Eukaryotic Cell

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Eukaryotic Cell **2013, 12(1):23. DOI: 10.1128/EC.00181-12.**
Published Ahead of Print 26 October 2012.

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Journals.ASM.org

Evidence that Two Pcl-Like Cyclins Control Cdk9 Activity during Cell Differentiation in *Aspergillus nidulans* Asexual Development

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Cyclin-dependent protein kinases (CDKs) are usually involved in cell cycle regulation. However, Cdk9 is an exception and promotes RNA synthesis through phosphorylation of the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNAPII). The CTD is comprised of repeating heptapeptides, in which serine residues at positions 2, 5, and 7 are of crucial importance. Ser5 phosphorylation causes transcription initiation and promoter escape. However, RNAPII pauses 20 to 50 bp downstream from the transcription start site, until Cdk9 phosphorylates Ser2. This event relieves the checkpoint and promotes the processivity of elongation. Here we present evidence that in the filamentous fungus *Aspergillus nidulans*, a Cdk9 homologue, PtkA, serves specific functions in conidiophore development. It was previously shown that PtkA interacts with two cyclins, PclA and the T cyclin PchA. Using yeast two-hybrid screens, we identified a third cyclin, PclB, and a kinase, PipA^{Bud32}. Both proteins were expressed in hyphae and in conidiophores, but interaction between each protein and PtkA was restricted to the conidiophores. Deletion of *pchA* caused a severe growth defect, and deletion of *pipA* was lethal, suggesting basic functions in PtkA-dependent gene transcription. In contrast, deletion of *pclB* in combination with deletion of *pclA* essentially caused a block in spore formation. We present evidence that the phosphorylation status of the CTD of RNA polymerase II in the conidiophore changes upon deletion of *pclA* or *pclB*. Our results suggest that tissue-specific modulation of Cdk9 activity by PclA and PclB is required for proper differentiation.

Eukaryotic development and differentiation rely to a large extent on differential gene expression, brought about by stage-specific transcription factors, which control the expression of certain sets of genes. In addition, epigenetic control is a common principle for gene expression control. This phenomenon was named the histone code, because posttranslational modifications of histone proteins control the accessibility of certain genome regions for regulatory proteins (1). Another regulatory principle is much less well understood and named the carboxy-terminal domain (CTD) code (2). This involves the cyclin-dependent kinase (CDK) Cdk9. Unlike other CDKs, Cdk9 does not regulate the cell cycle but, rather, promotes RNA synthesis (3). In higher eukaryotes, it has been shown that members of the Cdk9 kinase family interact with T cyclins and that this complex is a subunit of the positive transcription elongation factor b (P-TEFb) (4, 5). This complex phosphorylates the CTD of the large subunit of RNA polymerase II (RNAPII) and thus promotes the elongation phase during transcription (6). The CTD contains 52 heptad repeats in human and 26 in *Saccharomyces cerevisiae*. Each heptad repeat contains three serine residues, at positions 2, 5, and 7, and all three are amenable to phosphorylation. First, Ser5 is phosphorylated by CDK7 (7). This leads to initiation of transcription but is not sufficient for transcription of the entire gene. RNAPII, rather, pauses 20 to 50 bp downstream from the transcription initiation site until Cdk9 phosphorylates serine 2. With this event, the checkpoint can be overcome and transcription continues. The pausing is probably required for proper capping of the nascent mRNA. The highest Ser2 phosphorylation levels are normally reached 600 to 1,000 nucleotides downstream from the start site, and a dual-gradient model of increasing Ser2 and decreasing Ser5 phosphorylation has been suggested (8, 9). The role of Ser7 is less clear, although its phosphorylation has been shown in *S. cerevisiae* (7). Although the function of Cdk9 seems to be essential in regulation of global gene expression in all cells, it was shown that expression is highest in terminally differentiated cells, suggesting a role in specialized cellular functions. Not surprisingly, some diseases as well as cancer development were related to altered expression levels of Cdk9. Hence, human Cdk9 is regarded as a potential drug target in oncology, virology, and cardiology (3). Furthermore, there is first evidence for gene-specific modulation of gene expression by Cdk9 kinase (10). Likewise, in *Aspergillus nidulans* we do have evidence that a Cdk9 homologue specifically affects conidiophore development. A Cdk9 homologue (PtkA) was discovered in a targeted approach to identify the interaction partners of a Pcl-like cyclin. This cyclin was named *pclA* and was the first Pcl-like cyclin described in *A. nidulans* (11). PclA is important for spore formation during asexual development and interacts with the main cell cycle regulator *nimX* (*cdk1*) and may therefore have a second role in cell cycle regulation (12). The *pclA*-deletion mutant produced fewer spores, but vegetative growth was not affected. In contrast, deletion of the *cdk9* kinase gene *ptkA* is lethal (13). These results suggest that PtkA conidiophore-specific functions depend on the interaction with PclA.

Here, we performed a screening for further PtkA-interacting proteins and identified another cyclin as well as a kinase. The identified cyclin shows sequence similarities to members of the Pcl cyclin family of *S. cerevisiae* and was named PclB. The interaction is quite unusual, because the typical Cdk9-interacting proteins are members of the T-cyclin family. Deletion of *pclB* had only a minor effect on asexual spore formation. However, in combination with

Received 5 July 2012 **Accepted** 22 October 2012
Published ahead of print 26 October 2012
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doi:10.1128/EC.00181-12

TABLE 1 *A. nidulans* and *S. cerevisiae* strains used in this study

Strain	Genotype	Source or reference
FGSCA4	Glasgow wild type (*veA*$^{+}$)	Fungal Genetic Stock Centre, MO
TN02A3	*pyrG89 pyroA4 argB2 nkuA::argB veA1*	Fungal Genetic Stock Centre, MO
GR5	*pyrG89 wA3 pyroA4 veA1*	G. May, Houston, TX
RMS011	*pabaA1 yA2 ΔargB::trpCΔB trpC801 veA1*	17
SSNI30	*pyrG89 ΔargB::trpCΔB pyroA4 ΔpclA::argB veA1*	11
SKC7	*pclA*-deletion strain (*pyrG89 pyroA4 pclA::argB*) crossed to RMS011	This study
SKC9	TN02A3 transformed with *pchA*-deletion cassette	This study
SKC13	TN02A3 transformed with pKC24 [*alcA(p)::GFP::pipA177::pyro*]	This study
SKC19	GR5 transformed with pKC39 [*alcA(p)::YFP^{N}::pipA::pyro*] and pFB13 [*alcA(p)::YFP^{C}::ptkA::pyr4*]	This study
SKC20	GR5 transformed with pKC38 [*alcA(p)::YFP^{N}::pclB::pyro*] and pFB13 [*alcA(p)::YFP^{C}::ptkA::pyr4*]	This study
SKC27	TN02A3 transformed with *pipA::GFP::pyrG*-fusion construct	This study
SKC28	TN02A3 transformed with *pclB::GFP::pyrG*-fusion construct	This study
SKC34	TN02A3 transformed with *pclB* deletion cassette, *pyrG89 pyroA4 argB2 ΔnkuA::argB veA1 ΔpclB::pyrG*	This study
SKC40	SKC34 (*pclB* deletion) crossed to SKC9 (*pchA* deletion)	This study
SKC41	SKC34 (*pclB* deletion) crossed to SKC7 (*pclA* deletion)	This study
SKC44	GR5 transformed with pKC66 [*alcA(p)::GFP::ptkA_D173A::pyro*]	This study
SKC45	GR5 transformed with pKC71 [*alcA(p)::GFP::ptkA_D155G::pyro*]	This study
SKC46	GR5 transformed with pKC64 [*alcA(p)::GFP::ptkA_PITALRE::pyro*]	This study
SKC48	GR5 transformed with pKC87 [*alcA(p)::3× HA*[a]*::CTD::pyr4*]	This study
SKC49	TN02A3 transformed with *pipA* deletion cassette, *pyrG89 pyroA4 argB2 ΔnkuA::argB veA1 ΔpipA::pyrG*	This study
SKC50	GR5 transformed with pKC70 [*alcA(p)::GFP::ptkA_GAGA::pyro*]	This study
SKC51	GR5 transformed with pKC65 [*alcA(p)::GFP::ptkA_K54Q::pyro*]	This study
SKC72	GR5 transformed with pKC56 [*alcA(p)::mRFP::pclB::pyr4*] and pFB15 [*alcA(p)::GFP::ptkA::pyro*]	This study
SKC73	GR5 transformed with pKC55 [*alcA(p)::mRFP::pipA::pyr4*] and pFB15 [*alcA(p)::GFPptkA::pyro*]	This study
SKC77	SSNI17 transformed with pKC87 [*alcA(p)::3× HA::CTD::pyr4*]	This study
SKC79	SKC34 transformed with pKC87 [*alcA(p)::3× HA::CTD::pyr4*] and pNZ12 (pUMA::*pyro*)	This study
AH109	MAT**a**′ *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-His3 GAL2$_{UAS}$-GAL2$_{TATA}$-Ade2 URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ MEL1*	Clontech
Y187	MATα *ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112, gal4Δ met*$^{-}$ *gal80Δ MEL1 URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ MEL1*	Clontech

[a] HA, hemagglutinin.

the deletion of *pclA*, asexual spore formation was almost completely inhibited, despite normal vegetative growth. In addition to the new cyclin, we identified a *S. cerevisiae* Bud32-related kinase, PipA (14, 15). An interaction between Bud32 and a Cdk9 kinase has not been reported before in any other organism. In *A. nidulans*, PipA is essential for viability. Along with the already known interaction partners of PtkA, PclA and PipA are good future candidates for deciphering the CTD code during *A. nidulans* development.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Supplemented minimal medium (MM) and complete medium (CM) for *A. nidulans* were prepared as described by Hill and Käfer (16), and standard strain construction procedures are described by Hill and Käfer (16). A list of *A. nidulans* strains used in this study is given in Table 1. Standard laboratory *Escherichia coli* strain Top 10 F′ was used. Plasmids are listed in Table 2.

Molecular techniques. Standard DNA transformation procedures were used for *A. nidulans* (19) and for *E. coli* and *S. cerevisiae* (20). For PCR experiments, standard protocols were applied using a Biometra personal cycler (Biometra, Göttingen, Germany) for the reaction cycles. DNA sequencing was done commercially (Eurofins MWG Operon, Ebersberg, Germany). Genomic DNA was extracted from *A. nidulans* with a DNeasy plant minikit (Qiagen, Hilden, Germany). DNA analyses (Southern hybridizations) were performed as described by Sambrook and Russell (20).

