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Perturbation of protein kinase CK2 uncouples executive part of phosphate maintenance pathway from cyclin-CDK control[1]

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Abstract The budding yeast *Saccharomyces cerevisiae* encounters phosphate starvation by the transcription-regulated PHO pathway. We find that genetic perturbation of protein kinase CK2, a conserved tetrameric Ser/Thr phosphotransferase with links to cell cycle and transcription, affects expression of PHO pathway genes in a subunit- and isoform-specific manner. Remarkably, the genes encoding phosphate supplying phosphatases and transporters are significantly repressed, while the genes encoding components of the central pathway regulator complex, a cyclin-dependent kinase (CDK), a cyclin, and a CDK inhibitor, remain unaltered. Thus, perturbation of CK2 uncouples the executive part of the PHO pathway from its cyclin-CDK control complex.

Key words: Protein kinase CK2; PHO pathway; Gene expression; Cell cycle; *Saccharomyces cerevisiae*

1. Introduction

Availability of phosphate (P_i) is a prerequisite for the life of any cell. Eukaryotes such as the budding yeast *Saccharomyces cerevisiae* react to phosphate starvation by arrest of growth and cell division. When phosphate becomes limiting, *S. cerevisiae* responds by an increase of the production of high affinity phosphate transporters and secreted phosphatases in order to scavenge phosphate from environment. This response is mediated by components of the PHO pathway encoded by about 30 genes [1–3]. Several PHO pathway members are homologous to proteins that regulate the eukaryotic cell cycle, including a cyclin-dependent kinase (CDK; Pho85), a cyclin (Pho80) and a CDK inhibitor (Pho81). They form a regulator complex that determines transcription of the executive genes encoding phosphate transporters and phosphatases, such as Pho84 and Pho5, respectively. The Pho80/85 kinase phosphorylates the main transcriptional PHO gene activator, Pho4, when phosphate is abundant but not when it is limiting [4]. Hyperphosphorylated Pho4 is translocated into the cytoplasm and thus inactivated as a transcription factor. The phosphate level is sensed by a so far unknown, probably intracellular sensor and transmitted to Pho81 that tunes the Pho80/85 kinase accordingly [3]. However, Pho85 function is not limited to phosphate metabolism. Rather, in association with other cyclins, including Pcl1, Pcl2 and Pcl9, it also affects regulation of cell growth and division via additional substrates [5].

Protein kinase CK2 (also known as casein kinase 2) is a vital, highly conserved and pleiotropic serine/threonine phosphotransferase that occurs ubiquitously in the eukaryotic world. CK2 participates, among other functions, in signaling cascades linked to gene expression and cell cycle, including cell cycle re-entry [6]. CK2 is a tetramer composed of two catalytically active subunits, α, and two regulatory subunits, β, whereby stable β–β dimer intermediates complex into $\alpha_2\beta_2$ holoenzymes [7]. The subunits may occur in isoforms, giving rise to respective holoenzyme variants [8]. *S. cerevisiae* expresses two catalytic (Cka1; Cka2) and two regulatory (Ckb1; Ckb2) subunit isoforms that appear to build an obligatory heterotetramer of all four subunits [9] but may also participate in other constellations in transcription-related nuclear protein complexes [10,11]. Deletion of any of the four subunit genes (*CKA1*, *CKA2*, *CKB1*, and *CKB2*) individually or of *CKB1* and *CKB2* together has no overt phenotypic effect on normal media, but such strains exhibit varying degrees of sensitivity to Na^+ and Li^+. By contrast, simultaneous deletion of *CKA1* and *CKA2* is lethal, but can be complemented by catalytic subunits of other eukaryotes, including man [12,13], implying both an essential role of CK2's phosphotransferase activity and a conservation of CK2 over large evolutionary distances.

