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Functional Characterization of a New Member of the Cdk9 Family in *Aspergillus nidulans*▽†

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Cdk9-like kinases in complex with T-type cyclins are essential components of the eukaryotic transcription elongation machinery. The full spectrum of Cdk9/cyclin T targets, as well as the specific consequences of phosphorylations, is still largely undefined. We identify and characterize here a Cdk9 kinase (PtkA) in the filamentous ascomycete *Aspergillus nidulans*. Deletion of *ptkA* had a lethal effect in later stages of vegetative growth and completely impeded asexual development. Overexpression of *ptkA* affected directionality of polarized growth and the initiation of new branching sites. A green fluorescent protein-tagged PtkA version localized inside the nucleus during interphase, supporting a role of PtkA in transcription elongation, as observed in other organisms. We also identified a putative cyclin T homolog, PchA, in the *A. nidulans* genome and confirmed its interaction with PtkA *in vivo*. Surprisingly, the Pcl-like cyclin PclA, previously described to be involved in asexual development, was also found to interact with PtkA, indicating a possible role of PtkA in linking transcriptional activity with development and/or morphogenesis in *A. nidulans*. This is the first report of a Cdk9 kinase interacting with a Pcl-like cyclin, revealing interesting new aspects about the involvement of this Cdk-subfamily in differential gene expression.

Cyclin-dependent kinases (Cdks) are a large group of Ser/Thr protein kinases that are activated upon binding to a regulatory cyclin subunit. Initially, they have been characterized as key regulators of the cell cycle; however, more recently several Cdk subfamilies have been implicated in other processes such as transcriptional regulation and mRNA processing (25). The regulatory network that controls the initiation, elongation, and termination of the transcription of protein coding genes is extremely complex and has been mainly studied in mammalian cells and yeast. The phosphorylation state of the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNAPII) has emerged as one of the central components of this network, since the CTD serves as a platform for essential transcription-related proteins, including components of the chromatin remodeling and mRNA processing machinery (16, 21). The CTD is composed of heptad repeats with the consensus sequence YSPTSPS that has been evolutionary conserved in a wide range of organisms (49). However, the number of these repeats, as well as the degree of sequence conservation, differs greatly, ranging from 52 highly conserved repeats in mammals to 26 to 27 highly conserved repeats in yeast (48). In *Aspergillus nidulans* there are 19 heptad repeats; however, apart from one, they all contain one or more substitutions to the canonical sequence motif. The reason for this degeneracy is currently unknown. However, it has been proposed that individual organisms have evolved unique CTD functions that are reflected in a distinct array of heptad motifs, recognizing general core factors as well as species-specific factors (4). Due to reversible phosphorylation of the Ser-2 and Ser-5 residues within the consensus repeats, the CTD of the RNAPII cycles between a hypophosphorylated state that is competent of entering the preinitiation complex and a hyperphosphorylated state that is capable of processive elongation (19, 29).

Several Cdks, including the Cdk9 subfamily, and at least one protein phosphatase have been shown to control the CTD phosphorylation state (16, 21, 38). The human Cdk9 protein is the kinase subunit of the positive transcription elongation factor b (P-TEFb) and is required for the RNAPII to leave the promoter site and enter the transcriptional elongation phase (37). However, the exact mechanism of how P-TEFb controls transcriptional elongation is not fully understood. In *Drosophila* and mammalian cells, P-TEFb phosphorylates the CTD of RNAPII, primarily on Ser-2 within the heptad repeats, producing the hyperphosphorylated state of the CTD that is required for elongation (14, 39, 46). In addition, the negative transcriptional elongation factors DSIF and NELF are also phosphorylated by P-TEFb (37). In budding yeast, the functions of P-TEFb seem to be split between the essential Bur1/Bur2 complex and the nonessential Ctk1/Ctk2/Ctk3 complex, with both Bur1 and Ctk1 comprising sequence similarities to Cdk9 from higher eukaryotes (55). Both kinases can phosphorylate the CTD *in vitro*, but Ctk1 seems to be primarily responsible for Ser-2 phosphorylation during transcription, whereas Bur1 also has other targets like the elongation factor Spt4/Spt5 (24). A similar division of labor seems to exist in *Schizosaccharomyces pombe* between the essential SpCdk9/Pch1 and the nonessential Lsk1/Lsc1 complex (52). The full entity of Cdk9-targets, as well as the specific consequences of phosphorylations, especially for or-

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TABLE 1. *A. nidulans* strains used in this study

Strain	Genotype or construction[a]	Source or reference
FGSCA4	Glasgow wild type	FGSC[b]
TN02A3	*pyrG89*; *argB2* Δ*nkuA*::*argB*; *pyroA4*	27
GR5	*pyrG89*; *wA3*; *pyroA4*	53
SO451	*pyrG89*; *wA3*; *argB2*; *pyroA4*; Δ*nkuA*::*argB*; *sE15*	32
SFB1	TN02A3 transformed with pFB2; (*alcA(p)*::GFP-PtkA)	This study
SFB2	TN02A3 transformed with pFB2; (*alcA(p)*::GFP-PtkA)	This study
SFB19	GR5 transformed with pFB13 and pFB14; (*alcA(p)::*YFP^N-PclA and YFP^C-PtkA)	This study
SFB49	GR5 transformed with pFB25 (*alcA(p)*::GFP-PtkAK54Q)	This study
SFB51	SO451 transformed with *ptkA* deletion cassette (Δ*ptkA*, heterokaryotic strain)	This study
SFB54	SO451 transformed with *ptkA* deletion cassette (Δ*ptkA*, heterokaryotic strain)	This study
SFB55	SO451 transformed with *ptkA* deletion cassette (Δ*ptkA*, heterokaryotic strain)	This study
SFB69	GR5 transformed with pFB13 and pFB22 (*alcA(p)*::YFP^N-PchA and YFP^C-PtkA)	This study
SFB82	GR5 transformed with pFB15 (*alcA(p)*::GFP-PtkA; three ectopic integrations)	This study
SFB83	GR5 transformed with pFB15 (*alcA(p)*::GFP-PtkA; one ectopic integration)	This study
SCK9	TN02A3 transformed with pFB31 (*pchA* deletion plasmid, Δ*pchA*)	This study
SCK10	SCK9 transformed with pFB26 (*pchA*)	This study

[a] All strains are *veA1*.
[b] FGSC, Fungal Genetics Stock Center.

ganisms that lack a canonical CTD sequence, remains largely undefined.

In mammals, several cyclin subunits have been found to activate Cdk9-like kinases (T1, T2a, T2b, and K), with cyclin T1 being the predominant binding partner (11, 36). Unlike for cell cycle-related cyclins, the protein levels of the transcriptional cyclin T subfamily do not oscillate during different cell cycle phases. However, there is an upregulation of *cyclin T* expression during different developmental programs (13, 22, 41). Accordingly, the Cdk9 homologs in budding and fission yeast interact with regulatory cyclin partners that show significant sequence similarities to cyclin T proteins from higher organisms (34, 59). However, an upregulation of expression levels during certain cellular situations has not been reported.

Thus far, four Cdks have been characterized in the filamentous ascomycete *A. nidulans*. The Cdk1 homolog NimX in complex with the essential regulatory subunit $NimE^{cyclinB}$ represents the key cell cycle regulator (28). Together with another protein kinase, NimA, NimX is required for mitosis entry and septation (31, 33). It is noteworthy that *nimX* expression is upregulated during asexual development (60), which implies an interaction between developmental regulators and cell cycle regulators in *A. nidulans.* This was further emphasized by the discovery of a second cyclin interaction partner of NimX, the Pcl-like cyclin PclA (44, 45). As revealed by the expression pattern and the developmental defect of the deletion mutant, PclA function is limited to sporulation, where its exact role remains to be discovered. It was hypothesized that PclA in complex with NimX is required to accelerate cell cycle progression during the generation of conidiospores by rapid, repeated budding of the phialides (45). Apart from the cell cycle regulator $NimX^{cdk1}$, three more Cdks have been characterized in *A. nidulans*. The PITSLRE-type kinase NpkA is involved in a DNA damage signal transduction pathway and also plays a role in the regulation of cell cycle progression during S-phase (10). The PhoA and PhoB kinases are almost identical in sequence and exhibit a high degree of functional redundancy (3). They are closely related to the Pho85 kinase in *Saccharomyces cerevisiae*, which interacts with the members of the Pcl cyclin subfamily and is involved in a variety of cellular functions, including morphogenesis, metabolism, and transcription regulation (20). In *A. nidulans*, the nonessential PhoA kinase influences developmental decisions in response to environmental conditions (3). Deletion of the closely related PhoB kinase has no phenotype. However, in a Δ*phoA* background it is lethal and leads to defects in nuclear division and polarity (8). Both kinases form complexes with the cyclin AnPho80 (56) but, interestingly, the Pcl-like cyclin PclA does not interact with PhoA/PhoB (44).

