Integrity of a Zn finger-like domain in SamB is crucial for morphogenesis in ascomycetous fungi

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Genetic features determine the site of polarized growth in filamentous fungi and lead to hyphal tip extension or subapical branching. We have isolated the samB gene (suppressor of anucleate metulae) of Aspergillus nidulans which encodes a 66 kDa protein carrying an atypical Cys4 and an additional Cys2/His/Cys Zn finger motif at the carboxy-terminus. Such novel Zn finger-like domains have recently been found in several other developmental regulators in organisms ranging from yeast to man. Deletion of this domain at the carboxy-terminus of SamB led to premature hyphal ramiﬁcation, mislocalization of septa and suppression of the asporogenous phenotype of the developmental mutant ΔsamB deletion strain displayed an identical phenotype. A homologous gene in Saccharomyces cerevisiae was also characterized whose deletion resulted in a multi-budding phenotype; thus it was named MUB1. An underlying common mechanism for both genes in determination of the onset of polarized growth and its links to other cellular developmental processes is discussed.

Keywords: Aspergillus nidulans/budding/development/ filamentous fungi/Zn finger

Introduction

Fungi are able to grow by two alternative mechanisms, budding and filamentous growth. Cell growth is achieved accordingly by isotropic expansion of the cell or polarized tip extension. The two growth forms contribute to different extents to fungal morphogenesis in filamentous and in yeast-like organisms.

In filamentous species, polarized growth dominates during vegetative stages of the life cycle but isotropic expansion is important for the development of specialized cells for reproduction. Polarized growth requires a continuous supply of cell wall material at the hyphal apex, which is achieved by an active tipward vesicle transport (Gow, 1994). This organelle movement requires an intact actin cytoskeleton in combination with force-generating motor proteins like myosin (McGoldrick et al., 1995). Morphogenesis of filaments is normally accompanied by septation and subapical branching. In the ascomycetous fungus Aspergillus nidulans, the first septum is formed once a germinating hypha has reached a certain length and nuclei have completed the third round of mitoses (Wolkow et al., 1996) leading to eight nuclei. Septum formation occurs in the rear of the germtube close to the spore, resulting in 5–6 nuclei in the tip compartment and 2–3 in the spore. The molecular biology underlying these processes has just begun to be investigated. Several genes have been described which affect tip growth, branch formation or septation (Harris and Hamer, 1995; Harris et al., 1997).

In yeast, cell volume increases by isotropic growth which is followed by a temporary switch to polarized tip extension during budding (Cid et al., 1995). In Saccharomyces cerevisiae, bud site selection depends on the site of the previous bud (Chant, 1994). In haploid cells, daughter cells bud off near the site of the previous bud (axial budding) whereas in diploid cells daughter cells bud off alternately at both ends of the cell (bipolar budding). Axial and bipolar budding share some proteins but also require some specific components (Park et al., 1997). Structural genes, like chitin synthase 3 (Santos and Snyder, 1997), are as important as regulatory genes, like protein phosphatase 2 PPH22 (Evans and Stark, 1997). Three classes of proteins have been described which are involved in the establishment of polarity and thus bud site selection. One class of proteins localizes to the bud site and remains there during bud emergence and growth, e.g. Bem1 (Chenevert et al., 1992), Myo2 (Lillie and Brown, 1994) or Cdc42 (Ziman et al., 1993). Other proteins, like Cdc3 or Cdc11, assemble at the bud site but remain at the budding neck as the daughter cell grows (Ford and Pringle, 1991; Kim et al., 1991). A third class of proteins is involved in the onset of polarity without an asymmetric subcellular localization. It is not surprising that some of the regulatory genes coding for these proteins are conserved in organisms ranging from bacteria to man, because polarized growth is essential in all developing cells (Chant, 1994).

A switch from filamentous growth to yeast-like budding is found in fungi like A.nidulans during maturation of the asexual reproductive structure, the conidiophore (Timberlake, 1990, 1991). From a foot cell, a stalk develops by apical extension until it swells to a globose vesicle at the tip. In a budding-like process, primary sterigmata (metulae), secondary sterigmata (phialides) and conidiospores are produced. Besides the coordination of the two growth forms, nuclear distribution is essential for development. Nuclei migrate from the vesicle into the metulae, which remain uninucleate throughout further steps of development. All subsequent cell types are also uninucleate. Two mutants have been described, apsA and apsB (anucleate primary sterigmata), in which metulae fail to differentiate further because of a misdistribution of nuclei. The same mutations have only a slight effect on hyphal growth. Nuclei are clustered in the multinucleate hyphal cells of A.nidulans rather than evenly distributed like in the wild-type (Clutterbuck, 1994; Fischer and
Apsequent analysis (Figure 1).

