The *Aspergillus nidulans* cyclin PclA accumulates in the nucleus and interacts with the central cell cycle regulator NimX^{Cdc2}

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Abstract **The filamentous fungus *Aspergillus nidulans* reproduces asexually through conidiospores, which are continuously generated at morphologically differentiated structures, the conidiophores. In contrast to vegetative, multinucleate cells, spore formation requires a strict coordination of mitosis and cytokinesis. It was shown recently that the key regulator of the cell cycle in *A. nidulans* NimX^{Cdc2} and a G_1/S cyclin, PclA, are transcriptionally upregulated during development. Here we show that PclA accumulates in the nucleus and interacts with NimX^{Cdc2}. We propose that PclA modulates the kinase activity of NimX^{Cdc2} during spore formation. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: *Aspergillus*; Development; Cell cycle; Sporulation; Cyclin; Pcl; NimX

1. Introduction

The filamentous fungus *Aspergillus nidulans* is a model organism for the study of the cell cycle and for development [2,3]. For both processes numerous mutants were isolated in the past and subsequently analyzed at a molecular level. Although many of the components of the cell cycle such as the cyclin-dependent kinase (CDK) p34 homolog NimX^{Cdc2}, or the protein kinase NimA as well as their functional interactions are conserved among eukaryotes, *A. nidulans* has some interesting unique or fungal-specific features. One is that nuclear division and cell division are not strictly coordinated in vegetative cells. As a result, hyphal compartments contain several nuclei, which undergo nearly synchronous mitoses. The coordination of nuclear division and cytokinesis changes dramatically during asexual reproduction where cells with only one nucleus are produced [4–6]. Two layers of cells, metulae and phialides, emerge from an apically swollen conidiophore stalk and produce long chains (up to 100) of green pigmented, mitotically derived spores, the conidia, by repeated asymmetric divisions of the phialides. Conidia are arrested in the G_1 phase of the cell cycle until germination.

There is mounting evidence that conidiophore development in *A. nidulans* requires a fine-tuning of the cell cycle by modifying the activities of the components well known for their roles in vegetative cells. First, it was shown that the central regulator of asexual sporulation, BrlA, activates *nimX* and *nimE* [7]. In *A. nidulans* NimX^{Cdc2} is the only mitotic CDK and like in most eukaryotic cells activation of NimX^{Cdc2} and entry into mitosis requires binding of a B-type cyclin in interphase, which in *A. nidulans* is encoded by *nimE$^{cyclin B}$* [4,8]. Second, a specific *nimX* mutation, which makes NimX resistant to negative regulation by tyrosine phosphorylation, displayed a strong defect in conidiophore morphogenesis although the strain was not impaired in cell cycle progression [7]. Third, a temperature-sensitive mutation of the phosphotyrosine-phosphatase *nimT*, which is required for the entry into mitosis by activation of NimX^{Cdc2}, could be suppressed by multiple copies of NimE$^{Cyclin B}$. The suppression, however, was restricted to the vegetative growth phase, the strains were not able to correctly form the developmental cell types of the conidiophore and were inhibited in spore formation. As nuclear division is impaired in this strain a critical rate of nuclear division has been proposed to be required for development [4].

Recently, we have isolated the pcl-like cyclin PclA, which is specifically required for the fast, repetitive cell divisions of the phialides leading to long chains of mitogenic conidiospores on the conidiophore. Loss of function of *pclA* causes a great reduction in spore number. *pclA* is induced by BrlA and cell cycle-dependent transcribed. In previous experiments we found that it binds to a PSTAIRE-containing CDK. In *A. nidulans* two CDKs, the Pho85 homolog PhoA and the p34 homolog NimX^{Cdc2}, are known. By classical genetics we could exclude PhoA as partner for PclA during sporulation [9]. In this report we identified NimX as one interacting kinase.