Deletion of *pipA* and *pclB*. *pipA* flanking regions were amplified by PCR using genomic DNA and the primers AN2513_P1_LB (5′-CGTCAG GCCATTGAGAACCAC-3′) and AN2513-P3-KO (5′-gaagagcattgtttgagg cgGAGAGAGCGGTGATAGTGAGG-3′, where lowercase nucleotides indicate a linker) for the upstream region of *pipA* and AN2513-P4-KO (5′-atcagtgcctcctctcagacagGACAACGCCGCGCTCTTAGATG-3′) and Bud32_P6_rev (5′-GCGTGCTGTGAACAGGCAATTAG-3′) for the downstream region. The *pyrG* gene from plasmid pFNO3 (S. Osmani, OH) was amplified by PCR and used as the template together with *pipA* flanking regions for the fusion PCR. The deletion cassette was amplified with the fusion PCR method (21) with the primers AN2513_P2nested (5′-CTAACCGTGCCATCATTCGTACC-3′) and Bud32_P5_rev (5′-GG CAGCCGTCAACATTCAAGTC-3′). The resulting PCR product was transformed into *pyrG89*-auxotrophic *A. nidulans* strain TN02A3.

pclB flanking regions were amplified by PCR using genomic DNA and the primers AN10741_P1_LB (5′-GGTGCCGAGAAATGTCGAGGAC-3′) and AN10741-P3-KO (5′-gaagagcattgtttgaggcgCAGGGCGGGATGA AGGATGAAG-3′) for the upstream region of *pipA* and AN10741-P4-KO (5′-atcagtgcctcctctcagacagGCAAGTGCAGAGGTTACGGATG-3′) and Cyclin_P6_rev (5′-GCACGATATAATAGTGGCACCGC-3′) for the downstream region. The *pyrG* gene from plasmid pFNO3 (S. Osmani, OH) was amplified by PCR and used as the template together with *pipA* flanking regions for the fusion PCR. The deletion cassette was amplified with the fusion PCR method (21) with the primers AN10741_P2nested (5′-GCGATGGAGACGTCGATTTGACG-3′) and Cyclin_P5_rev (5′-C TTCGTCGGAGTAGTTCGCAGTG-3′). The resulting PCR product was transformed into *pyrG89*-auxotrophic *A. nidulans* strain TN02A3.

Transformants were screened by PCR for the homologous integration event. Single integration of the construct was confirmed by Southern blotting. One diploid strain with the homologous integrated deletion cassette and the remaining open reading frame (ORF) of *pipA* was selected and named SKC49. One *pclB*-deletion strain was selected from the transformants and named SKC34.

TABLE 2 Plasmids used in this study

Plasmid	Construction	Source or reference
pMCB17apx	*alcA*(*p*)::*GFP*, for N-terminal fusion of GFP to proteins of interest; contains *N. crassa pyr4*	V. Efimov (Piscataway, NJ)
pGBKT7	Yeast two-hybrid bait vector, Gal4-BD	Clontech
pGADT7	Yeast two-hybrid prey vector, Gal4-AD	Clontech
pGBT9	Yeast two-hybrid bait vector, Gal4-BD	Clontech
pDV7	GFP replaced N-terminal half of YFP in pCMB17apx	18
pDV8	GFP replaced C-terminal half of YFP in pCMB17apx	18
pFNO3	*ga5*::*GFP*::*pyrG* Kan^r Amp^r	Fungal Genetic Stock Centre, MO
pFB13	Full-length *ptkA* in pDV8	13
pFB15	Full-length *ptkA* in pCMB17apx	13
pKC3	Full-length *ptkA* in pGBT9	This study
pKC5	Truncated *ptkA_1/330* in pGBKT7	This study
pKC6	Truncated *ptkA_24/170* in pGBKT7	This study
pKC8	Full-length *pclA* in pGADT7	This study
pKC10	Full-length *pchA* in pGADT7	This study
pKC19	Full-length *pclB* in pGADT7	This study
pKC22	Full-length *pipA* in pGADT7	This study
pKC24	Truncated *pipA178* in pMCB17apx	This study
pKC38	Full-length *pclB* in pDV7	This study
pKC39	Full-length *pipA* in pDV7	This study
pKC55	Full-length *pclB* in pCMB17apx, with mRFP instead of GFP	This study
pKC56	Full-length *pipA* in pCMB17apx, with mRFP instead of GFP	This study
pKC64	Full-length PtkA with deletion of PITALRE in pCMB17apx, with *pyroA* instead of *pyr4*	This study
pKC65	Full-length PtkA with point mutation K54Q in pCMB17apx, with *pyroA* instead of *pyr4*	This study
pKC66	Full-length PtkA with point mutation D173A in pCMB17apx, with *pyroA* instead of *pyr4*	This study
pKC70	Full-length PtkA with point mutation G32AG34A in pCMB17apx, with *pyroA* instead of *pyr4*	This study
pKC71	Full-length PtkA with point mutation D155G in pCMB17apx, with *pyroA* instead of *pyr4*	This study
pKC87	CTD (1574–1746 aa) of the large subunit of RNA polymerase II in pSM14	This study

Tagging of proteins with mRFP and GFP under the control of the *alcA* or the natural promoter. To create an N-terminal monomeric red fluorescent protein (mRFP) fusion construct of PipA, the full-length *pipA* (starting from ATG) was amplified from genomic DNA, using the primers Bud32_Asc_for (5′-ggcataggcgcgccaATGCCACCAACGAACCGC-3′) and Bud32_Pac_rev (5′-gctacgttaattaaCTACCCAATCATGCTCCTCTTCC-3′) and cloned via AscI/PacI (the restriction sites are underlined) into the corresponding sites of pCMB17apx, yielding pKC55. Likewise, the truncated *pipA* ORF was amplified with the primers Bud32_Asc_for and Bud177_Bam_rev (5′-ggcataggatccCTACCCATGT ATAACACCCGC-3′) and cloned into pCMB17apx, yielding pKC24.

To create an mRFP fusion construct of PclB, the full-length *pclB* (starting from ATG) was amplified from genomic DNA, using the primers P7 Cyclin_Asc_for (5′-ggcataggcgcgccaATGAACAGTGGAGTCGGC-3′) and P8 Cyclin_Pac_rev (5′-gctacgttaattaaCTACCCAATCATGCTCCTCTTCC-3′) and cloned via AscI/PacI (the restriction sites are underlined) into the corresponding sites of pCMB17apx, yielding pKC56.

The N-terminal green fluorescent protein (GFP) fusion construct of PtkA in pCMB17apx used was described previously (13).

To create a C-terminal GFP fusion construct of PipA under the control of the natural promoter, *pipA* flanking regions were amplified by PCR using genomic DNA and the primers Bud32_P1_for (5′-GCATGGAATG ATGTCCATGGCG-3′) and Bud32-P3-rev (5′-ctccagcgcctgcaccagctccC CCAATCATGCTCCTCTTCCTTC-3′) for the upstream region of *pipA* and Bud32-P4-for (5′-atcagtgcctcctctcagacagTAGTTACCTATATAACA GGTATCGAATGG-3′) and Bud32_P6_rev (5′-GCGTGCTGTGAACAG GCAATTAG-3′) for the downstream region. The *gfp-pyrG* cassette from plasmid pFNO3 (S. Osmani, OH) was amplified by PCR and used as the template together with *pipA* flanking regions for the fusion PCR. The *gfp-pyrG* cassette was amplified with the fusion PCR method (21) with the primers Bud32_P2_for (5′-CTTCCCCTCCTCTCTTCTTCTC-3′) and Bud32_P5_rev (5′-GGCAGCCGTCAACATTCAAGTC-3′). The resulting PCR product was transformed into *pyrG89*-auxotrophic *A. nidulans* strain TN02A3.

To create a C-terminal GFP fusion construct of PclB under the control of the natural promoter, *pclB* flanking regions were amplified by PCR using genomic DNA and the primers Cyclin_P1_for (5′-GGATGGCTTG CCTAACAGCTCTTG-3′) and Cyclin_P3_rev (5′-ctccagcgctgcaccagctcc CGTCGGGTGTTCCATGTACCAATG-3′) for the upstream region of *pclB* and Cyclin-P4-for (5′-atcagtgcctcctctcagacagTGACTCTGGGTATG GTGACGTG-3′) and Cyclin-P6_rev (5′-GCACGATATAATAGTGGCA CCGC-3′) for the downstream region. The *gfp-pyrG* cassette from plasmid pFNO3 (S. Osmani, OH) was amplified by PCR and used as the template together with *pclB* flanking regions for the fusion PCR. The *gfp-pyrG* cassette was amplified with the fusion PCR method (21) with the primers Cyclin_P2_for (5′-GTCCGACCATGCCCGTTTCATC-3′) and Cyclin_P5_rev (5′-CTTCGTCGGAGTAGTTCGCAGTG-3′). The resulting PCR product was transformed into *pyrG89*-auxotrophic *A. nidulans* strain TN02A3.

Transformants were screened by PCR for the homologous integration event. Single integration of the construct was confirmed by Southern blotting. One *pipA-gfp* strain (SKC27) and one *pclB-gfp* strain (SKC28) were selected from the transformants.

Tagging of proteins with YFP^N/YFP^C for BiFC analyses. For bimolecular fluorescence complementation (BiFC) analyses, the GFP in pCMB17apx was replaced with the N-terminal half of yellow fluorescent protein (YFP^N) or the C-terminal half of yellow fluorescent protein (YFP^C), yielding pDV7 and pDV8, respectively, as described earlier (22). The YFP^C fusion construct of PtkA in pDV8, yielding pFB13, was described previously (13). The YFP^N fusion construct of PipA was generated by amplification of the complete ORF from genomic DNA with the primers Bud32_Asc_for and Bud32_Bam_rev (5′-ggcataggatccCTACCCAAT

CATGCTCCTC-3′). The resulting PCR product was then cloned via AscI/BamHI into the corresponding sites of pDV7, yielding pKC39. Using the same strategy, the YFP^N fusion of PclB was generated, using the primers Cyclin_Asc_for and Cyclin_Bam_rev (5′-ggcataggatccTCACGTCGGGTGTTCC-3′), yielding pKC38.

To create YFP^N fusion constructs of PclB and PipA under the control of the natural promoter, the same primers were used as described before in tagging of proteins with *gfp*. A *yfp^N-pyro* cassette was amplified by PCR and used as the template together with *pclB* or *pipA* flanking regions for the fusion PCR.

To create a YFP^C fusion construct of PtkA under the control of the natural promoter, *ptkA* flanking regions were amplified by PCR using genomic DNA and the primers PtkA_P4 (5′-GAAGGACGTTCTTGGCTGACC-3′) and PtkA_P6 (5′-ctccagcgcctgcaccagctccCCGGCGATACGGACCCCTG-3′) for the upstream region of *ptkA* and PtkA-P5 (5′-atcagtgcctcctctcagacagTGAAGCTACCGTCTACCATAAAAC-3′) and PtkA-P7 (5′-TGTGAAATCATCGCTCTTGCTC-3′) for the downstream region. A *yfp^C-pyrG*-cassette was amplified by PCR and used as the template together with *ptkA* flanking regions for the fusion PCR. The *yfp^C-pyrG* cassette was amplified with the fusion PCR method (21) with the primers ptkA_F1_neuer (5′-AAGGAAAGCCGATTCTTGCTGG-3′) and PtkA_KO_R1 (5′-GCGCGCCAAGTTTCGACCC-3′). The resulting PCR product of *ptkA-yfp^C-pyrG* was transformed into *A. nidulans* strain TN02A3 together with *pipA-yfp^N-pyro* or *pclB-yfp^N-pyro*.