In the present study we characterize a new Cdk, PtkA, in *A. nidulans* that displays sequence similarity with members of the Cdk9 subfamily in yeast and higher organisms. We found that PtkA plays an essential role in *A. nidulans* and, since it appears not to be involved in cell cycle regulation, most likely represents the first transcription-related Cdk to be characterized in filamentous fungi. The overexpression phenotype, as well as the identification of an unusual cyclin interaction partner, reveals interesting new aspects about a possible involvement of Cdk9-like kinases in differential gene expression.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Supplemented minimal medium (MM) and complete medium for *A. nidulans* were prepared as described earlier, and standard strain construction procedures were as described by Hill and Käfer (18). A list of *A. nidulans* strains used in the present study is given in Table 1. Standard laboratory *Escherichia coli* strains (XL1-Blue, Top10F′) were used. The plasmids are listed in Table 2.

Molecular techniques. Standard DNA transformation procedures were used for *A. nidulans* (61) and *E. coli* (43). For PCR experiments, standard protocols were applied by using a Biometra TRIO Thermoblock for the reaction cycles. DNA sequencing was done commercially (MWG Biotech, Ebersberg, Germany). Genomic DNA was extracted from *A. nidulans* by using a DNeasy plant minikit (Qiagen, Hilden, Germany). DNA analyses (Southern hybridizations) were performed as described previously (43).

Deletion of *ptkA* and *pchA*. The coding regions of *ptkA* were replaced by a deletion cassette containing the *pyrG* gene of *A. fumigatus*, which encodes the orotidine-5′-phosphate decarboxylase and complements the uridine/uracil auxotrophy that is imposed by the *pyrG89* mutations. The deletion cassette was generated by fusion PCR as described previously (50) and consists of the *pyrG* gene flanked by the upstream and downstream sequences (each ~1,500 bp) of the target genes. The following primers were used to generate the linear DNA

TABLE 2. Plasmids used in this study

Plasmid	Construction	Source or reference
pCMB17apx	*alcA(p)*::*GFP*, for N-terminal fusion of GFP to proteins of interest, contains *N. crassa pyr-4*	9
pCR2.1-TOPO	Cloning vector	Invitrogen
pDV7	GFP replaced by N-terminal half of YFP in pCMB17apx	51
pDV8	GFP replaced by C-terminal half of YFP in pCMB17apx	51
pSM14	GFP replaced by 3×HA in pCMB17apx	40
pNZ12	1.7-kb *pyroA* fragment in pCS1	62
pFB2	N-terminal 0.7-kb *ptkA* fragment in pCMB17apx	This study
pFB10	N-terminal *ptkA* cDNA fragment (aa 1 to 330) in pGBKT7	This study
pFB13	Full-length *ptkA* in pDV8	This study
pFB14	Full-length *pclA* in pDV7	This study
pFB15	Full-length *ptkA* in pCMB17apx	This study
pFB22	Full-length *pchA* in pDV7	This study
pFB25	Full-length *ptkA* with point mutation K54Q in pCMB17apx	This study
pFB26	Full-length *pchA* in pSM14	This study
pFB27	1.5-kb 5-flanking region of *pchA* with SfiI site in pCR2.1-TOPO	This study
pFB28	1.5-kb 3-flanking region of *pchA* with SfiI site in pCR2.1-TOPO	This study
pFB31	*pchA* deletion plasmid: flanking regions from pFB27 and pFB28 ligated with *pyroA* from pNZ12	This study
pCK1	N-terminal *ptkA* cDNA fragment (aa 24 to 330) in pGBKT7	This study
pCK2	Full-length *pclA* cDNA in pGADT7	This study
pCK3	Full-length *pchA* cDNA in pGADT7	This study

fragments for fusion PCR (the underlined segments are identical to the 5′ end or the 3′ end of the *pyrG* marker cassette): for the upstream region, ptkA-P3-for (GCGTACCGACACTGATTAGG) and ptkA-P1-rev (GAAGAGCATTGTTTGAGGCGTTTTCACCATTTGATACTGCGC); and for the downstream region, ptkA-P5-for (ATCAGTGCCTCCTCTCAGACAGTGAAGCTACCGTCTACCATAAAAC) and ptkA-P8-rev (AATGCGAAGCACCTGAAGC). The *pyrG* marker cassette was amplified by using the primers UP3 (GCCTCAAACAATGCTCTTCA) and UP2 (CTGTCTGAGAGGAGGCACTGAT) (32). The linear deletion fragment was then generated via fusion PCR by using the nested primers ptkA-P2-for (GCCCCTGTGATATTCTCTCG) and ptkA-P7-rev (TGTGAAATCATCGCTCTTGCTC). The deletion cassette was transformed into the Δ*nkuA* strain SO451 to increase the frequency of homologues integration (27).

In case of the *pchA* deletion the flanking regions were amplified by PCR using genomic DNA and the primers *pchA*-P3 (CTGACTCGCAGGAACATGAG) and *pchA*-P10-Sfi (CGGCCATCTAGGCCGATAAGGGACCAACCAGCAC) for the upstream region of *pchA* and the primers *pchA*-P9-Sfi (CGGCCTGAGTGGCCCGGACCTACCACGACAGTC) and *pchA*-P8 (GGAGCACCTACATCAGAACG) for the downstream region. The SfiI restriction sites are underlined. Both fragments were cloned into pCR2.1-TOPO to generate pFB-27 and pFB-28. In a three-fragment ligation, the *pyroA* gene from plasmid pNZ12 (62) was ligated between the *pchA*-flanking regions, resulting in plasmid pFB-31. The plasmid was transformed into the Δ*nkuA* strain TNO2A3. Primary transformants were screened by PCR for correct integration of the deletion cassette and the absence of the *pchA* gene.

Heterokaryon rescue analysis. The essential *ptkA* gene function was ascertained and analyzed by using the heterokaryon rescue technique as described elsewhere (32). In brief, conidia of the primary transformations were carefully removed from the surface and replica streaked on YAG and YAGUU plates. After 1 to 3 days of incubation at room temperature, the plates were observed and scored for growth either per eye or at low power (×40 magnification) in an Axiophot microscope (Carl Zeiss, Jena, Germany). On selective YAG plates almost no growth occurred, because the mononuclear conidia either lacked the nutritional marker gene or the essential *ptkA* gene function. On YAGUU plates, however, *pyrG*$^+$ Δ*ptkA*$^-$ conidia still could not form colonies, but the Δ*pyrG ptkA*$^+$ conidia were able to grow normally, thus indicating the heterokaryotic state of the primary transformants. This was confirmed by diagnostic PCR (see also Fig. 2B). Three individual Δ*ptkA* heterokaryotic strains were selected for further studies and propagated and stored as described previously (32).

Tagging of proteins with GFP and YFP^N/YFP^C. To create an N-terminal green fluorescent protein (GFP) fusion construct of PtkA, a 0.7-kb N-terminal fragment of *ptkA* (starting from ATG) was amplified from genomic DNA, using the primers ptkA-Efi-for (GGGGCGCGCCc ATGGGCATAGCGTCACTCGAACGG) and ptkA-Efi-rev (GGTTAATTAAACCTTGGTTGCTTATAAGTAGATTGGCAGCTGCGCTGGGTCAGCC), and cloned via AscI/PacI (the restriction sites are underlined) into the corresponding sites of pCMB17apx, yielding pFB2. Likewise, the complete *ptkA*-ORF was amplified with the primers ptkA-Efi-for and ptkAganz-Efi-rev (GGTTAATTAATCACCGGCGATACGGACCCC) and cloned into pCMB17apx, yielding pFB15. To generate a catalytically inactive mutant, the K54Q point mutation was introduced with the QuikChange XL mutagenesis kit (Stratagene, Heidelberg, Germany) using the primers ptkA-KQ-F1 (GATGGCTCCATCGTCGCGCTGCAAAAGATCCTCATGCATAATG) and ptkA-KQ-R1 (CATTATGCATGAGGATCTTTTGCAGCGCGACGATGGAGCCATC) (the mutation sites are underlined) and pFB15 as a template, yielding pFB25. All plasmids were verified by sequencing and then transformed into the uracil/uridine auxotrophic strain TNO2A3 (Δ*nkuA*). The integration events were confirmed by Southern blotting and microscopy (data not shown).