Results

Molecular cloning of samB

The lack of green spores on conidophores of A. nidulans apsA mutant strains leads to brown colonies easily distinguishable from green wild-type colonies. In the suppressor strain SM112 (apsA; samB), conidia production was restored due to the mutation of samB. The samB mutation had little effect on the phenotype in apsA+ strains which were not suitable for isolation of the gene by complementation. Therefore, the double mutant was used for cloning the samB gene. After successful complementation of the recessive mutation with the wild-type gene, an apsA-like phenotype was expected, which would appear in brown colonies among green non-complemented strains. In one co-transformation experiment using a chromosome VIII-specific library and the self-replicating plasmid pAD4ARP1, five strains with an apsA-like phenotype were isolated among 2000 transformants. After colony purification of the original transformant, the complementing hybrid plasmid was isolated. This plasmid complemented the samB defect with a high frequency, indicating that it contained the entire samB gene. Because severe rearrangements of the genomic DNA inserted into the pAD4ARP1 plasmid can occur, we isolated a cosmid from the same library by hybridization with restriction fragments from genomic DNA rather than from pAD4ARP1. We identified one cosmid which mapped to fragments from genomic DNA rather than from the same library by hybridization with restriction had little effect on the phenotype in apsA strain SMI12 (guishable from green wild-type colonies. In the suppressor mutant strains leads to brown colonies easily distin-

The lack of green spores on conidophores of A. nidulans apsB gene was also cloned and sequenced. This gene encodes a 121 kDa coiled-coil protein (R. Suelmann, N. Sievers, D. Galetzka, L. Robertson, W.E. Timberlake and R. Fischer, manuscript in preparation). To elucidate the molecular functions of apsA and apsB, a suppressor analysis was initiated which led to the discovery of at least two extragenic suppressors, samA and samB (Krüger and Fischer, 1996). While samA was dominant, the recessive locus samB, which was located on chromosome VIII, could be complemented by transformation with a wild-type library. In this study, samB was cloned, sequenced and the gene product localized subcellularly. We present evidence that samB encodes a protein, which is involved in regulation of morphogenesis rather than in nuclear distribution, and that its function is conserved in yeasts and filamentous ascomycetes.

Sequence of samB

The 4.1 kb insert of pMir25 was sequenced on both strands. To investigate whether the samB gene harbors any introns, the 4.1 kb fragment was used to isolate six corresponding cDNAs from a λgt10 cDNA library (kindly provided by G. May, Houston, TX). Sequence comparisons of three of the cDNAs with the sequence of the genomic fragment revealed no intron in the entire coding region; the cDNAs started at positions 633, 658 and 739. In the 600 bp upstream of the putative transcriptional start sites, three CAAT boxes and two TATAAA-like boxes were found. In addition, sequences preceding the start points contain CT-rich stretches. At the 3′ end of the cDNAs, polyadenylation sites 0.6 kb downstream of the stop codon at positions 3273, 3276 and 3282 were detected. Open reading frame (ORF) analysis revealed one region encoding a polypeptide of 590 amino acids (Figure 1). Translation started with a methionine at base pair 865. In the 232 bp region between the initiation of the longest cDNA and the first ATG, no further ATG-initiated ORFs were detected. The N-terminal 125 amino acids of the 590 amino acid polypeptide are hydrophobic, whereas the rest of the protein is hydrophilic. The protein is negatively charged (−0.758) at pH 7, with an average isoelectric point of 6.9. At the C-terminus, two Zn finger-like domains can be seen. One consists of a Cys/Cys–Cys/Cys and the other of a Cys/Cys–His/Cys sequence. This motif has also been found in several other eukaryotic proteins (Figure 2). In addition, three CDC28/CDC2-like phosphorylation sites (SPPR (CDC28 site of S. cerevisiae), PDSP and PTTP (CDC2 sites of Schizosaccharomyces pombe)) were identified in the middle region of the polypeptide (Figure 2). A search for SamB sequence similarity in the databases detected a hypothetical protein (YMR100w) in S. cerevisiae. The two fungal proteins shared sequence similarities at the N- and the C-termini, with 70% similarity (49% identity) and 75% similarity (62% identity), respectively. In addition to the conservation of the primary sequences of the genes, the secondary structure and hydrophilicity predictions, the CDC28 sites and the Zn finger-like domain were also similar in both proteins (Figure 2). These findings suggest conserved functions in A. nidulans and in S. cerevisiae.

The SamB protein resides in the cytoplasm

The analysis of the SamB protein revealed a hydrophobic region at the N-terminus and a Zn finger-like domain at the C-terminus. These domains suggested an association of the protein with a membrane or with the nucleus in the case the Zn finger domain functions in DNA binding. To test this, we analyzed the SamB protein in A. nidulans and localized the protein by secondary immunofluorescence in the cell (Figure 3). We introduced the hemagglutinin (HA) epitope of the human influenza virus into the BamHI restriction site of samB (pMir25). This construct complemented the samB defect in transformation experiments, indicating that the 33 additional amino acids inserted into SamB did not interfere with the biological function of the protein. A transformant with a single integration of the construct (SM152–7) was used to detect the SamB protein in vitro. Protein extracts were prepared and separated on a polyacrylamide gel. Using monoclonal anti-HA antibodies in a subsequent Western blot analysis,
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Fig. 1. Molecular analysis of samB. (A) Partial restriction map of the samB locus. The location of the transcript and the open reading frame are indicated. (B) Detection of the samB transcript in RNA isolated from hyphae of the A. nidulans wild-type (samB+/H11001) strain FGSC26. Twenty-five μg of total RNA were separated on a 1% agarose gel (glyoxal), transferred to a nylon membrane and processed for transcript detection. As a probe, we used the 32P-labeled EcoRV fragment of samB. (C) Sequence of the samB locus. The 4.1 kb EcoRI fragment was sequenced on both strands and compared with the cDNA sequence. The start sites of three cDNAs are marked above the sequence (*). Putative TATAAA-like and CAAT boxes are indicated (underlined). The poly(A) addition sites (*) were deduced from the cDNA sequences. The amino acid sequence of the open reading frame is indicated below the DNA sequence in the one-letter code. The point mutation leading to a stop codon detected in SMI20 is boxed. The sequence data reported here have been submitted to the DDBJ/EMBL/GenBank databases under accession No. AJ000996.

