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Protein phosphatase 2A is involved in hyphal growth of *Neurospora crassa*

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Abstract Cantharidin and calyculin A, natural toxins that are inhibitors of protein phosphatases 1 and 2A (PP1 and PP2A, respectively), inhibit *Neurospora crassa* hyphal growth. When *N. cassa* was grown in the presence of either drug, abnormalities were observed at hyphal tips. In addition, both drugs induced an increase in hyphal branching. Cantharidin inhibited *N. crassa* hyphal growth in a temperature-dependent manner, as the effect of the drug was more pronounced at 34° C than at 25° C. In addition to the drug-mediated inhibition of phosphatase activity, a genetic approach was used to determine the phenotypic consequences of reduced PP2A activity. Two strains with subnormal PP2A activity were constructed. The first, in which the original *pph-1* gene (encoding the PP2A catalytic subunit) was replaced with an ectopically integrated copy of *pph-1*, exhibited lower levels of *pph-1* transcript, lower PP2A activity and increased sensitivity to cantharidin. Similarly, in a second strain, in which the *pph-1* gene was cloned in an antisense orientation downstream of the inducible isocitrate lyase promoter, lower levels of *pph-1* transcript, as well as of PP2A activity, and a reduction in hyphal growth were observed. The results of this study indicate that PP2A, and probably other Ser/Thr phosphatases, are involved in the regulation of hyphal growth in *N. crassa*.

Key words Calyculin A · Cantharidin · *Neurospora crassa* · Okadaic acid · Protein phosphatase 2A

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Introduction

Serine/threonine protein phosphatases (PPs) are grouped into two structurally distinct families, the PPP family (PP1, PP2A and PP2B) and the PPM family (PP2C and pyruvate dehydrogenase phosphatase) (Barford 1996). Recently, additional members of the PPP family have been identified on the basis of molecular cloning and by genetic analysis (Cohen 1997).

PP2A enzymes play an essential role in the regulation of a wide range of cellular processes including metabolism, motility, cell division, growth signaling, and gene expression (Mumby and Walter 1993; Mayer-Yaekel and Hemmings 1994). The PP2A holoenzyme consists of a core complex, comprised of a 36-kDa catalytic subunit (PP2Ac) tightly associated with a 65-kDa regulatory subunit (regA). This dimeric core can be complexed with a third, variable, subunit (regB), which in higher eukaryotes has been shown to control enzyme activity and specificity (Mayer-Yaekel and Hemmings 1994). PP2A is mainly localized in the cytosolic fractions. PP2Ac has also been shown to associate with microtubules (Sontag et al. 1995).

Mutational studies in lower eukaryotes established the role that PP2A plays in controlling cell division, cell growth and morphogenesis. Disruption of the duplicate genes encoding PP2Ac, and a third related gene, in *Saccharomyces cervisiae* is lethal (Ronne et al. 1991). However, a high copy number of *PAM1*, a multicopy PP2A suppressor gene, can bypass the need for the PP2A catalytic subunit (Hu and Ronne 1994). Disruption of *ppa2*, one of the two PP2A genes in *Schizosac-*

charomyces pombe, leads to reduction in cell size, suggesting that mitosis may occur prematurely (Kinoshita et al. 1990). The linkage between PP2A activity and mitosis is further demonstrated by the observation that disruption of *ppa2* partially suppresses a thermosensitive mutation in the *cdc25* tyrosine phosphatase in *S. pombe*. The latter protein is required to activate the *cdc2* kinase at the start of mitosis, and it was therefore proposed that PP2A may inhibit cdc2-p by negatively affecting cdc25-p activity (Kinoshita et al. 1993). Furthermore, a block at the G_2/M stage of the cell cycle was observed in a *S. cerevisiae* mutant bearing a temperature-sensitive *pph21* gene. Since this block could be partially overcome by overproduction of a cyclin B, PP2A may provide an essential function by positively affecting cdc28 kinase, which is a homolog of the *S. pombe* cdc2 kinase (Lin and Arndt 1995). This notion is supported by results from studies utilizing *Xenopus* egg extracts, which have shown that inhibition of PP2A activity with okadaic acid leads to the activation of cdc2 kinase activity (Felix et al. 1990). In addition, PP2A is required for progression through mitosis and for the interaction of microtubules with chromosomal DNA in *Drosophila* (Snaith et al. 1996). Studies on *Drosophila* heterozygotes showed that partial deficiency of PP2A affects the Ras 1/Raf MAP kinase signal transduction cascade, and thereby influences photoreceptor cell fate (Wassarman et al. 1996).

