Kapl, a non-essential member of the Pse1p/Imp5 karyopherin family, controls colonial and asexual development in *Aspergillus nidulans*

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Asexual development in the filamentous fungus *Aspergillus nidulans* is governed by the timely expression and cellular localization of multiple transcription factors. Hence, factors mediating import and export across the nuclear pore complexes (karyopherins) are expected to play a key role in coordinating the developmental programme. Here we characterize Kapl, a putative homologue of the *Saccharomyces cerevisiae* Kap121/Pse1p karyopherin. Kapl is a non-essential importin- β -like protein located in the nucleus during vegetative growth and conidiophore development. The $\Delta kapl$ phenotype is aconidial with many aerial hyphae. This phenotype can be suppressed under abiotic stress. In this regard, it resembles that of the null allele of the bZIP transcription factor FlbB. However a $\Delta flbB$; $\Delta kapl$ double mutant exhibited an additive phenotype with totally impaired conidiation, unresponsive to abiotic stress. In contrast to $\Delta flbB$, the null $kapl$ mutant is not a fluffy-low-bristle expression mutant. Taken together the findings indicate that Kapl is required during asexual development, mediating the nuclear transport of factors acting in a different pathway(s) from those involving the upstream developmental activators.

Received 10 July 2009
Revised 28 August 2009
Accepted 1 September 2009

INTRODUCTION

In *Aspergillus nidulans*, the induction of the key transcription factor (TF), BrLA, activates the central developmental pathway of conidiation (Prade & Timberlake, 1993), which includes the sequential formation of the different cellular modules that form the conidiophore: foot-cell, stalk, vesicle, metulae and phialides (primary and secondary sterigmata), and finally asexual spores or conidia. Upstream developmental activators (UDAs) are expressed in vegetative cells, and are involved in sensing environmental stimuli and generating signals that activate *brLA* expression (Adams *et al.*, 1998; Kües & Fischer, 2006). Mutations in most UDA genes generate a phenotype commonly known as 'fluffy', which shows delayed or impaired conidiation (Etxebeste *et al.*, 2009, and references therein). Many UDAs are putative TFs (Adams *et al.*, 1998; Etxebeste *et al.*, 2008; Seo *et al.*, 2006; Wieser & Adams,

1995). The best-known member of this family is the bZIP-type TF FlbB, which is localized in the most apical nucleus of vegetative hyphae, and remarkably, in the *Spitzenkörper* (Etxebeste *et al.*, 2008, 2009; Garzia *et al.*, 2009). However, the mechanisms which regulate FlbB function, and that of other UDAs, remain to be clarified. In the case of those which are TFs, this includes nuclear transport.

In eukaryotes, macromolecule trafficking between cytoplasm and nucleus involves nuclear import and export pathways. This bidirectional transport occurs through nuclear pore complexes (NPC; Fried & Kutay, 2003), which are embedded in the nuclear envelope. Karyopherins are a family of nuclear transporters involved in this selective and active transport (Stewart, 2007). Most karyopherins belong to the superfamily defined by importin- β , and 22 and 15 such proteins have been identified in mammals and *Saccharomyces cerevisiae*, respectively (Mosammaparast & Pemberton, 2004; Strom & Weis, 2001). The genome of *Aspergillus nidulans* is predicted to contain 14 importin- β -type coding genes (Espeso & Osmani, 2008).

Our previous work showed noteworthy similarities between the FlbB and Pap1 or Yap1, two bZIP-type TFs

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Abbreviations: TF, transcription factor; UDA, upstream developmental activator.

A supplementary figure showing multiple alignments of Kapl-like karyopherins is available with the online version of this paper.

from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (Ettxebeste *et al.*, 2009; Glover & Harrison, 1995; Isoyama *et al.*, 2001). Moreover, FlbB was capable of binding the same target sequences as those recognized by Pap1 at the p25 gene promoter from *Schiz. pombe* (Ettxebeste *et al.*, 2009; Toda *et al.*, 1992). In addition, the FlbB amino acid sequence contained a conserved KRRG motif, also contained within the NLS recognized by karyopherin Kap121p/Pse1p at the *S. cerevisiae* regulator Pho4p (Kaffman *et al.*, 1998; Timney *et al.*, 2006). This work analyses the hypothetical requirement of the Kap121p/Pse1p homologue from *A. nidulans*, KapI, for the nuclear import of FlbB, and describes the role of this karyopherin in polarized hyphal growth, branching and conidiophore development.