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Fig. 2. Properties of the SamB protein. (A) Hydrophilicity prediction after Kyte–Doolittle. (B) Comparison of samB and the yeast homolog. Regions of high sequence similarity, the hydrophobic amino-terminus, the Zn finger-like domain and putative CDC28 sites are indicated. (C) SamB shares a Zn finger-like domain with the yeast protein Mub1 (accession No. gp Z49807), the Drosophila proteins DEAF-1 (Gross and McGinnis, 1996) and Nervy (Feinstein et al., 1995), the human MTG8 protein (Miyoshi et al., 1993) and the mouse RP-8 protein (accession No. gp U10903). The alignment was achieved using the Jothun Hein method (Meg Align program, Laser Gene Navigator, DNA STAR Inc., Madison, WI). The conserved cysteine and histidine residues are denoted in bold letters below the sequences.

we detected a specific band in SMI52-7 which expressed the SamB::HA fusion protein, as compared with FGSC26 which only expressed the non-tagged SamB protein. The calculated apparent Mₐ was 80 kDa, which is slightly higher than the predicted Mₐ of 70 kDa (66 kDa plus the HA epitope). The protein was not very abundant. Expression was also studied in an apsA deletion strain which was transformed with the SamB::3HA construct. Several independent transformants were analyzed on a Western blot. No significant difference could be detected in comparison with the expression in an apsA⁺ strain. The A.nidulans strains expressing SamB::3HA were also processed for immunostaining with monoclonal anti-HA antibodies and Cy3-labeled anti-mouse secondary antibodies for detection. Granular staining of the cytoplasm was observed (result not shown). A stronger signal was obtained with a strain (SMI52-10) with several integrations of the construct. An association with known subcellular structures such as microtubules or actin was not obvious (Figure 3). The protein was not clearly excluded from nuclei.

SamB is involved in fungal morphogenesis

The A.nidulans SamB protein showed significant homology to the hypothetical yeast protein YMR100w. To compare the phenotype of the samB mutant with the phenotype of a loss-of-function mutant of S.cerevisiae, we deleted the YMR100w coding region in the haploid yeast strain MH272-1da. We amplified the S.cerevisiae HIS3 marker gene using HIS-specific oligonucleotides with 50 bp overhangs at the 5' end derived from the flanking sequences of the coding region of YMR100w. The resulting PCR fragment was gel-purified and used for yeast transformation. In one of 13 transformants, the predicted gene replacement led to the deletion of the coding region (result not shown). The haploid transformant was viable. The name MUB1 (multi-budding) was assigned to the gene for the observed phenotype. The morphology of ΔMUB1 mutant cells was studied in detail and compared with the A.nidulans samB phenotype.

Comparison of the S.cerevisiae and A.nidulans phenotypes

The yeast deletion strain MK1α-H12 and the yeast wild-type strain MH272-1dx were grown in liquid culture at 30 and 37°C, and the budding pattern was analyzed in late exponential growth. We found that normal axial budding occurred in both haploid strains. In addition, bipolar and multi-budded cells were observed in which new buds were generated before cytokinesis was completed. The multi-budded cell clusters even remained when the cells were sonicated. The relative abundance of abnormal budded cells was increased in the MK1α-H12 deletion strain in comparison with the wild-type. The effect was more pronounced at 37°C (Figure 4, Table I).
Fig. 3. Immunological detection of the SamB protein in *A. nidulans*. (A) Introduction of the HA epitope into SamB. (B) Detection of SamB::HA (*) in crude protein extracts of the transformed strain SMI52-7. Germinated conidia were harvested and protein extracts prepared. Twenty-five μg of total protein were separated on a 10% SDS–PAGE and blotted onto a nitrocellulose membrane. The HA-tagged SamB protein was visualized using monoclonal anti-HA antibodies, affinity-purified anti-mouse secondary antibodies and a chemiluminescent detection system. Lane 1, FGSC26; lane 2, SamB::HA expressed in the *apsA*/*H11001* strain SMI52-7; lane 3, SamB::HA expressed in the *apsA* mutant strain SMI49-3. (C) Localization of SamB in vivo. Conidia of FGSC26 (left panel, a and b) and SMI52-10 (right panel, c and d) were germinated on cover slips and processed for secondary immunofluorescence. Monoclonal anti-HA antibodies and Cy3-labeled, affinity-purified secondary anti-mouse antibodies were applied. (a and c) Phase contrast, (b and d) *samB* fluorescence of the same germlings. Bar = 5 μm.