2. Materials and methods

2.1. Strains, culture conditions and plasmids

Supplemented minimal and complete media for *A. nidulans* were prepared as described and standard strain construction procedures were used [10]. Standard DNA transformation procedures were used for *A. nidulans* [11], *Escherichia coli* [12] and *Saccharomyces cerevisiae* [13]. For PCR experiments standard protocols were applied using a capillary Rapid Cycler (Idaho Technology, Idaho Falls, ID, USA) for the reaction cycles. We have used the following *A. nidulans* strains: SRF200 (*pyrG89*; *ΔargB::trpCΔB*; *pyroA4*; *veA1*), SSNI57 (SRF200 transformed with pSNI39 (*alcA::pclA::HA*; *argB*); *pyrG89 pyroA4*; *veA1*) [9]; SWTA (*pyrG89*; *pyroA4*; *veA1* containing pDC1 (*argB*)) [9]; SSNI58 (SRF200 transformed with pSNI116 (see below); *ΔargB::trpCΔB*; *pyroA4*; *veA1*; *NimX::FLAG*); SSNI62 (SSNI57 transformed with pSNI116; *pyroA4*; *veA1*; *alcA::pclA::HA(argB*)

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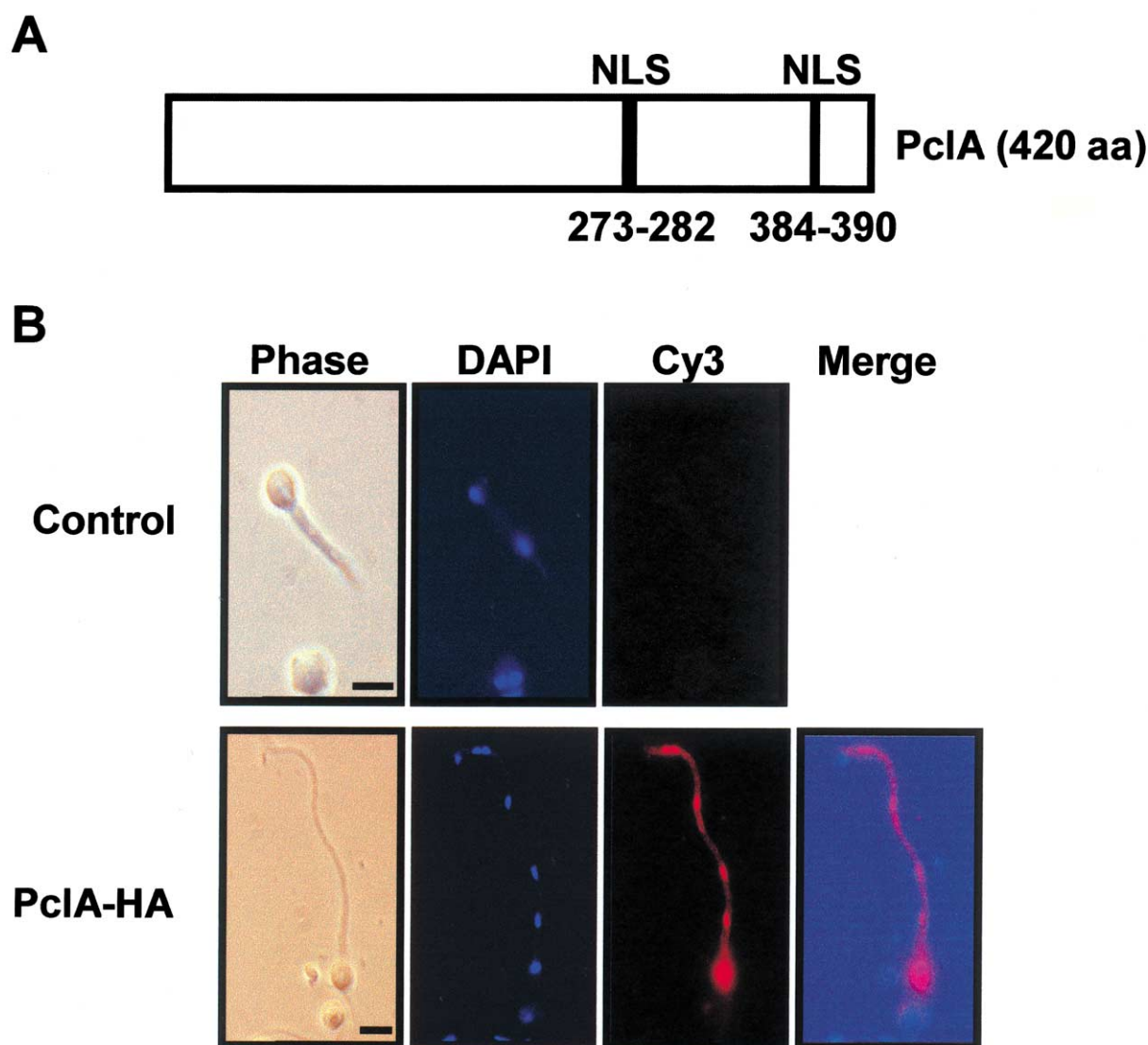[1] Present address: Chiron Behring GmbH and Co., 35037 Marburg, Germany.