Transformants were screened by PCR for the homologous integration event. One strain with *ptkA-yfp^C-pyrG* and *pipA-yfp^N-pyro* (SKC29) and one strain with *ptkA-yfp^C-pyrG* and *pipA-yfp^N-pyro* (SKC30) were selected from the transformants.

Insertion of mutations by site-directed mutagenesis. To introduce mutations in an N-terminal GFP fusion construct of PtkA, a QuikChange XL mutagenesis kit (Stratagene, Heidelberg, Germany) was used. To generate the K54Q point mutation, the following primers were used: ptkA-KQ-F1 (5′-GATGGCTCCATCGTCGCGCTGCAAAAGATCCTCATGCATAATG-3′) and ptkA-KQ-R1 (5′-CATTATGCATGAGGATCTTTTGCAGCGCGACGATGGAGCCATC-3′) (the mutation sites are underlined), yielding pKC65. To delete the PITALRE domain, the primers PtkA_PITALRE_f (5′-PHO-CTACTGAAAATGTTGTCCCACACC-3′, where PHO indicates phosphorylation of the primer) and PtkA_PITALRE_r (5′-PHO-CTTTGACGATCAGATAGCTGCC-3′) were used, yielding pKC64. To generate the G32AG34A point mutations, the primers PtkA_GAGA_f (5′-GCAAACTGGCGGAGGCCACCTTTGG-3′) and PtkA_GAGA_r (5′-CCAAAGGTGGCCTCCGCCAGTTTGC-3′) were used, yielding pKC70. For the D155G point mutation, the primers PtkA_D155G_f (5′-GTATCCTACACCGCGGCATGAAAGGCTAG-3′) and PtkA_D155G_r (5′-CTAGCCTTTCATGCCGCGGTGTAGGATAC-3′) were used, and for the mutation D173A, the primers PtkA_D173A_f (5′-CTGCAGATTGCCGCCTTCGGACTGGC-3′) and PtkA_D173A_r (5′-GCCAGTCCGAAGGCGGCAATCTGCAG-3′) were used, yielding pKC71 and pKC66, respectively.

All plasmids were verified by sequencing and then transformed into strain GR5.

Yeast two-hybrid analysis. The yeast two-hybrid screen was performed using a Matchmaker library construction and screening system (BD Clontech). For bait generation, the primers pGB-Y2H-F1 (5′-GGCATATGGGCATAGCGTCACTCGAACGG-3′) and pGB-Y2H-R1 (5′-GGATCCTCACCGGCGATACGGACCCCT-3′) were used to amplify a full-length *ptkA* cDNA fragment, the primers ptkA24/170_Nde (5′-GGCTACCATATGGAATTCGAGTTCTTAGGCAAACTGGG-3′) and ptkA24/170_Bam (5′-GGCTACGGATCCTCACTGCAGAATACCTTGGTTGCTTATAAG-3′) were used to amplify the truncated *ptkA* fragment from residues 24 to 170 (*ptkA_24/170*), and the primers ptkA_Bam_for (5′-GGATCCATGGGCATAGCGTCACTCG-3′) and ptkA995_Sal_rev (5′-GGGTCGACTCATGGCGGTGTCGAGAAATAA-3′) were used to amplify the truncated *ptkA_1/330*. The full-length *ptkA* and the truncated *ptkA_1/330* were cloned in the pGBT9 vector, and the truncated *ptkA_24/170* was cloned in the vector pGBKT7, which contains both the *GAL4* DNA binding domain and the *TRP1* marker (BD Clontech). cDNA was isolated from an *A. nidulans* wild-type strain, amplified, and cloned in the pGADT7-Rec vector, under the manufacturer's instructions. The pGADT7-Rec vector contains the *GAL4* DNA-AD and the *LEU2* marker (BD Clontech). pGBKT7- and pGBT9-associated plasmids were transformed in *S. cerevisiae* AH109 (mating type MAT**a**), and pGADT7-associated plasmids were transformed into *S. cerevisiae* Y187 (mating type MATα).

For yeast two-hybrid analysis, a *pipA* cDNA fragment was amplified with primers Bud32_Nde_for (5′-ggcatacatATGCCACCAACGAACCGC-3′) and Bud32_Bam_rev (5′-ggcataggatccCTACCCAATCATGCTCCTC-3′) and a *pclB* cDNA fragment was amplified with the primers Cyclin_Nde_for (5′-GGCATACATATGAACAGTGGAGTCGGCG-3′) and Cyclin_Bam_rev (5′-GGCATAGGATCCTCACGTCGGGTGTTCC-3′), and the amplicons were cloned in pGADT7-Rec.

Light and fluorescence microscopy. For live-cell imaging of germlings and young hyphae, cells were grown on coverslips in 0.5 ml MM plus 2% glycerol (derepression of the *alcA* promoter), MM plus 2% glucose (repression of the *alcA* promoter), or MM plus 2% threonine (activation of the *alcA* promoter). Cells were incubated at room temperature overnight. For pictures of conidiophores, spores were inoculated on glass slides coated with MM plus 2% glucose and 0.8% agarose and grown at 37°C for 1 to 2 days. Images were captured at room temperature using an Axiophot microscope (Zeiss, Jena, Germany). Images were collected and analyzed with an AxioVision system (Zeiss).

Gene expression analyses. Transferring development-competent vegetative hyphae from liquid medium to agar plates, thus inducing development by air exposure, synchronized asexual development of wild-type *A. nidulans* cells. At different time points, RNA was isolated. For that, the mycelium was harvested, dried, frozen in liquid N_2, and ground to a powder. Total RNA was isolated by using TRIzol reagent (Invitrogen, NV Leek, Netherlands) according to the manufacturer's instructions. For the expression analyses, quantitative real-time PCR was performed by using 100 ng of the isolated RNA for each reaction and an iScript one-step reverse transcription-PCR (RT-PCR) kit with SYBR green and the iCycler apparatus of Bio-Rad. For analysis of PclB, the primers Cyclin_for (5′-GGAGAGGGTGAACCTAGCCTTG-3′) and Cyclin_rev (5′-GAAGTTCCATGGCGTTCTCCG-3′) were used, and for analysis of PipA, the primers Bud_for (5′-CCGCGTCCCTTCCTTCTTACTC-3′) and Bud_rev (5′-GGGTTTTGACGGGCGGATTTTG-3′) were used. As the housekeeping gene, β-tubulin (*benA*) was used.

Protein extracts and Western blotting. To prepare protein extracts from vegetative hyphae, *A. nidulans* strains were incubated in liquid MM for 24 h at 37°C. For the isolation of proteins form asexual structures, hyphae were transferred to MM plates with cellophane and incubated for an additional 24 h at 37°C to induce asexual development. To induce the *alcA* promoter, the medium was supplemented with 0.2% glucose and 2% threonine. Mycelium was harvested by filtration through a Miracloth filter (Calbiochem, Heidelberg, Germany), dried, and immediately ground in liquid nitrogen. Afterwards, the mycelial powder was resuspended in protein extraction buffer (50 mM Tris-HCl [pH 8], 0.1% Triton X-100, 250 mM NaCl) containing 1 mM protease inhibitor cocktail recommended for fungal and yeast extracts (Sigma-Aldrich, Munich, Germany) and 1 mM phosphatase inhibitor cocktail 3 (Sigma-Aldrich, Munich, Germany). Samples were rotated for 20 min in a head-over-tail incubator. Cell debris was pelleted by centrifugation (5403-R centrifuge; Eppendorf, Hamburg, Germany) at 13,000 rpm and 4°C for 12 min. The supernatant was used for Western blotting. Equal amounts of protein extracts (75 μg) were loaded on 8% sodium dodecyl sulfate-polyacrylamide gels for detection of GFP-fusion proteins or on 10% SDS-gels for detection of the CTD. Proteins were blotted onto polyvinylidene difluoride membranes (Whatman; GE Healthcare, Little Chalfont, United Kingdom), and GFP-tagged proteins were analyzed with a monoclonal anti-GFP antibody (dilution, 1:8,000; product G1544; Sigma-Aldrich, Munich, Germany). The

TABLE 3 Identification of PtkA-interacting proteins with the PtkA full-length protein as bait

Broad Institute identification no.	Total no. of amino acids/size of isolated clone (amino acids)	No. of clones in screening	Conserved domain[a]	Assigned function in other organisms
AN6605.2	408/344	5	FAD-dependent oxidoreductase	
AN6827. 1	122/122	1	ssDNA binding protein Ssb3	Replication factor A protein 3: replication factor A is involved in eukaryotic DNA replication, recombination and repair
AN5438.2	422/422	3	Tetratricopeptide repeat protein	The tetratricopeptide repeat is a structural motif present in a wide range of proteins. It mediates protein-protein interactions and the assembly of multiprotein complexes
AN6600.2	723/604	1	Mis6 domain-containing protein	Mis6 is an essential centromere connector protein acting during G_1-S phase of the cell cycle
AN10741.1	241/241	1	Cyclin-dependent protein kinase complex component	This family includes many different cyclin proteins. Members include the G_1/S-phase-specific cyclin Pas1 and the phosphate system cyclin PHO80/PHO85
AN11303.1	92/92	1	ATP synthase e chain	This family consists of several ATP synthase e chain sequences which are components of the CF(0) subunit
AN4730.2	188/188	1	37S ribosomal protein S12	Ribosomal protein S12 is one of the proteins from the small ribosomal subunit
AN11317.1		4	Hypothetical protein	
AN7249.2		3	Hypothetical protein	

[a] FAD, flavin adenine dinucleotide; ssDNA, single-stranded DNA.

nontagged CTD was detected with the monoclonal antibody 8WG16 (dilution, 1:1,000; Covance, Princeton, NJ). Two monoclonal anti-phospho-CTD antibodies were applied to quantify CTD specifically phosphorylated at Ser2 (dilution, 1:1,000; antibody H5; Covance, Princeton, NJ) and CTD specifically phosphorylated at Ser5 (dilution, 1:1,000; antibody H14; Covance, Princeton, NJ).

RESULTS

Identification of novel interaction partners of the Cdk9 kinase PtkA. The cyclin-dependent kinase PtkA has been shown to interact with two cyclins, PclA (11) and PchA (13). Whereas *pchA* deletion caused a severe growth defect, *pclA* deletion had no effect on vegetative growth but had an effect only on sporulation. Because Cdk9 kinases are involved in several cellular pathways in higher eukaryotes, we anticipated that PtkA specificities might be determined by even more interaction partners in *A. nidulans*. To identify such new interacting proteins, we screened a cDNA library by using the Matchmaker library construction and screening kit (Clontech). Full-length PtkA (PtkA_1/545) was cloned into the standard yeast two-hybrid pGBKT7 vector, in which the constructs are strongly expressed. Corresponding yeast transformants, unfortunately, did not grow well and were unable to mate. This suggested an inhibitory function of the PtkA kinase. To avoid the toxic effect, we used instead pGBT9 as the vector, in which genes are expressed at lower levels. In addition, we constructed a truncated version of PtkA (PtkA_24/170) lacking a short piece of the N terminus and the kinase domain, located in the C terminus. This construct could be screened in the overexpression vector pGBKT7. In the screening experiment with PtkA_1/545, 120 yeast colonies grew on the selection medium (SD-Leu-Trp-His, where SD means synthetic dropout), from which 100 yeast colonies were able to grow again on the same medium. The screening with PtkA_24/170 led to the isolation of about 450 yeast colonies, two-thirds of which were able to grow again on selective medium. Plasmids from these colonies were isolated, and different plasmids were identified by restriction fragment length polymorphism analysis. After sequencing, nine different genes were identified from the screening with PtkA_1/545 (Table 3) and 10 were identified from the screening with PtkA_24/170 (Table 4), respectively. We have chosen two candidates, AN10741 and AN2513, for further analysis, because all other putative interacting proteins seemed to have metabolic functions. The protein for uncharacterized gene AN10741 was named PclB, and the second interacting protein, AN2513, was named PipA.