Fig. 4. Phenotypes of a *S. cerevisiae* wild-type strain (A) and the yeast deletion strain MK1α-H12 (B). (A and B) Low magnification view of two cultures in the late exponential growth phase (bar = 15 μm). Strains were grown at 37°C. Multi-budded cell clusters are indicated by arrows. (C-H) Yeast cells displaying different budding patterns (bar = 5 μm). The relative abundance of the different types in a wild-type culture and in the knock-out mutant culture are denoted in Table I.

Table I. Comparison of the budding pattern of a yeast wild-type strain and the *MUB1* deletion strain (MK1α-H12)

<table>
<thead>
<tr>
<th>Strain/growth temperature</th>
<th>Regular budding 0 or 1 bud</th>
<th>Regular bud site 2 buds</th>
<th>Multi-budded and/or irregular bud sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type/30°C</td>
<td>92.9</td>
<td>5.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Knock-out/30°C</td>
<td>80.8</td>
<td>14.3</td>
<td>4.9</td>
</tr>
<tr>
<td>Wild-type/37°C</td>
<td>78.1</td>
<td>18.7</td>
<td>3.2</td>
</tr>
<tr>
<td>Knock-out/37°C</td>
<td>55.9</td>
<td>28.5</td>
<td>15.6</td>
</tr>
</tbody>
</table>

The yeast strains were grown in liquid YPD medium at 30 and 37°C and analyzed in the late exponential growth phase. In each case, >250 cells were counted. A multi-budded complex was counted as one. Numbers are given in percent. For a description of the phenotypes see Figure 4.

carrying the *samB* mutation. The *samB* mutant was isolated originally as a suppressor of the *apsA* mutation which led to an increase of spore production of the oligosporogenous *apsA* strains (Krüger and Fischer, 1996). The phenotype of the *samB* mutation in an *apsA* strain with respect to asexual spore production or conidiophore development differed only slightly in comparison with a wild-type strain. This could have been due to only a partial loss of function of SamB after mutagenesis. To investigate the defect of the protein, we PCR-amplified the *samB* mutant allele and compared the sequence with the genomic DNA sequence of a wild-type strain. Twenty independent PCR clones of the mutant were pooled and used as templates for the sequencing reaction. This should allow for valid sequencing not disturbed by errors introduced during the amplification. It was found that in the mutant a point mutation at base pair 2519 changed the codon TGG (tryptophan) to the stop codon TAG. The observed transition from G to A is consistent with the method used (diethylsulfate as mutagen) (Krüger and Fischer, 1996). This transition results in the expression of a truncated protein in which the carboxy-terminus harboring the Zn finger-like domain is missing (expression of the protein has not been examined). The truncation should severely interfere with properties of the protein and can be considered non-functional. The multi-budding phenotype of the yeast mutant prompted us to re-investigate the phenotype of the *samB* mutant using germinating spores. In wild-type cells, spores produce a germ tube which is populated by nuclei through the coordinated action of nuclear division and nuclear migration (Suelmann et al., 1997). After the third round of mitosis, a septum is formed at the rear of the germ tube, separating the spore from the growing cell. The remaining spore usually produces a second germ tube opposite the first protrusion. In a wild-type population of germlings at the eight nuclei stage, >70% of the cells
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Table II. Phenotypes of young germlings of (A–E) a samB\(^{+}\) strain (FGSC26) and (A*–E*) a samB mutant strain (SMI20)

<table>
<thead>
<tr>
<th>Morphology</th>
<th>FGSC26 (%)</th>
<th>SMI20 (%)</th>
<th>Aberrant morphology [*]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>26.0</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>45.6</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>2.6</td>
<td>23.6</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>11.4</td>
<td>16.4</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>13.2</td>
<td>44.5</td>
<td></td>
</tr>
</tbody>
</table>

Conidia of the strains were inoculated on coverslips and grown in complete medium for 7 h in a humid chamber. For each strain, >100 germlings were counted. Numbers are given in percent. For a description of the phenotypes see Figure 5.

Fig. 5. Phenotypes of (a) wild-type germlings with one septum and (b–f) samB mutant strain SMI20 germlings of A. nidulans. Septa are indicated with an arrow, and branch initials are marked with an arrowhead. The relative abundance of the observed morphological types of the germlings is given in Table II. Bar = 5 μm.

Fig. 6. samB is not required for the normal distribution of actin or the deposition of cell wall material. Germlings of FGSC26 and the samB mutant strain SMI20 were analyzed by secondary immunofluorescence using anti-actin antibodies and Cy3-labeled secondary antibodies or FITC-labeled wheat germ agglutinin. (A) Phase contrast view. (B) Actin patches are localized predominantly at the hyphal tip (T) and at the septa (S). (C) Visualization of nuclei in germlings by DAPI staining. (D) New cell wall material is deposited mainly at the hyphal tip (arrowhead). Bar = 5 μm.