A

B

Fig. 1. In vivo localization of PclA cyclin by secondary immunofluorescence. A: Scheme of PclA. Two putative nuclear localization signals as predicted with the computer program PSORT (http://www.nibb.ac.jp) are indicated. The numbers represent the amino acids. B: Conidia of the untagged wild-type control strain SWTA (upper row) and the PclA–HA carrying strain SSNI57 were germinated in liquid media and subsequently subjected to secondary immunofluorescence microscopy as described in Section 2. The DNA of the cells was stained with DAPI. Pictures were merged with the computer program Adobe Photoshop.

and *NimX::FLAG(ura4)*). *S. cerevisiae* strain EGY48 (*Matα, his3, trp1, ura3, LexA*$_{op(x6)}$-*LEU2*) was used for two-hybrid analysis. Standard protocols were followed for the preparation of yeast media. Standard laboratory *E. coli* strains (XL-1 blue, Top 10 F′) were used. The following plasmids were constructed: for two-hybrid analysis the complete open reading frames (ORFs) of *pclA* and *nimX* were amplified with the primers 2H-*pclA*-f (5′-GAATTCAACCGAA-CAGCGCTG-3′) or with the primers 2H-*nimX*-f (5′-GTCGACATG-GAAAACTACCAG-3′) and 2H-*pclA*-r (5′-GTCGACATGGAAA-ACTACCAG-3′) and 2H-*nimX*-r (5′-GTCGACTTAGTGAAAGC-CATT-3′), respectively, and subsequently cloned into pCR®2.1-TOPO vector (Invitrogen, Leek, The Netherlands). Afterwards the genes were inserted into the two-hybrid vectors pLexA and pB42AD (Clontech, Heidelberg, Germany), respectively. For FLAG-tagging of NimX, the *nimX* ORF including the natural promoter was amplified with the primers *nimX* 5′-1 (5′-CGTCCTAGTCGAAGA-TAGAAG-3′) and *nimX* 3′-FLAG (5′-TTACTTGTCATCGTC-GTCCTTGTAGTCGTGAAAGCCATTGCGGCGAGC-3′) with genomic DNA as template and cloned into pCR®2.1-TOPO vector (Invitrogen). Afterwards the *pyr4* gene from *Neurospora crassa* [14] was inserted as auxotrophy marker to make pSNI116.

2.2. Two-hybrid assays

Yeast two-hybrid strain EGY48 was transformed with the reporter plasmid p8op-lacZ and afterwards simultaneously with combinations of activation (AD) and binding domain (BD) vectors and plated on SD+leu. Colonies were resuspended in water at equal densities, spotted onto SD$_{GAL/RAF}$+leu and SD$_{GAL/RAF}$−leu and scored for differential growth on the selective medium.