Relations between CK2 and the PHO pathway have not been described yet. We report here that CK2 gene deletions in *S. cerevisiae* affect the expression of PHO pathway genes at cell cycle re-entry in a subunit- and isoform-specific manner concerning degrees of repression and persistence. Using transcript profiling by oligonucleotide arrays and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, we find genetic perturbation of CK2 to specifically repress genes encoding phosphate supplying phosphatases and P_i transporters, while genes encoding components of the central cyclin-CDK regulator complex are unaffected. In the regulatory CK2 subunit mutant, expression of the transcription activator Pho4 is also reduced, providing a possible explanation for the strong and persistent PHO gene repression detected. However, while PHO gene expression is rather unaffected in the *cka2*Δ strain, we see temporary repression in the *cka1*Δ mutant despite unaltered *PHO4* transcript levels. Consistent with these isoform- and subunit-specific CK2 transcriptional effects,

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Pho4 is phosphorylated by CK2 in vitro in a β-dependent and α-specific manner.

2. Materials and methods

2.1. Strains, growth conditions, synchronization

The CK2 mutant strains *ckb1*Δ *ckb2*Δ (YAPB10-2c, *MAT***a** *CKA1 CKA2 ckb1-Δ1::HIS3 ckb2-Δ1::LEU2*; [14]), *cka1*Δ (JC4-1a, *MAT***a** *cka1-Δ1::HIS3 CKA2 CKB1 CKB2*; [15]) and *cka2*Δ (RPG22-1b, *MAT***a** *CKA1 Δcka2::TRP1 CKB1 CKB2*; [12]) as well as their respective wild-types (YPH499, *MAT***a** *CKA1 CKA2 CKB1 CKB2* and YPH250, MATa *CKA1 CKA2 CKB1 CKB2*) were grown in YPD medium at 30°C, shaken at 230 rpm.

Pheromone-based synchronization was performed as previously described by Spellman et al. [16]. Briefly, yeast cells were grown to early-log phase (OD_{600} 0.2) and arrested by the addition of α-pheromone (12 μg/ml), followed by 2 h cultivation. After centrifugation, arrest release was provided by resuspension of the cell pellet in fresh (pheromone-free) medium to an OD_{600} of 0.18. During further cultivation samples were taken 0, 7 and 14 min after release by adding culture aliquots to sterile ice. Cells were immediately collected by centrifuga-