These results indicated that the samB mutation could contain no septum or one septum close to the spore. No branch formation was initiated in these cells (Table II). In contrast, in the samB mutant strain, the SMI20 morphology of the germlings was changed. They appeared curved and shorter than the corresponding wild-type germlings. The proportion of germinated spores with no septum or one septum was only 15%. On the other hand, >80% showed an aberrant morphology with branched germ tubes without any septum or germinated spores with one long and one short germ tube. The timing of septum formation was not abnormal but septa were often mislocalized, e.g. at the rear of the shorter germ tube (Figure 5). These results indicated that the samB mutation could affect the determination of polarity. Since actin is important for polarized growth, we analyzed the subcellular distribution of this cytoskeletal protein in samB mutant strains (Yang et al., 1997). It was found that the pattern was indistinguishable from the wild-type, where actin has a patch-like distribution at the hyphal tip and at septa (Figure 6). This suggests that SamB is not involved in the polarization of cortical actin deposition. The number of nuclei and their distribution was also not different in the two strains.

Determination of the samB null phenotype

Deletion of MUB1 in yeast resulted in viable haploid strains, indicating that the mutation was not lethal. To determine whether the truncated SamB protein in which the Zn finger-like domain was missing was non-functional, we deleted the samB gene in an apsA mutant strain (SMI16). We introduced the pyr4 marker gene of Neurospora crassa into pMir25 and thereby replaced 81% of the coding region. The corresponding plasmid pMir45 was used to transform the apsA mutant strain SMI16. From 600 transformants, 20 strains produced more spores than the recipient strain, three of which displayed a stable suppressor phenotype. The colonies were colored due to spore production, and changed color to brown after
Fig. 7. Deletion of the \textit{samB} gene in \textit{A nidulans} and description of the phenotype. (A) Construct to delete the genomic copy of \textit{samB}. Partial restriction map of pMir25 and pMir45 in which \textit{pyr4} replaced 80\% of the open reading frame of \textit{samB}. The PCR fragments obtained in (B) and the probe used in (C) are indicated. The \textit{EcoRV} restriction site of \textit{samB} was destroyed upon insertion of \textit{pyr4}. (B) PCR amplification of the \textit{samB} region with pMir45 as template (lane 2), genomic DNA isolated from the deletion strain SMI55 (lane 3) and pMir25 (lane 4). The PCR fragments were cut with \textit{XhoI} and also analyzed on the agarose gel. pMir45 (lane 5), genomic DNA of SMI55 (lane 6) and pMir25 (lane 7). Lanes 1 and 8 are lambda marker DNA (\textit{EcoRI}). (C) Southern blot of a wild-type strain (lane 1) and the \textit{samB} deletion strain SMI55 (lane 2). Genomic DNA was isolated, digested with \textit{XhoI}, separated on a 1\% agarose gel and hybridized to the probe indicated in (A). As a control, the 3.8 kb \textit{XhoI} restriction fragment of the \textit{apsA} locus was used as a probe. (D) Phenotypic analysis of an \textit{apsA}, the \textit{samB} deletion and a wild-type strain. Conidiophores were observed in a phase contrast microscope.