2.3. Secondary immunofluorescence and microscopy

For microscopy conidia were inoculated in media containing 2% ethanol or threonine for induction of the *alcA* promoter. After growth for 12–15 h germlings were subjected to secondary immunofluorescence essentially as described before [15,16].

2.4. Protein extracts, immunoprecipitation and Western blotting

Protein extraction and immunoprecipitation procedures were essentially performed as described before [9]. For the induction of the *alcA* promoter an overnight glucose grown culture was filtered through a layer of miracloth, washed with a large volume of water and transferred to induction medium containing 2% threonine for 4 h. All strains, SWTA, SSNI57, SSNI58 and SSNI62, were treated the

same way, although the NimX–FLAG construct in SSNI58 is not under the control of the *alcA* promoter. Protein extracts were prepared by grinding the mycelium in liquid nitrogen and suspension of the powder in protein extraction buffer (20 mM Tris–HCl, pH 8; 0.05% Triton X-100; 150 mM NaCl; supplemented with the fungal protease inhibitor cocktail from Sigma (Sigma, St. Louis, MO, USA) and 2 mM phenylmethylsulfonyl fluoride (PMSF)). Crude extracts were cleared from cell debris by two subsequent centrifugation steps (10 000 rpm, 15 min, HB-6 rotor). All steps were performed on ice or at 4°C. For Western blot analysis monoclonal antibodies against the HA (see above) or the FLAG epitope (ANTI-FLAG® M2, Sigma) were used with a dilution of 1:2500. For the detection of the primary antibody we used the ECL system according to the manufacturer's protocol with a dilution of the secondary antibody of 1:10 000 (Amersham Pharmacia, Freiburg, Germany). Immunoprecipitation experiments were performed in the protein extraction buffer adjusted to 300 mM NaCl. As a control for co-immunoprecipitation experiments we used a monoclonal antibody against a synthetic peptide of the human $p62^{c-myc}$ protein (anti-*c-myc*, clone 9E10, Sigma).

2.5. Kinase assay

Protein extraction for kinase activity assays was performed in extraction buffer (20 mM Tris–HCl (pH 8), 0.05% Triton X-100) supplemented with complete protease inhibitors (Roche), 5 mM benzamidine, 2 mM PMSF, 5 mM ethylenediaminetetra-acetate (pH 8), 60 mM β-glycerolphosphate, 15 mM ethylene glycol-β(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (pH 8), 500 μM sodium vanadate, 10 mM sodium fluoride, and 15 mM *p*-nitrophenyl phosphate. Immunoprecipitation procedures were performed as described before [9] followed by two washing steps in KAB buffer (50 mM Tris–HCl (pH 7.5), 10 mM MgCl$_2$, 1 mM dithiothreitol). The immunoprecipitate was adjusted to 25 μl in KAB buffer. Kinase reaction was started by addition of 5 μl of histone H1 substrate mixture (2.5 μl of 1 mM ATP, 2 μl of histone H1 (10 mg/ml), 0.5 μl of [γ-^{32}P]ATP (6000 Ci/mmol)). Probes were incubated for 15 min in a 30°C water bath, the kinase reaction was stopped with 10 μl of preheated 4× sodium dodecyl sulfate (SDS)–protein sample buffer (100°C), the probes denatured at 100°C for 5 min, separated by SDS–polyacrylamide gel electrophoresis (PAGE) and the gel was dried. Kinase reaction products, phosphorylated histone H1, were visualized by autoradiography and quantified with a PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA).

3. Results and discussion

To gain more insight into the function of PclA we determined the subcellular localization of a PclA–HA fusion protein. Since we were not able to detect any fluorescence in strains expressing the construct under the control of the natural promoter, we used strain SSNI57, in which the natural promoter was replaced through the inducible *alcA* promoter [9]. We detected PclA–HA by secondary immunofluorescence with monoclonal anti-HA and Cy3-labeled secondary anti-mouse antibodies. PclA–HA localized in rapidly growing cells predominantly to the nucleus (Fig. 1). This is in agreement with the prediction of the computer program PSORT (http://www.nibb.ac.jp), which identified two putative nuclear localization signals in PclA.