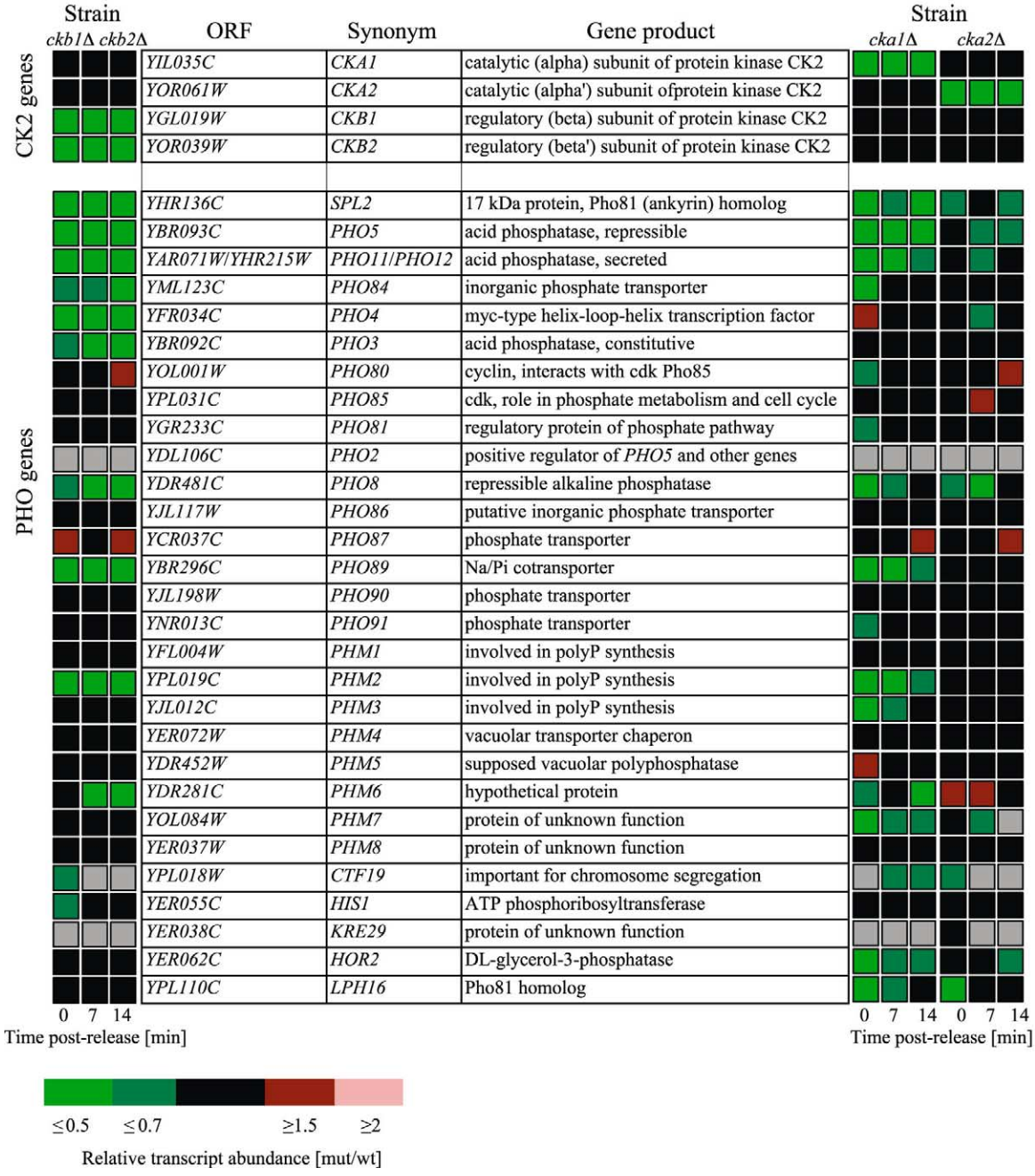

Fig. 1. Several PHO genes exhibit altered expression profiles in regulatory and catalytic CK2 subunit deletion strains at cell cycle (re-)entry. Transcript profiles of α-pheromone-synchronized yeast strains were determined at 0, 7 and 14 min post-pheromone release by hybridization to oligonucleotide arrays comprising the whole yeast genome. Relative transcript abundance of PHO genes and CK2 genes in the indicated CK2 mutants (mut) compared to the respective wild-type (wt) according to color scale provided at bottom line (black, comparable expression; green, repression; red, elevation), gray indicates absence of transcripts. Encoding nearly identical proteins, *PHO11* and *PHO12* transcripts are detected by the same oligonucleotide probe set.

tion and pellets were flash-frozen in liquid nitrogen. Synchrony was verified by fluorescence-activated cell sorting (FACS) analysis as previously described by Nash et al. [17].

2.2. Sample preparation, hybridization, scanning, data processing

The following procedures were performed as previously described in detail by Ackermann et al. [18]. Briefly, total RNA was isolated applying the hot phenol protocol followed by mRNA concentration using oligo(dT)-cellulose (Ambion). 3 μg mRNA were reversely transcribed using SuperScript™ Choice System (Gibco BRL) for first and second strand cDNA synthesis. 1 μg cDNA was transcribed in vitro using biotinylated ribonucleotides (ENZO BioArray™ High Yield™ RNA transcript labeling kit, ENZO Diagnostics). After fragmentation, 15 μg biotin-labeled cRNA were taken for hybridization on oligonucleotide arrays (YG-S98 Arrays, Affymetrix). Staining and washing steps were carried out on a GeneChip Fluidics Station 400 (Affymetrix). Arrays were scanned on a HP GeneArray™ scanner (Affymetrix).