METHODS

Strains, standard techniques and culture conditions for *A. nidulans*. The strains of *A. nidulans* employed in this study (Table 1) were obtained by crossing or transformation following standard protocols (Ettxebeste *et al.*, 2008). Strain BD212 was obtained by meiotic recombination between strains TN02A25 and MAD2159. BD212 was then crossed with BD143, BD167 and BD185, to obtain strains BD223, BD230 and BD231, respectively. Strain MAD2446 expresses protein H1::mCh (*hhoA* locus; Ramon *et al.*, 2000). Strains MAD2334 and MAD2654 were obtained by the transformation of BD167 (Ettxebeste *et al.*, 2008) and MAD2446, respectively, with the fusion PCR products to generate C-terminal *mCherry* or *gfp*-tagging of the KapI protein by gene replacement (Ettxebeste *et al.*, 2008; Yang *et al.*, 2004).

Phenotypic analyses under stress growth conditions were done using *Aspergillus* minimal medium (MMA) as in Ettxebeste *et al.* (2008). Strains were point inoculated and cultivated at 37 °C for 72 h in appropriately supplemented MMA. For carbon or nitrogen starvation studies, either glucose or nitrate/ammonium content was reduced to one-tenth. For other stresses KCl (0.6 M), NaH₂PO₄ (0.5 M), sorbitol (1.2 M) or sucrose (1.0 M) was added to MMA. Experiments in liquid media were performed essentially as described by Garzia *et al.* (2009). Briefly, strains were cultivated in supplemented MMA for

18 h at 37 °C, and then the mycelium was transferred to different stress conditions (see text) in MMA and cultured for an additional 20 h.

Samples for the microscopic analysis of FlbB, KapI and histone H1 localization, both in vegetative hyphae and during conidiophore development, were prepared essentially as described previously (Ettxebeste *et al.*, 2009).

For the quantification of conidiospore production five solid MMA plates were point inoculated with each strain and incubated for 72 h at 37 °C. Conidia from 1 cm² at the centre of each colony were collected and counted using a haemocytometer. Values reported are the means $\pm$ SEM of the five replicates.

Light and fluorescence microscopy. *In vivo* imaging of strains expressing GFP and mCherry-labelled FlbB, KapI or H1 proteins was performed using a Leica DMI6000b microscope, equipped with a 63 $\times$ Plan Apo 1.4N.A. objective and a 100 W mercury lamp. GFP was observed using a specific filter (excitation 470 nm, emission 525 nm). For observation of mCherry (mCh) a Txred (excitation 562 nm, emission 624 nm) filter was used. Images were recorded with an ORCA-ER digital camera (Hamamatsu Photonics) and processed with Metamorph (Universal Image) software.

Liquid cultures or samples for *G* and μ measurements were examined with a Nikon Optiphot microscope under bright-field optics. Colony surface examination on solid cultures was performed with a Nikon SMZ800 binocular microscope. In both cases images were recorded with a Nikon FX-35DX or Nikon Coolpix 8400 Digital camera.

Measurement of kinetic parameters. Radial extension rate (K_r ; radial extension/time, in $\mu\text{m h}^{-1}$) was determined by established methods (Trinci, 1974; Trinci *et al.*, 1994). Briefly, colony radii were calculated from digital images of colonies taken at regular intervals during cultivation as above (mean of five radii). K_r values were obtained as the slope of the radius versus time.

Branching frequency was measured in terms of the hyphal growth unit (*G*: mycelium length/number of tips; Trinci, 1974; Trinci *et al.*, 1994). In germlings (G_g), 10³ conidia were loaded onto 1 cm² squares of nitrocellulose membrane placed on the surface of solid minimal medium, thus only allowing for bi-dimensional growth. After 10 h of culture, the membranes were deposited on a slide, covered with a coverslip and observed with a Nikon AFX-DX microscope under bright-field optics. The branching frequency was also measured at the