We speculated previously that PclA might be involved in cell cycle regulation during conidiation and since it was shown before that NimX^{Cdc2} is a nuclear protein, the detection of PclA in this organelle was a first indication that the two proteins could form a complex. However, we were not able to detect NimX–PclA interaction in the two-hybrid system (data not shown). This might have been due to an incorrect conformational or phosphorylation status of the two *A. nidulans* proteins in yeast. Especially cyclin–cyclin-dependent kinase interactions are often regulated by post-translational phosphorylation (for a review see [17]). Another possibility for the failure of this heterologous experiment could be that the
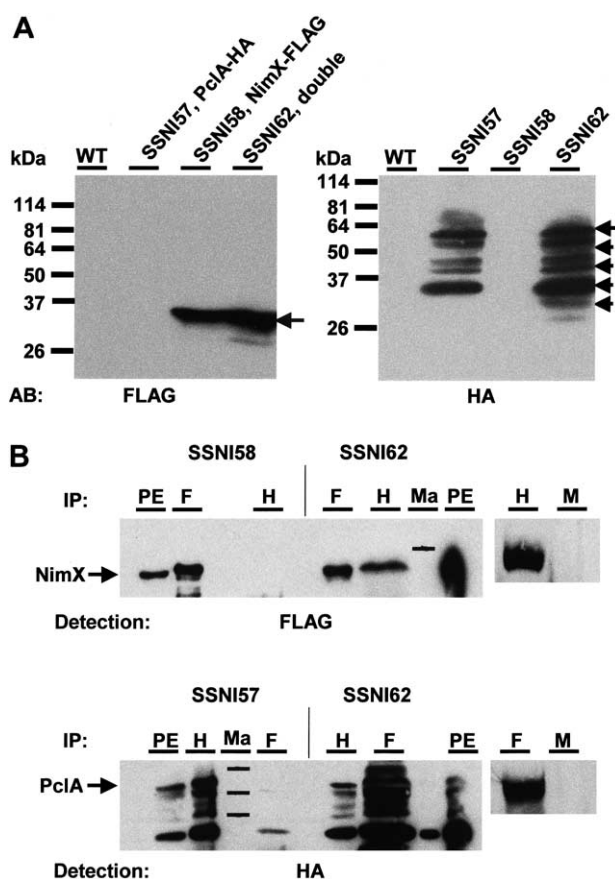

Fig. 2. Co-immunoprecipitation of PclA and NimX. A: Detection of PclA–HA and NimX–FLAG in protein extracts. The wild-type strain SWTA (WT), the PclA–HA carrying strain (SSNI57), the NimX–FLAG strain (SSNI58) and the PclA–HA/NimX–FLAG carrying strain SSNI62 were induced for 4 h on threonine medium and protein extracts were prepared. For the Western blot in each lane 100 μg of total protein was analyzed and the epitopes detected with a monoclonal anti-HA (left panel) or a monoclonal anti-FLAG antibody (right panel). PclA–HA and NimX–FLAG are indicated with an arrow. The dotted arrows point to degradation products of PclA–HA. B: Co-immunoprecipitation (IP) of NimX and PclA. 10 mg protein extract of SSNI57, SSNI58 and SSNI62 was precipitated using monoclonal anti-FLAG (=F), anti-HA (=H) or anti-myc (M) antibodies (AB). Protein was eluted from the protein G-agarose beads (50 μl) with 60 μl double-concentrated loading buffer by boiling for 5 min. If precipitation and detection were performed with the same AB, 3 μl (ca. 3% of precipitate) of the eluate was analyzed for the precipitated proteins, and in case precipitation and detection were with different AB, 45 μl (ca. 40% of precipitate) was subjected to the Western blot analysis. ABs for the detection were used as indicated. In addition to the precipitated proteins, crude protein extracts (PE) (25 μg, ca. 0.25% input) of the corresponding strains were loaded. The labels in the marker lanes (Ma) correspond to 37 kDa (upper panel) or 81, 64 and 49 kDa (lower panel). The X-ray films for the detection of the signals were exposed for 5 s.

interaction between the two proteins is only transient and rather weak.

