A type 2A protein phosphatase gene from *Aspergillus nidulans* is involved in hyphal morphogenesis

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Abstract A PCR-based approach, using degenerate oligonucleotide primers, was used to isolate fragments of two genes encoding type 2A protein phosphatases from the filamentous fungus, *Aspergillus nidulans*. The complete genomic sequence of one of these genes, *pphA*, was isolated and characterised. The *pphA* gene was predicted to encode a 329-residue protein which is about 85% identical to mammalian protein phosphatase 2A. Ectopic expression of the wild-type *pphA* gene slightly inhibited growth in some transformants; but a mutant form of *pphA*, in which R259 was mutated to Q, led to slow growth, delayed germ tube emergence and mitotic defects at low temperature. These results indicate that the *pphA* gene plays an important role in hyphal growth.

Introduction

The reversible phosphorylation of serine, threonine and tyrosine residues is extensively employed in eukaryotic systems as a versatile mechanism for regulating the biological activity of enzymes and other proteins (Cohen 1989). Protein phosphatases, which remove phosphate groups from phosphorylated amino acid residues, are important regulatory proteins involved in numerous cellular processes. The protein serine/threonine phosphatases comprise two distinct gene families, PPP and PPM. The PPP family comprises the Mg\(^{2+}\)-dependent protein phosphatases, including PP2C. The PPP family includes the biochemically-related type 1, type 2A and type 2B phosphatases (usually referred to as PP1, PP2A and PP2B – or calcineurin – respectively). These proteins, originally defined on the basis of biochemical characteristics, have since been shown to be structurally related and probably share a common evolutionary origin. More recently, many other phosphatases have been discovered in the PPP family, including PP4 (originally termed PPX), which is located in the centrosome in both mammalian cells and *Drosophila* (reviewed by Cohen 1997).

PP2A has been implicated in a wide range of cellular functions in a diverse number of species (reviewed by Goldberg 1999). The PP2A holoenzyme consists of a core complex, comprising a 36-kDa catalytic subunit (PP2Ac), associated with a 65-kDa structural subunit (PR65/A). This dimeric core can be complexed with a third, variable regulatory subunit (B). The B subunit may control both the activity and specificity of the phosphatase complex (Mayer-Yaekel and Hemmings 1994; reviewed by Wera and Hemmings 1995). PP2Ac also forms complexes with other proteins, including protein kinase signalling complexes (Millward et al. 1999).

Fission yeast contains two closely related PP2Ac genes that have partially overlapping functions with PP1 (Kinoshita et al. 1990). In budding yeast, there are two PP2A-like genes, *PPH21* and *PPH22*, and a PP2A-related gene, *PPH3* (Ronne et al. 1991; Sneddon et al. 1990). Genetic and molecular analysis of PP2A mutants suggest functions in cell wall integrity, actin cytoskeleton organisation and mitosis (Evans and Stark 1997; Lin and Arndt 1995; Ronne et al. 1991), sister chromatid cohesion (Minshull et al. 1996) and glycogen metabolism (Clotet et al. 1995). Like yeasts, mammalian cells contain multiple PP2A genes but at least some of these are essential: mice lacking one of the PP2A genes die as young embryos (Gotz et al. 1998). In human cells, alterations in the C- and N-termini of PP2A produce dominant, interfering mutations (Evans et al. 1999).
PP2A plays a significant role at the G2/M transition in a number of cell types, including *Xenopus* cells (reviewed by Lee (1995)) and yeast cells (Kinoshita et al. 1993). A significant number of cancer cell lines have mutated PP2A genes (Wang et al. 1998), suggesting these proteins may act as tumour suppressors. PP2A inhibits telomerase function in cell extracts (Li et al. 1997). Reduced levels of PP2A activity affect microtubule function during mitosis in *Drosophila* (Snait et al. 1996) and mutations in regulatory subunits implicate PP2A in pattern formation (Uemura et al. 1993). PP2A genes have been isolated from plants (Arino et al. 1993); and mutations in regulatory subunits affect axin signalling (Garbers et al. 1996). PP2A therefore plays diverse and important roles in eukaryote cellular morphogenesis and development.

Both PP1 and PP2B have been shown to play essential functions during hyphal growth in *Aspergillus*. The *binG* gene, encoding a PP1 (Doonan and Morris 1989; Doonan et al. 1991), plays multiple roles in cellular morphogenesis and mitosis. At a non-permissive temperature, *binG11* mutants showed a pleiotropic phenotype, where cells were arrested in mitosis and cell polarity was affected. Examination of cell wall composition showed that mutant cells grown at elevated temperatures contained less chitin than the wild-type cells, suggesting a role for the *binG* phosphatase in chitin synthesis (Borgia 1992). However, deletion of the *binG* gene (Hughes et al. 1996) is not lethal and probably another PP1 gene exists which can compensate for any loss of *binG* function.