For bimolecular fluorescence complementation (BiFC) analyses, the GFP in pCMB17apx was replaced with the N-terminal half (YFP^N) or the C-terminal half (YFP^C) of yellow fluorescent protein, yielding pDV7 and pDV8, respectively, as described earlier (51). To create a C-terminal YFP^C fusion construct of PtkA, the AscI/PacI fragment from pFB15 was subcloned into the corresponding sites of pDV8, yielding pFB13. The N-terminal YFP^N fusion construct of PclA was generated by amplification of the complete open reading frame (ORF) from genomic DNA with the primers pclA-Efi-for (GGGGCGCGCCCATGGATCTCAACCGAACAGCGC) and pclAganz-Efi-rev (GGTTAATTAACTAAACGCGAGGCCGCATCCGGTGG). The resulting PCR product was then cloned via AscI/PacI into the corresponding sites of pDV7, yielding pFB14. Using the same strategy, the N-terminal YFP^N fusion of PchA was generated, using the primers pchA-Efi-for (GGGCGCGCCCATGGCGTCCCAACCGCCAGTCAAA) and pchA-Efi-rev (GGTTAATTAACTACAATTCCCCTTCTTCACTTCCAC), yielding pFB22.

Yeast two-hybrid analysis. A yeast two-hybrid analysis was performed using the Matchmaker Two-Hybrid system (Clontech, Mountain View, CA). For bait generation, an N-terminal *ptkA* cDNA fragment corresponding to amino acids (aa) 1 to 330 was amplified with the primers pGB-ptkA-for (GGCATATGGGCATAGCGTCACTCG) and pGB-ptkA-rev2 (GGGATCCTCATGGCGGTGTCGAGAAATAAGGATG) and cloned via NdeI/BamHI (restriction sites are underlined) into the pGBKT7 vector, which contains the GAL4 DNA-BD and the *TRP1* marker, yielding pFB10. Since the expression of this construct turned out to have toxic effects on yeast cells, a shortened bait construct was generated, containing a *ptkA* cDNA fragment corresponding to the conserved kinase catalytic domain (aa 24 to 330). This construct was generated by restriction digestion of pFB10 with EcoRI and BamHI and subcloned into pGBKT7, yielding pCK1. The complete *pclA* cDNA was amplified using the primers pclA-prey-for (GGCATATGGATCTCAACCGAACAGCGC) and pclA-prey-rev (GGGGATCCCTAAACGCGAGGCCGCATCC) and then cloned using NdeI/BamHI into the vector pGADT7, which contains the GAL4 DNA-AD and the *LEU2* marker, yielding pCK2. Likewise, the complete *pchA* cDNA was amplified by using the primers pchA-pGADT7-for (GGCATATGATGGCGTCCCAACCGCCAGTCA) and

pchA-pGADT7-rev (GGCCCGGGCTACAATTCCCCTTCTTCACTTC) and cloned into pGADT7 using NdeI/XmaI, yielding pCK3. pGBKT7-derived plasmids were transformed into the yeast strain AH109 (mating type *MAT***a**), and pGADT7-derived plasmids were transformed into the yeast strain Y187 (mating type *MAT***a**). The system uses three reporter genes (*HIS3*, *ADE2*, and *LacZ*) under the control of the GAL4-responsive UAS. Protein interactions were analyzed on selective plates lacking tryptophan, leucine, and histidine (triple-dropout medium [TDO]) or lacking tryptophan, leucine, adenine, and histidine (quadruple-dropout medium [QDO]).

Protein extracts and Western blotting. To prepare protein extracts, *A. nidulans* strains were incubated in liquid MM for 24 h at 37°C. To induce the *alcA* promoter, this medium was supplemented with 0.2% glucose and 2% threonine. The mycelium was harvested by filtration through Miracloth (Calbiochem, Heidelberg, Germany), dried, and immediately ground in liquid nitrogen. Afterward, the mycelial powder was resuspended in protein extraction buffer (50 mM Tris-HCl [pH 8], 0.1% Triton X-100, 250 mM NaCl) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride) and rotated for 20 min in a head-over-tail incubator. Cell debris was pelleted by centrifugation (Eppendorf Centrifuge 5403-R; Eppendorf, Hamburg, Germany) at 13,000 rpm and 4°C for 12 min. The supernatant was used for Western blotting. Equal amounts of samples protein extracts (150 μg) were loaded on a 8% sodium dodecyl sulfate-polyacrylamide gel and blotted onto nitrocellulose membranes from Schleicher & Schuell (Dassel, Germany). For detection, a monoclonal anti-GFP antibody (product G1544 [Sigma-Aldrich, Munich, Germany]; dilution 1:4,000) was used.

Light and fluorescence microscopy. For live-cell imaging of germlings and young hyphae, cells were grown on coverslips in 0.5 ml of MM plus 2% glycerol (derepression of the *alcA* promoter and thus moderate gene expression), MM plus 2% threonine (activation of the *alcA* promoter and thus high gene expression), or MM plus 2% glucose (repression of the *alcA* promoter). For pictures of young hyphae, cells were incubated at 25°C for 1 day. For nuclear staining, coverslips were mounted on microscope slides with mounting medium containing DAPI (4′,6′-diamidino-2-phenylindole) and VectorShield (Vector Laboratories, Burlingame, CA). Images were captured at room temperature by using an AxioImager microscope (Zeiss, Jena, Germany). Images were collected and analyzed by using the AxioVision system (Zeiss).

Gene expression analyses. Transferring development-competent vegetative hyphae from liquid medium to agar plates, thus inducing development by air exposition synchronized the asexual development of wild-type *A. nidulans* cells. At different time points, the RNA was isolated. For that, the mycelium was harvested, dried, frozen in liquid N_2, and ground to a powder. The total RNA was isolated by using TRIzol (Invitrogen, NV Leek, Netherlands) according to the manufacturer's instructions. After removal of DNA contaminations with DNase I (Invitrogen), cDNA was synthesized from 1 μg of RNA from each sample by using a SuperScript first-strand synthesis kit (Invitrogen). The resulting cDNA was then used as a template for PCRs with the primers ptkA-F1 (AAGGAAAGCCGATTCTTGCTGG) and ptkA-R1 (CGGGCAGCGCTAGAAATGC) for *ptkA* expression analysis, pclA-F3 (AAACAGTACCCACGCTTTGC) and pclA-R3 (AAGGTGGACGTATCCACTGC) for *pclA* expression analysis, and pchA-F2 (CCAACGCTACCAACACCCAC) and pchA-R2 (CTTCTTCACTTCCACCACCCTC) for *pchA* expression analysis. To check for genomic DNA contaminations, all reverse transcription-PCRs (RT-PCRs) were performed in the absence of reverse transcriptase; however, no bands were produced.

RESULTS

PtkA is a novel member of the Cdk9 family. The developmental Pcl-like cyclin PclA forms a complex with the main cell cycle regulator NimX; however, in contrast to yeast, it does not interact with the Pho85 homologues kinases PhoA and PhoB. To further investigate the functional role of PclA in *A. nidulans*, we aimed to identify other possible kinase interaction partners by performing a BLASTP search of the *A. nidulans* genome at the Broad Institute (Cambridge, MA) using the NimX sequence. We revealed a novel gene that encodes a predicted protein with 544 aa (gi/14530079/emb/CAC42219.1). The N-terminal part of the coding sequence (aa 24 to 326) comprises a kinase catalytic domain (17), and sequence comparison with several Cdks revealed extensive similarities, clearly grouping the new protein into this superfamily (Fig. 1). Within the cyclin-binding region the new protein comprises a characteristic PITALRE motif instead of the PSTAIRE motif present in cdc2-like kinases. Thus, it was designated PtkA, for PITALRE kinase in *A. nidulans*.