PP1 and PP2A, whose catalytic subunits are structurally related (Mumby and Walter 1993), are specifically inhibited by a variety of natural toxins (Holmes and Boland 1993). Among them are two diarrehtic shellfish poisons – okadaic acid (which is also a strong tumour promoter) (Cohen et al. 1990) and the phosphorylated polyketide calyculin A (Ishihara et al. 1989). A third natural toxin – cantharidin, which has been shown to be toxic to mammals (Li et al. 1993) – is extracted from the blister beetle *Cantharis vericatoria* (Honkanen 1993). In fission yeasts, a mutant lacking *ppa2* activity was hypersensitive to okadaic acid (Kinoshita et al. 1993). Okadaic acid and calyculin A have been shown to bind to the same site on PP2Ac (Takai et al. 1995). Li and Casida (1992) isolated a cantharidin-binding protein from mouse liver cytosol which was identified as PP2A. They also demonstrated that okadaic acid is a potent inhibitor of cantharidin (which is structurally unrelated to okadaic acid) binding to this protein. Cantharidin has been shown to inhibit *N. crassa* hyphal growth (Yatzkan and Yarden 1995). This may well be a consequence of inhibition of PP2Ac by this drug, since in vitro studies have shown that purified PP2Ac of *N. crassa* is sensitive to cantharidin (Szoor et al. 1995). In this study we have analyzed some of the morphological effects of these inhibitors on *N. crassa* growth, and utilized a genetic approach to study the effects of altered PP2A activity in vivo.

Materials and methods

N. crassa strains, media and growth conditions

Wild-type *N. crassa* strains 74-OR23-1A (FGSC987) and ORS-6a (FGSC 2490), as well as the *pph-1* disrupted heterokaryon, strain 3922 (Yatzkan and Yarden 1995), were used throughout this study. Procedures used in culturing and other manipulations are described by Davis and de Serres (1970). Cultures were maintained on 1.5% agar slants containing Vogel's minimal medium N (Vogel 1956) with 1.5% sucrose. Strains were grown either in liquid Vogel's media or on solid media in petri dishes (90 or 150 mm) or in race tubes (30 cm in length and 18 mm in diameter). When appropriate, the medium was supplemented with either hygromycin B (Calbiochem or Boehringer Mannheim), benomyl (DuPont), cantharidin (Sigma) or calyculin A (Sigma), at appropriate concentrations. Benomyl was filter-sterilized and added to sterile media at approximately 50° C. Stock solutions of the other drugs were prepared in appropriate solvents (DMSO and DMF for calyculin A and cantharidin, respectively). Media containing the phosphatase inhibitors were prepared and stored under conditions of minimal illumination. DNA-mediated transformation of *N. crassa* was carried out as described by Vollmer and Yanofsky (1986). In the antisense expression experiments the medium was supplemented with either 1% glucose or 50 mM sodium acetate (inducer) as sole carbon sources.

For cell wall regeneration assays, 50 µl of a wild-type protoplast suspension (10^6/ml) was added to 2 ml of liquid Vogel's medium with 1.5% sucrose and 1 M sorbitol. To test the effects of cantharidin, the medium was supplemented with either 50, 100 or 250 µM concentrations of the drug. The protoplasts were shaken at 80 rpm for 10 h at 34° C.

The number of conidia was determined as follows: the fungal colony area was measured and the petri dish was washed with either 5–10 or 15–20 ml of sterile water (for small and large petri dishes, respectively). Conidia were collected and counted in a haemocytometer.

