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c-Jun and RACK1 homologues regulate a control point for sexual development in *Aspergillus nidulans*

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Summary

Amino acid limitation results in impaired sexual fruit body formation in filamentous fungi such as *Aspergillus nidulans*. The starvation signal is perceived by the cross-pathway regulatory network controlling the biosynthesis of translational precursors and results in increased expression of a transcriptional activator encoded by a *c-Jun* homologue. In the presence of amino acids, the gene product of the mammalian *RACK1* homologue *cpcB* is required to repress the network. Growth under amino acid starvation conditions permits the initiation of the sexual developmental programme of the fungus, but blocks fruit body formation before completion of meiosis. Accordingly, arrest at this defined control point results in microcleistothecia filled with hyphae. Addition of amino acids results in release of the block and completion of development to mature ascospores. The same developmental block is induced by either overexpression of *c-Jun* homologues or deletion of the *RACK1* homologue *cpcB* of *A. nidulans* in the presence of amino acids. Therefore, the amino acid starvation signal regulates sexual development through the network that also controls the amino acid biosynthetic genes. Expression of the *RACK1* gene suppresses the block in development caused by a deletion of *cpcB*. These data illuminate a connection between metabolism and sexual development in filamentous fungi.

Introduction

Filamentous fungi are a diverse group of organisms with high nutritional versatility that can therefore easily adapt to different environmental conditions and quickly colonize large areas. Extension of hyphal tips enables the fungus to forage for new resources when nutrients are depleted near the centre of the fungal colony. In addition, the ability to produce asexually or sexually derived spores facilitates dispersal by air or water. *Aspergillus nidulans* and *Neurospora crassa* are major fungal model organisms that have been used primarily for fundamental physiological, biochemical or genetic studies and have been compared with their unicellular relative *Saccharomyces cerevisiae*. Both filamentous fungi have contributed to our understanding of the mechanisms controlling eukaryotic growth, differentiation and development.

Amino acids as precursors for proteins can either be synthesized or acquired from the environment by using corresponding uptake systems. In ascomycetes, the biosyntheses of protein precursor molecules as amino acids or charged tRNAs are co-ordinately controlled by a complex transcriptional network, which has been studied in the unicellular yeast *S. cerevisiae* as well as in the filamentous fungi *A. nidulans* and *N. crassa.* This network has been described as 'general amino acid control' in yeast or as 'cross-pathway control' in filamentous fungi (Carsiotis and Jones, 1974; Piotrowska, 1980; Hinnebusch, 1992). Starvation for a single amino acid resulting in specific uncharged tRNAs induces a signal transduction pathway. A sensor kinase located at the ribosome is activated when uncharged tRNAs are recognized (Marton *et al*., 1997; Sattlegger *et al*., 1998). Phosphorylation of the translational initiation factor eIF2 results in increased translation of the mRNA for a transcriptional activator that is a member of the *c-Jun* family (Struhl, 1988). These transcriptional activators, including Gcn4p from yeast (Hinnebusch, 1992), CpcAp from *Aspergillus* (Wanke *et al*., 1997) and CPC1p from *Neurospora* (Paluh *et al*., 1988), are functionally interchangeable between the different fungi (Paluh and Yanofsky, 1991; Wanke *et al*., 1997). They bind to the same upstream DNA binding site as c-Jun in mammalian cells (John *et al*., 1996). Expression of the activator increases transcription of more than 50 genes involved in the biosynthesis of amino acids (Hinnebusch, 1992), purines (Mösch *et al*., 1991) or encoding aminoacyl-tRNA synthetases (Mirande and Waller, 1988). The regulatory network is repressed in the presence of amino acids. One essential protein for repression of the network has been identified in yeast (Hoffmann *et al*., 1999). The corresponding gene locus *CPC2* encodes two gene products (Qu *et al*., 1995). Besides the protein coding region, the small nucleolar RNA U24 is located within one intron of the precursor

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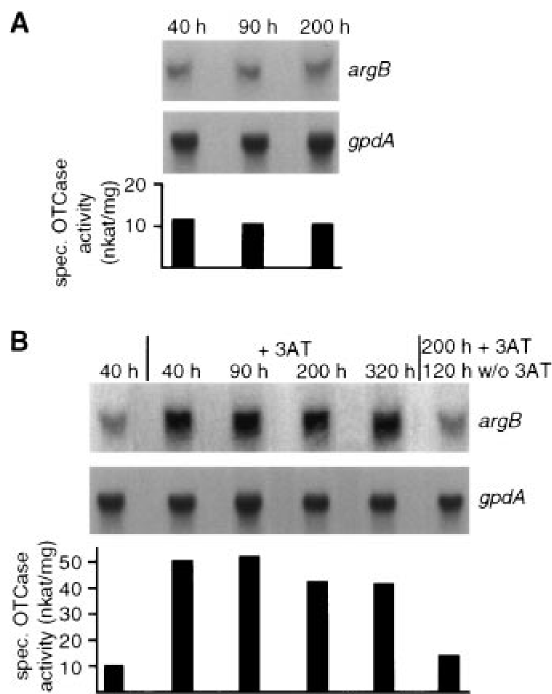

Fig. 1. mRNA levels and enzyme activities of the cross-pathway control-regulated gene *argB* of *A. nidulans* during sexual development under non-starvation and amino acid starvation conditions.A. Strain A234 was incubated on solid medium under non-starvation conditions, and total RNA and total proteins were isolated at the indicated time points for Northern blot analysis of the *argB* gene and determination of specific OTCase activities respectively. The activities are given in nkat mg^{-1} and are shown as the average values of four independent measurements. Standard deviations did not exceed 15%. The *gpdA* gene was used as reference in Northern blot analysis.B. Strain A234 was incubated on solid minimal medium with 5 mM 3AT for 200 h to induce amino acid starvation conditions. RNA and enzyme analyses after 40 h, 90 h, 200 h or 320 h were performed as in (A). The left row shows the 40 h value without amino acid limitation for comparison. In the right row, mycelium was transferred to fresh minimal medium without 3AT after 200 h and incubated for an additional 120 h.

mRNA. This metabolically stable snoRNA is normally associated with nucleolar proteins in snoRNPs (Maxwell and Fournier, 1995). U24 contains two regions that are complementary to the 25S rRNA and is required for site-specific 2′-*o*-methylation of rRNA (Kiss-Laszlo *et al*., 1996). Translation of the mature mRNA results in a Gβ-like protein consisting of seven WD repeats. The deduced amino acid sequences of homologue genes from different organisms show high identities (60–100%). These genes include fungal as well as mammalian genes, e.g. the *N. crassa cpc-2* (Müller *et al*., 1995) or the rat *RACK1* (Ron *et al*., 1994). RACK1 has originally been identified as a putative intracellular receptor for activated protein kinase C (Ron and Mochly Rosen, 1995).

Fruit bodies are the most complex structures in filamentous fungi that exhibit a sexual cycle. Initiation of cleistothecia formation, the fruit bodies of *A. nidulans*, is affected by several environmental factors, including oxygen limitation, illumination or the phosphate concentration (Yager, 1992; Bussink and Osmani, 1998). Only a few regulatory genes that affect sexual development have been identified. For example, the *veA1* gene product seems to be a light-dependent regulator protein, which represses asexual development and promotes cleistothecia formation (Mooney and Yager, 1990). Mutations in the *veA1* gene lead to a prolonged sexual development. *stuA* and *medA* are also regulatory genes that affect both asexual and sexual development (Clutterbuck and Timberlake, 1992; Wu and Miller, 1997). The regulatory gene *nsdD*, isolated in a mutant screen for sexual development, seems to act specifically in the sexual programme (Han *et al*., 1994; Chae *et al*., 1995).