The catalytic domain of PtkA shares 44% identical amino acids with the human Cdk9 protein. Highest similarity was found with Cdk9 from *S. pombe* (56% identical and 70% similar residues), followed by the Crk1 kinase in *Candida albicans* with 48% identities and 67% similarities. Both are functional orthologs of the Bur1/Sgv1 kinase from *S. cerevisiae* that shares 44% identical and 59% similar residues with PtkA. Based on these sequence similarities, we deduced PtkA as a new member of the Cdk9 family in *A. nidulans*. Outside the catalytic domain, there is only sequence conservation with homologue kinases in closely related filamentous fungi (e.g., *Aspergillus niger* and *fumigatus*, *Neurospora crassa*, and *Penicillium marneffei*), but almost no sequence similarities were found with noncatalytic domains of Cdk9 kinases from other organisms.

Generation of a *ptkA* deletion strain. To analyze the function of the deduced Cdk9 homologue PtkA in *A. nidulans*, we constructed a *ptkA* deletion strain by replacing the coding sequence with a gene deletion construct obtained by fusion PCR (SFB54 [see Materials and Methods]). Transformation of the deletion construct in a KU70-deficient recipient strain resulted in the generation of heterokaryons, implying an essential cellular function of *ptkA* in *A. nidulans*. The deletion could only be maintained in a heterokaryotic state, where two genetically nonidentical nuclei are present in a common cytoplasm, with the essential *ptkA* gene provided by the undeleted nucleus, and the nutritional marker provided by the nucleus containing the deletion construct (32). The heterokaryon formation was verified by diagnostic PCR, using primer sets that confirm the presence of both, the correctly integrated deletion cassette and the parental *ptkA* gene (Fig. 2B). In addition, we tested for the heterokaryotic state by plating conidia obtained from the heterokaryon on selective plates. Since conidia are mononuclear, only those with the Δ*ptkA*::*pyrG*-containing nucleus would be able to grow. However, due to the lack of the *ptkA* gene, positive transformants only produced very small colonies that are barely visible per eye (Fig. 2A).

PtkA is essential for effective vegetative growth and asexual development in *A. nidulans*. To examine the growth characteristics of Δ*ptkA* cells, conidia of the heterokaryotic primary transformants were streaked onto nutritionally selective (YAG) or nonselective (YAGUU) plates, incubated for several days at room temperature, and observed at low magnification (Fig. 3A). On nonselective plates, the undeleted, Δ*pyrG* conidia exhibited normal growth, with the projection of long germ tubes after 1 day and the appearance of conidia after 3 to 4 days. On selective plates, Δ*pyrG* conidia are unable to germinate, whereas, in contrast, the deleted *pyrG*$^+$ conidia grow to the extent allowed by the lack of the essential *ptkA* gene. After 1 day, the projection of short germ tubes was observed that continued hyphal growth up to the formation of very small colonies, barely visible per eye. After 4 days, hyphal growth stopped. Asexual conidia could not be observed, indicating that *ptkA* is required for effective vegetative growth and asexual development in *A. nidulans.* Next, we examined a possible effect of the *ptkA* deletion on the nuclear state. For that, the conidia of deletion transformants were incubated in liquid medium lacking uracil/uridine on coverslips and incubated

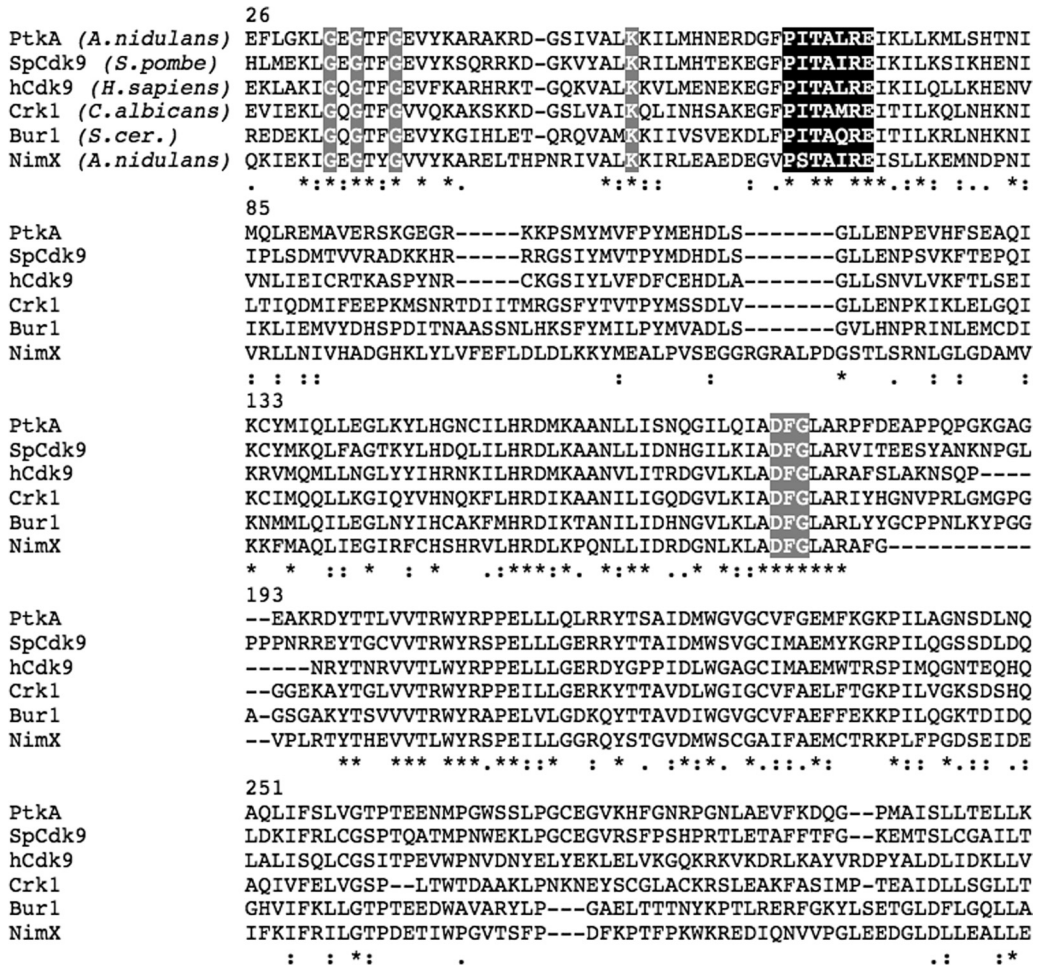

FIG. 1. Alignment of Cdk9 homologs from different species as indicated. Identical residues are marked with asterisks; chemical conserved residues are marked with dots. Residues important for ATP hydrolysis and phosphate transfer, including the conserved Lys 54, are highlighted in gray, the conserved PITALRE motif within the cyclin binding box is highlighted in in black. The alignment was performed with CLUSTAL W using standard parameters.

overnight. DAPI staining of Δ*ptkA* cells revealed no significant differences in morphology, number, or distribution of nuclei compared to the wild type (Fig. 3B). This indicates that PtkA, similar to other members of the Cdk9 family, is not involved in regulation of the nuclear state or mitosis but possibly in transcriptional regulation.