To further study a potential interaction between NimX and PclA we performed co-immunoprecipitation experiments with cell extracts of an *A. nidulans* strain that carried epitope-tagged versions of the respective proteins. To create this strain the PclA–HA expressing strain SSNI57 was transformed with pSNI116 carrying NimX–FLAG under the control of the natural promoter. Four transformants were checked in a Western blot with the anti-FLAG antibody, and we detected in all transformants a specific signal of the expected molecular
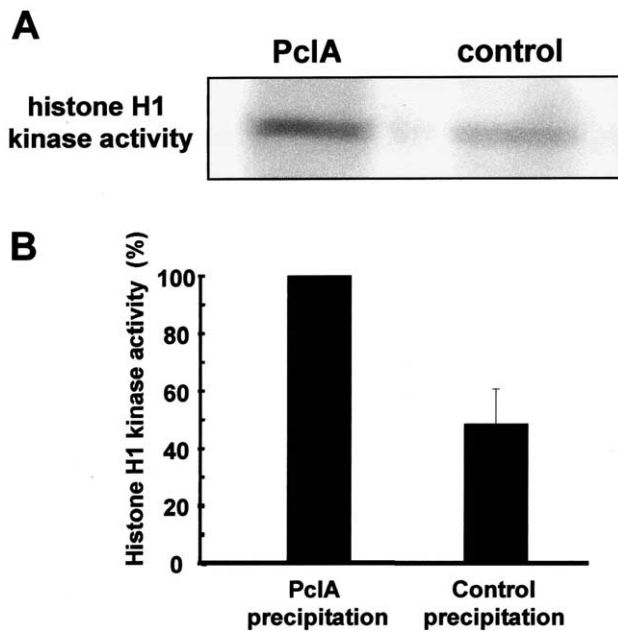
A

B

Fig. 3. Determination of the kinase activity in the PclA precipitate. A: Precipitation of PclA::HA with the anti-HA antibody was performed as described in Fig. 2. The immunoprecipitates were analyzed for histone H1 kinase activity as described in Section 2. After kinase reaction, the probes were denatured at 100°C for 5 min, and separated by SDS–PAGE. As a control, the same amount of protein extract was precipitated with an unspecific antibody and treated identically. After drying of the gel kinase reaction products, phosphorylated histone H1, were visualized by autoradiography. B: The ^{32}P signal obtained in (A) was quantified with a PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA). Histone H1 kinase activity of PclA–HA immunoprecipitate was set as 100%.

mass of 37 kDa for a NimX–FLAG fusion protein (results not shown). We analyzed cell extracts of one of these strains (SSNI62) for co-expression of PclA–HA and NimX–FLAG in a Western blot. In addition to the NimX–FLAG signal, the PclA–HA protein was strongly expressed after 4 h of growth in induction medium. Some degradation products of PclA were visible, probably due to overexpression of PclA (Fig. 2A). As controls we analyzed SSNI57 and SSNI58, which express only one epitope-tagged protein, PclA–HA or NimX–FLAG, respectively.

For testing NimX–PclA interaction in strain SSNI62 by co-immunoprecipitation we first precipitated PclA–HA with a monoclonal anti-HA antibody. Associated NimX was detected subsequently by Western blot analysis with a monoclonal anti-FLAG antibody (Fig. 2B, upper panel). To verify this interaction, we also precipitated NimX–FLAG using the anti-FLAG antibody and detected the associated PclA–HA cyclin with the anti-HA antibody (Fig. 2B, lower panel). As a control, the same amount of cell extract of the same strain was precipitated with the identical amount of unspecific monoclonal anti-myc antibody. In the experiment no signal was obtained, demonstrating that the PclA–NimX interaction was specific. Furthermore, we could not detect co-precipitation either when we used the strains with only one tagged protein (SSNI57, SSNI58).