The resulting image data were analyzed using the GeneChip Expression Analysis software (Micoarray Suite version 4.0; Affymetrix). Expression deviations above two-fold were considered as significant.

2.3. Quantitative RT-PCR

cDNA was synthesized in a 50 μl reverse transcription reaction containing 5 μg total RNA, 2.25 μg oligo(dT)$_{18}$ primer, 10×RT buffer (Qiagen), 0.5 mM each dNTP and 10 U reverse transcriptase (Omniscript, Qiagen). The reaction was incubated at 37°C for 1 h. cDNA was PCR-amplified using the QuantiTect SYBR Green PCR Kit (Qiagen), combining 2×QuantiTect SYBR Green PCR Master Mix, 20 pmol sense and antisense primer, 2 μl of the RT reaction and nuclease-free H_2O at 20 μl. The following primer sequences were used, s and as indicating sense and antisense, respectively: *PHO4*s (GATTTAGAGCCCAAGAGTAG), *PHO4*as (CTGTTGTGGTTCTCTTGACT), *PHO5*s (TGTAAATGAATACGACACAA), *PHO5*as (CACACCACGAGAATAAAGTA), *PHO11*s (GGAAAGTTATCTGACATTGA), *PHO11*as (GAA-ATACCATAATCACCAGG), *PHO84*s (GAGTTCCGTCAATAAAGATA) and *PHO84*as (CAAT-CTTCTTCTTTCCAGAG). Thermal cycling was performed using the ABI Prism 7700 Sequence Detector (Perkin-Elmer) with an initial activation step of 15 min at 95°C and 45 cycles of 15 s at 94°C, 30 s at 59.3°C and 30 s at 72°C. Amplification rates were measured automatically and initial amounts of gene-specific templates were determined.

2.4. Phosphorylation assay

15 pmol recombinant yeast Pho4 protein were incubated with 1 pmol recombinant human CK2 subunits or Pho80/85, respectively, in 25 μl kinase reaction buffer (50 mM Tris–Cl pH 7.5, 12 mM $MgCl_2$, 100 mM NaCl, 40 μM ATP, 10 μCi [γ^{32}]ATP) at 37°C for 40 min. 20 μl of the reaction mix were pipetted on P81 Whatman paper stripes that – after air drying – were washed in 0.5% H_3PO_4 (2×10 min) and shortly immersed in acetone. After air drying, radioactivity was determined in a scintillation counter.

3. Results and discussion

In order to analyze gene expression specifically at cell cycle (re-)entry, we synchronized *S. cerevisiae* wild-type and CK2 deletion strains of regulatory subunits (*ckb1*Δ *ckb2*Δ) and catalytic subunits (*cka1*Δ and *cka2*Δ) by α-pheromone treatment, verified synchrony by FACS analysis, and determined the gene expression profiles on oligonucleotide arrays at 0, 7, and 14 min after pheromone release corresponding to M/G1 and early G1 phase [16].

3.1. Effect of genetic perturbation of regulatory CK2 subunits on PHO gene expression

The *ckb1*Δ *ckb2*Δ mutant deviated from wild-type by a striking repression of 11 genes out of the 30 related to phosphate metabolism (Fig. 1). These genes are known to be regulated by the PHO pathway and/or the cell cycle [2,16], except for one, *PHO4*, whose regulation is hitherto unknown. Functionally, the genes relate to distinct sections of the PHO pathway. *PHO3*, *5*, *8*, *11*, and *12* encode phosphatases, *PHO84* and *89* high affinity P_i transporters, *SPL2* a putative CDK inhibitor, and *PHM2* and *PHM6* are probably involved in polyphosphate metabolism. *PHO4* encodes a transcription factor playing a main role in PHO gene regulation. Interestingly, the arrays did not indicate any significant expression deviations for *PHO80*, *85*, and *81*, encoding the components of the cyclin-CDK regulator complex.