PtkA overexpression affects hyphal morphology. In order to study the essential function of PtkA in more detail, we constructed an *A. nidulans* strain expressing the only *ptkA* copy as a GFP fusion protein under the control of the conditional *alcA* promoter (SFB1 and SFB2 [see Materials and Methods]). Under repressing conditions (grown on glucose) the mutant showed a pronounced effect on vegetative growth, leading to very small colonies compared to the wild type and thus reflecting the phenotype of the deletion mutant to a weaker extent (Fig. 4A). Under derepressing conditions (glycerol) the wild-type morphology was restored, providing an internal control for the functionality of the N-terminal GFP fusion construct. Under inducing conditions (threonine), the phenotype of the *alcA(p)GFPptkA* colonies was also very similar to that of the wild type. However, individual hyphae of the mutant showed different defects in polarized growth (Fig. 4B and C). Most notably, a high proportion of mutant hyphae displayed a curvy phenotype under inducing conditions (56%). Partially, this effect could be due to the pourer carbon source threonine since the wild-type strain also displayed an increased proportion of curved hyphae in threonine medium (32%). However, the increase in the mutant strain under overexpressing conditions was still significantly higher (Fig. 4C), indicating that an increased abundance of PtkA somehow interferes with the directionality of polarized growth. In addition, under inducing conditions lateral and apical branching was also much more frequently observed in mutant hyphae than in the wild type. To confirm that these effects are really due to the overexpression of *ptkA* and not an individual characteristic of SFB1, two *A. nidulans* strains were generated that ectopically express one (SFB83) or two (SFB82) additional *ptkA* copies under *alcA* control. These strains also displayed high proportions of curvy hyphae under inducing conditions, as well as lateral branching and hyphal tip splitting. (Fig. 4B and C). Hyperbranching was even more pronounced in strains with additional ectopic *ptkA* copies compared to SFB1. These results point to a role of PtkA

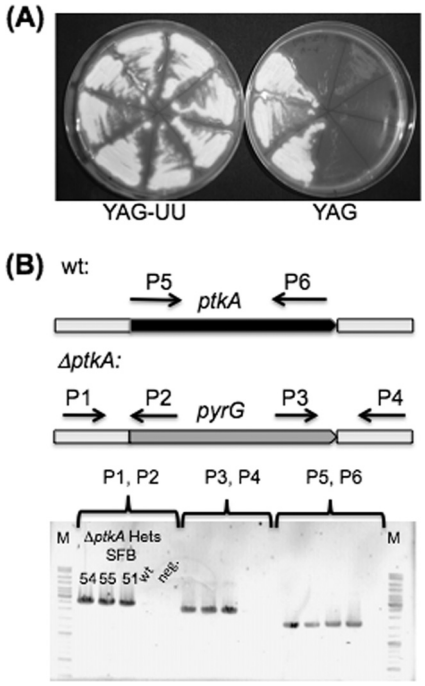

FIG. 2. Essential function of *ptkA* in *A. nidulans*. (A) Heterokaryon rescue analysis of *ptkA* deletion strains. Conidia from six primary transformants obtained after transformation of a *ptkA* deletion cassette were replica streaked on selective YAG and unselective YAGUU plates. For four of the six strains tested, the ability of untransformed conidia to grow on unselective plates and the inability of *ptkA* conidia to grow on selective plates indicate the essential function of *ptkA* in *A. nidulans*. (B) Heterokaryon formation was verified with diagnostic PCR using the primer pairs as indicated in the scheme. The heterokaryotic Δ*ptkA* strains SFB 54, 55, and 51 (lanes 1 to 3 for each primer pair) contain both the wild-type and the deletion allele, whereas the wild-type SO451 (lane 4 for each primer pair) only contains the wild-type *ptkA* allele.

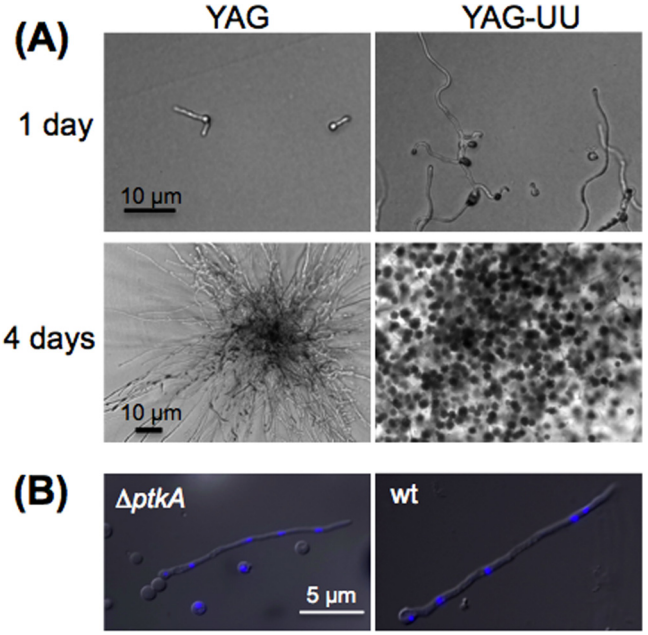

FIG. 3. Phenotypic characterization of the *ptkA* deletion strain. (A) The growth characteristics of the Δ*ptkA* mutant were recorded by plating the conidia of a Δ*ptkA* heterokaryotic strain on selective YAG or unselective YAGUU plates. Cells were observed after 1 to 3 days at low power (×20 to ×40 magnification). Deleted conidia were able to germinate and form germ tubes on selective YAG plates, although delayed compared to the undeleted conidia on unselective plates. This indicates that *ptkA* is not strictly essential for early stages of polarized growth. However, the Δ*ptkA* mutant was unable to continue vegetative growth further than the 3 days stage and never underwent asexual development and conidia formation. (B) Representative germlings of the *ptkA* deletion mutant and the wild type after incubation in selective medium and DAPI staining. No differences could be seen in nuclear morphology, number, and distribution, indicating that *ptkA* is not involved in the regulation of the nuclear state or mitosis.

in maintaining the directionality of hyphal growth and the regulation of the initiation of new branching sites.

Active PtkA is concentrated in the nuclei but changes localization during mitosis. To investigate the subcellular localization of PtkA, the *alcA(p)GFP-ptkA* strain was grown in glycerol medium and observed in a fluorescence microscope. The protein clearly localized inside the nucleus, which was confirmed by costaining with DAPI (Fig. 5A). However, in some hyphae, a faint cytoplasmic staining was also visible, indicating that the protein may shuttle between the nucleus and the cytoplasm. For human Cdk9 it has been shown that nuclear localization is regulated by autophosphorylation since mutants that either lack kinase activity or certain phospho-acceptor sites at the C termini fail to enter the nucleus (26). To investigate whether PtkA localization is regulated by a similar mechanism, we generated a PtkA point mutant where a strictly conserved lysine residue (K54, see Fig. 1) that is essential for ATP-binding, phosphate transfer, and kinase regulation (17, 42) was exchanged against glutamine. We constructed an *A. nidulans* strain expressing the GFP-labeled PtkA-K54Q mutant under the control of the *alcA* promoter and examined localization of the fusion protein under inducing conditions (Fig. 5B). The inactive kinase still enters the nucleus. However, a much bigger proportion of the protein resided in the cytoplasm compared to the active kinase. To exclude the possibility that the cytoplasmic localization of the mutant PtkA is caused by overexpression and leakage from the nucleus, we compared the protein levels of the mutant and the wild-type GFP fusion by Western blot analysis (Fig. 5B). We found a lower protein level for the inactive PtkA than for the wild type, indicating that the catalytic activity of PtkA and possibly autophosphorylation is indeed required for the correct localization of the protein.

In addition, the localization of GFP-PtkA was observed by time-lapse confocal microscopy throughout the cell cycle (see Fig. S1 in the supplemental material). When mitosis started the fluorescence signal dispersed into the cytoplasm. After the completion of mitosis, indicated by the doubling of nuclei number, it relocalized back into the nucleus. Since *A. nidulans* undergoes a partially open mitosis (30), the mitosis specific dispersal of PtkA indicates that it does not remain associated with nuclear components during nuclear division.