In *A. nidulans*, amino acid starvation does not significantly affect hyphal growth or the development of conidiophores that carry the asexual spores (Martinelli, 1976). However, *A. nidulans* strains carrying mutations in amino acid biosynthetic genes for arginine or tryptophan biosynthesis, such as *argB* or *trpC*, have been described as being impaired in the formation of fruit bodies (Käfer, 1977; Serlupi-Crescenzi *et al*., 1983; Eckert *et al*., 1999). In *N. crassa*, a mutation in the *RACK1* homologue *cpc-2* affects not only the genetic network of amino acid biosynthesis, but also the formation of protoperithecia, the female sexual organs of this fungus, via an unknown mechanism (Müller *et al*., 1995).

In this paper, we explain these previous observations at a molecular level and show that an arrest in the formation of fruit bodies in the filamentous fungus *A. nidulans* can be induced by cross-pathway control upon the signal 'amino acid starvation'. Both the *c-Jun* homologue *cpcA* as well as the *RACK1* homologue *cpcB* are involved as regulators of a control point of sexual development in filamentous fungi.

Results

Amino acid starvation specifically arrests cleistothecia formation in A. nidulans

Amino acid auxotrophic mutants of *A. nidulans* are known to be affected in their sexual development, resulting in an inability to form fertile ascospores (Käfer, 1977; Serlupi-Crescenzi *et al*., 1983; Eckert *et al*., 1999). Wild-type *A. nidulans* was grown in the presence or absence of amino acid limitation to compare sexual development and the induction of the cross-pathway control regulatory network of amino acid biosynthesis. Amino acid starvation was induced by the addition of the histidine analogue 3-amino-1,2,4-triazole (3AT), resulting in histidine starvation (Klopotowski and Wiater, 1965).

The mRNA levels and enzyme activities of the amino

acid biosynthetic gene *argB* were determined during development in the presence or absence of 3AT (Fig. 1A and B). *argB* encodes the ornithine transcarbamoylase (OTCase, EC 2.1.3.3) involved in arginine biosynthesis and was used as an example to monitor the induction of the cross-pathway control (Käfer, 1977; Piotrowska, 1980). Expression of *argB* was constantly low during cleistothecia development under non-starvation conditions (Fig. 1A). Amino acid starvation resulted in up to fivefold increased mRNA levels and enzyme activities during development compared with non-starvation conditions (Fig. 1B). Similar results were found for a second cross-pathway-regulated gene *trpC* (data not shown).

When amino acids were not limited, hyphae started to form nests on the colony surface 60 h after spore inoculation with approximately 200 nests cm^{-2}. Additional incubation for 30 h resulted in small, soft and lightly coloured microcleistothecia, which are the primordia of mature cleistothecia. The diameter of these structures was approximately 20 μm, and they were filled with short swollen hyphae. The microcleistothecia were located within the nests and surrounded by specific auxiliary hülle cells (Hermann *et al*., 1983). Mature fruit bodies were formed within 200 h. They had a size of 150 μm and were filled with 40 000–80 000 fertile red ascospores (Fig. 2A).

Initiation of fruit body formation, including the number of nests formed cm^{-2}, and the first steps of development were comparable between *A. nidulans* grown in the presence or absence of amino acid limitation. However, amino acid starvation resulted in an arrest of further development after approximately 90 h when microcleistothecia had been formed (Fig. 2B). Additional days of cultivation did not release this block, and microcleistothecia were still filled with hyphae even after 200 h. Hyphae and ascospores were discriminated by fluorescence microscopy using 4,6-diamidino-2-phenylindole (DAPI; data not shown). The dye stains nuclei of hyphae but not of ascospores because of the ascospore wall, which interferes with the staining (Vagvölgyi and Ferenczy, 1991).

Microcleistothecia blocked in their development by amino acid limitation were analysed further to distinguish whether the arrest is irreversible or whether development can be completed in a changed environment. Therefore, microcleistothecia that were grown for 200 h under amino acid starvation conditions were shifted to non-starvation medium and cultivated for an additional 120 h. During this time, microcleistothecia were released from the block and completed development, resulting in normal cleistothecia with fertile ascospores (Fig. 2B). Simultaneously, *argB* expression was decreased, indicating that the cross-pathway control was turned off (Fig. 1B).

Supplementation with histidine suppressed the effect of 3AT, resulting in mature cleistothecia (data not shown), suggesting that the effect was not 3AT specific. In addition, amino acid starvation induced by the amino acid analogue 5-methyl-tryptophan, a false feedback inhibitor of tryptophan biosynthesis, resulted in the same arrest in cleistothecia formation as 3AT. These data indicate that cleistothecia development is specifically blocked at the microcleistothecia state when *A. nidulans* cells are cultivated under amino acid starvation conditions.

Expression of the c-Jun *homologue* cpcA *blocks cleistothecia formation in* A. nidulans *in the middle of development*

Amino acid starvation prevented the development of *A. nidulans* fruit bodies beyond the microcleistothecia stage. This could result from a deficiency in amino acids as building blocks for this organ or be a response of the organism to the signal 'amino acid starvation'. Therefore, we analysed whether the same block at the microcleistothecia state could also be induced in the presence of amino acids. Amino acid starvation induces the cross-pathway control in filamentous fungi. At the end of this signal transduction, the *c-Jun* homologue *cpcA* is expressed. We overexpressed this gene isolated from the same genus (*cpcA* from *A. niger*) (Wanke *et al*., 1997), from another filamentous fungus (*cpc-1* from *N. crassa*; Paluh *et al*., 1988) or from the unicellular yeast *S. cerevisiae* (Hinnebusch, 1992). Neither *A. niger* nor *S. cerevisiae* are able to form fruit bodies. Each of the three open reading frames (ORFs) was expressed under the control of the inducible *alcA* promoter of *A. nidulans*, which is induced in the presence of ethanol in the

Fig. 2. Amino acid starvation and sexual development in *A. nidulans*.
A. Sexual development of *A. nidulans* wild-type strain A234 is shown under non-starvation conditions. On solid minimal medium, small microcleistothecia were formed (μC) after 90 h (90 h). Mature cleistothecia (C) were developed after 200 h (200 h). Microcleistothecia of the 90 h stage and cleistothecia of the 200 h stage are shown by arrows and were isolated (second lane). Both structures were squeezed and analysed by Nomarski optics (third and fourth lanes).
B. Sexual development of *A. nidulans* A234 is shown under amino acid starvation conditions and after release from amino acid starvation. Strain A234 was incubated for 200 h on medium containing 5 mM 3AT causing histidine starvation. The 90 h developmental stage (upper lane) corresponded to development without starvation. After 200 h, only microcleistothecia were visible. Transfer of 200 h culture to fresh medium without 3AT for an additional 120 h resulted in mature cleistothecia (200 h 3AT + 120 h w/o 3AT). Further amino acid starvation had the same phenotype as that after 200 h. Microcleistothecia and cleistothecia were isolated and analysed as described in (A). Scale bars represent 10 μm.