PclB is a homologue of *Saccharomyces cerevisiae* Pcl7 and the second Pcl-like cyclin identified in *A. nidulans*. The *pclB* full-length cDNA was found once in the yeast two-hybrid screen with *ptkA_1/545* as bait. The ORF (AN10741) is 726 bp long, contains no introns, and codes for a protein with 242 amino acids. PclB displays 30% identical residues compared to *S. cerevisiae* Pcl7. If chemically similar amino acids are included, the degree of similarity increases to 60%. *S. cerevisiae* Pcl7 forms a complex with the kinase Pho85/Pho80 (23), and its expression is regulated during the cell cycle (24). Because of the homology to Pcl7 and because it is the second member of the Pcl cyclin family to have been found in *A. nidulans*, we named the protein PclB. PclB is also conserved in other filamentous fungi, but none has yet been analyzed. *A. nidulans* PclB contains a predicted cyclin box (49 to 219 aa), a PEST domain (82 to 92 aa), and a putative destruction box (169 to 177 aa). Furthermore, it contains a predicted Cdk phosphorylation site (87 to 93 aa) and a nuclear export signal, located behind the cyclin box (208 to 223 aa) (Fig. 1A).

PipA, a new putative serine/threonine kinase. The second interacting protein (AN2513) was named PipA (Fig. 1A), which stands for PtkA-interacting protein A. The open reading frame

TABLE 4 Identification of PtkA-interacting proteins using a truncated version of PtkA as bait

Broad Institute identification no. Total	Total no. of amino acids/size of isolated clone (amino acids)	No. of clones in screening	Conserved domain	Assigned function in other organisms
AN9297.2	590/388	12	Myosin cross-reactive antigen	Members of this family are thought to have structural features in common with the beta chain of the class II antigens
AN0369.1	459/311	1	Methionine aminopeptidase 2B	Metallopeptidase family
AN0451.2	215/185	6	C-8 sterol isomerase	C-8 sterol isomerase, catalyzes a reaction in ergosterol biosynthesis
AN03223.1	796/569	4	6-Phosphofruktokinase alpha subunit	Catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate, a key regulatory step in the glycolytic pathway
AN7044.2		1	Histidinol phosphatase	The polymerase and histidinol phosphatase (PHP) domain is a putative phosphoesterase domain
AN3847.2		1	Oxidoreductase	
AN3586.2		1	Ubiquinone biosynthesis monooxygenase	
AN7432.2		1	Ornithine decarboxylase antizyme	
AN4202.1		1	Ribosomal protein L16a	Ribosomal protein L13 is one of the proteins from the large ribosomal subunit
AN2513.2	285/118	1	Serine/threonine-protein kinase Bud32	Bud32 is a subunit of the KEOPS/EKC complex, which plays a role in transcription and maintenance of telomeres

was also not disrupted by any intron. PipA is a protein of 286 aa with a predicted protein kinase domain (26 to 239 aa), a PEST domain (188 to 200 aa), and a nuclear localization signal (269 to 283 aa). Homologous proteins are also found in other filamentous fungi but have not yet been characterized. PipA shows homology to the kinase Bud32 of *S. cerevisiae* (36% identical and 49% conserved). In *S. cerevisiae*, Bud32 comprises part of the KEOPS/EKC complex, which is known to play a role during transcription and to be important for maintenance of the telomeres and separation of the chromosomes (15, 25). Furthermore, Bud32 is an atypical serine/threonine kinase, because it is shorter and some conserved domains are missing compared to the sequences of other known kinases (14, 26). Deletion of *BUD32* causes slower growth, reduced survival of the cells in the stationary growth phase, inhibition of sporulation of homozygous diploids, as well as inhibition of meiotic divisions (26, 27).

Interaction of PtkA with PipA and PclB is restricted to the head of the conidiophore. To verify the interaction of PtkA with PipA and PclB, a yeast two-hybrid assay with different bait constructs was performed (Fig. 1B). Full-length PtkA was used as bait, as were two truncated versions, PtkA_24/170 and PtkA_1/330. The truncated PtkA_24/170 contains a large part of the kinase domain and most of the conserved domains, and the PtkA_1/330 construct includes the whole kinase domain. Interaction of PipA and PclB with PtkA could be shown with the three different bait constructs, showing that the kinase domain of PtkA is sufficient for the interaction with these proteins. To confirm these results and to analyze the localization of the PtkA-PipA and PtkA-PclB complexes *in vivo*, we used bimolecular fluorescence complementation (BiFC) (Fig. 1C). To this end, the N-terminal half of YFP was fused to PipA and PclB and the C-terminal half of YFP was fused to PtkA. Strains expressing PtkA-YFP^{C} and PipA-YFP^{N} or PclB-YFP^{N} showed a strong YFP signal, which was restricted to metulae, phialides, and conidia. The same results were obtained when constructs were expressed from the natural or the *alcA* promoter. No signal could be detected in hyphae, either in the cytoplasm or in nuclei, despite the presence of all three proteins (see below). In comparison, PclA and PchA interactions with PtkA were detected in the nuclei of hyphae (13).

PclB and PipA reside in the cytoplasm and in the nuclei of hyphae and conidiophores. Because the interaction of PtkA with PclB and PipA was restricted to the conidiophore, we tested whether expression of the two proteins was also restricted to this developmental phase (Fig. 2). To this end, *A. nidulans* strains which contained GFP-PtkA in combination with mRFP-PipA or mRFP-PclB were constructed. All proteins were C-terminally tagged at their native locus. To generate these constructs, a fusion PCR method was used (21). The fusion PCR was set up with two flanking sequences and a *GFP-pyrG* cassette derived from pFNO3. Transgenic *A. nidulans* colonies were tested for homologous integration of the constructs by PCR and Southern blotting (results not shown). All proteins were detectable in hyphae and in conidiophores in the cytoplasm and also in nuclei. In addition to the subcellular localization using the native promoter (Fig. 2A and C), the tagged proteins were also expressed from the *alcA* promoter (Fig. 2B and D). After moderate induction of the promoter, clear signals which unambiguously demonstrated nuclear localization of some fraction of the proteins were observed. GFP-PtkA could mainly be observed in the nuclei of hyphae, in metulae, phialides, and spores, with only faint signals observed in the cytoplasm. mRFP-PipA appeared in metulae, phialides, and spores, with strong signals observed in nuclei and the cytoplasm (Fig. 2D). The mRFP-PclB fusion protein had the same localization pattern as

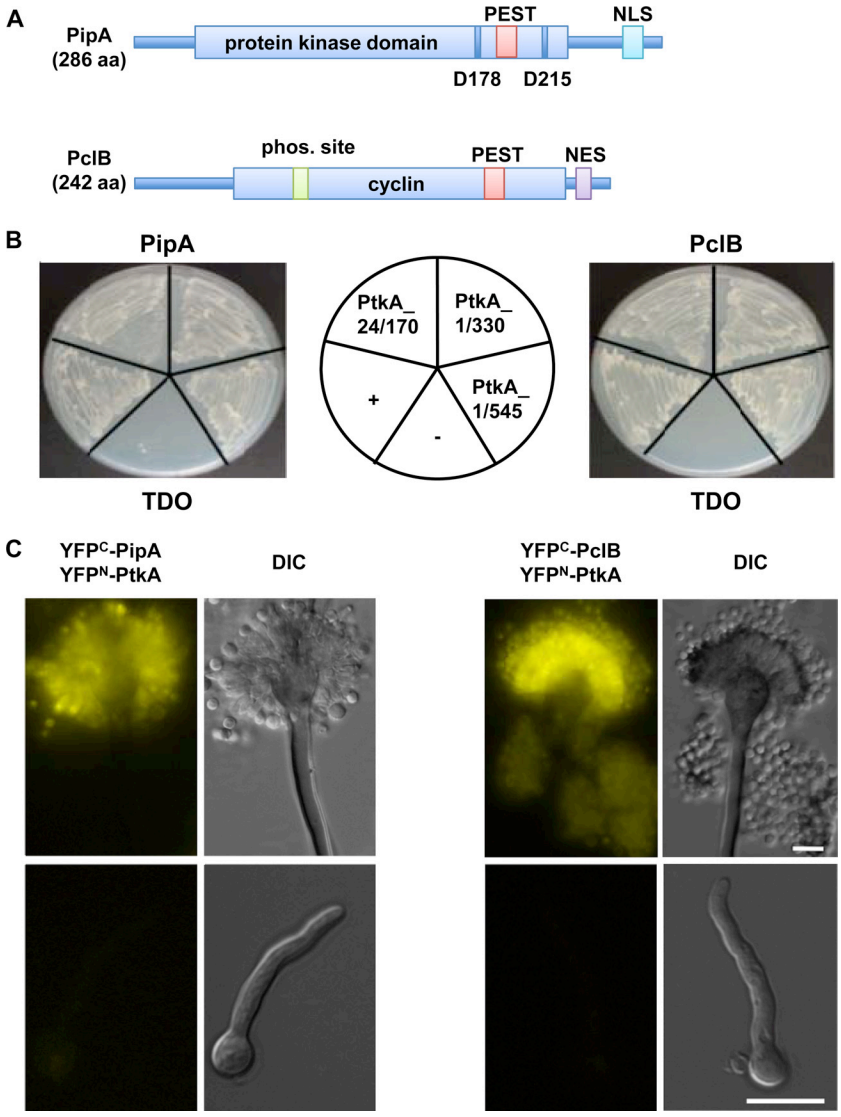

FIG 1 Isolation of two new PtkA-interacting proteins. (A) Scheme of the two proteins. Conserved domains of PipA and PclB. The PipA protein is 286 aa long and contains a kinase domain, a PEST domain, and a nuclear localization signal (NLS). PclB is 242 aa long and contains a cyclin domain, a PEST domain, and a destruction box. The PEST domain and a destruction box are both signals for degradation of proteins. The nuclear export signal (NES) is a sign for localization of the protein in the nucleus and possible export in the cytoplasm. (B) Yeast two-hybrid assay with PtkA and PipA or PclB. The interaction of PipA and PclB with PtkA was tested with three different PtkA constructs, the full-length PtkA_1/545 and two truncated constructs, PtkA_1/330 and PtkA_24/170. TDO, triple dropout. (C) BiFC analyses to confirm the interaction of PtkA with PipA and PclB. In SKC19 expressing PtkA tagged with the N-terminal half of YFP and PipA tagged with the C-terminal half, a YFP signal was detected in the head of the conidiophore but not in hyphae. Strain SKC20 expressing YFP^N-PtkA and YFP^C-PclB also showed a YFP signal, which was restricted to the conidiophore and was not observed in the hyphae. DIC, differential inference contrast.