PtkA interacts with the cyclin T homolog PchA and the Pcl-like cyclin PclA. Since PtkA is a Cdk, an integral part of its regulation is the interaction with a cyclin partner. Different members of the transcriptional cyclin family have been established to activate Cdk9 in metazoans, including cyclin T1, T2a,

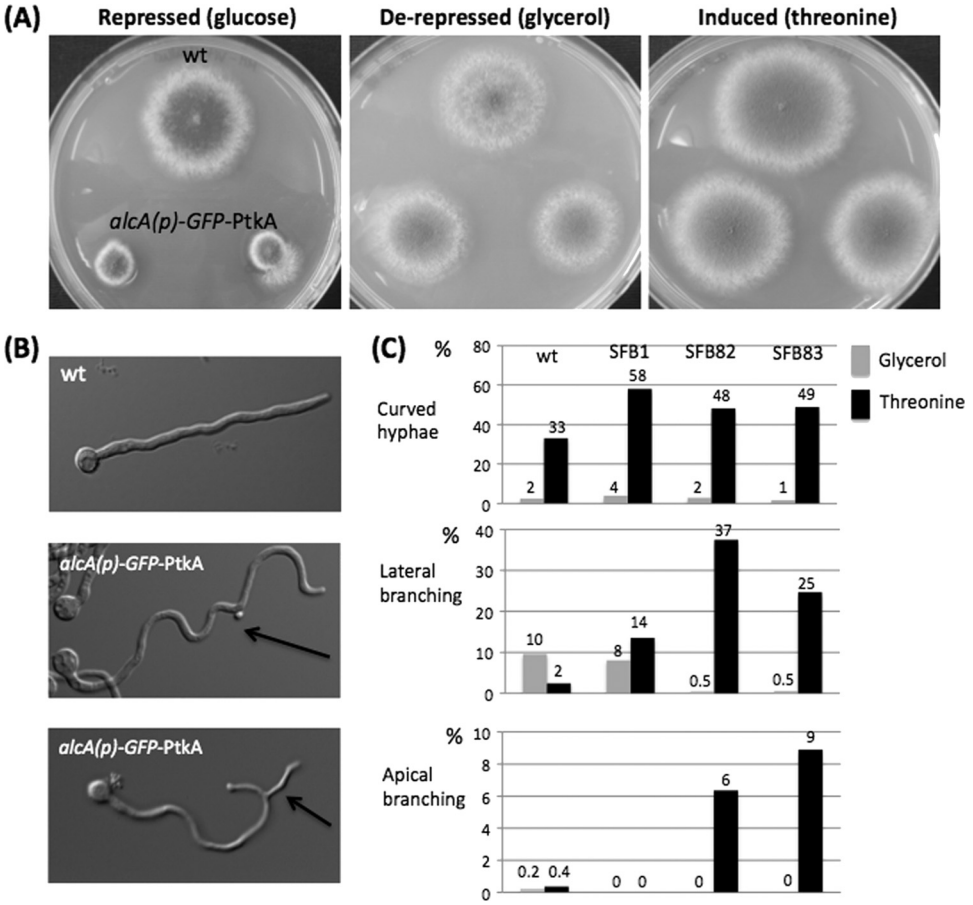

FIG. 4. Phenotypic characterization of conditional *ptkA* mutant strains. (A) Colonies of wild-type (TNO2A3) and *alcA(p)*::*GFP-ptkA* (SFB1 and SFB2) strains. Strains were grown for 2 days on MM agar plates containing glucose (repressing conditions), glycerol (derepressing conditions), or threonine (inducing conditions) as a carbon source. (B) Hyphae of the wild type and strains SFB1, SFB82, and SFB83 with threonine as a carbon source using differential interference contrast. The arrows indicate phenotypic effects of *ptkA* overexpression (curvy hyphae in the upper picture and lateral branching in the lower picture). Hyphae are 3 to 5 μm in diameter. (C) Quantification of the phenotypic effects of *ptkA* overexpression. The proportions of curved hyphae and hyphae displaying lateral and apical branching were determined for wild type (GR5) and the strains SFB1, SFB82, and SFB83 under derepressing (glycerol medium) and inducing (threonine medium) conditions. For each strain and growth medium, at least 200 germlings were counted.

T2b, and cyclin K, with cyclin T1 being the predominant binding partner (11, 35). Accordingly, the *S. pombe* Cdk9 interacts with the cyclin T-homolog Pch1. To identify possible cyclin interaction partners for PtkA, we searched the *A. nidulans* genome for cyclin T homologs and found an uncharacterized ORF (AN4981.2), encoding a predicted 513-aa protein (Fig. 6A). Within the N-terminal half, including the predicted cyclin box fold (aa 51 to 145), the protein sequence is closely related to the Pch1 cyclin from *S. pombe* (40% identical and 63% similar residues); therefore, it was named PchA. Lower sequence similarities exist to the N-terminal halves of the human transcriptional cyclins K (32% identical, 49% similar residues) and T1 (29% identities, 32% similarities). Based on the sequence characteristics, we considered the new protein a likely cyclin partner of PtkA. We tested this by performing yeast two-hybrid experiments and indeed found an interaction between the catalytic domain of PtkA and PchA (Fig. 7A). To confirm this result and to analyze the localization of the PtkA-PchA complex *in vivo*, we used the BiFC method (1). For that, the N-terminal half of YFP (YFP^N) was fused to PchA and the C-terminal half (YFP^C) was fused to PtkA. Strains expressing only PchA-YFP^N or PtkA-YFP^C did not show any YFP fluorescence (not shown). However, the expression of both fusion proteins together produced a signal inside the nuclei (Fig. 7B), in agreement with the GFP-PtkA localization observed before (Fig. 5A). Almost no fluorescence signal was detected in the cytoplasm, indicating that the interaction of PtkA with PchA is confined to the nucleus.

To further analyze the function of the newly identified cyclin PchA, we generated a deletion strain by replacing the coding sequence with a deletion cassette (see Materials and Methods). Diagnostic PCR and Southern blotting (Fig. 6B and see Fig. S2 in the supplemental material) verified the correct integration and the absence of the *pchA* gene, and one of the strains (SCK9) was selected for further analysis. The deletion mutant displayed a severe growth defect with colony sizes of ca. 10% compared to wild-type colonies and a severe reduction in conidial production. All phenotypes were rescued after transformation with the *pchA* gene (SCK10, Fig. 6C). These results are in agreement with the effects of the deletion of

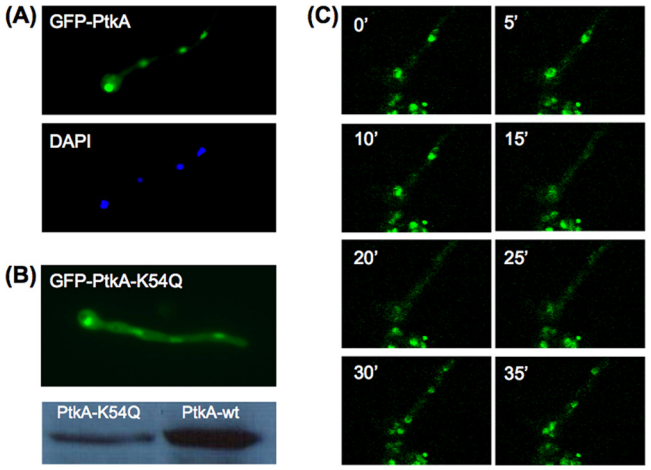

FIG. 5. PtkA localization. The strains SFB1 and SFB49 were grown on minimal medium with glycerol as carbon source. Hyphae are 3 to 5 μm in diameter. (A) GFP-PtkA accumulates in the nuclei, as confirmed by costaining with DAPI, but also produces a faint signal in the cytoplasm. (B) The inactive point mutant GFP-PtkA-K54Q is still able to enter the nucleus; however, a much bigger proportion resides in the cytoplasm compared to the wild-type protein (upper panel). Expression levels of mutant and wild-type proteins are compared using Western blot analysis with an anti-GFP antibody (lower panel). (C) PtkA changes location during mitosis. N-terminal GFP-tagged PtkA (SFB1) was imaged by time-lapse confocal microscopy as cells passed through the cell cycle. During interphase, GFP-PtkA predominantly localizes in the nucleus but disperses into the cytoplasm during mitosis and is reimported into the daughter nuclei after completion of mitosis. A video file (see Video S1 in the supplemental materiall) displays the full time course collected at 1-min intervals over 40 min.

cyclin T homologue genes in *S. cerevisiae* or *S. pombe*, which also resulted in severe growth defects or lethality, a finding comparable to the phenotype of *ptkA* repression (12, 59). Since in *A. nidulans* the deletion of the kinase *ptkA* is lethal, whereas the deletion of *pchA* is not, we concluded that there are one or more other cyclins interacting with PtkA that have not been identified in our genome search and that allow for survival in the absence of *pchA*, albeit with severe defects.

Our original aim was to identify kinase interaction partners for the developmental cyclin PclA in addition to the already-reported interaction with NimX (44). Therefore, we again performed yeast-two hybrid experiments with the full-length PclA protein and indeed found a strong interaction with the catalytic domain of PtkA (Fig. 7A). To confirm this result, we used BiFC analysis, which also supported the formation of PtkA-PclA complexes *in vivo* (Fig. 7B). The hypothesis that PchA and PclA are the two main interaction partners of PtkA was further supported by the fact that the generation of a *pclA-pchA* double mutant was unsuccessful, indicating a lethal phenotype comparable to the *ptkA* deletion.