Light microscopy

Samples were viewed with a Zeiss Axioscope epifluorescence microscope. For cell wall visualization, a drop of 10 µg/ml Calcofluor (Fluorescent brightener 28, Sigma) was applied to fungal samples spotted on a microscope slide. For nuclear staining, fungal samples were grown on a thin layer of 1.5% agarose medium. The mycelial samples were fixed for 10 min with 1:3 (v/v) acetic acid:ethanol. The sample was then washed with sterile water and was stained for 10 min with a 0.1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI, Sigma) solution. Subsequently, the sample was washed twice and a cover slide was placed on top of the sample. Photographs were taken either with Fujichrome 100 ASA film or using an Applitech CCD camera coupled to NIH Image software.

Nucleic acid isolation and manipulations

Genomic DNA was isolated as described by Yatzkan and Yarden (1995). Restriction endonuclease-digested *N. crassa* DNA samples were electrophoresed and Southern blot analysis was carried out as described by Sambrook et al. (1989). The blots were probed with [α^{32}P]dCTP hexamer-labeled DNA probes (Prime-A-Gene, Promega).

RNA was isolated from 100-mg samples (quick-frozen in liquid nitrogen), which were transferred to a 2-ml screw-cap tube containing 1 ml of TRI reagent (Sigma) and 2 g of zirconium beads (Biospec Products). Cells were disrupted by two 30-s rounds of shaking (4200 rpm) in a bead beater (Biospec Products), and total RNA was extracted according to the TRI reagent protocol. Subsequently, poly(A)$^+$ -RNA was isolated with the PolyA Tract mRNA isolation kit (Promega) and resolved on a formaldehyde-

agarose gel (Sambrook et al. 1989). The RNA was then transferred to a Magnacharge NT Nylon membrane (MSI). Appropriate RNA probes were generated using phage T3/T7 RNA polymerases as described by Sambrook et al. (1989). Northern hybridization was performed in the presence of 100 mM PIPES pH 6.8, 10 × Denhardt's solution, 500 mM NaCl, 0.2%, w/v, SDS, 250 μg/ml yeast tRNA and 20% formamide at 68° C. The most stringent washes were carried out at 68° C with 0.2 × SSC and 0.1%, w/v, SDS. RT-PCR assays were performed with the Promega RT-PCR system kit and in accordance with the manufacturer's instructions. DNA modification and cloning procedures were carried out as described by Sambrook et al. (1989).

Constructs for altered expression of *pph-1* in *N. crassa*

Two plasmids, designated pEY40 and pEY36, were constructed in order to alter *pph-1* expression in *N. crassa*. pEY40, used for ectopic integration of *pph-1*, was prepared as follows: a 2.3-kb *Pst*I-*Pvu*II fragment, containing the entire *pph-1* coding region flanked by approximately 700 bp upstream of the putative initial ATG codon, and 290 nucleotides downstream of the putative stop codon (which include the proposed polyadenylation site), was isolated from pEY239 (Yatzkan and Yarden 1995). This fragment was cloned into the *Pst*I/*Eco*RV site of pBT6, resulting in the formation of the 8-kb pEY40 construct [pBT6 contains the *Hin*dIII-*Sal*I fragment from pBT3 (Orbach et al. 1986), inserted into a pBluescript SK$^-$ vector].

A second plasmid (pEY36) was used for induced antisense *pph-1*expression. First, pEY54 was constructed by cloning a 2.5-kb *PstI-Hind*III fragment of the *acu-3* promoter, isolated from pIAT, into pEY53 (a pBluescript SK$^-$ plasmid containing the 1.4-kb *Hpa*I Hyg^r cassette isolated from pCB 1004). A 1.85-kb *Bst*XI fragment containing the *pph-1* gene was isolated from pEY239 (Yatzkan and Yarden 1995), and, following treatment with T4 DNA polymerase to create blunt ends, was cloned into the unique *Sma*I site of pEY54. The resulting pEY36 construct contained the *pph-1* gene in a 3′ to 5′ orientation under the transcriptional regulation of the *acu-3* promoter (Fig. 1). Sequence analysis of the promoter gene junction was carried out in order to confirm the proper *acu-3* promoter-*pph-1* antisense orientation.