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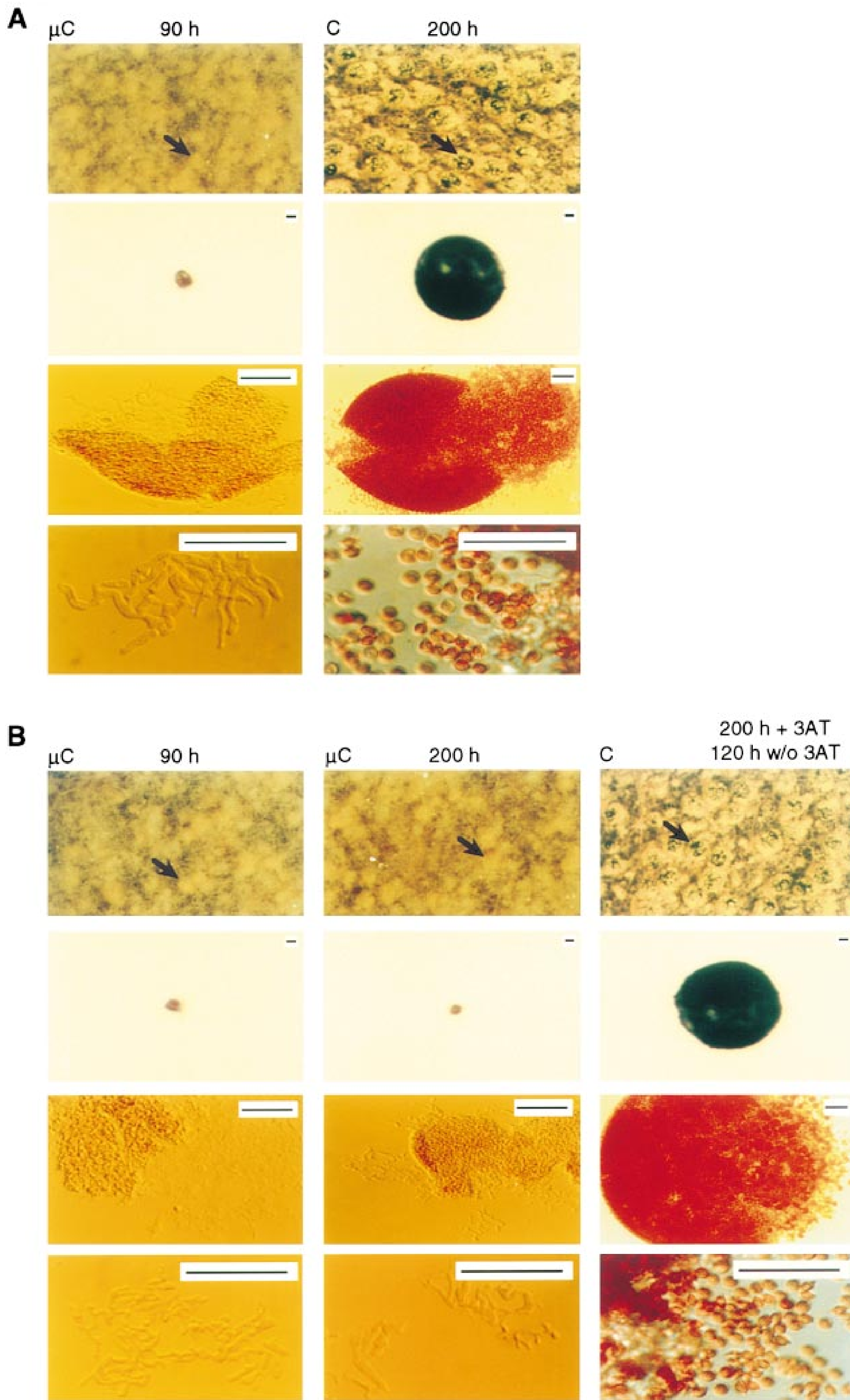
A
μC
90 h
C
200 h
B
μC
90 h
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C
200 h + 3AT
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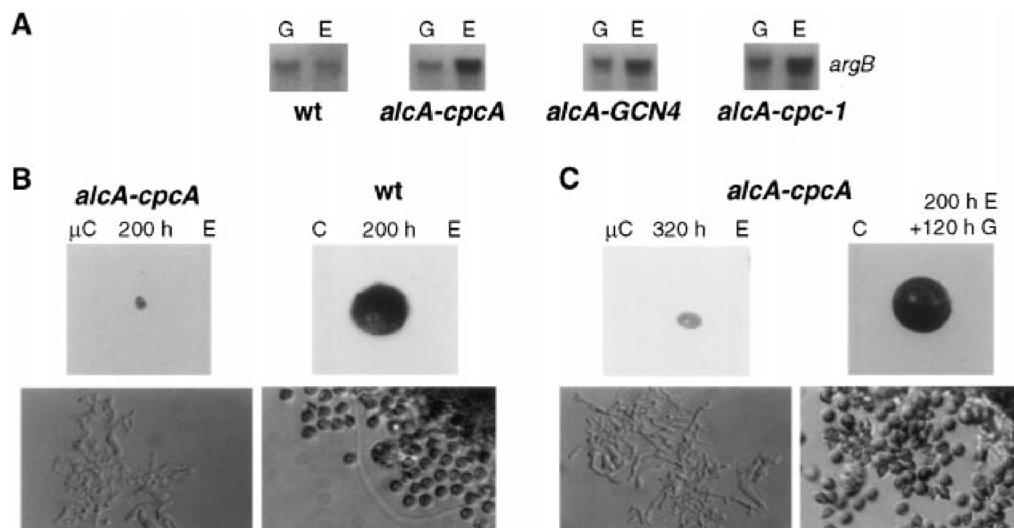

Fig. 3. Block in sexual development in strains overexpressing the *c-Jun* homologues *cpcA*, *GCN4* or *cpc-1*.
A. Transcription of the cross-pathway-controlled *argB* gene activated by heterologous c-Jun-like proteins was analysed. Strains containing the *alcA* promoter fused to *cpcA* from *A. niger* (*alcA-cpcA*), *GCN4* (*S. cerevisiae*, *alcA-GCN4*) or *cpc-1* from *N. crassa* (*alcA-cpc-1*) were grown overnight under non-inducible conditions (2% glucose, G) or under inducing conditions (2% ethanol, E). RNA amounts were equalized using the *gpdA* gene as probe (not shown). The wild-type (wt), transformed with an empty expression plasmid, did not induce cross-pathway control in the presence of ethanol and is shown as control.
B. Analysis of cleistothecia formation under *alcA*-inducing conditions of strains GR5 and GR5 + *alcA-cpcA* was performed as described in the legend to Fig. 2. Isolated fruit bodies and the developmental products within the fruit bodies are shown. Overexpression of *cpcA* causes a block in sexual development at the time point of microcleistothecia (μC). Note that a *cpcB*Δ mutation causes the same developmental block as shown here for overexpression of c-Jun-like transcription factors.
C. The block in development of the *alcA-cpcA* strain can be released by transfer of the strain to glucose in which *cpcA* is not expressed, resulting in mature cleistothecia (C).

cultivation medium (Waring *et al*., 1989). In all resulting strains, induction of the expression of the *c-Jun* homologues by 2% ethanol as sole carbon source resulted in a strongly increased mRNA level of the arginine biosynthetic gene *argB* in comparison with glucose as sole carbon source. Therefore, the gene products of all three *c-Jun* homologues are able to induce the cross-pathway control in *A. nidulans* (Fig. 3A).

Hyphal growth and asexual development resulting in equal amounts of conidia were not affected in comparison with wild type by overexpressing any of these cross-pathway control activator proteins. However, overexpression of each *c-Jun* homologue resulted in a block in fruit body formation at the microcleistothecia state. The development of the fruit bodies could not be distinguished from the development of wild-type *A. nidulans* grown under conditions of amino acid limitation (Fig. 3B).

The block in the fruit body developmental programme could be reversed and the development could be completed when overexpression of the *c-Jun* homologues was turned off by transfer of the culture to fresh medium containing glucose instead of ethanol as carbon source. Additional incubation on ethanol for 120 h as a control could not release the block on sexual development (Fig. 3C). These data suggest that the 'amino acid starvation' signal is transferred to the cross-pathway control, which activates the expression of *cpcA* and, subsequently, induces a block in the middle of fruit body development.