***pchA* and *pclA* are differentially expressed during asexual development.** Next, we examined the expression profiles of *ptkA* and its interacting cyclins during different phases of development. For that, asexual development of the wild-type strain FGSCA4 was synchronized by air exposition of a liquid culture, and the total RNA was isolated at different time points of development (Fig. 8A). RT-PCR experiments with *ptkA* primers gave similar bands for all time points (Fig. 8B), indi-

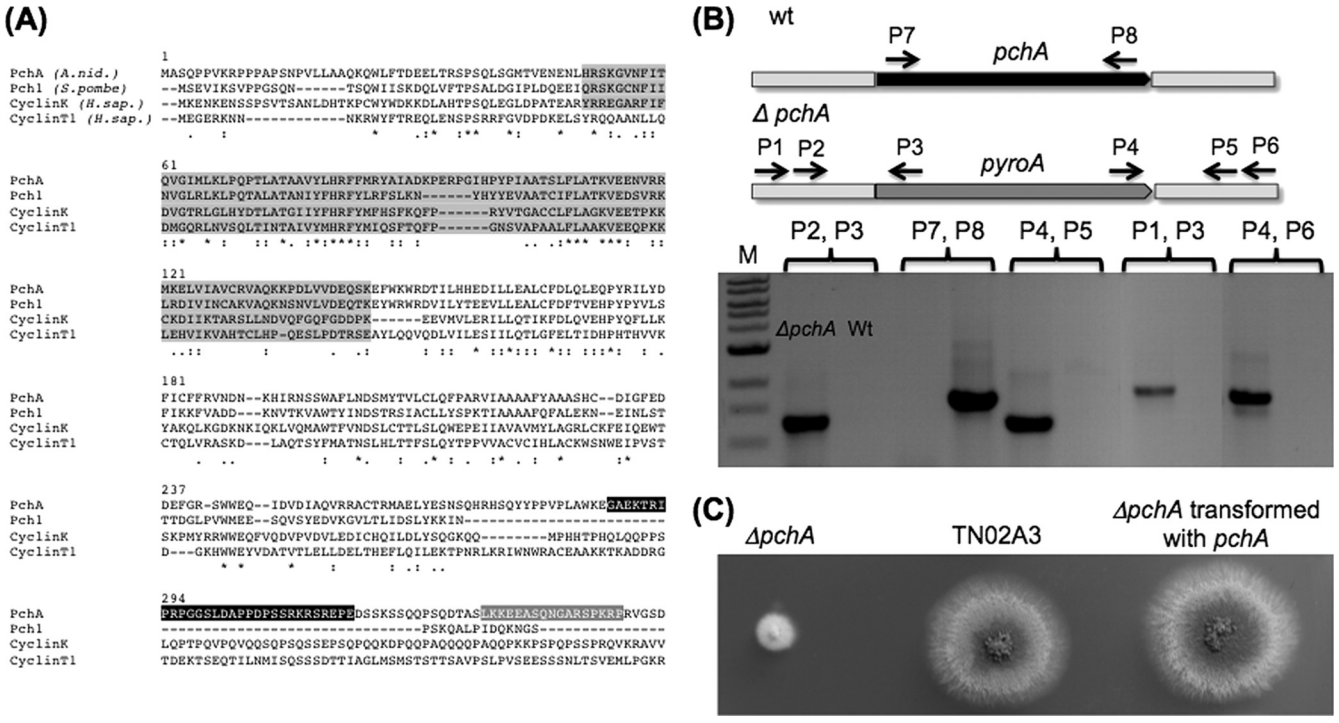

FIG. 6. Characterization of the newly identified cyclin PchA. (A) Alignment of cyclin T homologs from different species as indicated. Identical residues are marked with asterisks; chemical conserved residues are marked with dots. The N-terminal cyclin box is shaded in light gray. The putative PEST and NLS motifs in the PchA sequence are highlighted in black or dark gray, respectively. The alignment was done with CLUSTAL W using standard parameters. (B) Confirmation of the *pchA* deletion event by diagnostic PCR using the primer pairs as indicated in the scheme. (C) Comparison of colony growth of the *pchA* deletion strain (SCK9), the wild type (TNO2A3), and the deletion strain recomplemented with *pchA* (SCK10). Colonies were grown for 3 days on MAG/UU.

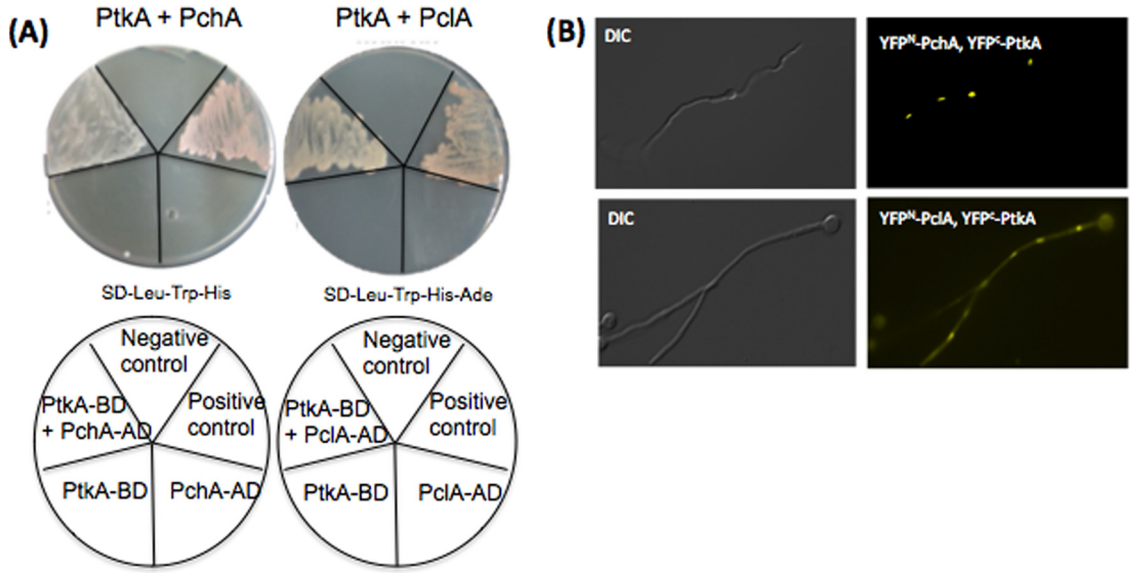

FIG. 7. Interaction of PtkA and the cyclins PchA and PclA. (A) Yeast two-hybrid interaction between the GAL4 DNA-binding domain fused to the catalytic domain of PtkA (PtkA-BD) and the GAL4 activation domain fused to full-length PchA (PchA-AD) or PclA (PclA-AD). To test for interaction, the mated yeasts were grown on nutritionally selective SD/−Leu/−Trp/−His or SD/−Leu/−Trp/−His/−Ade plates. (B) BiFC analysis of PtkA with PchA or PclA. In SFB69 expressing PtkA tagged with the N-terminal half of YFP and PchA tagged with the C-terminal half, a YFP signal was detected within the nuclei. For PtkA-PclA complexes a nuclear localization pattern and a fainter signal in the cytoplasm was observed in SFB19 expressing PtkA tagged with YFP^N and PclA tagged with YFP^C. Hyphae are 3 to 5 μm in diameter.