The expressional alterations detected by the DNA arrays were substantiated without any exception by quantitative RT-PCR, regarding both the genes affected and their degree of deviation at the individual time points (Fig. 2A). Furthermore, unaltered expression of the cyclin-CDK complex components was confirmed (not shown).

How can PHO gene repression in *ckb1*Δ *ckb2*Δ be explained? As all repressed PHO genes, except *PHO3*, have one or more Pho4-binding sites in their promoter region and are transcriptionally induced in response to P_i limitation [2], the repression of *PHO4* would be a logical explanation. Transcription of *PHO4* occurs constitutively and independently from phosphate conditions [19] but is mechanistically poorly defined. Possibly, a sequence within the *PHO4* promoter region (-C**AAGGGTGTCAAAAAA**-) might play a role, which considerably resembles the consensus sequence of the zinc finger DNA-binding protein Mig1 (-**AAGGGGCGTAAAAATA**-) [20]. Mig1 has been found to mediate glucose repression of the salt tolerance gene *ENA1* [21], and interest-

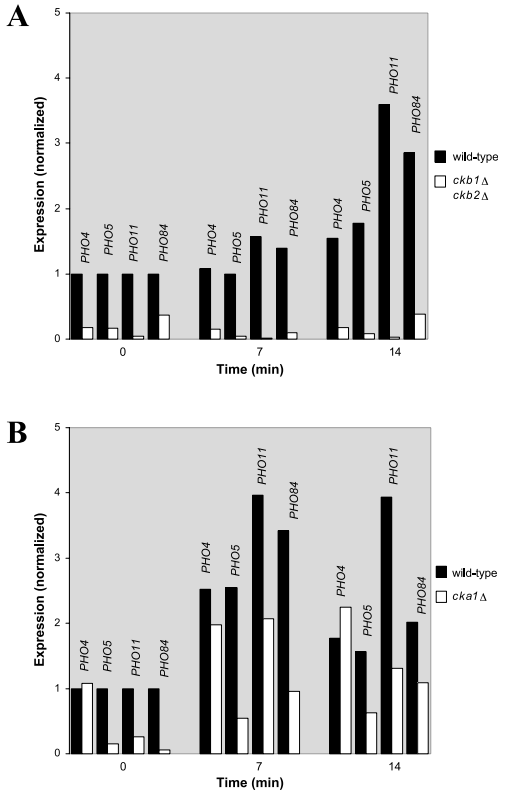

Fig. 2. Verification of PHO gene repression in early cell cycle of CK2 mutants by quantitative RT-PCR. Column diagrams showing PHO gene transcript levels in CK2 mutants *ckb1*Δ *ckb2*Δ (A) and *cka1*Δ (B) compared to respective wild-type. Note: As a reference, wild-type transcript levels at 0 min have been set to 1 for each gene. Thus, comparing transcript levels of different genes with each other is not possible.