We analysed further whether the expression of the different *c-Jun* homologues could still block development after passing the microcleistothecia state. For this purpose, we compared the development of *c-Jun* homologue-overexpressing strains grown on glucose medium for 50 h or 120 h and subsequently shifted to ethanol medium. This corresponded to an induction of the cross-pathway control after 50 h and 120 h of development respectively. Induction after 50 h resulted in a block of development and the formation of microcleistothecia, whereas induction after 120 h resulted in the completion of development and the formation of mature ascospores (data not shown). These data indicate that the microcleistothecia state of fruit body formation correlates with a control point of development within a narrow time window. Proceeding beyond this control point requires that the cross-pathway control is not induced by the signal 'amino acid limitation'. When the control point has been passed, further development cannot be stopped any more by the cross-pathway control.

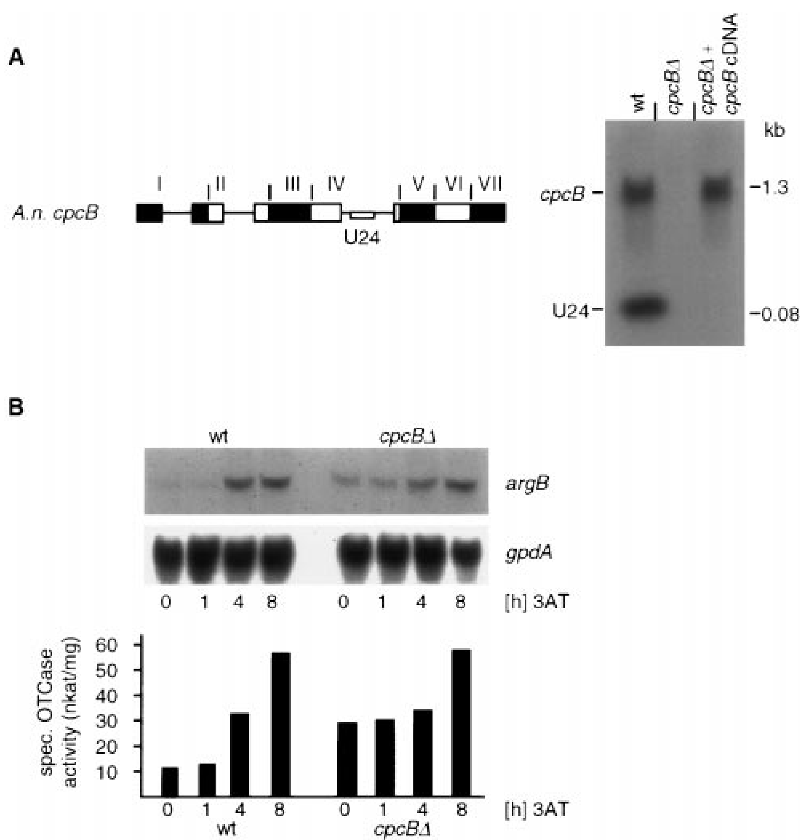

Fig. 4. Gene structure and analysis of *cpcB* of *A. nidulans.*
A. *cpcB* of *A. nidulans* encodes two transcripts. Exons and the snoRNA U24 are shown as boxes and introns as lines. Alternative black and white boxes indicate the boundaries of seven WD repeats. Repeats are numbered above the boxes. Northern analysis of a wild-type strain (wt) revealed both transcripts when probed with a fragment containing the last exon and the last intron of *cpcB*, whereas in a *cpcB* deletion mutant (*cpcBΔ*) or in a *cpcB* deletion mutant complemented with the *cpcB* cDNA (*cpcBΔ* + *cpcB* cDNA), no transcript or only the *cpcB* transcript, respectively, was detectable.
B. Total RNA and total proteins from *cpcB* wild-type A234 (wt) and the *cpcB* mutant strain AGB20 (*cpcBΔ*) grown on liquid minimal medium or minimal medium containing 10 mM 3AT to induce amino acid limitation were isolated at the indicated times. Expression of the *argB* gene was analysed by Northern blot (top) and by measuring OTCase activities (nkat mg^{-1} protein) from protein extracts (bottom). The *gpdA* gene was used as internal standard. The enzyme activities are an average of three independent measurements. Standard deviations did not exceed 15%.

Deletion of the RACK1 *homologue* cpcB *of* A. nidulans *constitutively induces the cross-pathway control and blocks fruit body formation at the microcleistothecia state*

Amino acid starvation and expression of the transcriptional activators of the cross-pathway control result in a developmental block in the middle of fruit body formation. We wondered whether a subtle induction of the cross-pathway control would already be sufficient to block fruit body formation. In yeast, the *RACK1* homologue *CPC2* is required to repress the amino acid regulatory network in the presence of amino acids (Hoffmann *et al*., 1999). Thus, a deletion of a homologue gene in *A. nidulans* should result in slightly increased transcription of the cross-pathway control-regulated genes. Therefore, we wanted to know whether the fruit body formation programme would be able to pass the microcleistothecia control point under such circumstances.

The *RACK1* homologue gene fragment of *A. nidulans*, *cpcB*, was amplified from genomic DNA using degenerate primers designed within conserved regions of known Gβ-like proteins. The amplified 670 bp DNA fragment exhibits approximately 80% identities to RACK1-encoding genes and hybridized to a single locus on chromosome IV of an *A. nidulans* chromosome-specific library (Brody *et al*., 1991). Isolation of a 4.5 kb full-length *Eco*RI genomic clone and the cDNA revealed an ORF of 948 bp interrupted by three introns (Fig. 4A). The deduced *A. nidulans* protein consists of seven WD repeats of identical structure and length as the RACK1 protein. The sequence of 316 amino acids showed 64% (*CPC2* of *S. cerevisiae*) to 92% (*cpc-2* of *N. crassa*) identity to Gβ-like protein sequences, but less than 30% identity when compared with other classes of WD proteins, e.g. β-subunits of heterotrimeric G-proteins (26% identities to the Gβ-subunit SfaD of *A. nidulans*; Rosen *et al*., 1999). The three introns of the *A. nidulans* gene *cpcB* (GenBank accession no. AF176775) are located at identical positions in the ORF to the last three introns of the *cpc-2* gene of *N. crassa* (data not shown). The last intron, which is also conserved in the yeast *CPC2* gene, shelters the U24snoRNA (Qu *et al*., 1995). The expression of the *cpcB* mRNA as well as of a U24snoRNA in *A. nidulans* was demonstrated by Northern blot analysis (Fig. 4A). The complete ORF of *cpcB* was deleted, and the resulting *cpcBΔ* mutant strain was viable and showed neither *cpcB* mRNA nor U24snoRNA expression. Expression of the *cpcB* cDNA in this mutant resulted only in the expression of the *cpcB* mRNA without expressing any snoRNA U24.