Table 1. Strains used in this study

Strain	Genotype	Source
TN02A3	<i>pyrG89; $\Delta nkuA::argB$; argB2; pyroA4; veA1</i>	Nayak <i>et al.</i> (2006)
TN02A25	<i>pyrG89; $\Delta nkuA::argB$; argB2; pabaB22; riboB2; veA1</i>	Nayak <i>et al.</i> (2006)
MAD2159	<i>pyrG89; $\Delta nkuA::argB$; argB2; pyroA4; $\Delta kapI::pyrG$; veA1</i>	This study
MAD2334	<i>pyrG89; $\Delta nkuA::argB$; argB2; pyroA4; flbB::gfp::pyrG; KapI::mCh::pyroA; veA1</i>	This study
MAD2446	<i>pyrG89; wA4; inoB2, pyroA4; hhoA::mCh::pyroA; (Myc)3-PacC(PacC900)</i>	This study
MAD2654	<i>pyrG89; wA4; inoB2, pyroA4; hhoA::mCh::pyroA; kapI::gfp::pyrG; (Myc)3-PacC(PacC900)</i>	This study
BD143	<i>pyrG89; $\Delta nkuA::argB$; argB2; $\Delta flbB::pyrG$, pyroA4; veA1</i>	Ettxebeste <i>et al.</i> (2008)
BD167	<i>pyrG89; $\Delta nkuA::argB$; argB2; flbB::gfp::pyrG, pyroA4; veA1</i>	Ettxebeste <i>et al.</i> (2008)
BD185	<i>pyrG89; $\Delta nkuA::argB$; argB2; gfp::flbB, pyroA4; veA1</i>	Ettxebeste <i>et al.</i> (2008)
BD212	<i>pyrG89; $\Delta nkuA::argB$; argB2; pabaB22, $\Delta kapI::pyrG$; veA1</i>	This study
BD223	<i>$\Delta nkuA::argB$; $\Delta flbB::pyrG$, pyroA4; $\Delta kapI::pyrG$; veA1</i>	This study
BD230	<i>pyrG89; $\Delta nkuA::argB$; flbB::gfp::pyrG, pyroA4; $\Delta kapI::pyrG$; veA1</i>	This study
BD231	<i>pabaA1, pyrG89; $\Delta nkuA::argB$; gfp::flbB, $\Delta kapI::pyrG$; veA1</i>	This study

peripheral region of mature colonies (G_p) by determining the average hyphal length between any two branching points. This was done by measuring lactophenol-blue-stained hyphae from the apical tip backwards and scoring the number of branching points (Reeslev & Kjoller, 1995; Vesentini *et al.*, 2006). In all cases, G values were obtained as the mean of 20 measurements. The specific growth rate (μ) was calculated from dry weight measurements of biomass collected at different incubation times in liquid cultures (Trinci, 1974; Trinci *et al.*, 1994).

Isolation and manipulation of nucleic acids. DNA and RNA samples for Southern and Northern blot experiments were isolated and manipulated as described previously (Etxebeste *et al.*, 2008; Garzia *et al.*, 2009).

RESULTS

Kapl is related to importin 5 and Kap121p/Pse1p nuclear transporters

In silico searches had already identified gene *kapI/AN5717.3* as the putative homologue of the *S. cerevisiae* nuclear transporter Kap121p/Pse1p (Espeso & Osmani, 2008). The *AN5717.3* locus maps within contig 98 of chromosome V, encoding a 1096 amino acid protein called KapI. An importin- β N-terminal domain (Pfam, PF03810) is predicted between amino acids 30 and 111 (grey rectangle in Fig. 1a), which might be involved in RanGTPase interaction (Chook

Fig. 1. Domain structure and evolutionary analysis of KapI. (a) Domain structure of the KapI chain. Predicted Ran-binding and HEAT domains are represented by light and dark grey filled rectangles, respectively, with amino acid sequence coordinates indicated above. (b) Phylogenetic tree of KapI putative homologues. Analyses were conducted using MEGA software, version 4 (neighbour-joining method, with a bootstrap of 100 000 replicates and amino p-distance substitution model). To root the tree *A. nidulans* KapA (importin α) and KapB (importin β) proteins were used. On the right are indicated the three main branches from the tree: KapI-, Pse1p- and Imp5-like proteins. Abbreviations: Afum, *Aspergillus fumigatus*; Aory, *Aspergillus oryzae*; Cimm, *Coccidioides immitis*; Acao, *Ajellomyces capsulatum*; Pnod, *Phaeosphaeria nodorum*; Sscl, *Sclerotinia sclerotiorum*; Bfuc, *Botryotinia fuckeliana*; Mgri, *Magnaporthe grisea*; Ncra, *Neurospora crassa*; Pans, *Podospira anserina*; Cglo, *Chaetomium globosum*; Spom, *Schizosaccharomyces pombe*; Scer, *Saccharomyces cerevisiae*; Calb, *Candida albicans*; Psti, *Pichia stipitis*; Dhan, *Debaryomyces hansenii*; Xlae, *Xenopus laevis*; Hsap, *Homo sapiens*; Mmus, *Mus musculus*. *A. nidulans* KapI (Anid-KapI) is marked by a black rectangle.