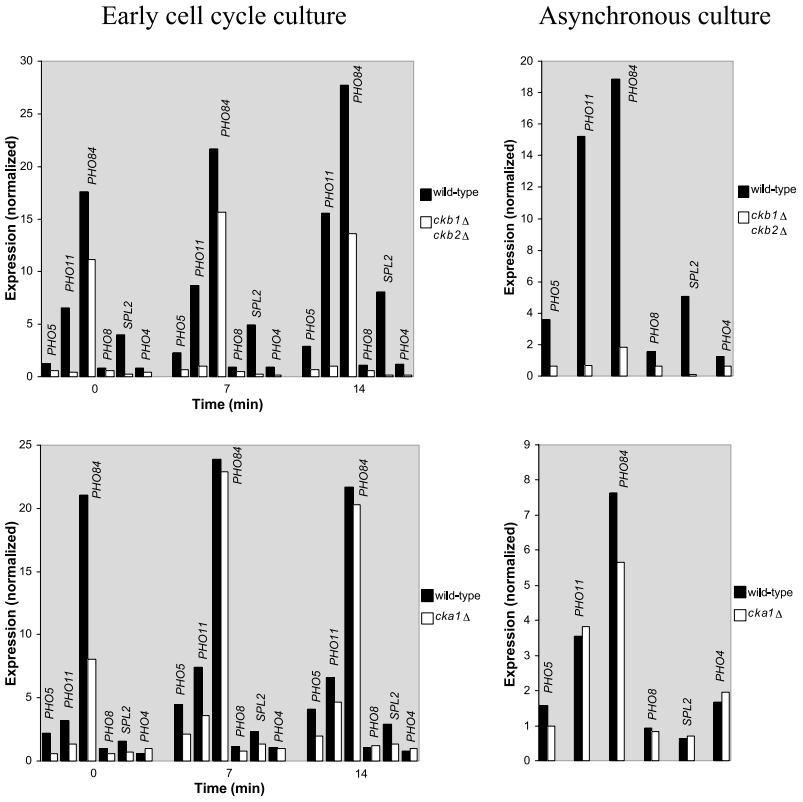

Fig. 3. Persistence of PHO gene repression in CK2 mutants at cell cycle entry depends on the deleted subunit. Column diagrams showing PHO gene expression in early cell cycle (left) and asynchronous cultures (right) of CK2 mutants and respective wild-types.

ingly, *ckb*Δ strains are defective in their ability to induce *ENA1* in response to high external Na^+ concentrations [9].

3.2. Effect of genetic perturbation of catalytic CK2 subunits on PHO gene expression

When we compared the transcript profiles of wild-type and catalytic CK2 subunit deletion strains, expression characteristics in the *cka1*Δ strain resembled that of the *ckb1*Δ *ckb2*Δ mutant (Fig. 1): nine of the 11 PHO genes were also repressed and another four in addition (*PHM3*, *PHM7*, *HOR2*, *LPH16*), while the regulator complex genes (*PHO80*, *81*, *85*) were unaffected. However, in contrast to the *ckb*Δ strain, *PHO4* transcript levels were unaltered. *PHO4* expression was also unaffected in the *cka2*Δ mutant but only two PHO genes (*PHO8*, *LPH16*) – and each at only a single time point – were repressed. This indicates strong isoform specificity of CK2 and is consistent with isoform-specific effects reported for various processes in yeast, including cell cycle events, cell polarity, and gene expression [9,18] as well as in mammalian cells [22].

The quantification by RT-PCR confirmed that the repression degrees in *cka1*Δ were lower than in *ckb1*Δ *ckb2*Δ and that *PHO4* expression was unaltered (Fig. 2B). Thus, reduced *PHO4* transcription as an explanation for PHO gene repression, as considered for *ckb1*Δ *ckb2*Δ, does not suffice. It rather argues for additional mechanisms associated with a regulatory subunit-controlled Cka1 activity.

3.3. PHO gene repression in the regulatory subunit mutant persists but is temporary in the catalytic subunit mutant

To find out whether or not CK2-linked deviations in PHO gene expression are restricted to cell cycle re-entry, we compared the early cell cycle transcript profiles with those of asynchronous, permanently cycling cultures (Fig. 3). In *ckb1*Δ *ckb2*Δ, *PHO4* as well as nine of the executive PHO genes repressed at cell cycle entry (all except *PHM6* and *PHO89*), were also significantly repressed in the asynchronous culture, whereas the regulator complex genes, *PHO80* and *85*, were unaltered. In contrast to the regulatory subunit mutant, PHO gene repression was much less pronounced or no longer evident in the asynchronous *cka1*Δ culture; i.e. the effect of *CKA1* deletion on PHO gene expression in early cell cycle is temporary.

Collectively, CK2-linked PHO gene alterations do not seem to be restricted to cell cycle re-entry, and whether they are transient or not, apparently depends on the nature of the deleted CK2 subunit(s).