The transcript levels of the *argB* gene as cross-pathway control indicator of the *cpcB* wild type and the *cpcB*

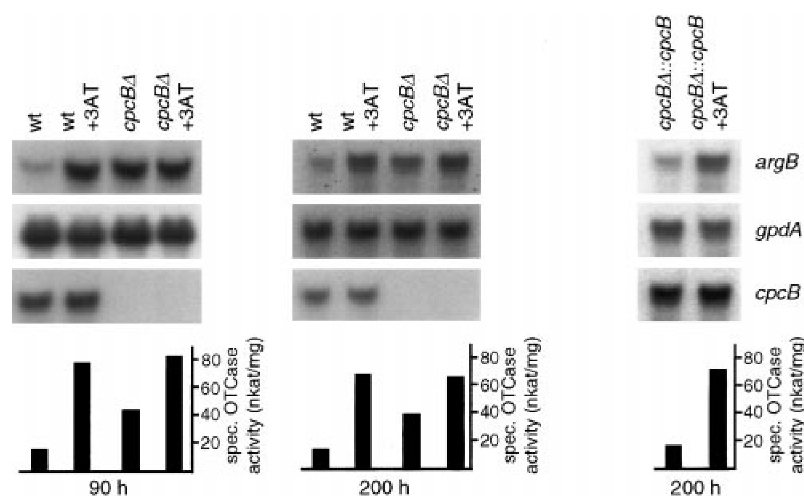

Fig. 5. Influence of *cpcB* on cross-pathway control. The induction of the cross-pathway control during sexual development was analysed using the expression of *argB* as an indicator. Wild-type strain A234 (wt), the *cpcB* mutant strain (*cpcBΔ*) and the *cpcB* mutant strain complemented by a *cpcB* cDNA clone (*cpcBΔ*::*cpcB*) were grown on solid minimal medium under non-starvation or amino acid starvation (+3AT) conditions. After 90 h or 200 h, mycelia were harvested, and total RNAs and total proteins were isolated. *argB* and *cpcB* mRNA were visualized by Northern hybridization and autoradiography; *gpdA* was used as an internal standard. Specific OTCase activities of the *argB* gene product were measured in crude protein extracts and are given as nkat mg^{-1}. Indicated values are averages of at least four independent measurements. Standard deviations were less than 15%.

deletion strain were determined. The *cpcBΔ* mutant strain showed a 2.5-fold higher basal *argB* mRNA level than the wild-type strain cultivated in liquid minimal medium. Amino acid starvation induced by 3AT led to an approximately fivefold increase in *argB* mRNA after 8 h in wild-type cells and to a twofold increase in the mutant strain, resulting in equal steady-state levels in both strains (Fig. 4B). Specific enzyme activities of the *argB* gene product OTCase were measured in both strains to examine whether the increased mRNA levels corresponded to increased enzyme levels (Fig. 4B). Under non-starvation conditions, approximately threefold increased specific enzyme activities were measured in the absence of the *cpcB* gene in comparison with the wild-type situation. Amino acid starvation conditions resulted in comparable OTCase activity in both strains, corresponding to a sixfold induction for the wild type and a twofold induction for the mutant strain. Similar results were also found for other cross-pathway control-regulated genes, e.g. *trpC*. The repressing effect of *cpcB* is specific for genes regulated by the cross-pathway network. The specific activity of the enzyme isocitrate dehydrogenase (EC 1.1.1.41), which is not affected by the cross-pathway control (Kelly and Hynes, 1982), is not influenced by the *cpcB* gene (data not shown).

These results show that the *cpcB* gene of *A. nidulans* has the same function in the cross-pathway control as its homologue in yeast (Hoffmann *et al.*, 1999). It is required for the repression of the regulatory network in the presence of sufficient amounts of amino acids. The cross-pathway transactivation activity is not affected by the CpcB protein under amino acid starvation conditions.

The development patterns of the *cpcBΔ* and *cpcB* wild-type strains were compared under non-starvation conditions. The germination of the mutant spores was retarded, and the germination rate was reduced. After germination, the growth rate of the hyphae and the formation of asexual spores were similar to those of the wild type. However, the sexual development was impaired in the mutant strain. The initial steps of fruit body formation were identical for the first 80–90 h in wild-type and mutant strains. After the formation of microcleistothecia, the *cpcBΔ* mutation caused a block in cleistothecia formation at the same distinct reproducible time point of fruit body development as was observed in wild-type cells under amino acid starvation or after overexpression of different *c-Jun* homologues (data not shown). However, in the *cpcBΔ* mutant strain, this block was irreversible and independent of the physiological conditions. The cross-pathway control was constitutively derepressed in the *cpcBΔ* mutant strain during development at an intermediate level and was not affected by the morphological changes of the fungal fruit body formation (Fig. 5).

The status of the cross-pathway control was tested for the *cpcBΔ* mutant strain after transformation with *RACK1* homologue genes. Retransformation of the *cpcB* cDNA into the mutant strain resulted in comparable *argB* mRNA levels and enzyme activities as found for the wild-type strain under non-starvation and amino acid starvation conditions (Fig. 5). In addition, expression of

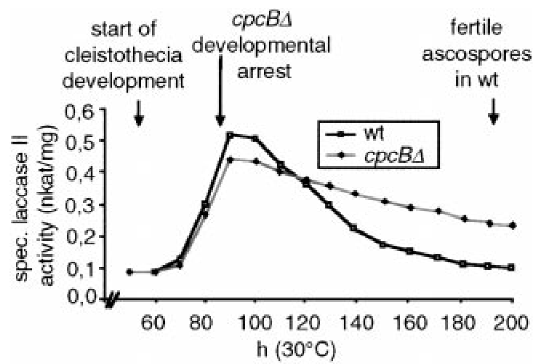

Fig. 6. Specific laccase II activity during cleistothecia formation of *A. nidulans*. *cpcB* wild-type strain A234 (wt) and the *cpcB* mutant strain AGB22 (*cpcBΔ*) were incubated on solid minimal medium under oxygen limitation. Laccase II activity could be detected in both strains because both organisms are unable to express laccase I activities as a result of the *yA2* mutation. Cultures were isolated at the indicated time points, and specific laccase II activities were determined in crude protein extracts and are given in nkat mg^{-1} protein. Activities of four independent measurements are shown as average values with standard deviations of less than 20%.

only the *cpcB* cDNA of *A. nidulans* encoding the Gβ-like protein in the *cpcB* deletion strain complemented the developmental phenotype, suggesting that the presence of CpcBp without the snoRNA U24 is sufficient for the effect.

These data show that an *A. nidulans* strain exhibiting a constitutively induced cross-pathway control is unable to pass the microcleistothecia control point of fruit body development. This inability is independent of the presence or absence of amino acids and cannot be compensated for by changing the environmental conditions.

Microcleistothecia are surrounded by functional auxiliary hülle cells

Deletion of the *RACK1* homologue *cpcB* of *A. nidulans* resulted in constitutive incompetence to complete cleistothecia development. Therefore, we used a laccase I-deficient *cpcBΔ* mutant strain to analyse whether the block in development at the microcleistothecia state might result from non-functional auxiliary hülle cells (Champe and Simon, 1992). This specific cell type surrounds the young cleistothecium and provides support for development including laccase II enzyme activity, which is exclusively correlated with cleistothecia formation (Hermann *et al*., 1983; Scherer and Fischer, 1998). Specific enzyme activity could be detected during wild-type fruit body formation approximately 50 h after spore inoculation, and the activity peaked during the subsequent 40 h. Afterwards, laccase II activity decreased and was no longer detectable after approximately 170 h. The *cpcBΔ* mutant strain formed hülle cells normally, and laccase II activity was similar to that of the wild type for the first 90 h (Fig. 6). As in wild-type cells, laccase II activity started decreasing after approximately 90 h, but the rate of decrease was lower than in the wild-type strain. After completion of wild-type cleistothecia formation after 200 h, the amount of hülle cells was reduced. The block in cleistothecia formation in the *cpcBΔ* mutant strain resulted in the persistence of most hülle cells still active, with corresponding amounts of laccase II enzyme activity (Fig. 6). These results indicate that the *cpcBΔ* mutant strain is unaffected in sexual development during the first 90 h and produces intact hülle cells, which are able to