Discussion

Filamentous growth or reproduction by budding are the two important strategies for an increase of fungal biomass. In this study we have characterized a gene which is involved in filamentous growth and sporulation in \textit{A nidulans} and in budding in \textit{S cerevisiae}. Mutagenesis and deletion of this gene, \textit{samB}, in \textit{A nidulans}, or of its homolog \textit{MUB1} in \textit{S cerevisiae}, led to increased hyphal branch or bud formation, respectively. We showed that the reason for the non-functional SamB protein in the original \textit{samB} mutant strains is a truncation of the C-terminus, which contains an evolutionarily conserved Zn finger-like domain. This suggests that expression of the aberrant protein in \textit{A nidulans} would lack an essential domain. That the \textit{samB} mutant strains indeed display the phenotype of a null mutant was confirmed by constructing a \textit{ΔsamB} strain in which 80\% of the gene was replaced by \textit{pyr4}. The deleted region contains the functional part of \textit{samB} because the 3 kb \textit{BamHI–EcoRI} restriction fragment complemented the mutation in \textit{trans}. This showed that the region upstream of \textit{BamHI} was not important for biological activity. In haploid \textit{S cerevisiae} strains, deletion of the \textit{samB} homolog \textit{MUB1} also resulted in viable cells. The question arises of whether there is a common mechanism underlying the increased branching frequency in \textit{A nidulans} and the initiation of premature bud formation in yeast. These two phenomena could be explained by a common mechanism if the homologous prolonged incubation. This effect was due to lysis of mature spores as it was observed with the original \textit{samB} mutant strain. PCR and Southern analyses of the putative \textit{samB} deletion strains revealed that a double cross-over event had occurred as predicted from the integration pattern of the constructs (Figure 7). One strain, SM155, was analyzed further phenotypically. No difference was observed with respect to suppression of the \textit{apsA} mutation, spore production, lysis of the conidia and germination pattern of the spores (results not shown).
proteins would be directly or indirectly triggering initiation of polarized growth. For a direct involvement of SamB in determining the site of polarized growth, the protein should be localized at the site of branch or bud initiation. Although SamB was found in the cytoplasm, cycling of the protein between the cytoplasm and the bud site could take place as observed for the yeast protein Bud1 (Park et al., 1997). However, in our experiments, we found no evidence for such a behavior of SamB. In a second scenario, SamB could determine the location of tip growth indirectly. The detection of a Zn finger-like domain at the carboxy-terminus of the protein strongly suggests such a function. The Zn finger-like domain in SamB significantly differs from classical DNA-binding Zn finger motifs (Harrison, 1991), but the evolutionary conservation of such domains implies an important function (Figure 2). A novel Cys–His protein motif has been proposed for this atypical Zn finger-like domain (Feinstein et al., 1995). The motif was found in several regulatory proteins such as DEAF-1, involved in development of Drosophila (Gross and McGinnis, 1996) or in RP-8 in mammals, which is related to programed cell death. For all these proteins, interaction with DNA was proposed because of the presence of the Zn finger-like domains. However, contact to target DNA sometimes requires additional proteins, as was shown for the Drosophila DEAF-1 protein. It also could be that the function of the two Zn fingers of SamB are distinct, with only one interacting directly with DNA as shown for the glucocorticoid receptor (Wright, 1992). Whether the Zn finger-like domain of SamB or an interacting protein could be responsible for DNA binding of SamB remains to be investigated. SamB could then, through DNA binding, regulate downstream genes required for the localized induction of growth. Although the cytoplasmic localization of SamB speaks against a DNA-binding function of SamB, it could be that SamB is relocalized from the cytoplasm to the nucleus upon certain stimuli. This has been reported for the transcriptional activator Yap-1 in S. cerevisiae (Kuge et al., 1997). Another possibility is that only a certain fraction of SamB localizes in the nucleus. Such a distribution has been suggested for the Drosophila protein Ci (Motzney and Holmgren, 1995). The challenge will now be to ascertain if, and how, SamB binds to DNA and which are the downstream regulated target genes.

The question arises in which pathways SamB could be involved in the regulation of bud site or branch site selection, and how these are linked with other cellular functions like development. One indicative sequence motif is the putative CDC28 phosphorylation site detected several times in samB and in MUB1. CDC28 is a protein kinase in S. cerevisiae necessary at START of the cell cycle and triggering several other different pathways (Marini et al., 1996). One component of this pathway is protein kinase C (PKC). This kinase is required for bud growth, and a knock-out of the gene leads to autolysis when cells develop buds. This phenomenon is also known for other mutants with defects in polarized growth (Evans and Stark, 1997). Interestingly, A. nidulans samB, apsA double mutants also display a lysis phenotype during development of conidiaospores which are produced in a budding-like process (Krüger and Fischer, 1996). The phenotype is less obvious in samB mutants with an apsA wild-type allele and, in addition, in ΔMUB1 yeast mutants no lysis was observed. It is very likely, therefore, that significant lysis in yeast would only occur in a double mutant in which the apsA homolog, NUM1, is also deleted.

Given that samB is involved in the regulation of branching in A. nidulans hyphae, the question remains of how samB and apsA might be linked. In apsA mutant strains, metulae remain anucleate and thus development stops at that stage. However, the mutation is leaky and some metulae receive a nucleus and continue development. The defect in nucleating metulae could be due to a failure of nuclei to enter the cells or it could be due to a defect in fixation of the nuclei in the metulae. In the latter case, nuclei could enter the cells temporarily without being trapped. In samB, apsA double mutants, the number of nucleated metulae is increased. This, together with the result that lysis of the conidiaospores only occurs in a samB, apsA double mutant, suggests a functional interaction of the two genes. However, we found that samB also suppressed an apsA deletion mutation, which supports the idea of a bypass suppression. A bypass of the apsA function could in theory act either downstream of ApsA in the nuclear positioning pathway, or could activate another system that is able to mediate sporulation in the absence of ApsA. Since in samB mutant strains nuclear positioning seems not to be affected, it is more likely that SamB increases the number of spores by a mechanism other than by rescuing the apsA phenotype. If in apsA mutants the lack of trapping of nuclei would account for the developmental block, mutagenesis of samB could lead to earlier septum formation at the bases of the metulae and thereby to an increased number of metulae with trapped nuclei. These metulae would continue development and thus produce spores. In accordance with this latter explanation, we found that SamB is also involved in septum formation in germings. In samB mutants, septa are mislocalized. In addition to this effect of the mutation on septation, it also could be that SamB has a specific function during development, distinct from the function in hyphae. Interestingly, the samB mutation also affects sexual development of A. nidulans, leading to self-sterile strains.

They initiate fruit body formation, but no viable ascospores are produced. The spores rather lyse and form a red liquid inside the cleistothecia.