Phosphatase assays

Mycelia from cultures grown in 50 ml of Vogel's N medium were collected by filtration on Whatman No. 1 filter paper over a Büchner funnel. Samples were quick-frozen in liquid nitrogen. The frozen samples were powdered by grinding in liquid nitrogen and were suspended in a two volumes of extraction buffer (50 mM TRIS-HCl pH 7.4, 1 mM EDTA, 0.1 mM EGTA, 0.1% v/v, β-mercaptoethanol, 5 mM benzamidine, 200 μM PMSF, 1 mM *o*-phenanthroline). The mixture was centrifuged at 15 000 × *g* for 15 min at 4° C and the supernatant was filtered through glass wool. ^{32}P-labeled phosphorylase *a* was prepared as described by Krebs and Fischer (1962). To distinguish between PP1 and PP2A activity, aliquots of diluted samples were mixed with either 2 nM okadaic acid or 100 U of inhibitor-2, respectively. The reaction was initiated by the addition of 1 mg/ml ^{32}P-phosphorylase *a* and 5 mM caffeine to a total volume of 30 μl, and was terminated after a 10-min incubation at 30° C by the addition of 100 μl of 10% trichloroacetic acid (TCA). TCA-soluble radioactivity was determined by using a Wallac 1409 Liquid Scintillation Counter. Activity is expressed in units, where one unit of activity is defined as that releasing 1 μmol of P_i from the ^{32}P-phosphorylase *a* in 1 min under the reaction conditions described.

The activity inhibited by okadaic acid was attributed to PP2A and that inhibited by inhibitor-2 was considered to be PP1 (Cohen et al. 1989).

Protein concentrations were determined according to Read and Northcote (1981) with bovine serum albumin standards.

Paired *t*-test statistical analysis was used to compare PP2A specific activities measured in the wild-type and 406 strains. Analysis of covariance was used to fit a semilogarithmic curve of specific activity versus cantharidin concentration for each strain, using experiment as a blocking factor. The slopes and intercepts of the curves were compared using the *t*-test. Three-way (experiment, strain, medium) ANOVA with interaction was used to analyze statistically the differences in PP2A activity measured under the various experimental conditions.

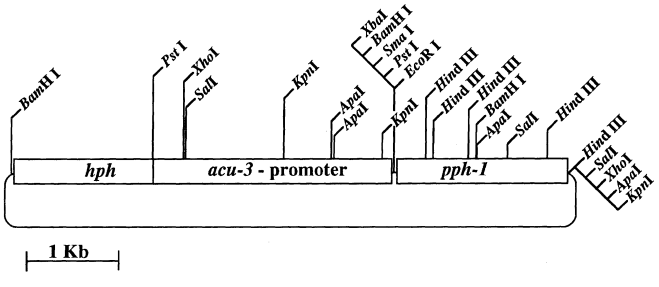

Fig. 1 Structure and partial restriction map of the pEY36 plasmid used in the *pph-1* antisense experiment. pEY36 contains the hygromycin B phosphotrasferase gene (driven by the *A. nidulans trpC* promoter) as a selectable marker, the promoter of the *N. crassa* isocitrate lyase gene (*acu-3*), and the *pph-1* gene in 3′ to 5′ orientation

Results

Effects of PP inhibitors on hyphal morphology

Okadaic acid, one of the most specific inhibitors of PP2A known, was chosen to study the possible involvement of PP2A in regulating *N. crassa* hyphal morphology. Unexpectedly, and despite the fact that okadaic acid has been shown to inhibit the growth of *S. pombe* (Kinoshita et al. 1993), the drug did not inhibit hyphal growth of the fungus even at 200 nM, a concentration which is two orders of magnitude higher than the concentration which completely inhibited PP2A activity in vitro (Szoor et al. 1995). We therefore used cantharidin and calyculin A for drug-mediated inhibition of PP2A throughout this study. The drawback of using these inhibitors lies in the fact that, even though they inhibit PP2A, they can also affect PP1 activity (in contrast to the higher PP2A specificity of okadaic acid).