PipA. It was also found in nuclei, but the protein did not specifically accumulate in these organelles (Fig. 2B).

Besides the protein localization, the expression of *pipA* and *pclB* was tested during different stages of development (Fig. 3). Asexual development of the wild-type strain FGSCA4 was synchronized by air exposure of mycelium grown in liquid culture, and total RNA was isolated at different time points and used as the template for quantitative real-time PCR experiments. Whereas *pipA* and *pchA* appeared to be constitutively expressed throughout development, the expression of *pclB* changed during development. The expression after 5 h postinduction of asexual development was higher than that in hyphae and decreased with time to the lowest level after 9 h. After that time point, the mRNA level increased again, with a maximum occurring after 24 h. The high levels of *pclB* and *pipA* expression during the formation of the conidiophores are in agreement with the results of the interaction and localization studies and thus imply a function of these proteins during later stages of asexual development. The analysis was repeated with strains containing tagged versions of the proteins, and similar expression patterns were obtained (data not shown).

***pipA* is important for normal growth and development.** In order to account for the putative lethality of *pipA* deletion, we first tried to downregulate *pipA* using the *alcA* promoter (Fig. 4A). A ca. 500-bp fragment was amplified from the region 5′ of the *pipA* open reading frame and cloned in frame with GFP under the control of the *alcA* promoter (pMCB17apx). Integration of the circu-

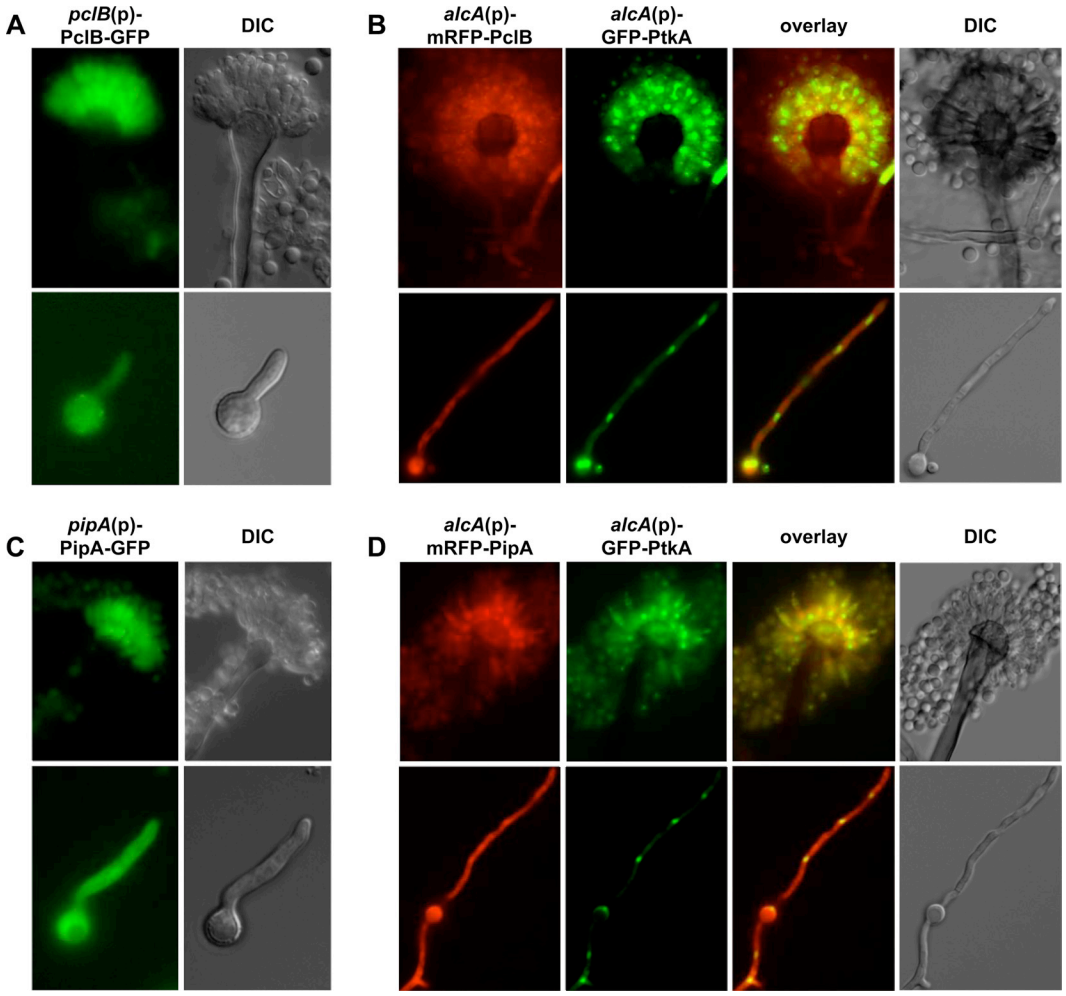

FIG 2 Localization of PipA and PclB. (A) PclB tagged with GFP expressed from the natural promoter localized to the head of the conidiophore and mainly to the cytoplasm of hyphae. (B) Localization of PclB expressed from the *alcA* promoter. Strain SKC72 was grown on minimal medium with glycerol as the carbon source. mRFP-tagged PclB mainly localized to the cytoplasm, but it also localized in the cytoplasm of hyphae and conidiophores. Localization in the nucleus was confirmed by an overlay with the GFP signal of PtkA. (C) PipA tagged with GFP expressed from the natural promoter localized to the head of the conidiophore and mainly to the cytoplasm of hyphae. (D) Localization of PipA expressed from the *alcA* promoter. Strain SKC73 was grown on minimal medium with glycerol as a carbon source. mRFP-tagged PipA localized to the nucleus and the cytoplasm of hyphae and conidiophores. The localization in the nucleus was confirmed by an overlay with the GFP signal of PtkA.

lar plasmid in the *pipA* locus results in duplication of the 500-bp fragment and placement of the full-length gene under *alcA* promoter control. Growth of the strain on medium containing glucose led to repression of the *alcA* promoter and caused a decrease in asexual spore formation, although the morphology of the conidiophores did not differ from wild type. The reduced spore production resulted in loss of the green color of wild-type colonies and an increased production of aerial hyphae. Furthermore, most of the vegetative hyphae showed a curved phenotype with multiple branches under repressing conditions. Under derepressing conditions (medium with glycerol), the strain showed wild-type morphology, proving the functionality of the N-terminal GFP fusion construct. Under inducing conditions (medium with threonine), asexual development was completely blocked. The produced hyphae showed a phenotype similar to that of hyphae formed under repressing conditions, as they developed more branches and curves, whereas the wild type grew straight. These results suggest that *A. nidulans* hyphal growth and asexual development are very sensitive to changes in PipA concentration.

***pipA* is an essential gene.** To further analyze the function of *pipA*, we aimed to construct a *pipA*-deletion strain by replacing the coding sequence with a deletion construct obtained by fusion PCR (Fig. 4A). Protoplasts of a KU70-deficient recipient strain were produced and transformed with the deletion construct. Transformants were tested for the loss of the ORF and homologous integration of the deletion cassette. The correct integration of the deletion cassette was confirmed by PCR and Southern blotting (Fig. 4B and C). However, the ORF of *pipA* was still detectable by PCR and Southern blotting. This was a first indication that *pipA* deletion is lethal and that only heterokaryotic or dikaryotic strains survived. In order to study whether the strain was a diploid, medium with benomyl was used to induce haploidization. The putative diploid strain was inoculated in the center of a plate and incubated for several days. Benomyl destabilizes microtubules and

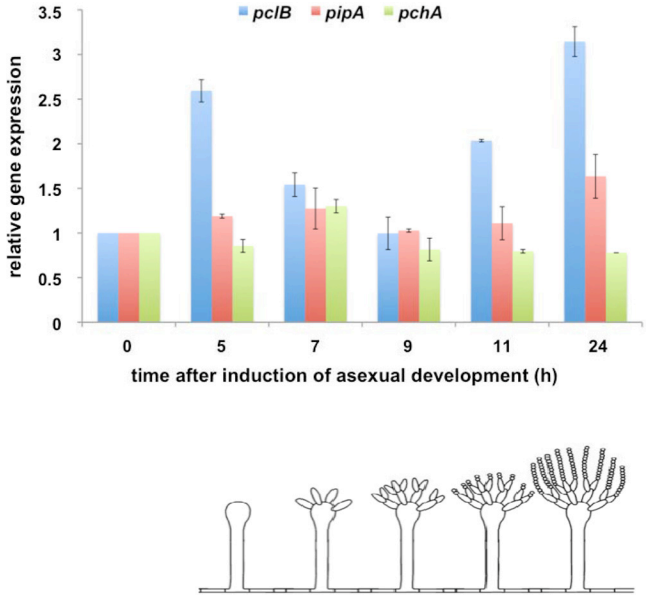

FIG 3 Gene expression analyses by quantitative RT-PCR. Total RNA was isolated after different time points, indicated by the scheme below the graph, to monitor gene expression during development. Transcripts were quantified relative to β-tubulin (*benA*). The expression level during the asexual development (5 h to 24 h) was compared to the expression during vegetative growth (0 h).

causes random losses of chromosomes during mitosis. Because aneuploid *A. nidulans* strains are rather unstable, the number of chromosomes is rapidly reduced to the haploid set in aneuploid nuclei. The progeny may therefore represent different genotypes, which may be visible by sector formation in colonies. Although we obtained sectoring colonies, we were unable to isolate homokaryotic *pipA*-deletion strains but always obtained either wild-type or still diploid strains. These results suggest that *pipA* is an essential gene.

Genetic interaction between *pclB* and *pclA*. To analyze the function of *pclB*, a deletion strain was produced, as was a strain expressing full-length *pclB* as a GFP fusion protein under the control of the inducible *alcA* promoter (Fig. 5A). Deletion of *pclB* was confirmed by Southern blotting and PCR. The strain did not show any discernible phenotype with regard to hyphal growth. However, spore production was significantly reduced. The strain expressing *pclB* under *alcA* promoter control also showed no differences in vegetative growth, regardless of repression or induction of *pclB*. These results show that *A. nidulans* growth is not sensitive to different PclB protein concentrations.

To investigate the genetic interaction between *pclB* and the other known PtkA-interacting cyclins, PclA and PchA, corresponding double-deletion mutants were produced (Fig. 5B). The *pclA*-deletion mutant was characterized by a reduced number of asexual spores (11). The *pclB pclA* double-deletion strain displayed an additive effect of the *pclA*- and the *pclB*-deletion phenotypes, and even fewer spores were produced. Furthermore, most of the conidiophores showed abnormal morphologies but were still able to produce some spores. The *pclB pchA* double-deletion strain produced the same tiny colonies as the *pchA* mutant. Conidiophore morphology was severely impaired, and spore formation was almost completely blocked (Fig. 5C).