The catalytic subunit of PP2B in *Aspergillus* is encoded by a single gene, *cnaA*, which is essential for cell growth (Rasmussen et al. 1994). *cnaA* + mRNA levels varied in a cell cycle-dependent manner with a peak just after mitosis, possibly in GI prior to DNA replication. Disruption of the *cnaA* + gene was lethal, indicating that the gene is essential for growth. Staining the nuclei of disruptant cells with 4', 6-diamidino-2-phenylindole (DAPI) revealed small nuclei, suggesting that cells were arrested in an early phase of the cell cycle. Taken together with transcript accumulation data, this suggests that PP2B may play a role in GI. However, conidia lacking the *cnaA* + gene were usually able to complete one, or at most, two nuclear divisions.

To date, no PP2A genes from *Aspergillus nidulans* have been characterised, although PP2A-like activity has been identified in cell extracts (Doonan et al. 1991). A PP2A gene has recently been isolated from another filamentous fungus, *Neurospora crassa*, where it is implicated in hyphal growth (Yatzkan and Yarden 1995). To ascertain what, if any, role PP2A plays in cellular morphogenesis in *Aspergillus*, we sought and isolated homologues of PP2A. We identified two such genes by PCR, isolated a full length copy of one gene, *pphA* + and made a dominant, interfering mutation in this gene, which suggests that PP2A is required for normal mitotic progression and for hyphal morphogenesis.

### Materials and methods

#### Strains and media

The wild-type strain of *Aspergillus nidulans* used was GR5 (wa2, ppyG89, pyroA4). The *Escherichia coli* strain DH5α served as plasmid host. DNA was extracted as described by Sambrook et al. (1989) for *E. coli*. Media for propagation and genetic manipulation of *A. nidulans* were as described by Osmani et al. (1987).

#### Isolation of pphA + gene

The sequence of degenerate oligonucleotides used for DNA amplification by PCR were (forward) c110 5'-TA(TC)GGTT-TT(TC)TA(TC)GA(TC)GA(AG)TG-3' and (reverse) c111 5'-TA- CCTT(TC)CCITAT(AG)TT(GA)ACC-3' (where I indicates inosine residues). Genomic DNA was prepared from strain GR5 as described by Osmani et al. (1988). First-strand cDNA was a gift from Dr. Arundhati (Hughes et al. 1996). For PCR, 100 ng of DNA was amplified in a mixture containing 10 mM Tris-HCL, 50 mM KCl, 3 mM MgCl2, 0.2 mM dNTPs, 100 pmol of each primer and 2.5 U of Taq polymerase. The cycle parameters were as follows: 94 °C for 1 min, 47 °C for 1 min and 72 °C for 1 min. After 40 cycles, the mixture was treated with Klenow at 37 °C for 20 min. The mixture was extracted with phenol/chloroform and ethanol-precipitated; and a PCR fragment with the expected size (400 bp) was then gel-purified and ligated with EcoRI-digested pBEC (Stratagene). Inserts from the resultant clones were sequenced.

A genomic clone was isolated by screening a λ-DNA library (gift of G May) with a radiolabelled insert from pBA1. EcoRI fragments containing the *A. nidulans pphA* + gene were cloned into pUC19. Nucleotide sequencing was performed using the dideoxy nucleotide chain termination method and fluorescent primers in an automatic ABI DNA sequencer, following the manufacturer’s instructions. Computer analysis of DNA sequences utilised the Wisconsin GCG and BLAST packages.

#### Southern and Northern blot analysis

Genomic DNA was isolated from mycelia as described (Osmani et al. 1988). Samples (1–5 μg) of DNA were digested with different restriction enzymes, separated on 0.8% agarose gels and transferred onto nitrocellulose filters. Hybridisation with 32P-labelled probes was performed at 65 °C in 5 × SSC, 1% (w/v) SDS and filters were washed at 65 °C in 2 × SSC, 0.1% (w/v) SDS. Total mRNA was extracted from liquid-grown cultures using Trizol reagent (Gibco-BRL). Briefly, approximately 100 mg of tissue were harvested by filtration, washed thoroughly in ice-cold water and homogenised in 1 ml Trizol, according to the manufacturer’s instructions. Total RNA was resuspended in sterile water and a 12-μg sample was run on a 1.0% (w/v) agarose gel containing 0.7 M formaldehyde and then blotted to Hybond-N (Amersham). The rRNA bands were visualised as a loading control using 0.03% (w/v) methylene blue in 0.5 M sodium acetate (pH 5) and photographed. Blots were hybridised with 32P-labelled probes in Church’s buffer at 65 °C overnight, washed three times in 2 × SSC, 0.1% (w/v) SDS at 65 °C and then washed once in 1 × SSC, 0.1% (w/v) SDS at 65 °C. Autoradiographs were scanned into Adobe Photoshop and relative amounts of *pphA* + transcript were estimated after normalising to the loading control.

#### Over-expression of *pphA*

To facilitate cloning of the *pphA* + gene into the inducible expression plasmid, pAL3 (Waring et al. 1989), a *KpnI* site was introduced by PCR upstream of the first codon, using primers c276 (5'-TTTGGTTACCTCACGAGACCCCTTCGCCC-3') and c272 (5'-TAGGGATTTTCAAGGCA-3') which were previously synthes-