In conclusion, our results clearly demonstrate the key role of SamB or its homolog Mub1 in the regulatory pathway of bud site selection, branch initiation and developmental processes. Nevertheless, further investigation is required to solve the remaining questions of how SamB interacts, if it does, with DNA and which other downstream processes are affected.

Materials and methods

Strains, plasmids and growth conditions

Supplemented minimal and complete media for A. nidulans were prepared as described, and standard strain construction procedures were used (Käfer, 1977). A list of A. nidulans strains used in this study is given in Table III. For the growth of S. cerevisiae strains (Table IV) in liquid culture,YPD medium (2% bacto-peptone, 1% yeast extract, 2% glucose) was used. For growth on solid media, YPD was supplemented with 1.5% agar. Escherichia coli strains and the plasmids and cosmids used in this study are listed in Table V.
### Table III. Aspergillus nidulans strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGSC26</td>
<td>biA1; veA1</td>
<td>Fungal Genetic Stock Center, KS</td>
</tr>
<tr>
<td>GR5</td>
<td>pyrG&lt;sup&gt;89&lt;/sup&gt;; wA3; pyroA4; veA1</td>
<td>G.May, Houston, TX</td>
</tr>
<tr>
<td>AJC1.17</td>
<td>biA1; apsA36</td>
<td>Clutterbuck, 1994</td>
</tr>
<tr>
<td>SMI</td>
<td>pyrG&lt;sup&gt;89&lt;/sup&gt;; wA3; apsA36; pyroA4</td>
<td>this study, from cross AJC1.17×GR5</td>
</tr>
<tr>
<td>SMI12</td>
<td>biA1; apsA36; samB; veA1</td>
<td>Krüger and Fischer, 1996, ATCC 201038</td>
</tr>
<tr>
<td>SMI20</td>
<td>biA1; samB; veA1</td>
<td>this study, transformation of SMI12</td>
</tr>
<tr>
<td>SMI28</td>
<td>pyrG&lt;sup&gt;89&lt;/sup&gt;; biA1; apsA36; pyroA4; samB; veA1</td>
<td>this study; from cross GR5×SMI12</td>
</tr>
<tr>
<td>SMI49-3</td>
<td>pyrG&lt;sup&gt;89&lt;/sup&gt;; biA1; apsA36; pyroA4; samB; veA1; samB:&lt;i&gt;3&lt;/i&gt;HA; pyr4</td>
<td>this study; transformation of GR5 with pMiR40 and pRG1</td>
</tr>
<tr>
<td>SMI52-7</td>
<td>pyrG&lt;sup&gt;89&lt;/sup&gt;; wA3; pyroA4; samB; veA1; samB:&lt;i&gt;3&lt;/i&gt;HA; pyr4</td>
<td>this study; transformation of GR5 with pMiR40; one integration at least two integrations</td>
</tr>
<tr>
<td>SMI52-10</td>
<td>pyrG&lt;sup&gt;89&lt;/sup&gt;; wA3; pyroA4; samB; veA1; samB:&lt;i&gt;3&lt;/i&gt;HA; pyr4</td>
<td>this study; see SMI52-7, but</td>
</tr>
<tr>
<td>SMI55</td>
<td>pyrG&lt;sup&gt;89&lt;/sup&gt;; biA1; wA3; apsA36; pyroA4; ΔsamB::pyr4</td>
<td>this study, transformation of SMI16 with pMiR45</td>
</tr>
</tbody>
</table>

### Table IV. Saccharomyces cerevisiae strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH272-1Δα</td>
<td>Mat α, leu2, ura3, rme1, trp1, his3, GAL+; HMLα</td>
<td>M.Hall, Basel, Switzerland</td>
</tr>
<tr>
<td>MK1α-H12</td>
<td>Mat α, leu2, ura3, rme1, trp1, his3, GAL+, HMLα, DMUB1::HIS3</td>
<td>this study</td>
</tr>
</tbody>
</table>

### Table V. Plasmids and cosmids used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Construction</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAD&lt;sub&gt;2&lt;/sub&gt;-Arp1</td>
<td>Self-replicating AMA1-sequence and the pyrG gene of A. nidulans; ampicillin and ori for E. coli expression</td>
<td>P.Punt, A.J. Zeist, The Netherlands</td>
</tr>
<tr>
<td>pMiR25</td>
<td>4.1 kb samB-complementing EcoRI restriction fragment in pBlueScript KS+</td>
<td>this study</td>
</tr>
<tr>
<td>pMiR45</td>
<td>2 kb SamI–PvuII pyr4-containing fragment of pRG1 cloned into K&lt;sub&gt;R&lt;/sub&gt;-EcoRV of pMiR25, thereby replacing most of the samB open reading frame</td>
<td>this study</td>
</tr>
<tr>
<td>GTMP1</td>
<td>3×HA-containing plasmid with pBlueScript KS+ as vector</td>
<td>A.P. Mitchell, New York</td>
</tr>
<tr>
<td>pMiR40</td>
<td>3×HA in BamHI site of pMiR25</td>
<td>this study</td>
</tr>
<tr>
<td>pRG1</td>
<td>pyr4 gene containing plasmid; pyr4 from Neurospora crassa</td>
<td>Waring et al., 1989</td>
</tr>
<tr>
<td>YDP-H</td>
<td>HIS3 in a derivative of pUC9</td>
<td>Berben et al., 1991</td>
</tr>
<tr>
<td>SL10E10</td>
<td>samB-containing genomic fragment of Chr.VIII in cosmid vector LORIST2</td>
<td>Fungal Genetic Stock Center, KS</td>
</tr>
</tbody>
</table>