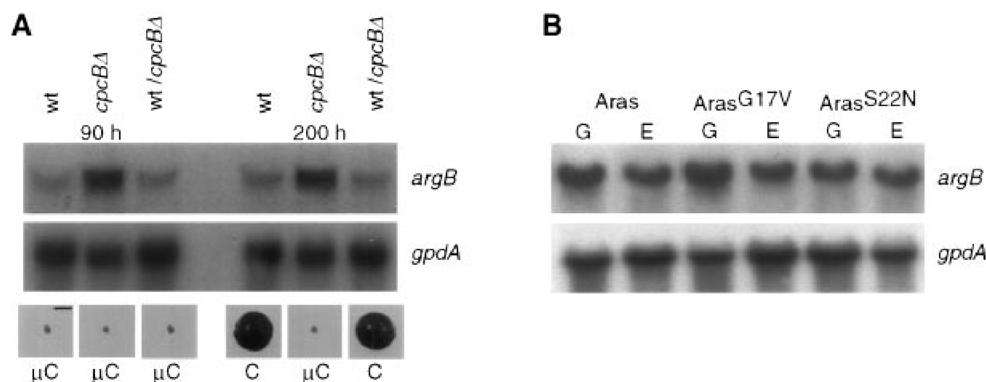

Fig. 7. Cross-pathway control in homokaryotic and heterokaryotic *A. nidulans* strains carrying different *cpcB* and *Aras* alleles.
A. Homokaryotic wild-type strain A234 (wt), the *cpcBΔ* mutant strain (*cpcBΔ*) and the A234/*cpcBΔ* heterokaryon (wt/*cpcBΔ*) were grown on solid minimal medium under oxygen limitation to induce sexual development. After 90 h or 200 h, cultures were harvested, and total RNAs were isolated. RNA amounts were equalized using the *gpdA* gene as internal standard. The cross-pathway control was analysed by monitoring the *argB* transcript levels. Fruit bodies were isolated after 90 h and 200 h and are shown below the mRNA levels. Mature cleistothecia (C) were fertile, whereas microcleistothecia (μC) were filled with hyphae and were sterile. Scale bar represents 100 μm.
B. The *A. nidulans* strains A986, A987 and A988 carrying an *alcA* promoter-driven wild-type *Aras* open reading frame (Aras), a constitutively active Aras^{G17V} allele (Aras^{G17V}) or an inactive GDP-bound form Aras^{S22N}, respectively, were grown overnight in glucose-containing medium. Mycelia were harvested, halved and shifted to fresh medium containing glucose (G) or ethanol (E) as sole carbon source. After an additional 8 h of incubation, mycelial RNA was isolated and probed against the *argB* gene. The *gpdA* gene was used as internal standard.

provide the developing cleistothecia with auxiliary functions such as laccase II activity.

The mammalian RACK1 *gene complements an* A. nidulans cpcB *mutation and permits the completion of sexual development*

Homokaryotic *A. nidulans* strains carrying a deletion in the *RACK1* homologue *cpcB* were unable to overcome the microcleistothecia state of sexual development. One intact copy of *cpcB* in a heterokaryotic *A. nidulans* strain was sufficient to complement the phenotype. $cpcB^{+}/cpcB\Delta$ strains completed sexual development, resulting in wild-type cleistothecia with normal amounts of fertile ascospores. In addition, one copy of the *cpcB* gene in a heterokaryotic *A. nidulans* strain was sufficient to reduce the expression of cross-pathway-regulated genes, e.g. *argB*, to levels found in wild type (Fig. 7A). These data indicate that the *cpcB* wild-type allele is dominant to a *cpcB* deletion.

Gβ-like proteins encoded by *RACK1*-like genes are highly similar throughout the eukaryotic kingdom. Therefore, we tested whether the mammalian *RACK1* gene permits the completion of the sexual life cycle of the *A. nidulans cpcB* mutant strain. *alcA* promoter-driven expression of *RACK1* restored the developmental defect in a *cpcBΔ* mutant strain. This resulted in normal-sized cleistothecia with 40 000–80 000 fertile ascospores after 200 h, as shown for the wild type in Fig. 3B. In addition, the *RACK1* protein was able to complement all growth phenotypes caused by the *cpcB* deletion.

One putative transcription factor specifically affecting the sexual development represents the *nsdD* gene product (Chae *et al*., 1995). Deletion of *nsdD* leads to a complete loss of fruit body formation. The transcript levels of *nsdD* were determined in a *cpcB* deletion mutant as well as in the wild type starved of amino acids by the addition of the analogue 3AT. Northern hybridization experiments revealed that *nsdD* transcription is not regulated by the cross-pathway control and is not affected by the presence or absence of the *cpcB* gene (data not shown).

The *Aras* gene encodes another protein important for developmental processes during the *A. nidulans* life cycle (Som and Kolaparthi, 1994). *Aras* alleles encoding constitutively active or inactive G-proteins were overexpressed to analyse whether there are effects on cross-pathway control-regulated genes. Overexpression of the *Aras* alleles tested resulted in an acleistothecial phenotype but did not affect the mRNA level of the cross-pathway-regulated gene *argB* (Fig. 7B), indicating that the Aras protein influences sexual development by a cross-pathway control-independent mechanism.

These results show that at least one copy of a *RACK1*-like gene encoding a Gβ-like protein has to be present in one of the nuclei of *A. nidulans* for a functional sexual cycle. The origin of this *RACK1*-like gene can be from any eukaryote, including mammalian cells.

Discussion

The growth and development of eukaryotic organisms is strongly influenced by environmental conditions. Specific conditions can induce or repress developmental processes. How such specific conditions control development on a molecular level and how the regulatory processes involved therein can be compared between different species from fungi to mammals are topics of intensive study.

In this paper, we show on a morphological and molecular level that starvation for amino acids leads to a specific block during early sexual development of the fungus *A. nidulans*. This specific developmental block could be induced by three different conditions, all leading to or mimicking amino acid starvation and thus activation of the cross-pathway control: (i) by applying the drug 3AT to the culture medium; (ii) by overexpressing members of c-Jun-like transcription factors; and (iii) by a chromosomal deletion of the *RACK1* homologue *cpcB*.

c-Jun-like proteins

c-Jun-like transcription factors represent a subgroup of the AP-1 family of proteins and have been shown to stimulate the expression of specific classes of genes in response to a wide variety of extracellular stimuli. In fungi, the yeast protein Gcn4p (Hinnebusch, 1997) and its counterparts from filamentous fungi, CPC1p from *Neurospora* (Paluh and Yanofsky, 1991) and CpcAp from *Aspergillus* (Wanke *et al*., 1997), represent the best studied members of the c-Jun subfamily. The fungal proteins regulate the availability of precursors for translation and co-ordinately activate the transcription of at least 50 genes involved in either amino acid and purine biosynthesis or in charging of tRNAs (Mirande and Waller, 1988; Mösch *et al*., 1991; Hinnebusch, 1992). In addition, at least the *GCN4*-mediated regulation in the yeast *S. cerevisiae* seems to extend even further into metabolism by regulating components of several multienzyme complexes at the entry and within the citric acid cycle of the carbon metabolism (Zaman *et al*., 1999).

It has also been shown that AP-1 transcription factors, such as Gcn4p in yeast or c-Jun in mammals, are activated in a remarkably similar manner through a Ras-dependent signalling pathway to protect against damage to cellular components other than DNA (Engelberg *et al*., 1994). Although a connection between the cross-pathway control and Aras protein function was not found in *A. nidulans*, defects in these transcription factors reduce the

fitness of a fungus and its ability to survive in a hostile environment. Accordingly, a strain of the parasitic fungus *Cryphonectria parasitica*, whose *c-Jun*-like *cpCPC1* gene is impaired, is ineffective in colonizing chestnut plant tissue (Wang *et al*., 1998). However, the morphological and developmental consequences of a functioning c-Jun-dependent response to environmental conditions are barely known. In this respect, it is interesting that *A. nidulans* can grow and develop asexual spores normally when stress conditions such as amino acid starvation are applied or mimicked by overexpression of *c-Jun*-like homologues, but is impaired in developing the more complex and energy-consuming fruit bodies. This links developmental signalling events to metabolic conditions on a molecular level and shows the strong functional conservation of c-Jun-like transcription factors.