& Blobel, 1999). Domain searches also predicted the presence of five HEAT (huntingtin-elongation-A subunit-TOR) motifs (Groves *et al.*, 1999) distributed throughout KapI as indicated in Fig. 1(a) (dark rectangles). This agrees with reports describing members of the importin- β superfamily to contain multiple HEAT repeats (Petosa *et al.*, 2004).

Phylogenetic analyses revealed that KapI homologues are found among eukaryotes ranging from fungi to metazoans. Three main branches are clearly differentiated from the phylogenetic tree (Fig. 1b). One corresponds to importin 5 (Imp5) karyopherins from higher eukaryotes. The remaining two branches arise from a common origin and differentiate yeast Kap121p/Pse1p-like proteins and filamentous fungal KapI-like proteins. The high degree of amino acid sequence conservation among these proteins (Supplementary Fig. S1), with a similarity higher than 50% in all cases, suggests that the mechanisms governing Pse1p/Imp5/KapI nuclear import pathways are highly conserved. However, while Pse1p has been described as an essential nuclear transporter of *S. cerevisiae* (Seedorf & Silver, 1997), the strain carrying the null allele of *kapI* in *A. nidulans* was viable (Fig. 2a).

KapI has a role in polar growth and branching

The $\Delta kapI$ strain showed a significantly lower extension rate (K_r $215.9 \pm 3.3 \mu\text{m h}^{-1}$) than the wild-type strain (K_r

$261.8 \pm 2.9 \mu\text{m h}^{-1}$), representing a 17.5% reduction (Table 2, Fig. 2a). An analysis of the branching frequency, measured as the average length of hyphae between any two branches (G , μm), showed that the mutant strain had about twice the branching frequency of the wild-type, both in germlings (G_g ; Table 2, Fig. 2b) and at the periphery of mature colonies (G_p ; Table 2, Fig. 2c). G_g decreased from $82.9 \pm 1.6 \mu\text{m}$ in the wild-type to $38.7 \pm 0.6 \mu\text{m}$ in the mutant strain ($n=20$ germlings for each strain), while G_p decreased from $303.9 \pm 15.6 \mu\text{m}$ to $150.8 \pm 6.2 \mu\text{m}$ ($n=10$ hyphae in two colonies of each strain). In order to determine whether the increase observed in the branching frequency could account for the recorded differences in K_r alone without effecting changes in specific growth rate (μ), this kinetic parameter was measured in both wild-type and $\Delta kapI$ strains. The mutation resulted in a 20% reduction of μ , from $0.0852 \pm 0.0169 \text{ h}^{-1}$ in the wild-type to $0.0687 \pm 0.0079 \text{ h}^{-1}$ in the $\Delta kapI$ strain (Table 2). Therefore, the reduced radial extension rates of $\Delta kapI$ mutant colonies was due to the combined effect of increased branching rate and reduced specific growth rate.

KapI is required for proper asexual development

Although some conidiophore development was detectable at the centre (aged part) of $\Delta kapI$ colonies after 72 h of

Fig. 2. Branching pattern of the $\Delta kapI$ mutant. (a) Comparison of colonies from the $\Delta kapI$ mutant (right) and its isogenic wild-type strain (left) grown for 72 h on solid MMA at 37 °C. Scale bar, 2 cm. (b) Comparison of hyphal morphology in the two strains after 10 h culture at 37 °C below pieces of nitrocellulose dialysis membrane (see Methods). The $\Delta kapI$ mutant shows higher branching (lower G). Scale bar, 30 μm . (c) Photomicrographs showing the peripheral region of wild-type and $\Delta kapI$ mutant colonies in solid medium. The latter shows higher hyphal density and branching, giving decreased G values in comparison to the wild-type strain (see Table 2). Scale bar, 100 μm .