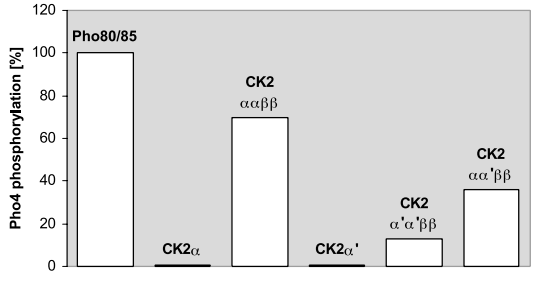

Fig. 4. Pho4 is phosphorylated by CK2 in vitro. Column diagram showing phosphorylation efficiency of Pho4 by different human CK2 subunit combinations compared to Pho80/85 as determined by kinase assays.

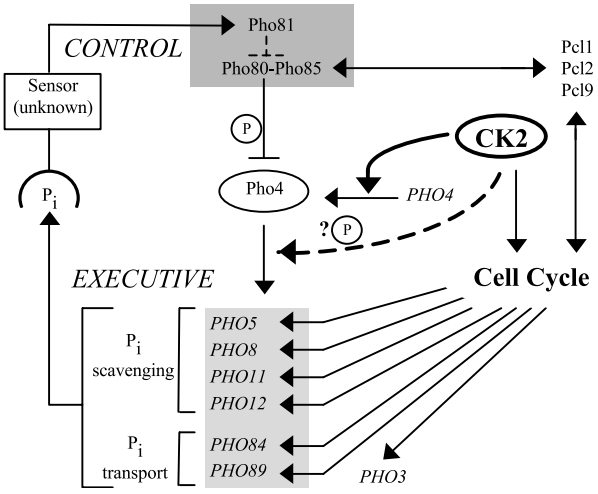

Fig. 5. Schematic overview of links between the PHO pathway, protein kinase CK2 and the cell cycle. For details see text.

3.4. CK2 phosphorylates Pho4 in vitro

Pho4 is phosphorylated and thereby regulated by Pho80/85 kinase [4]; whether it is also a substrate of CK2 is unknown. Therefore, we performed kinase assays with different combinations of recombinant human CK2 subunits and recombinant Pho4 protein. As a positive control, phosphorylation of Pho4 by Pho80/85 was run in parallel. While no phosphorylation by the individual catalytic CK2 subunits (α or α′) alone was detected, the $\alpha_2\beta_2$ holoenzyme significantly phosphorylated Pho4 (Fig. 4). Remarkably, phosphorylation efficiency by holoenzyme $\alpha'_2\beta_2$ was below 20% of that by $\alpha_2\beta_2$, and, consistently, by holoenzyme $\alpha\alpha'\beta_2$ roughly half of it. The result demonstrates that Pho4 is a possible substrate of CK2. The strong isoform preference for CK2 holoenzymes containing the catalytic subunit α and the absolute dependence on the presence of β subunits are consistent with the isoform-specific PHO gene repression characteristics described above. Thus, a Ckb1/Ckb2-controlled, Cka1-specific Pho4 phosphorylation might be conceivable as an additional tuning device of PHO gene transcription.

3.5. Conclusion

Our data reveal for the first time a link between protein kinase CK2 and the PHO pathway (Fig. 5). The deletion of CK2 subunits causes repression of genes encoding phosphate supplying phosphatases and transporters while genes encoding components of the central cyclin-CDK regulator complex are unaltered. Thus, perturbation of CK2 uncouples the executive part of the PHO pathway from its phosphate level-sensing and cell cycle-related regulatory part; i.e. whatever the central regulator might try to tell, the addressed executive genes will not respond. The effect shows pronounced CK2 subunit dependence. It is persistent when the regulatory subunits are deleted, but restricted to cell cycle entry when one of the catalytic subunits is deleted. While the former might be explained by reduced *PHO4* transcription, the latter cannot, as it occurs despite unaltered *PHO4* levels. Since these isoform- and subunit-specific effects are consistent with the β-dependent and α-specific phosphorylation of Pho4 by CK2 in vitro, the idea of CK2-mediated Pho4 phosphorylation tuning PHO gene transcription might be conceivable.

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