The precipitated proteins of SSNI62 were not only tested for the presence of the epitopes but also for kinase activity. We used histone H1 and γ-^{32}P-labeled ATP as substrates for a kinase reaction and analyzed the ^{32}P-labeled protein on SDS–polyacrylamide gels (Fig. 3). Quantification of the signal re-

vealed a two-fold higher kinase activity in the precipitated fraction in comparison to the control. This demonstrates that precipitation of PclA pulls down NimX^{Cdc2} as an active kinase. This is in contrast to *S. cerevisiae* where pcl cyclins do not interact with Cdc28 but rather resembles the properties of a pcl cyclin, Pas1, recently characterized in *Schizosaccharomyces pombe* [18–20].

Given that the interaction between PclA and NimX detected in hyphae is of functional importance during development two roles for this interaction appear to be likely. (i) The interaction of the two proteins could modulate the NimX activity and trigger the cell cycle in order to adapt the speed of nuclear division to the speed of the rapid spore production. (ii) On the other hand, the NimX kinase appears not only to control the cell cycle but also septation. It was found that an active allele of NimX caused septum formation in the conidiophore stalk, although the stalk is not compartmentalized in wild-type [1,7]. PclA could regulate cytokinesis in phialides via NimX activity modulation and thus coordinate the cell cycle and cell divisions during spore formation. It will be the challenge of future research to unravel the exact role of NimX during the development of the spore producing structures of *A. nidulans*.

References

[1] Kraus, P. and Harris, S.D. (2001) Genetics 195, 557–569.
[2] Morris, N.R. and Enos, A.P. (1992) Trends Genet. 8, 32–37.
[3] Adams, T.H., Wieser, J.K. and Yu, J.-H. (1998) Microbiol. Mol. Biol. Rev. 62, 35–54.
[4] O'Connell, M.J., Osmani, A.H., Morris, N.R. and Osmani, S.A. (1992) EMBO J. 11, 2139–2149.
[5] Doonan, J.H. (1992) J. Cell Sci. 103, 599–611.
[6] Mirabito, P.M. and Osmani, S.A. (1994) Dev. Biol. 5, 139–145.
[7] Ye, X.S., Lee, S.-L., Wolkow, T.D., McGuire, S.-L., Hamer, J.E., Wood, G.C. and Osmani, S.A. (1999) EMBO J. 18, 6994–7001.
[8] Osmani, A.H., van Peij, N., Mischke, M., O'Connell, M.J. and Osmani, S.A. (1994) J. Cell Sci. 107, 1519–1528.
[9] Schier, N., Liese, R. and Fischer, R. (2001) Mol. Cell. Biol. 21, 4075–4088.
[10] Käfer, E. (1977) Adv. Genet. 19, 33–131.
[11] Yelton, M.M., Hamer, J.E. and Timberlake, W.E. (1984) Proc. Natl. Acad. Sci. USA 81, 1470–1474.
[12] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1999) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[13] Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1995) Short Protocols in Molecular Biology, John Wiley and Sons.
[14] Waring, R.B., May, G.S. and Morris, N.R. (1989) Gene 79, 119–130.
[15] Fischer, R. and Timberlake, W.E. (1995) J. Cell Biol. 128, 485–498.
[16] Geißenhöner, A., Sievers, N., Brock, M. and Fischer, R. (2001) Mol. Genet. Genomics 266, 672–685.
[17] Morgan, L.W. and Feldmann, J.F. (1997) Genetics 146, 525–530.
[18] Tanaka, K. and Okayama, H. (2000) Mol. Biol. Chem. 11, 2845–2862.
[19] Espinoza, F.H., Ogas, J., Herskowitz, I. and Morgan, D.O. (1994) Science 266, 1388–1391.
[20] Measday, V., Moore, L., Ogas, J., Tyers, M. and Andrews, B. (1994) Science 266, 1391–1395.