Table 2. Radial extension rate (K_r), hyphal growth unit in germlings (G_g) and at the periphery of the colony (G_p), and specific growth rate (μ) of wild-type and $\Delta kapI$ strains

All parameters were calculated as described in Methods and are means $\pm$ SEM of multiple measurements as described in Results.

Strain	K_r ($\mu\text{m h}^{-1}$)	G_g (μm)	G_p (μm)	μ (h^{-1})
TN02A3	261.8 ± 2.9	82.9 ± 1.6	303.9 ± 15.6	0.0852 ± 0.0169
$\Delta kapI$	215.9 ± 3.3	38.7 ± 0.6	150.8 ± 6.2	0.0687 ± 0.0079

culture, their pattern of conidiation was notably reduced (Fig. 2a, Fig. 3, first row). Conidia production was reduced by two orders of magnitude compared to the isogenic wild-type strain ($2.18 \times 10^5 \pm 0.76 \times 10^5$ and $3.97 \times 10^7 \pm 0.56 \times 10^7$ conidia cm^{-2} respectively; $n=5$ for each strain).

Since the conidiation pattern of $\Delta kapI$ was similar to that shown by mutants from the upstream developmental pathway of conidiation (Etxebeste *et al.*, 2008, 2009; Garzia *et al.*, 2009), we analysed the aconidial phenotypes of single and double null $kapI$ and $flbB$ strains on solid media under different stress conditions which are known to promote conidiation (Etxebeste *et al.*, 2008, 2009; Garzia *et al.*, 2009). In synthetic complete minimal medium, both $\Delta flbB$ and $\Delta kapI$ strains showed an aconidial phenotype, although the former was more prominently fluffy (Fig. 3, first row). A similar conidiation pattern of both $\Delta flbB$ and $\Delta kapI$ single mutant strains was observed when the concentration of carbon or nitrogen was reduced to 1/10 (Fig. 3, rows 2–4). Divergent responses were observed under salt stress: KCl reversion of the $\Delta flbB$ aconidial phenotype (Etxebeste *et al.*, 2008) was not observed in the $\Delta kapI$ colony, but conidiation was induced in both strains in the presence of an extracellular excess of dihydrogen phosphate anion (Fig. 3, rows 5 and 6). Under osmotic stress, induced by addition of sorbitol or sucrose, the $\Delta kapI$ strain did not show the autolytic phenotype exhibited by the $\Delta flbB$ mutant in the central region of the colony. Conversely, conidiation was induced (Fig. 3, rows 7 and 8). Under the conditions analysed, the double ($\Delta kapI$; $\Delta flbB$) strain showed an additive phenotype, displaying an extreme aconidial phenotype even in high-phosphate medium. Under osmotic stress, the central autolytic region was also visible in the double null as in the single $\Delta flbB$ strain.

The conidiation patterns of $\Delta kapI$ and double mutant strains were also assessed in liquid cultures (see Methods) (Fig. 4). Cultures transferred to MMA maintained an undifferentiated vegetative growth pattern (Fig. 4, first row). Under glucose starvation, both $\Delta kapI$ and wild-type strains generated simplified conidiophores (Etxebeste *et al.*, 2008; Garzia *et al.*, 2009; Fig. 4, second row), consisting of phialide-like structures with a single conidium at their end; however, the $\Delta flbB$ mutant produced narrow hyphae, approximately 1.5 μm in diameter, from which single

conidium-like structures rarely emerged (Fig. 4, second row; Etxebeste *et al.*, 2008).

Nitrogen starvation revealed additional phenotypic differences between $\Delta flbB$ and $\Delta kapI$ strains. Whereas no conidiation response and an autolytic phenotype were found in $flbB$ mutants (Fig. 4, third row; Etxebeste *et al.*, 2008), the $\Delta kapI$ strain produced anomalous conidiophores in which some metulae were abnormally lengthened and did not differentiate into phialides (Fig. 4, third row). Salt stress (0.6 M KCl) induced the formation of fully developed conidiophores in the wild-type strain while the $\Delta kapI$ strain could not induce conidiation, as occurred with all $flbB$ mutants previously tested (Fig. 4, fourth row; Etxebeste *et al.*, 2008). On the other hand, for the wild-type and mutant strains, addition of 0.5 M dihydrogen phosphate anion caused the formation of conidiophores with abnormally swollen vesicles, twice the size of those found in standard medium (Fig. 4, fifth row; $n=20$). Most notably, under any growth condition, cells from the double mutant strain did not differentiate into conidiophores, implying the requirement of both FlbB and KapI for the development of any class of conidiating structures. The additive phenotype of the double null strain in either solid or liquid media suggested that FlbB and KapI might be acting on different regulatory pathways mediating conidiophore development.