Previously, we demonstrated that cantharidin can inhibit ($IC_{50} = 200$ μM) *N. crassa* hyphal growth (Yatzkan and Yarden 1995). In the present study, a series of in vivo inhibition assays was performed in order to study the effects of cantharidin and calyculin A on the morphology of *N. crassa* hyphae. When the wild type was grown on solid medium supplemented with 220 or 320 μM cantharidin, at optimal (34° C) and sub-optimal (25° C) temperatures (Fig. 2), the reduction in growth rate was correlated with the cantharidin concentration, and was more pronounced at the higher temperature. At the lower temperature, the shape of the colony was more uniform and little sectoring was observed. Similar effects on growth rates were observed when the wild type was grown on solid medium containing 250, 750 nM or 1.25 μM calyculin A (data not shown).

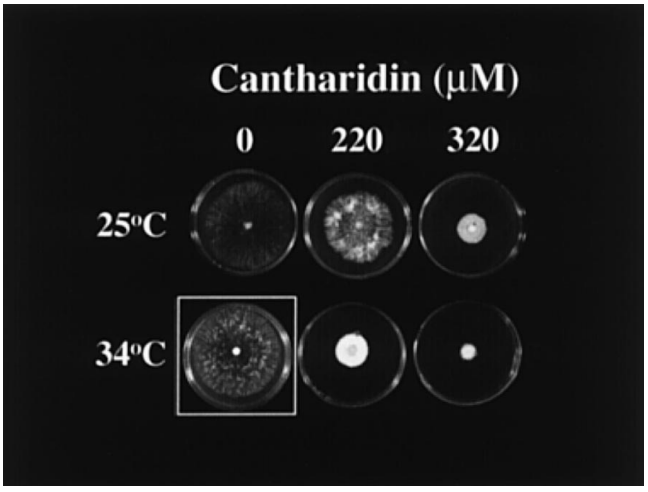

Fig. 2 *N. crassa* hyphal growth on solid medium supplemented with cantharidin at 25° C and 34° C, 27 h after inoculation at the center of the petri dish. Cantharidin was dissolved in DMF and was added to pre-sterilized Vogel's minimal salts medium to prepare the concentrations indicated. The petri dish in which the fungus was grown at 34° C in unsupplemented medium (marked by the *white frame*) was photographed 21 h after innoculation, as the colony had overun the dish by 27 h

In order to determine the extent of the morphological changes caused by PP1 and PP2A inhibitors in vivo, we conducted a microscopic analysis of wild-type hyphae grown under inhibitory conditions (Fig. 3). Cantharidin and calyculin A affected hyphal growth of *N. crassa*, particularly the branching frequency and the morphology of hyphal tips. The latter phenomenon was characterized by the presence of split tips and cytoplasmic leakage from regions distal to the hyphal tips (Fig. 3A–B). Significant swelling and multiple branching occurred at some of the affected tips (Fig. 3C–D). Hyphal tips at which cytoplasmic leakage had occurred were devoid of nuclei. However, at swelling tips, bright DAPI staining was observed, indicating a high concentration of nuclei at the hyphal tip. Hyphae showing abnormal morphology were stained with Calcofluor (Fig. 3B). Following Calcofluor treatment, staining was evident at the hyphal cell wall, as well as at the cross walls. However, staining at the hyphal tips was not as intense as that observed in the control cultures. As the effect of PP1 and PP2A inhibitors on hyphal tip morphology could be indicative of interference with cell wall biosynthesis, we examined the effect of cantharidin on cell wall regeneration by wild-type protoplasts. Protoplast regeneration and germ-tube formation were negatively correlated with the concentration of cantharidin. After 10 h of growth on medium without inhibitor, intense Calcofluor staining was observed at the regenerated cell wall and at the hyphal tip of the germ tube. In samples which were grown in the presence of cantharidin, the cell wall regeneration was very poor, with weak Calcofluor staining. No germ-tube formation or Calcofluor staining were observed in samples which were grown in the higher (250 μM) concentration of cantharidin. Under these conditions most cells contained one to several vacuoles which occupied most of the cell space. These results indicate an increased sensitivity of regenerating protoplasts to the PP1 and PP2A inhibitor, which may impair cell wall biosynthesis, as has been shown by Borgia (1992) in the case of the *A. nidulans* PP1-encoding gene *bimG*.