### Molecular techniques

Standard DNA transformation procedures were used for *A. nidulans* (Yelton et al., 1984), *S. cerevisiae* (Ausbel et al., 1995) and *E. coli* (Sambrook et al., 1989). For cloning *samB* by complementation, SMI28 was crossed to GR5 and a uracil auxotrophic strain was selected from the progeny. This strain, SMI28, was co-transformed with an *A. nidulans* wild-type genomic library of chromosome VIII constructed in a cosmid vector (Promega, Madison, WI). Five ml of the resulting DNA was transformed by electroporation into *E. coli* XL1-blue cells, which were streaked onto LB agar plates supplemented with 100 μg/ml ampicillin. The transformed clones were analyzed for plasmids, and restriction fragments which again complemented the *samB* mutation. Isolation of the original cosmid from the cosmid library (in *E. coli*) and screening of a DNA library (in phages) was done by colony and plaque hybridization, respectively (Sambrook et al., 1989). DNA sequencing was performed with the automatic sequencer ALFexpress® (Pharmacia Biotech, Freiburg) and Cy5-labeled primers. RNA was isolated with TRIZol (Gibco BRL), according to the manufacturer’s protocol. Northern hybridization was performed as described by Sambrook et al. (1989). Plasmids were constructed using standard procedures (Sambrook et al., 1989) and amplified in *E. coli* XL-1blue or TOP10F+ (Invitrogen, Leek, The Netherlands). Polymerase chain reactions (PCRs) were generated with Taq polymerase (Gibco BRL). For construction of the HA epitope-tagged *samB* gene, the HA epitope coding region which codes for three repeats of HA (3×HA) was amplified by PCR using the plasmid GTEP1 as template and the primers Bglhem5 (5′-GAAAGATCTTTTACCCATACGATGTTCCT-3′) and Hemhgl3 (5′-GTCGAGTCAGGCTGCAGACCTGAAC-3′) with BglII restriction sites added. The product was cleaved with BglII and cloned into the BamHI site of pMiR25. Deletion of the YMR100w coding region was performed following the method of Baduin et al. (1993).
The disruption construct was amplified by PCR using the plasmid YDp-H containing the HIS3 gene and the primers Pros (5' -CACGGTTATA-GAGCTATTTGGAATACCAAAAGAGGAAAGCT-CTGGGCTCCTGCTAG-3') and Termdis (5' -CCCTTGACTTAC-TCAGGATGTCTTTCATCTCCTATTCTCTGTTCAGAA-GAGCAGC-3'), derived from the HIS3 sequences used in the protocol and the published sequence of the flanking region of YMR100w (accession No. S55086).

**Biochemical and immunological techniques**

For protein extraction, Western blot analysis and immunostaining of young germlings, the procedures described in Suelmann et al. (1997) and Fischer and Timberlake (1995) were used. Monoclonal anti-HA (BabCo, Richmond, CA), anti-actin (ICN Pharmaceuticals Inc., Costa Mesa, CA) antibodies and aceton powder-treated appropriate secondary antibodies were used for the detection. Acetone powder treatment of antibodies was performed as described (Harlow and Lane, 1988).

**Microscopy**

Fluorescence microscopy was performed with a Zeiss Axiosvert microscope with the appropriate Zeiss filter combinations for Cy3, fluorescein isothiocyanate (FITC) or 4',6-diamidino-2-phenylindole (DAPI) and a 3CCD INTEAS seecon video camera system (INTAS, Göttingen, Germany). To visualize distribution of nuclei, mounting of the specimens was in VECTASHELD mounting medium with DAPI (1.5 μg/ml) (Vector Laboratories, Burlingame, CA. For staining with fluorescently labeled wheat germ agglutinin (WGA), germlings were fixed in 5% formaldehyde for 30 min and rinsed three times in phosphate-buffered saline (PBS) buffer and incubated with 25 μg/ml of fluorescein isothiocyanate (FITC)-labeled WGA (Sigma Chem. Comp.) in PBS for 45 min (Lehmler et al., 1997).

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We thank Dr. A.P. Mitchell (Columbia University, NY) for the provision of the HA-containing plasmid GTEP1 and Dr. S. Rospert and Dr. M. Hall (Biozentrum Basel, Switzerland) for sending us the yeast strains and Fischer and Timberlake (1995) were used. Monoclonal anti-HA (BabCo, Richmond, CA), anti-actin (ICN Pharmaceuticals Inc., Costa Mesa, CA) antibodies and aceton powder-treated appropriate secondary antibodies were used for the detection. Acetone powder treatment of antibodies was performed as described (Harlow and Lane, 1988).

**References**


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