FlbB nuclear import does not require KapI

In agreement with its predicted karyopherin function, KapI showed a nuclear localization (Fig. 5a). The KapI::GFP fusion protein had a distribution rather similar to that described for the *S. cerevisiae* Pse1p karyopherin, as punctate accumulations in the nucleus, some of which were probably in close relationship with the nuclear envelope (Seedorf & Silver, 1997). All nuclei displayed the same localization for KapI::GFP, predominantly nuclear, with some fluorescence also appearing in the cytoplasm (Fig. 5a).

Given that preliminary results pointed to FlbB as a possible cargo for KapI (see Introduction), we analysed the localization of the chimeric FlbB::GFP protein in a $\Delta kapI$ background. Similar localization of FlbB::GFP was observed in either wild-type or $\Delta kapI$ backgrounds (Fig. 5b). KapI (as a KapI::mCh fusion protein) localization barely coincided with that of FlbB::GFP (Fig. 5c). KapI located mainly at the nuclear periphery of the most apical nucleus (see graph in Fig. 5d), where nuclear FlbB is predominantly observed. In addition, this karyopherin was absent from the tip of the cell, where FlbB also accumulates (Fig. 5e). Neither pull-down assays nor bimolecular fluorescence complementation (BiFC) analyses provided any evidence of interaction between KapI and FlbB (not shown). These experiments showed that FlbB nuclear import and tip accumulation were independent of the KapI transport pathway.

Fig. 3. Analysis of conidiation capacity and colony morphology of *Δkapl*, *ΔflbB* and double null mutants in solid media. The photographs on the left show the phenotypes of wild-type, *Δkapl*, *ΔflbB* and the double null (*ΔflbB; Δkapl*) strains grown for 72 h at 37 °C on solid MMA (row 1), MMA with 1/10 the normal concentration of glucose (row 2), ammonium (row 3) or nitrate (row 4), and on MMA with KCl (0.6 M; row 5), NaH_2PO_4 (0.5 M; row 6), sorbitol (1.2 M; row 7) or sucrose (1.0 M; row 8). Scale bar, 2 cm. The bar diagrams on the right show spore production of each strain in each medium assayed (means $\pm$ SEM of five replicates).

Fig. 4. Stress induction of conidiating structures in liquid media, in mycelia from wild-type, $\Delta kapI$, $\Delta flbB$ and the double null ($\Delta flbB$; $\Delta kapI$) strains. Strains were cultured for 18 h at 37 °C in MMA, followed by transfer for an additional 20 h to MMA without glucose (row 1) or nitrate (row 2), or with KCl (0.6 M; row 3) or NaH_2PO_4 (0.5 M; row 4). Arrowheads indicate the structures formed in each case. Scale bar, 30 μm .

***brlA* is expressed in the absence of KapI activity**

UDA factors are positively required for *brlA* transcription (see Introduction) and some of the mutants in this regulatory pathway are called *flb* due to the fluffy phenotype associated with a low *brlA* expression level (Wieser *et al.*, 1994). In contrast to $\Delta flbB$ (Etxebeste *et al.*, 2008), or to other *flb* mutants such as $\Delta flbE$ (Garzia *et al.*, 2009), we detected *brlA* transcription in the $\Delta kapI$ deletion strain (Fig. 6). This result supports our assumption that *kapI* is not a member of the *flb* gene class, and therefore strongly backs our conclusion that KapI must exert its role on the regulation of conidiation by acting in a pathway different from that mediated by the Flb proteins.