RACK1*-like proteins*

The *RACK1*-like genes of fungi have – as the *c-Jun*-like homologues – originally been isolated by analysing metabolic processes. Mutations of the *RACK1*-like *Neurospora cpc-2* as well as the yeast *CPC2* gene affect the regulatory network controlling translational precursors (Müller *et al*., 1995; Hoffmann *et al*., 1999). In addition, *CPC2* (also named *ASC1*) has been found to be a suppressor of a *hap1 hem1*-deficient yeast strain with defects in anaerobic and haem-deficient growth (Chantrel *et al*., 1998). In *S. cerevisiae*, we have shown that Cpc2p is required for repression of Gcn4p activity in the absence of amino acid starvation (Hoffmann *et al*., 1999). Accordingly, we found here that a deletion of *cpcB* in *A. nidulans* leads to activation of cross-pathway-regulated genes and to a developmental block morphologically identical to the block occurring as a consequence of overexpression of *c-Jun* homologues or of applying amino acid starvation conditions. This analogy suggests the existence of a *c-Jun*-like homologue in *A. nidulans*, which, as in *S. cerevisiae*, is regulated by CpcB.

The mechanism leading to genetic repression of the c-Jun-like homologues by the *RACK1* homologue in *S. cerevisiae* and possibly also in *A. nidulans* and other organisms is still unknown. A hint may be given by the deduced three-dimensional structure of the RACK1-like proteins. These proteins represent the Gβ-like protein subfamily of Trp-Asp (WD) repeat proteins. WD proteins normally contain four to 16 repeats (Smith *et al*., 1999). Crystal structures of β-subunits of heterotrimeric G-proteins indicate that reiterated WD motifs form a symmetric doughnut-shaped structure, called a β-propeller. Each blade is made up of a small four-stranded twisted β-sheet, which might facilitate the assembly of multiprotein complexes (Sondek *et al*., 1996; Smith *et al*., 1999). In a search for such interaction partners of Gβ-like proteins, interactions have been identified in mammalian cells. There, RACK1 has originally been identified as a receptor of protein kinase C, suggesting a function in signal transduction (Ron and Mochly Rosen, 1995). Selective interactions have also been shown with a cAMP-specific phosphodiesterase (Yarwood *et al*., 1999), with the cytoplasmic, membrane-associated Src tyrosine kinases (Chang *et al*., 1998) or the integrin β-subunit domains (Lilienthal and Chang, 1998). These data suggest that RACK1-like proteins are able to recruit different proteins and therefore act as scaffolds for multiple signalling complexes. As the mammalian RACK1 protein is able to complement developmental defects of *A. nidulans*, such interactions with signalling molecules may also be conserved. Their identification could possibly lead closer to an explanation of how signalling events are coupled to the cross-pathway control and to development. Whether the function of *cpcB* is affected by the snoRNA U24 remains to be shown. A comparison of all genomic sequences of *RACK1*-like genes argues for a conserved localization of this snoRNA within one intron of Gβ-like genes.

Environmental stimuli and fruit body formation in A. nidulans

Cleistothecia formation in *A. nidulans* is a complex, time-, energy- and material-consuming developmental programme. The temporal location of the developmental control point described here is an interesting feature of fruit body formation. It is surprising that the signal transduction pathway that activates the *c-Jun*-like *cpcA* in response to the metabolic signal 'amino acid starvation' does not block cleistothecia formation at the initiation of the process, but within a narrow time window in the early middle of development. It is still unclear whether there is a cross-talk between the initiation point of sexual development, which is regulated by external stimuli (Zonnefeld, 1975; Champe *et al*., 1987; Yager, 1992; Yager *et al*., 1998), and the amino acid control point in the middle of development, although connections between the NsdD protein that is important for the initiation point of sexual development (Chae *et al*., 1995) and the cross-pathway control were not observed. It is also unknown how the deprivation of other nutrients might affect fruit body formation. The asexual programme seems to be induced by glucose and nitrogen starvation depending on the expression of regulatory genes such as *acoD* (Adams *et al*., 1992) or *brlA* (Skromne *et al*., 1995). Genes involved in sexual development and affected in their expression by an activated cross-pathway control are not known. Future studies should identify such genes to give further insights into the cross-talk between amino acid biosynthesis and sexual development.

At the time point of the block in development at the microcleistothecia state, hyphae are short and swollen within the fruit bodies. Such a morphological phenotype is described for the state of dikaryotic hyphae (Pontecorvo *et al.*, 1953). Therefore, an induced cross-pathway control seems to arrest the sexual development before or at an early time point after entry into karyogamy and meiosis. The biological advantage of a block in fruit body formation after amino acid starvation seems to be an economical consequence of an anticipated lack of building material. Whereas hyphal growth exploring new areas is hardly affected by stress conditions (Martinelli, 1976), the formation of complicated sexually reproductive structures is highly sensitive to external stimuli.

Experimental procedures

Strains, media and genetic techniques

A. nidulans strains A234 (*yA2*; *pabaA1*; *veA1*), A986 (*liA1*; *argB*$^+$*::palcA:ArasG17V*; *veA1*), A987 (*liA1*; *argB*$^+$*::PalcA:ArasS22N*; *veA1*), A988 (*liA1*; *argB*$^+$*::palcA:Aras*; *veA1*) and the specific *A. nidulans* cosmid library (Brody *et al.*, 1991) were provided by the Fungal Genetic Stock Center (FGSC, University of Kansas, USA). The *A. nidulans Aras* gene is integrated by an *alcA* promoter–*Aras* expression cassette in strain A988. Strain A986 contains a constitutively active *Aras*^{G17V} allele driven by the *alcA* promoter, while in strain A987, the *alcAp-Aras*^{S22N} allele is blocked in its constitutively inactive GDP-bound form (Som and Kolaparthi, 1994). Strain GR5 (*wA3*; *pyrG89*; *pyroA4*; *veA1*) was obtained from G. May (Houston, TX, USA). The diploid *A. nidulans* strain AGB67 was constructed from the haploid strains A234 and GR5 as described previously (Roper, 1952). Cultivation of *A. nidulans* strains was performed at 30°C on minimal medium (Bennett and Lasure, 1991). Transformation was carried out as described previously (Punt and van den Hondel, 1992). Transformants were selected either for the presence of the *ble* gene of *Streptoalloteichus hindustanus* causing phleomycin resistance on minimal medium containing 10 μg ml^{-1} phleomycin (Cayla, F) or on medium without uridine for the presence of the prototrophic marker *pyrG*. Expression of the *alcA* promoter was induced on media with 2% ethanol as sole carbon source. Amino acid starvation was induced by the addition of the histidine analogue 3-amino-1,2,4-triazole (3AT) at a concentration of 5 mM to solid medium and 10 mM to liquid medium. Cultures on 3AT plates were transferred at 2-day intervals to fresh 3AT plates because of the degradation of the analogue after prolonged incubation.

