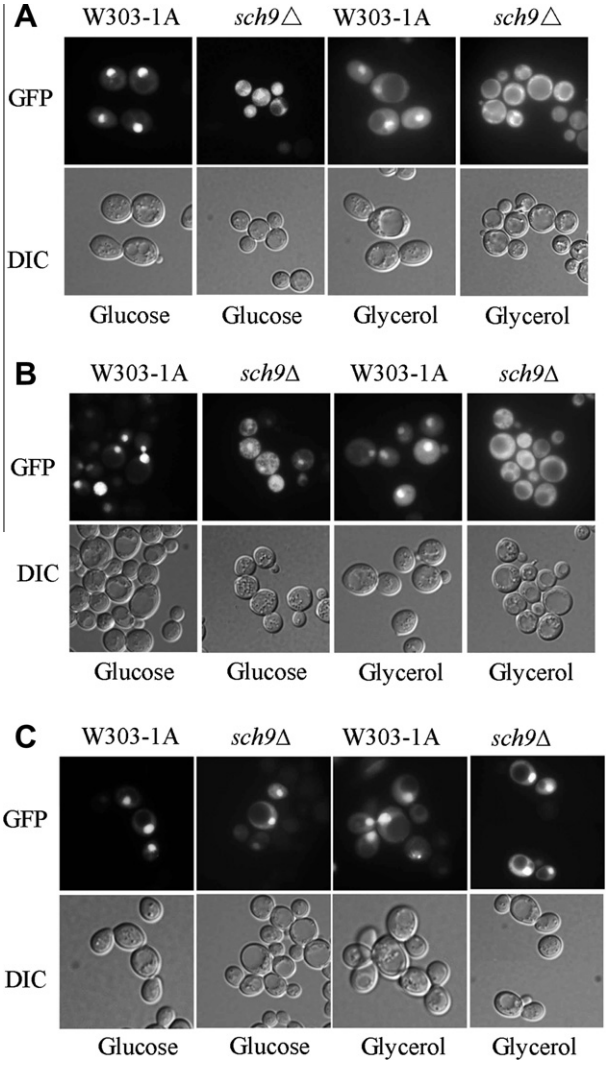

Fig. 5. Effect of Sch9 on localization of catalytic subunits of PKA. TPK1-GFP, TPK2-GFP, TPK3-GFP strains were grown until exponential phase of growth on YPD or YPG. (A) Localization of Tpk1-GFP in wild type and *sch9*Δ cells. Cells carrying single-copy plasmid *YCplac22-TPK1-GFP* were observed directly without fixation. (B) Localization of Tpk2-GFP in wild type and *sch9*Δ cells. Cells carrying single-copy plasmid *YCplac22-TPK2-GFP* were observed directly without fixation. (C) Localization of Tpk3-GFP in wild type and *sch9*Δ cells. Cells carrying multi-copy plasmid *YEplac112-TPK3-GFP* were observed directly without fixation.

whether feedback inhibition of PKA on cAMP signaling caused lower glucose-induced cAMP signal in *sch9*Δ strains, we examine phosphorylation level of Cdc25 in *sch9*Δ cells.

As shown in Fig. 6A, Cdc25 was phosphorylated immediately after addition of 100 mM glucose to glycerol-grown wild type cells, while Cdc25 was hyper-phosphorylated in *sch9*Δ cells before and after addition of 100 mM glucose. These results indicate that deletion of *SCH9* resulted in high PKA activity which caused hyper-phosphorylation of Cdc25, while hyper-phosphorylated Cdc25 have lower GEF (Guanine Nucleotide Exchange Factor) activity on Ras, thus glucose-induced cAMP signal decreased in *sch9*Δ cells. Our results demonstrate that protein levels of Tpk2 increased markedly in *sch9*Δ cells (Fig. 3B), we want to know whether PKA phosphorylate Cdc25 via Tpk2. As indicated in Fig. 6B and C, Cdc25 was phosphorylated in wild type cells, *tpk1*Δ cells, *tpk3*Δ cells, *tpk1*Δ*sch9*Δ cells and *tpk3*Δ*sch9*Δ cells after addition of 100 mM glucose, but Cdc25 was not phosphorylated in *tpk2*Δ cells and *tpk2*Δ*sch9*Δ cells. These indicate that PKA phosphorylate Cdc25 via Tpk2.

4. Discussion

4.1. The physiological role of the inhibition of PKA activity by Sch9

Our research confirmed and extended previous work which suggested that deletion of *SCH9* enhanced PKA activity [14] by showing: (1) Bcy1 predominately localized in nucleus in glycerol-grown *sch9*Δ cells; (2) large part of catalytic subunits of PKA transferred from nucleus into cytoplasm in *sch9*Δ cells; (3) higher protein stability of Tpk2 resulted in higher protein level of Tpk2 in *sch9*Δ cells. Compartmentalization of signal transduction pathways is an important additional level of control to ensure sufficient specificity in cAMP-PKA signaling. In this way, signaling molecules of the same pathway are brought into close vicinity, thereby increasing the probability that they only affect each other appropriately. Bcy1 (inhibitor of Tpks) predominantly localize in nucleus in *sch9*Δ cells, while more Tpks transferred from nucleus into cytoplasm at different levels. So liberated catalytic subunits are not restricted by the regulatory subunits and consequently competent to phosphorylate substrates preferentially located nearby (e.g., fructose-1,6-bisphosphatase, trehalase), all these lead to high PKA activity phenotype of *sch9*Δ cells.