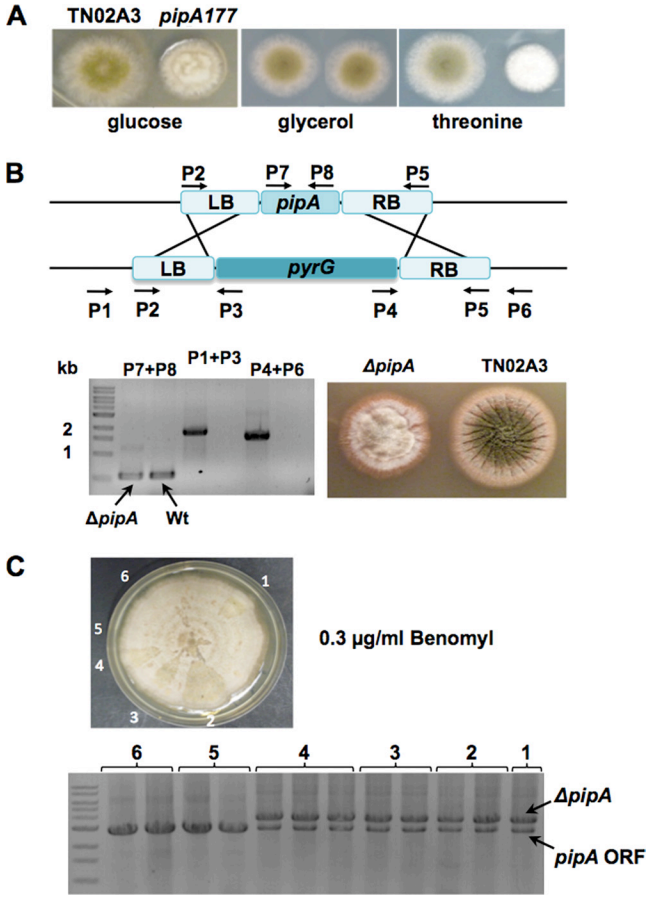

FIG 4 Characterization of PipA. (A) Colonies of wild-type strain TN02A3 and strain SKC13 [*alcA*(*p*)::*GFP*::*pipA177*] on MM with different carbon sources, as indicated. Strains were grown for 2 days on MM agar plates containing glucose (repressing conditions), glycerol (derepressing conditions), or threonine (inducing conditions). (B) Deletion strategy and confirmation of the homologous integration of the deletion cassette by diagnostic PCR using the primer pairs indicated in the scheme. Comparison of the Δ*pipA*/*pipA* diploid strain (SKC49) and the wild-type (Wt) strain (TN02A3). Colonies were grown for 3 days on yeast agar glucose. LB, left border; RB, right border. (C) Haploidization of SKC49 on medium with 0.3 μg/ml benomyl. The *pipA* mutant grown on medium with benomyl produced several sectors. Spores were collected from different sectors, and corresponding colonies were tested by PCR for the *pipA*-knockout or wild-type situation. Some colonies were haploid after growing on benomyl medium but contained only the *pipA* ORF (sectors 5 and 6), whereas other colonies were still diploid (sectors 1 to 4).

The conserved domains of PtkA are essential for the localization and the ability to interact with other proteins. To investigate if certain domains in PtkA are important for the subcellular localization and the ability to interact with other proteins, five important domains were mutated. The exchange of the conserved sequences was performed by site-directed mutagenesis using a plasmid containing *ptkA* with N-terminal *GFP* under the control of the *alcA* promoter. The conserved glycine-rich region, which is important for the correct binding and positioning of ATP, was mutated by the exchange of two glycines to alanine (G32A and G34A) (28). The conserved lysine (K54) is needed for the transfer of phosphate to the substrate and was mutated to a glutamine (K54Q) (29). The invariable PITALRE sequence, important for cyclin binding, was deleted (30). The next important amino acid is

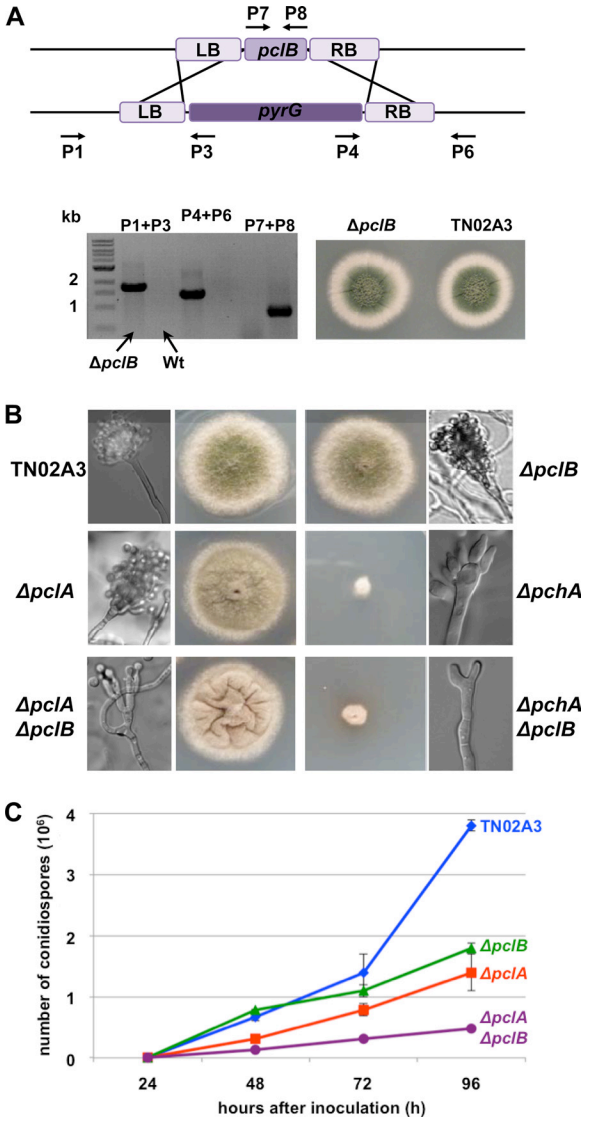

FIG 5 Characterization of the cyclin PclB. (A) Deletion strategy and confirmation of *pclB* deletion by diagnostic PCR using the primer pairs indicated in the scheme. Comparison of the *pclB*-deletion strain (SKC34) and the wild type (TN02A3). Colonies were grown for 3 days on MM. LB, left border; RB, right border. (B) Characterization of double-deletion mutants of the different cyclins interacting with PtkA. Conidiophore stalks are about 8 μm in diameter. (C) Quantification of asexual spores of wild-type (TN02A3), the *pclA*- and *pclB*-deletion strains, and the *pclA pclB* double-deletion strain. Strains were grown on MM agar plates, and spores were counted after 24, 48, 72, and 96 h.

the aspartate of the DMKAAN domain, which facilitates the release of the substrate after the phosphate transfer and was exchanged to a glycine (D155G) (31). In the conserved DFG domain, which is needed for the transfer of the phosphate residue and the binding of ATP, aspartate was changed to alanine (D173A) (32). PtkA-GFP was detected in the nuclei of hyphae and conidiophores, with faint GFP signals detected in the cytoplasm. All mutated PtkA-GFP fusion proteins mainly localized in the cytoplasm and only partly localized in the nuclei. To exclude localization artifacts due to the degradation of the GFP-PtkA fusion proteins, protein integrity and abundance were analyzed in Western blots. All GFP-PtkA constructs were present in similar amounts independent of the mutation of different domains of PtkA (Fig. 6B). The localization results thus indicate an important role of all conserved domains for proper localization and activity of PtkA.

To test the relevance of the different domains for the interaction of PtkA with other interacting proteins (PclA, PclB, PchA, and PipA), yeast two-hybrid analyses were performed using the same modified PtkAs. To select for the incorporation of the two plasmids and for the interactions, different selection media were used. Although the presence of both plasmids could be demonstrated, no interaction was detected (data not shown). In accordance with the loss of the normal localization pattern, the mutation of the conserved domains also inhibited the interaction with the different interaction partners.

Evidence for a possible role of Pcl cyclins in controlling the phosphorylation status of the CTD of the large subunit of RNA polymerase II. Given that Cdk9 kinases are involved in the regulation of RNA polymerase II, we anticipated that *A. nidulans* PtkA fulfills a similar function and that the identified PtkA interaction partners may control PtkA activity and thereby control RNA polymerase II. The control of RNA polymerase II activity should involve the phosphorylation status of the CTD. Several approaches were undertaken to test this hypothesis. Three antibodies (for human and yeast) which can be used to analyze the phosphorylation status of RNA polymerase II are commercially available. The antibody 8WG16 recognizes the unphosphorylated form. The antibody H5 is derived against phosphorylated serine 2 (Ser2) and H14 is derived against serine 5 (Ser5) in the heptad repeat of the CTD. One obvious experiment to analyze the phosphorylation status is, of course, to quantify the amounts of RNA polymerase and the phosphorylated forms in cell extracts of *A. nidulans*. However, the protein concentrations of RNA polymerase appeared to be below our detection limit. Therefore, we aimed at the establishment of an *in vitro* phosphorylation assay. To this end, we successfully expressed PclA, PclB, PtkA, and the CTD in *E. coli*. Unfortunately, most proteins were insoluble and thus could not be used in the assay. Next, we overexpressed the CTD (pKC87) in *A. nidulans* wild type and *pclA* (SSNI30) and *pclB* (SKC34) mutant strains. Strains were incubated in liquid medium for 24 h at 37°C and then induced for asexual development, and after an additional 24 h, protein extracts were prepared. The CTD could be detected in all protein samples prepared from vegetative hyphae (Fig. 7A and B) or conidiophores (Fig. 7C and D). Interestingly, the concentration of the CTD was very low in wild-type conidiophores, whereas the protein was very abundant in *pclA*- or *pclB*-deletion strains, suggesting that the presence of either cyclin renders the CTD rather unstable (Fig. 7C and D), indicating a connection between Pcl cyclins and the CTD.