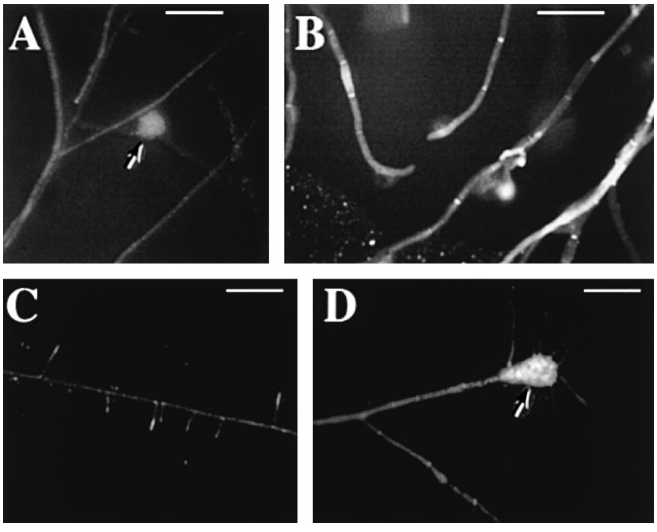

Fig. 3A–D Effect of PP1 and PP2A inhibitors on *N. crassa* growth. **A**, **B** Cytoplasmic leakage at hyphal tips in *N. crassa* grown in the presence of 320 μM cantharidin. **C**, **D** Abnormal hyphal tips and frequent branching of *N. crassa* grown in the presence of 750-nM calyculin A Samples were stained with calcofluor (**B**) or DAPI (**A**, **C**, **D**) prior to epifluorescence microscopy. Images were captured on an Applitec CCD camera. The brightly stained regions (marked by *arrows*) are indicative of the presence of nuclei in regions of cytoplasmic leakage (**A**) or hyphal swelling (**D**). *Bar* 100 μm

Decreased expression of an ectopically-integrated copy of *pph-1*

In a previous study, based on RIP and insertional inactivation experiments, we demonstrated that *pph-1*, which encodes PP2Ac in *N. crassa*, is an essential gene (Yatzkan and Yarden 1995). To analyze further the role of this gene and its product in *N. crassa* hyphal growth, we constructed strains which exhibited different growth phenotypes as a consequence of reduced *pph-1* expression levels. Two different approaches were used in order to obtain such strains. Our first approach was based on the availability of a heterokaryon strain harboring nuclei containing wild-type and disrupted *pph-1* genes (strain 3922). We transformed strain 3922 with pEY40, which contains an intact copy of *pph-1* together with a dominant selectable marker conferring Ben^r. Transformant selection was carried out in the presence of the two selectable markers, Hyg^r for the disrupted *pph-1* gene and Ben^r for the intact, ectopic copy of *pph-1*. Among 12 transformants which were transferred through four serial passages in order to obtain homokaryons, five (401, 406, 407, 408 and 4011) were found to have lost the

native copy of *pph-1* (Fig. 4). Transformant 406 was used for further experiments. We assume that sufficient (though not necessarily optimal) expression levels of the ectopic copy of *pph-1* enabled the purification of such homokaryons. Northern analysis was performed on poly(A)$^+$RNA isolated from the wild-type and 406 strains in order to determine if the ectopic copy of *pph-1* was transcribed similarly to the native, wild-type, copy of the gene (Fig. 5). In contrast to the single 1.5-kb *pph-1* transcript present in the wild-type RNA sample, multiple transcripts were detected by the *pph-1* probe in the 406 strain RNA sample. These results indicate that *pph-1* transcript formation and stability are impaired in the 406 strain. *act-1* (Bruno et al. 1996) transcription in the 406 strain was similar to that observed in the wild type (Fig. 5, bottom panel).