Furthermore we demonstrated that the higher protein levels of Tpk2 in *sch9*Δ cells were due to higher protein stability (Fig. 4). The effect of Sch9 deletion on the stability of Tpk2 protein might result from direct modification of the Sch9 protein by Tpk2, generating a more stable protein, or from modification of a protease(s), which is responsible for the degradation of Tpk2 protein and makes it less effective with respect to Tpk2 degradation.

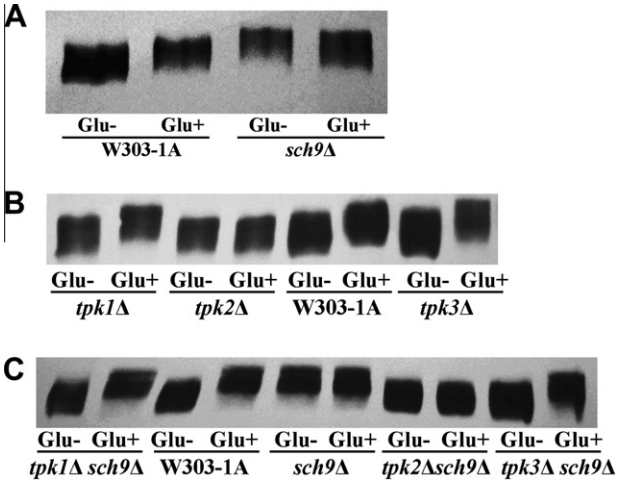

Fig. 6. Effect of *SCH9* deletion on phosphorylation of Cdc25: Glu-, extract from glycerol-grown cells; Glu+, extract from glycerol-grown cells after addition of 100 mM glucose for 30s. Proteins were extracted, equal amounts of cellular protein (50 μg) were separated on 7% SDS–PAGE gels. Phosphorylated Cdc25 had lower mobility than unphosphorylated Cdc25 on the blot. (A) Deletion of *SCH9* results in hyper-phosphorylation of Cdc25. (B) Effects of *TPK1*, *TPK2* or TPK3 deletion on expression of Cdc25 in wild type cells. (C) Effects of *TPK1*, *TPK2* or *TPK3* deletion on expression of Cdc25 in *sch9*Δ.

4.2. Sch9 regulated localization and phosphorylation of regulatory subunits Bcy1

Bcy1 predominantly localized in nucleus of rapidly glucose-grown cells, but distributed over nucleus and cytoplasm in glycerol-grown cells [12]. We found that cytolasmic localization of Bcy1 were largely absent in glycerol-grown *sch9*Δ cells (Fig. 2). It was reported that Zds1 interact with Bcy1 N-terminal domain, in ethanol-grown *zds1*Δ cells cytolasmic localization of Bcy1 was largely absent, while overexpression of *ZDS1* led to increased cytoplasmic Bcy1 localization [27]. Whether Sch9 regulate localization of Bcy1 directly or via Zds1 needs to be clarified. We also found that phosphorylation of Bcy1 was deficient in *sch9*Δ mutant. According to Griffioen and his colleagues' research, Bcy1 modification was dependent on Yak1 kinase, and in ethanol-grown *yak1*Δ cells the Bcy1 remained nuclear [27]. Whether Sch9 regulate phosphorylation of Bcy1 directly or via Yak1 or other unknown protein also needs to be investigated.

4.3. Feedback inhibition systems of PKA on cAMP accumulation

Previous studies on feedback control of cAMP level showed that deletion of the two phosphodiesterase genes in an $RAS2^{val19}$ 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 [28]. This indicates that high PKA activity in some way is required for efficient breakdown of cAMP by the phosphodiesterases [29]. Studies on the low-affinity cAMP phosphodiesterase Pde1 and the high-affinity cAMP phosphodiesterase Pde2 have revealed their specific functions in controlling agonist-induced cAMP signaling [8,9].

Our studies demonstrate that Cdc25 was hyper-phosphorylated in *sch9*Δ cells (Fig. 6A). It was reported recently that hyper-phosphorylated Cdc25 had lower GEF (Guanine Nucleotide Exchange Factor) activity on Ras in vitro, and phosphorylation of Cdc25 by activated PKA was a feedback inhibition mechanism on cAMP signaling [26]. Thus the hyper-phosphorylated Cdc25 had feedback inhibition on cAMP synthesis and caused the lower glucose-induced cAMP signal in *sch9*Δ cells. Our research extended previous work by showing Cdc25 was not phosphorylated in *tpk2*Δ cells and *tpk2*Δ*sch9*Δ which suggested that feedback inhibition of PKA on Cdc25. was through Tpk2.

Authors' contribution

Aili Zhang and Yubao Shen contributed equally to this work.

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