Cellular localization of KapI is maintained during conidiophore development

As KapI is required for conidiophore development, we followed KapI::GFP localization in the different cell modules that form the conidiophore: stalk, vesicle, metulae, phialides and conidia (Fig. 7). For this purpose we constructed a strain expressing both KapI::GFP and the histone H1 fused to the monomeric Cherry fluorescent protein (mCh). We used H1::mCh as an *in vivo* positional marker of nuclei. In our conditions for induction of asexual development (Etxebeste *et al.*, 2009), we tracked the distributions of nuclei during the different stages of conidiophore morphogenesis, which

Fig. 5. Distribution of KapI and FlbB in vegetative cells. (a) KapI::GFP localization in nuclei and cytoplasm from vegetative hyphae. Scale bar, 5 μm. (b) FlbB::GFP localization (left panels) compared to that observed in a $\Delta kapI$ genetic background (right panels). The arrowhead and the arrow indicate the tip and the nucleus, respectively. Scale bar, 5 μm. (c) KapI::mCh (red) and FlbB::GFP (green) localization in vegetative hyphae. Vertical bars and numbers in the bottom right panel indicate the start and end points for the line profiles shown in the graphs in (d) and (e). Scale bar, 5 μm. (d, e) KapI::mCh (red) and FlbB::GFP (green) relative fluorescence intensities at the hyphal tip and the apical nucleus, respectively.

Fig. 6. *brlA* expression in vegetative and asexual cycles. *brlA* expression at 18 h of vegetative growth and 6, 12, 24 and 48 h after the induction of asexual development for $\Delta kapl$ and wild-type isogenic strains. Loading control is shown by the ethidium-bromide-stained ribosomal 40S subunit.

were comparable to those observed before using electron microscopy (Mims *et al.*, 1988). Nuclei were evenly distributed along the stalk and concentrated at the vesicle due to the presence of large vacuoles (Fig. 7a). In these conditions, KapI was observed in all the nuclei, from the multinucleate stalk and vesicle to the mononucleate metulae, phialides and conidia, in all cases following a similar location to that described above in vegetative cells (see Fig. 7b for a general view and Fig. 7c for higher magnifications). The presence of KapI in the nucleus during all the stages of conidiophore formation is consistent with the requirement of this nuclear transport pathway during the process of conidiation.

Fig. 7. KapI localization in conidiophores. (a) Localization of KapI::GFP (green) and H1::mCh (red; positional marker for nuclei) in an immature conidiophore bearing a stalk, vesicle and metulae. KapI is accumulated mainly at the nuclear envelope but is not excluded from the nucleoplasm. The region of fluorescence exclusion seen in most nuclei is probably the nucleolus. 'Vac' in the top left panel indicates the vacuole-like structures in the stalk and vesicle. Scale bar, 10 μ m. (b) Localization of KapI::GFP (green) and H1::mCh (red) in a mature conidiophore. KapI is also observed in phialides and conidia. The arrows marked V, M+P and C in (a) and (b) indicate the vesicle, metulae plus phialides and conidium, respectively, that are shown magnified in (c). Scale bar, 20 μ m. (c) Magnification of the differentiated cells marked in (a) and (b). Scale bars: 4 μ m for the pictures showing the vesicle, 5 μ m for metulae and phialides, and 3 μ m for the conidium.

DISCUSSION

In this work we report the characterization of KapI, the *A. nidulans* Imp5/Kap121p/Pse1p karyopherin homologue. This family of transporters controls general and varied processes in all eukaryotic organisms. In humans, Imp5 regulates, for example, neuronal differentiation and degeneration by the import of p35 protein (Fu *et al.*, 2006), chromosome structure by the import of core histones (Baake *et al.*, 2001), and specific DNA recombination processes through RAG-2 binding (Ross *et al.*, 2003). In yeast, Kap121p/Pse1p is an essential protein that regulates multiple pathways such as the phosphate stress response via Pho4p import (Kaffman *et al.*, 1998), the oxidative stress response via Yap1 (Isoyama *et al.*, 2001), membrane biogenesis by Pdr1 (Delahodde *et al.*, 2001), and both mating response and pseudohyphal transition mediated by Ste12 (Leslie *et al.*, 2002). From our study in *A. nidulans*, evidence of different levels of control has emerged. KapI does not exclusively participate in essential import or export pathways in this fungus. However, it may participate in the regulation of the nuclear transport of an undetermined protein required for polarized growth. Our recent work on this topic showed the involvement of the TF FlbB not only in conidiation but also in branching. While FlbB is involved in branching, but not in extension growth (Etxebeste *et al.*, 2009), KapI is required for both processes; nevertheless KapI is not essential in either process.