Isolation of the cpcB genomic and cDNA clone

Deduced Gβ-like protein sequences were aligned to identify specific and highly conserved amino acid regions. Amino acids GTTTRRFVGH in the third and PNRYWLCA in the sixth WD repeat were chosen to design the degenerated primers AC2F (5′-ACYCGYCGYTTCGTYGGYCACAC-3′) and AC2R (5′-GCRCAVAGCCAGTARCGG TTRGG-3′) to isolate and clone a 670 bp *cpcB* gene fragment from *A. nidulans* genomic DNA. This 670 bp DNA fragment was [α-^{32}P]-ATP and used as a probe in Southern analysis of genomic DNA of *A. nidulans* and to identify a genomic clone from a sublibrary of 4–5 kb *Eco*RI genomic DNA fragments in pBluescript SK+. The isolated plasmid pME1600 contained the complete *cpcB* gene and approximately 1.5 kb flanking sequences on both sides of the gene.

The cDNA was isolated by reverse transcribing the *cpcB* mRNA from total mRNA using a *cpcB*-specific primer binding at the UAG stop codon. The *cpcB* cDNA was then amplified by polymerase chain reaction (PCR) and cloned under the control of *cpcB* promoter and terminator sequences into the *Eco*RI site of pME1565, resulting in plasmid pME1599.

Plasmid construction

For the *cpcB* deletion cassette, a 1.85 kb *Eco*RI–*Stu*I DNA fragment from upstream of the *cpcB* ORF and a 1.2 kb *Eco*RV–*Eco*RI DNA fragment from downstream of the ORF were cloned in front of and behind the phleomycin resistance cassette in plasmid pAN8-1 (Punt and van den Hondel, 1992) respectively. This yielded plasmid pME1598. For deletion of the *cpcB* gene, this plasmid was cut with *Bam*HI and *Cla*I and transformed into *A. nidulans* strain AGB67.

The rat *RACK1* gene was isolated from a kidney cell line of rat (Z. Ngugen, unpublished) and cultivated in Dulbecco's modified Eagle medium (DMEM) cell media (ICN) supplemented with 5% bovine serum albumin (BSA). To isolate the corresponding cDNA, RNA was isolated with TRIZOL reagent (Gibco BRL), and *RACK1* cDNA was synthesized using Superscript reverse transcriptase (Gibco BRL) and a *RACK1*-specific primer binding downstream of the UAG stop codon. The ORF of *RACK1* cDNA was then amplified by PCR using *Pfu* polymerase (Stratagene). *RACK1* cDNA was inserted into the *Kpn*I site of the plasmid pME1565, resulting in plasmid pME1458. Plasmid pME1565 contains the *pyrG* gene as selectable marker and was constructed by exchange of the green fluorescence protein-encoding gene of pMCB32 (Fernandez-Abalos *et al.*, 1998) with the multiple cloning site of pBluescript SK+ (Stratagene). In plasmid pME1458, the *RACK1* ORF is surrounded by the *alcA* promoter and *his2B* terminator sequences.

The *cpcA* ORF of *A. niger* was amplified from a cDNA clone as template (Wanke *et al.*, 1997). Genomic DNA of *S. cerevisiae* was used as template to amplify the *GCN4* ORF. The *cpc-1* ORF of *N. crassa* was amplified in a PCR reaction using as forward primer the complete sequence of the first exon and binding at the beginning of the second. All ORFs were blunt ended with Klenow fragment and cloned into the *Sma*I restriction site downstream of the inducible *alcA* promoter of plasmid pME1565. Resulting overexpression plasmids for *cpcA*, *cpc-1* and *GCN4* were named pME1603, pME1604 and pME1605 respectively.

Strain constructions

Plasmid pME1598 carrying the *cpcB* disruption cassette was cut with *Bam*HI and *Cla*I and transformed into the diploid strain AGB67. Conidia of transformants were grown for 8 h

and tested for homologous or ectopic integration using three independent primers in parallel PCR experiments. One primer was located in the *cpcB* ORF, which should be deleted after homologous integration. A second primer corresponded to the *trpC* terminator of the *cpcB* disruption cassette, and a third primer hybridized to the *A. nidulans* DNA downstream of the 3′ genomic DNA fragment used for the construction of the *cpcB* disruption. Homologous recombination resulted in PCR products of 1.2 kb in length, whereas a 1.6 kb fragment was amplified after ectopic integration. Five heterozygotic *cpcBΔ* mutants out of 1800 transformants tested were isolated and characterized further by Southern hybridization experiments. Strain AGB19 exclusively contained the *cpcB* deletion and was haploidized on plates containing 50 $\mu g\ l^{-1}$ benomyl. Resulting haploid *cpcBΔ* mutants were retested in PCR and Southern hybridization experiments and resulted in the *cpcBΔ* strain AGB20.

For the isolation of the *A. nidulans cpcBΔ* mutant strain AGB21 containing an additional *pyrG* marker, *cpcBΔ* strain AGB20 (*cpcBΔ*; *pabaA1*; *veA1*) was crossed with GR5. The resulting ascospores were tested for growth on plates containing phleomycin (20 $\mu g\ ml^{-1}$) and 5-fluoro-orotic acid (70 $\mu g\ ml^{-1}$; Toronto Research Chemicals) to select for a *cpcBΔ*; *pyrG89* genotype.

The *cpcBΔ yA2* strain AGB22 was constructed by crossing of the *cpcBΔ* mutant strain AGB21 with the isogenic *yA* mutant strain A234. Ascospores were selected on plates containing phleomycin (20 $\mu g\ ml^{-1}$), and yellow-spored colonies were isolated.

cpcA, *cpc-1* and *GCN4* overexpression strains were constructed by transformation of plasmids pME1603, pME1604 and pME1605, respectively, into the *A. nidulans* strain GR5. Transformants were tested for single integration by Southern hybridization analysis.

The heterokaryotic *cpcB/cpcBΔ A. nidulans* strain was constructed by crossing *cpcB* wild-type strain A234 with the *cpcBΔ* mutant strain AGB21 and stabilized by selection against the auxotrophic markers *pabaA1* and *pyrG89* respectively.

Recombinant DNA techniques

Recombinant DNA techniques were used as described previously (Sambrook *et al.*, 1989). DNA sequencing was performed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using custom oligonucleotides (Gibco BRL) and the T7 sequencing kit (Pharmacia).

For Northern blot analysis, total RNA was isolated at the indicated time points with TRIZOL reagent (Gibco BRL). Total RNA (20 $\mu g\ lane^{-1}$) was separated on a formaldehyde agarose gel, electroblotted onto a nylon membrane (BiodyneB, PALL) and hybridized with [α-^{32}P]-ATP DNA fragments according to the oligolabelling technique (Feinberg and Vogelstein, 1984). The RNA ladder of Gibco BRL was used as the RNA size standard.

Protein methods

Protein contents were estimated as described previously (Bradford, 1976). Laccase activity of crude extracts was determined (Clutterbuck, 1972) using the chromogenic substrate DMP (Fluka). Initial rates were corrected for spontaneous oxidation of substrate. The OTCase protein activity of the *argB* gene product was measured in crude extracts as described earlier (Davis, 1962). Crude extracts were made from cultures grown overnight in liquid medium and from cultures grown on a Miracloth filter on plates inoculated with 10^4 conidia. Plates were sealed with Parafilm to induce sexual development by oxygen limitation.

Microscopic techniques

A. nidulans strains were grown on solid medium under oxygen limitation at 30°C to induce cleistothecia formation. Oxygen limitation was induced by taping Petri dishes with Parafilm. Full-size cleistothecia and colonies were viewed using a Zeiss Stemi 2000-C binocular (Jena). Developmental stages of cleistothecia formation were analysed by a Zeiss Axiovert S100 microscope (Jena) using Nomarski-DIC optics. Fluorescence microscopy with DAPI (Sigma) was performed as described previously (Pringle *et al.*, 1991).

Nucleotide sequence accession number

The DNA sequence of the 4.5 kb *Eco*RI genomic fragment containing the complete *cpcB* gene has been submitted to the GenBank sequence database (accession no. AF176775).

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