In order to determine the phosphorylation status of the CTD, phosphorylation of Ser2 and Ser5 of the CTD was compared between wild type and the deletion mutants. The intensities of all bands in the Western blot were quantified and normalized using the signals obtained with the anti-CTD antibody (8WG16) in the wild-type strain. Afterwards, the values obtained with the phosphorylation-specific antibodies were compared between the wild-type and the *pcl*-deletion strains. In hyphae, the phosphorylation of Ser2 decreased slightly in the absence of PclA or PclB, whereas Ser5 phosphorylation increased almost two times in the case of *pclA* deletion (Fig. 7A and B). The experiment was repeated four times. In conidiophores, phosphorylation of Ser2 in the *pclA*-de-

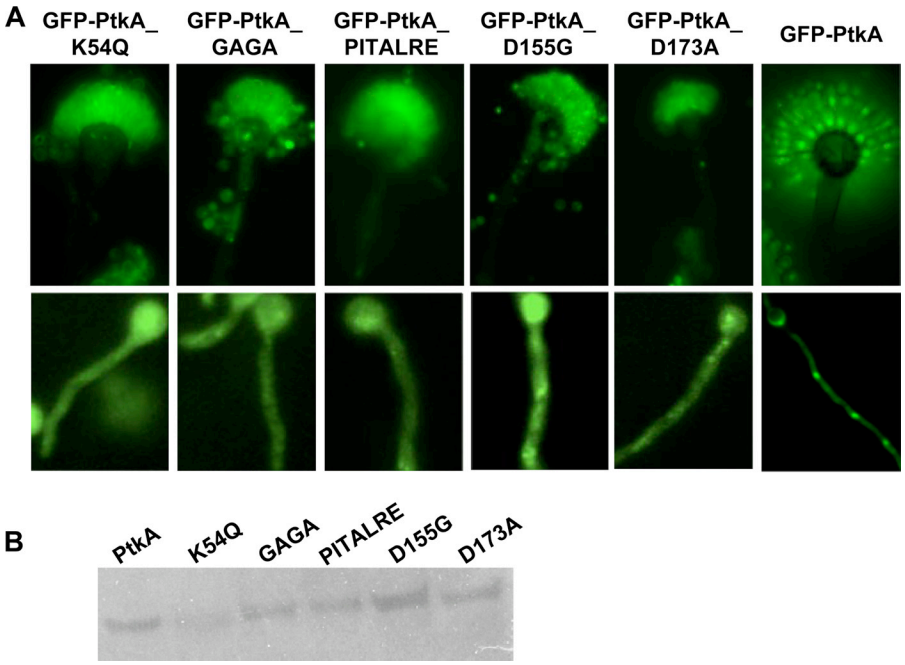

FIG 6 Conserved domains of PtkA are necessary for nuclear localization and the ability to interact with its partners. (A) Localization of PtkA and different mutated versions of PtkA in hyphae and conidiophores. Only the unmodified PtkA protein localized within nuclei. (B) Detection of GFP-PtkA fusion proteins by Western blotting. Equal amounts of protein were analyzed with the anti-GFP antibody.

letion strain increased in comparison to that in the wild type, whereas phosphorylation of Ser5 was reduced (Fig. 7C and D). In comparison, the phosphorylation pattern in the Δ*pclB* strain resembled the pattern obtained in vegetative hyphae; Ser2 phosphorylation was reduced and Ser5 phosphorylation was increased (Fig. 7C and D). The experiment was repeated several times, but in many cases, the CTD concentration in wild type was below the detection limit. Therefore, those samples could not be normalized. Taken together, our results strongly suggest a relation between Pcl cyclins and RNA polymerase II.

DISCUSSION

It has been shown in different organisms that transcription is regulated by a complex which is called P-TEFb in mammalian cells and consists of the Cdk9 kinase and a member of the T-cyclin family (33). This complex is important for the phosphorylation of the CTD of the large subunit of RNA polymerase II and thus regulates the transition from the initiation to the elongation phase (6, 34). In *C. elegans*, it has been shown that Cdk9 is essential for the expression of genes during early development (35). A gene-specific function of Cdk9 was also demonstrated in mammalian cells, because the inhibition of Cdk9 resulted in up- and down-regulation of certain genes and not in a breakdown of the whole RNA synthesis (10). However, the exact mechanism by which Cdk9 exerts a specific effect on the expression of certain genes remains enigmatic.

Here we studied the *A. nidulans* Cdk9 homologue PtkA and identified two new interaction partners of PtkA, in addition to the previously characterized T cyclin, PchA and the Pcl-like cyclin PclA (11, 13). Hence, in total, four different Cdk9 interaction partners which are likely to control PtkA activity alone or in combination are now known. Deletion of *pchA* severely impaired vegetative growth, and *pipA* deletion was lethal, suggesting that it has basic functions in the transcription machinery. In agreement with that, PchA interacted with PtkA in nuclei of hyphae *in vivo* (13). PipA did not interact with PtkA in hyphae. However, PipA could serve a PtkA-independent function in hyphae, because the *S. cerevisiae* PipA homologue Bud32 is part of the KEOPS/EKC complex, which is also known to be involved in transcription. In addition, it plays a role in telomere maintenance and mitosis (15, 25). Our interaction results with PtkA in the conidiophore indicate that PipA may additionally play specific functions during development.

Most interestingly, however, is the finding that two cyclins, PclA and PclB, apparently contribute to the functioning of PtkA in conidiophore development. Deletion was not lethal but specifically affected spore production. This could be due to an interaction with NimX (Cdk1), as has been shown before (12, 36). However, a second possibility is that PtkA controls the phosphorylation status of RNA polymerase II, as it has been shown for other Cdk9 kinases. Indeed, we found that the phosphorylation status of the CTD of the large subunit of RNA polymerase II changes upon deletion of either *pclA* or *pclB*. The most drastic change was that the CTD was hardly detectable in conidiophores, whereas in *pclA*- and *pclB*-deletion strains, the protein appeared as a strong band in Western blots. This suggests instability of the CTD in conidiophores and could be explained by different phosphorylation states of the protein in the absence of either PclA or PclB. In addition, we found that Ser2 phosphorylation increased and Ser5 phosphorylation decreased after *pclA* deletion. In contrast, the phosphorylation pattern was opposite in *pclB*-deletion strains. We take these results as first evidence that PtkA—in combination with different Pcl cyclins—controls the phosphorylation status of RNA polymerase II. Hence, our results suggest a new level of regulation of asexual development. *A. nidulans* is an attractive model for studying eukaryotic

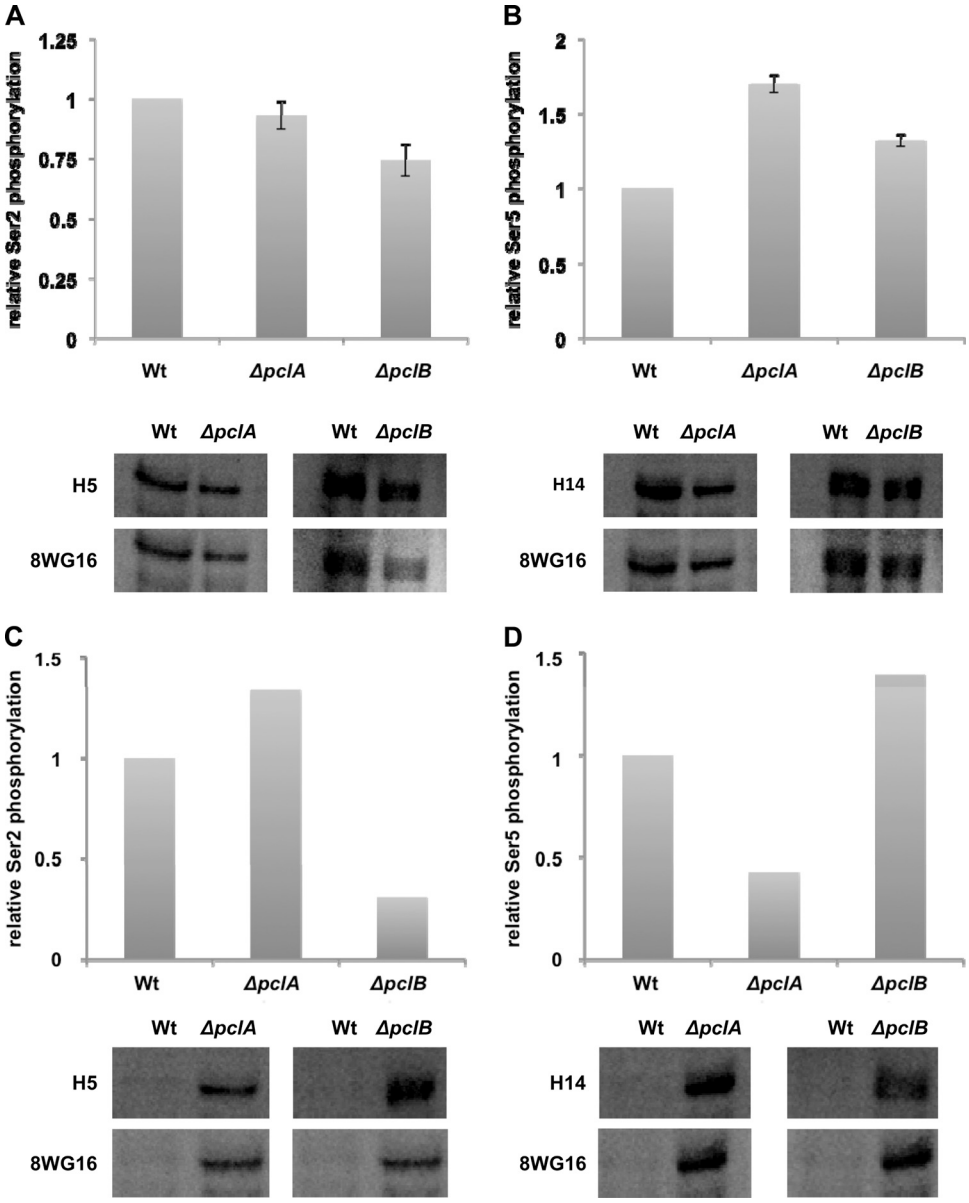

FIG 7 Analysis of the phosphorylation status of the CTD of RNA polymerase II in wild-type and *pcl*-deletion strains. Equal amounts of protein were analyzed for the CTD concentration with antibody 8WG16 and for Ser2 and Ser5 phosphorylation with the antibodies H5 and H14, respectively. The intensities of the bands in the Western blot were quantified using ImageJ software. (A, B) Analysis of hyphae. The values represent the means of four independent experiments. The bars represent the standard deviations. (C, D) Analysis of conidiophores. A representative experiment is displayed. The amount of CTD in wild type was often below the detection limit. The following strains were used: SKC48, SKC77, and SKC79.

development (37, 38). Using developmental mutants and complementation approaches for gene isolation, a number of important regulators as well as differentially regulated proteins have been identified and a central cascade of transcription factors has been characterized. One of the central master regulators is a Cys_2His_2 zinc finger transcription factor called *bristle* (*brlA*). Corresponding mutants fail to develop conidiophores beyond the stalk stage (39, 40). The BrlA protein controls downstream genes like a second APSES transcription factor, AbaA (41). This protein is important for the sterigma differentiation during conidiophore development, because *abaA*-null mutants produce aconidial conidiophores, which are not able to produce sporogenous phialides. One of the early developmental mutants had a mutation for conidial pigmentation. The isolation of the genes revealed a laccase (*yA*) and a polyketide synthase (*wA*) (42, 43). Such transcriptionally regulated genes were important tools for investigating the functioning of the transcriptional regulators.

Besides transcriptional induction of developmental genes, control of gene expression through microRNAs was shown for some genes, such as *brlA* and *stuA* (44–46).

Most likely, chromatin remodeling is also important in development of *A. nidulans*. There is already ample evidence for an important role of the histone code in the control of secondary metabolism (47, 48).

Here, we present evidence that modulation of RNA polymerase II through the Cdk9 kinase PtkA is involved in developmental control. Of course, one of the major challenges for future research will be the identification of target genes whose activity is controlled in a PtkA-dependent manner. *A. nidulans* appears to be a nice model system with many possibilities to unravel the CTD code and its interplay with the histone code.