Notably, KapI also plays an important role in asexual differentiation. Although $\Delta kapI$ is not an *flb* mutant, as *brlA* is expressed, the absence of this nuclear importer in the fungal cell largely prevents normal conidiophore production. Differences were found between the aconidial phenotype of null $\Delta kapI$ and the fluffy phenotype of $\Delta flbB$ in response to different conidiation-inducing stimuli. Moreover, the $\Delta kapI$ mutant has in common with other fluffy mutants such as $\Delta fluG$ that it does not participate in the autolytic process in which FlbB is involved in response to osmotic stress induced by sorbitol or sucrose (Etxebeste *et al.*, 2009). These results lead us to propose that KapI and FlbB may act in different regulatory pathways, or that the latter is transported to the nucleus by more than one karyopherin. The additive phenotype of the double null ($\Delta kapI$; $\Delta flbB$) mutant, in which conidiation is completely blocked, suggests that both pathways must be coordinated to activate conidiation in response to inducing media conditions.

In diverse transport processes, Kap121p/Pse1p requires coordination with Kap123p (Isoyama *et al.*, 2001; Rout *et al.*, 1997; Schaper *et al.*, 2005; Seedorf & Silver, 1997). In *A. nidulans*, a redundancy between KapI and KapJ (Kap123p putative homologue) could be also proposed. However, the absence of evidence precludes the assumption of a concerted action between those two karyopherins in the conidiation regulatory pathway, as the $\Delta kapJ$ strain does not display an aconidial phenotype and no interaction has been detected between KapJ and FlbB in pull-down analyses (our unpublished results).

It is important to underline the high number of TFs that participate in conidiation signalling, either in the UDA pathway or in the central regulatory pathway governed by *brlA* (Kües & Fischer, 2006). Four putative TFs have been previously characterized in the first pathway: FlbB, FlbC and FlbD (Adams *et al.*, 1998), and SfgA, a hypothetical repressor of Flb factors in the genetic cascade that leads to *brlA* induction (Seo *et al.*, 2006). The results obtained in this work lead us to propose that KapI is not the importer of FlbB. In addition, as *brlA* is expressed in the null $\Delta kapI$ background but not in *flbD* null (Wieser & Adams, 1995) or *flbC* mutants (Wieser *et al.*, 1994), we discard the possibility of an exclusive import of those two TFs by KapI.

The role of KapI is also extended to the production of the different modules that form the conidiophore. We observed its presence at the nuclear envelope in stalks, vesicles, metulae, phialides and conidia. It may be postulated that KapI could regulate general pathways during these stages, but also conidiation-specific processes, especially at the metula stage, where its participation seems to become essential. Under nitrogen-starvation conditions in liquid cultures, the $\Delta kapI$ mutant had abnormally elongated metulae without any differentiation into phialides, while the wild-type strain produced fully developed conidiophores. We have recently described that a control point already exists at the metula–phialide transition stage and that conidiophore development in a wild-type strain can be reverted by changing media conditions, generating hyphae-like structures that can be indefinitely elongated (Etxebeste *et al.*, 2009). Besides *BrlA*, other previously characterized TFs regulate the central developmental cascade of conidiation, as for example the bZIP-like *AbaA* (Sewall *et al.*, 1990) or *StuA* (Dutton *et al.*, 1997). Their mutants produce aberrant conidiophores altered at the metula or phialide stage. KapI could participate in the control point proposed at the metula stage, having a key role in the import of any of these TFs or unidentified additional TFs. Future identification of KapI interactors will constitute a novel approach to the understanding of this important dispersion mechanism of economically and medically important *Aspergillus* species.

ACKNOWLEDGEMENTS

This work was supported by the Spanish Ministerio de Educación y Ciencia through grants BFU2006-04185 and BFU2004-03499/BMC to E.A.E. and U.O.U., respectively, and by the University of The Basque Country (UPV/EHU) for grant GIU08/32 to U.U. A.G. and E.H.-G. held predoctoral fellowships from the Basque Government. A.M.-I. held a predoctoral FPI fellowship from the Ministerio de Educación y Ciencia. O.E. was a contract researcher of the CIC Nanogune, and currently holds a research contract associated with grant BFU2006-04185 at the CIB.

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Edited by: N. L. Glass