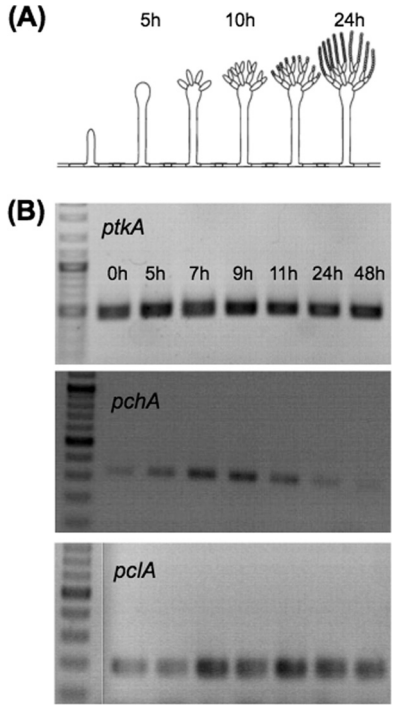

FIG. 8. Expression profiles of *ptkA*, *pchA*, and *pclA* during asexual development. (A) Developmental time course of synchronized *A. nidulans* cells. The total RNA was isolated after different time points as indicated. (B) Semiquantitative RT-PCR analysis using specific primers for *ptkA*, *pchA*, and *pclA*. Similar bands for all time points indicate constitutive expression of *ptkA* during development. In contrast, the expression of both cyclins *pchA* and *pclA* becomes upregulated after 7 h. For *pclA* the expression level stays high for the rest of the time course, but for *pchA* it goes down again at 11 h, indicating that PchA is not needed for later stages of development. To check for genomic DNA contaminations, all RT-PCRs were run in the absence of reverse transcriptase, but no bands were produced (not shown).

cating that *ptkA* is constitutively expressed throughout development. In contrast, both cyclins exhibited differential expression profiles. *pchA* mRNA levels were unregulated between 7 and 11 h of development, which is about when the conidiophores are formed. Interestingly, after 11 h the expression went down again, indicating that PchA may be not required for later stages of development. *pclA* mRNA levels also increased after 7 h of development but remained at that level for the whole time course. This is in agreement with Northern blot analysis results published earlier (45) and reflects the role of PclA during the generation of conidiospores.

DISCUSSION

In this study we identified and characterized the Cdk PtkA in the filamentous ascomycete *A. nidulans*. Isolated by screening of the *A. nidulans* genome for sequences related to the Cdc2 homolog NimX, the protein sequence reveals extensive similarities to Cdk9-like kinases, including the characteristic PITALRE motif in the cyclin-binding box, and thus clearly grouped into this subfamily. In complex with T-type cyclins, Cdk9-like kinases are involved in the stimulation of transcriptional elongation, chromatin remodeling, and mRNA processing in various organisms (2, 37, 55). We show here that deletion of *ptkA* in *A. nidulans* causes lethality during vegetative growth and, since no asexual spores are produced, can only be propagated in a heterokaryon. The germination of Δ*ptkA* spores is delayed, but germ tubes otherwise showed no morphological defects, which would account for the detrimental effect of *ptkA* deletion in later growth stages. This either means that PtkA does not become essential until a certain cell size has been reached, possibly reflecting an increased need for transcriptional activity to continue cellular growth, or that, alter-

natively, PtkA might be sufficiently stable so that a small amount of protein from the heterokaryotic phase supports growth for a limited time. The growth characteristics of a strain carrying a conditional *ptkA* allele confirm the essential role of PtkA in *A. nidulans*, since under repressing conditions only very small colonies are produced. The absence of any recognizable defect in nuclear morphology and/or nuclei number in the *ptkA* deletion mutant, which would be expected in case of a functional role in cell cycle regulation, supports the view that PtkA is a transcription-related rather than cell cycle-related Cdk.

A GFP-labeled PtkA version accumulated inside the nuclei. Since no nuclear localization signal was identified in the protein sequence, we suggest that nuclear entry depends on the interaction with either a cyclin binding partner or a target protein. In addition, we discovered that the nuclear localization is partly dependent on the kinase activity, since an inactive GFP-labeled point mutant produced a fluorescent signal in both nuclear and cytoplasmic compartments. A similar effect was described for human Cdk9, where nuclear accumulation is dependent on catalytic activity, as well as phosphoacceptor sites in the C terminus (26). This indicates that autophosphorylation is required for the correct localization of hCdk9, and we suggest a similar mechanism for PtkA. During mitosis, the kinase gets dispersed in the cytoplasm. Since *A. nidulans* undergoes a partially open mitosis (6, 7), this indicates that PtkA does not remain associated with nuclear structures during nuclear division. A similar localization pattern was also observed for RNAPII (47), possibly reflecting the general downregulation of transcription during mitosis (57, 58).

Based on the phenotypes associated with the *ptkA* and *pchA* deletions, the protein sequence characteristics, and the localization studies, we hypothesize a role of PtkA in controlling transcriptional activity in *A. nidulans*, although direct proof for this is still missing and subject of our current investigations. Different transcriptional T-type cyclins (cyclins T1, T2a, T2b, and K) have been established to activate Cdk9 kinase activity in various organisms. Accordingly, we identified a novel cyclin protein in *A. nidulans*, PchA, that comprises significant sequence similarities to the cyclin T-homolog Pch1 from *S. pombe* and the human cyclins T1 and K. Using yeast-two-hybrid and BiFC analysis, we show that PchA interacts with PtkA *in vivo*, suggesting PchA to be an activating cyclin for PtkA in *A. nidulans.* Accordingly, the deletion of *pchA* resulted in a severe growth defect and the complete absence of asexual development, which is comparable to the effects of *cyclin T* deletions in yeast cells (12, 59).

Surprisingly, we identified the Pcl-like cyclin PclA as a second cyclin interaction partner for PtkA *in vivo*. In yeast, members of the Pcl cyclin family interact with the Pho85-kinase and are involved in a variety of cellular processes, including environmental signaling, cell cycle regulation, and polarized growth (20). Although the targets of the different Pho85-Pcl complexes include a number of transcription factors, the Pcl cyclins are not transcription related in the sense of a direct involvement in the transcriptional machinery. We show here in *A. nidulans* that PtkA is an interacting kinase of PclA, which is to our knowledge the first report of a Cdk9-like kinase to form a complex with a canonical cyclin. Since PclA is essential for spore generation, this may indicate a functional role of PtkA/PclA complexes in asexual development. Furthermore, both cyclins PclA and PchA are upregulated during spore generation, and the complete lack of asexual development in the *ptkA* and *pchA* deletion mutants supports an involvement of PtkA in this developmental program.

We hypothesize that PtkA links the regulation of transcriptional activity with cellular processes such as development or morphogenesis in *A. nidulans*. This idea is supported by the phenotype of *ptkA*-overexpressing strains that show a significantly increased proportion of curved and hyperbranched hyphae, indicating a functional role of PtkA in the initiation and directionality of polarized growth. The exact mechanism of how PtkA regulates these processes is not elucidated here; however, the nuclear localization of PtkA rather than at hyphal tips or the cytoskeleton indicates that the kinase does not directly influence spatial organization of the cytoskeleton but acts indirectly via gene expression. A similar function has been proposed for the Cdk9 homolog Crk1 in the dimorphic fungus *Candida albicans*. A *crk1* deletion mutant is viable but shows defects in hyphal development due to an impaired induction of hyphal specific genes (5).

There are several indications that Cdk9-like kinases are not simply supporting global transcription but are rather involved in differential gene expression in response to certain stimuli. For example, recent microarray analyses in yeast and human cells after specific inhibition of Cdk9 revealed that certain genes involved in different pathways such as stress response or cell division are especially dependent on Cdk9 activity, whereas others are much less dependent or even independent (15, 52). However, the mechanism of how different gene sets are distinguished with respect to Cdk9 recruitment or activity has not yet been elucidated. One possibility is the local chromatin environment, since in budding yeast Bur1 was found to regulate histone ubiquitination and methylation (23, 55)—a need that could vary at different gene loci (52). Alternatively, several activating factors are known to recruit the P-TEFb complex, such as, for example, the HIV transactivator protein Tat, thereby stimulating transcription of the respective genes (37). Notably, only one of the four known hCdk9-interacting cyclins can bind to Tat (54), suggesting that different Cdk9/cyclin complexes are specifically recruited to certain genes (14). In the present study we show that the Cdk9 homolog PtkA in *A. nidulans* interacts with at least two different cyclins, including PclA. Thus far, Pcl-like cyclins are not known to activate Cdk9-like kinases. However, since they are involved in the response to a large variety of internal and external cues, it is conceivable that this cyclin family plays a role in the differential control of transcriptional activity. Further work is required to confirm this hypothesis, including the elucidation of the mechanism how PtkA/cyclin complexes activate transcription in *A. nidulans*, the identification of genes to which they are recruited, and the discovery of possible other cyclin binding partners.

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