ACKNOWLEDGMENTS

This work was funded through the Baden-Württemberg Stiftung and the German Science Foundation (DFG FOR1334). C.K. was a fellow of the Landesgraduiertenförderung of the Karlsruhe Institute of Technology (KIT).

REFERENCES

1. **Jenuwein T, Allis CD.** 2001. Translating the histone code. Science **293**:1074–1080.
2. **Zhang DW, Rodriguez-Molina JB, Tietjen JR, Nemec CM, Ansari AZ.** 2012. Emerging views on the CTD code. Genet. Res. Int. **2012**:347214. doi:10.1155/2012/347214.
3. **Wang S, Fischer PM.** 2008. Cyclin-dependent kinase 9: a key transcriptional regulator and potential drug target in oncology, virology and cardiology. Trends Pharmacol. Sci. **29**:302–313.
4. **Peng J, Marshall NF, Price DH.** 1998. Identification of a cyclin subunit required for the function of Drosophila P-TEFb. J. Biol. Chem. **273**:13855–13860.
5. **Peng J, Zhu Y, Milton JT, Price DH.** 1998. Identification of multiple cyclin subunits of human P-TEFb. Genes Dev. **12**:755–762.
6. **Marshall NF, Peng J, Xie Z, Price DH.** 1996. Control of RNA polymerase II elongation potential by a novel carboxyl-terminal domain kinase. J. Biol. Chem. **271**:27176–27183.
7. **Czudnochowski N, Bösken CA, Geyer M.** 2011. Serine-7 but not serine-5 phosphorylation primes RNA polymerase II CTD for P-TEFb recognition. Nat. Commun. **3**:842. doi:10.1038/ncomms1846.
8. **Buratowski S.** 2009. Progression through the RNA polymerase II CTD cycle. Mol. Cell **36**:541–546.
9. **Egloff S, Murphy S.** 2008. Cracking the RNA polymerase II CTD code. Trends Genet. **24**:280–288.
10. **Garriga J, Xie H, Obradovic Z, Grana X.** 2010. Selective control of gene expression by CDK9 in human cells. J. Cell. Physiol. **222**:200–208.
11. **Schier N, Liese R, Fischer R.** 2001. A Pcl-like cyclin of *Aspergillus nidulans* is transcriptionally activated by developmental regulators and is involved in sporulation. Mol. Cell. Biol. **21**:4075–4088.
12. **Schier N, Fischer R.** 2002. The *Aspergillus nidulans* cyclin PclA accumulates in the nucleus and interacts with the central cell cycle regulator NimX(Cdc2). FEBS Lett. **523**:143–146.
13. **Bathe F, Kempf C, Osmani A, Osmani AH, Hettinger S, Wohlmann E, Fischer R.** 2010. Functional characterization of a new member of the Cdk9 family in *Aspergillus nidulans*. Eukaryot. Cell **9**:1901–1912.
14. **Facchin S, Lopreiato R, Stocchetto S, Arrigoni G, Cesaro L, Marin O, Carignani G, Pinna LA.** 2002. Structure-function analysis of yeast piD261/Bud32, an atypical protein kinase essential for normal cell life. Biochem. J. **364**:457–463.
15. **Srinivasan M, Mehta PYY, Prugar E, Koonin EV, Karzai AW, Sternglanz R.** 2011. The highly conserved KEOPS/EKC complex is essential for a universal tRNA modification, t6A. EMBO J. **30**:873–881.
16. **Hill TW, Käfer E.** 2001. Improved protocols for *Aspergillus* minimal medium: trace element and minimal medium salt stock solutions. Fungal Genet. Newsl. **48**:20–21.
17. **Stringer MA, Dean RA, Sewall TC, Timberlake WE.** 1991. *Rodletless*, a new *Aspergillus* developmental mutant induced by directed gene inactivation. Genes Dev. **5**:1161–1171.
18. **Takeshita N, Higashitsuji Y, Konzack S, Fischer R.** 2008. Apical sterol-rich membranes are essential for localizing cell end markers that determine growth directionality in the filamentous fungus *Aspergillus nidulans*. Mol. Biol. Cell **19**:339–351.
19. **Yelton MM, Hamer JE, Timberlake WE.** 1984. Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. Proc. Natl. Acad. Sci. U. S. A. **81**:1470–1474.
20. **Sambrook J, Russell DW.** 1999. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
21. **Szewczyk E, Nayak T, Oakley CE, Edgerton H, Xiong Y, Taheri-Talesh N, Osmani SA, Oakley BR.** 2006. Fusion PCR and gene targeting in *Aspergillus nidulans*. Nat. Protoc. **1**:3111–3120.
22. **Zekert N, Veith D, Fischer R.** 2010. Interaction of the *Aspergillus nidulans* microtubule-organizing center (MTOC) component ApsB with gamma-tubulin and evidence for a role of a subclass of peroxisomes in the formation of septal MTOCs. Eukaryot. Cell **9**:795–805.
23. **Measday V, Moore L, Retnakaran R, Lee J, Donoviel M, Neiman AM, Andrews B.** 1997. A family of cyclin-like proteins that interact with the Pho85 cyclin-dependent kinase. Mol. Cell. Biol. **17**:1212–1223.
24. **Lee M, O'Regan S, Moreau JL, Johnson AL, Johnston LH, Goding CR.** 2000. Regulation o the Pcl7-Pho85 cyclin-cdk complex by Pho81. Mol. Microbiol. **38**:411–422.
25. **Hecker A, Lopreiato R, Graille M, Collinet B, Forterre P, Libri D, van Tilbeurgh H.** 2008. Structure of the archaeal Kae1/Bud32 fusion protein MJ1130: a model for the eukaryotic EKC/KEOPS subcomplex. EMBO J. **27**:2340–2351.
26. **Briza P, Bogengruber E, Thur A, Rutzler M, Munsterkotter M, Dowes IW, Breitenbach M.** 2002. Systematic analysis of sporulation phenotypes in 624 non-lethal homozygous deletion strains of *Saccharomyces cerevisiae*. Yeast **19**:403–422.
27. **Sartori G, Mazzotta G, Stocchetto S, Pavanello A, Carignani G.** 2000. Inactivation of six genes from chromosomes VII and XIV of *Saccharomyces cerevisiae* and basic phenotypic analysis of the mutant strains. Yeast **16**:255–265.
28. **Wierenga RK, Hol WG.** 1983. Predicted nucleotide-binding properties of p21 protein and its cancer-associated variant. Nature **302**:842–844.
29. **Carrera AC, Alexandrov K, Roberts TM.** 1993. The conserved lysine of the catalytic domain of protein kinases is actively involved in the phosphotransfer reaction and not required for anchoring ATP. Proc. Natl. Acad. Sci. U. S. A. **90**:442–446.
30. **Grana X, De Luca A, Sang N, Fu Y, Claudio PP, Rosenblatt J, Morgan DO, Giordano A.** 1994. PITALRE, a nuclear CDC2-related protein kinase that phosphorylates the retinoblastoma protein in vitro. Proc. Natl. Acad. Sci. U. S. A. **91**:3834–3838.
31. **Gibbs CS, Zoller MJ.** 1991. Rational scanning mutagenesis of a protein kinase identifies functional regions involved in catalysis and substrate interactions. J. Biol. Chem. **266**:8923–8931.
32. **Taylor SS, Knighton DR, Zheng J, Ten Eyck LF, Sowadski JM.** 1992. Structural framework for the protein kinase family. Annu. Rev. Cell Dev. Biol. **8**:429–462.
33. **Zhu Y, Pe'ery T, Peng J, Ramanathan Y, Marshall NF, Marshall T, Amendt B, Mathews MB, Price DH.** 1997. Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation in vitro. Genes Dev. **11**:2622–2632.
34. **Prelich G.** 2002. RNA polymerase II carboxy-terminal domain kinases: emerging clues to their function. Eukaryot. Cell **1**:153–162.
35. **Shim EY, Walker AK, Shi Y, Blackwell TK.** 2002. CDK-9/cyclin T (P-TEFb) is required in two postinitiation pathways for transcription in the *C. elegans* embryo. Genes Dev. **16**:2135–2146.
36. **Ye XS, Lee S-L, Wolkow TD, McGuire S-L, Hamer JE, Wood GC, Osmani SA.** 1999. Interaction between developmental and cell cycle regulators is required for morphogenesis in *Aspergillus nidulans*. EMBO J. **18**:6994–7001.
37. **Adams TH, Wieser JK, Yu J-H.** 1998. Asexual sporulation in *Aspergillus nidulans*. Microbiol. Mol. Biol. Rev. **62**:35–54.
38. **Etxebeste O, Garzia A, Espeso EA, Ugalde U.** 2010. *Aspergillus nidulans* asexual development: making the most of cellular modules. Trends Microbiol. **18**:569–576.
39. **Aguirre J.** 1993. Spatial and temporal controls of the *Aspergillus brlA* developmental regulatory gene. Mol. Microbiol. **8**:211–218.
40. **Prade R, Timberlake WE.** 1993. The *Aspergillus nidulans brlA* regulatory locus consists of two overlapping transcription units that are individually required for conidiophore development. EMBO J. **12**:2439–2447.
41. **Andrianopoulos A, Timberlake WE.** 1994. The *Aspergillus nidulans abaA* gene encodes a transcriptional activator that acts as a genetic switch to control development. Mol. Cell. Biol. **14**:2503–2515.
42. **Aramayo R, Timberlake WE.** 1993. The *Aspergillus nidulans yA* gene is regulated by *abaA*. EMBO J. **12**:2039–2048.
43. **Mayorga ME, Timberlake WE.** 1990. Isolation and molecular characterization of the *Aspergillus nidulans wA* gene. Genetics **126**:73–79.
44. **Han S, Adams TH.** 2001. Complex control of the developmental regula-

tory locus *brlA* in *Aspergillus nidulans.* Mol. Genet. Genomics **266**:260–270.
45. **Han S, Navarro J, Greve RA, Adams TH.** 1993. Translational repression of *brlA* expression prevents premature development in *Aspergillus.* EMBO J. **12**:2449–2457.
46. **Wu J, Miller BL.** 1997. *Aspergillus* asexual reproduction and sexual reproduction are differentially affected by transcriptional and translational mechanisms regulating *stunted* gene expression. Mol. Cell. Biol. **17**:6191–6201.
47. **Bok JW, Chiang YM, Szewczyk E, Reyes-Dominguez Y, Davidson AD, Sachez JF, Lo HC, Watanabe K, Strauss J, Oakley BR, Wang CC, Keller NP.** 2009. Chromatin-level regulation of biosynthetic gene clusters. Nat. Chem. Biol. **5**:462–464.
48. **Nützmann HW, Reyes-Dominguez Y, Scherlach K, Schoeckh V, Horn F, Gacek A, Schümann J, Hertweck C, Strauss J, Brakhage AA.** 2011. Bacteria-induced natural product formation in the fungus *Aspergillus nidulans* requires Saga/Ada-mediated histone acetylation. Proc. Natl. Acad. Sci. U. S. A. **108**:14282–14287.