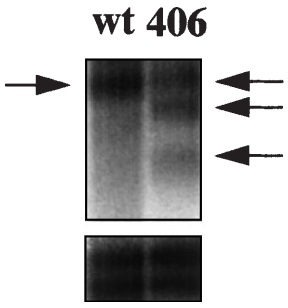

Fig. 5 Northern analysis of *pph-1* transcript levels in wild-type and 406 strains of *N. crassa*. Five micrograms of poly (A)$^+$ RNA was loaded in each lane. pEY17, a clone containing a 1900-bp fragment of *pph-1*, was linearized with *Xba*I and was used as a template for preparing the RNA probe with T3 RNA polymerase. In the left lane, a single band representing the native *pph-1* transcript, as present in wild-type strains, is marked. In the right lane, multiple *pph-1* transcripts of various lengths (disrupted *pph-1*, ectopic *pph-1* and partially degraded *pph-1* transcripts) can been observed. An *act-1* probe was used to verify loading of similar amounts of RNA

PP2A activity and phenotypic changes in the 406 strain

In order to examine the significance of the lower *pph-1* expression levels observed in strain 406, we carried out protein phosphatase assays on cell-free extracts prepared from wild-type and 406 strains. The PP2A activity of strain 406 was 0.08 ± 0.03 mU/mg – about 50% lower than that measured in the wild-type extracts-0.17 ± 0.03 mU/mg ($P < 0.05$) – while the PP1 activities of the two strains were not significantly different ($P \gg 0.05$).

Cantharidin and calyculin A have been shown to inhibit PP1 and PP2A activity in vitro with IC_{50} values of 500 nM and 40 nM (Li et al. 1993) and 0.5–2 nM and 0.1–1 nM (Ishihara et al. 1989), respectively. Apparently homogenous preparations of *N. crassa* PP1 and PP2A catalytic subunits were inhibited by cantharidin with IC_{50} of 300 nM and 60 nM, respectively (B. Szöőr, unpublished results). In order to determine if a reduction in PP2A activity is accompanied by increased sensitivity to a PP2A inhibitor, the 406 strain was grown in the presence of cantharidin. Cycloheximide, a protein synthesis inhibitor, was used as a non-specific control. When grown on minimal medium or on medium supplemented either 100 μM cantharidin of 70 nM cycloheximide, the relative growth rate of strain 406 was 78, 25 and 73%, respectively, when compared to growth of the wild-type on identical media (Fig. 6). Thus, increased sensitivity to cantharidin, but not to cycloheximide, accompanies the reduced PP2Ac expression in strain 406.

As transcription of *pph-1*in the 406 strain was qualitatively altered and the strain was more sensitive to PP2A inhibitors, we carried out experiments in order to determine if the strain 406 PP2A enzyme was more sensitive to cantharidin in vitro. PP2A specific activity was measured in cell-free extracts in the presence of various cantharidin concentrations (up to 1 mM). In all samples tested, similar cantharidin concentrations had a

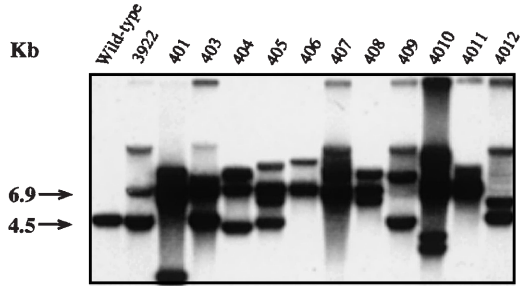

Fig. 4 Southern blot analysis of pEY40 transformants. Genomic DNA samples from wild type, the 3922 heterokaryon strain and 11 transformants were digested with *Kpn*I. The DNA was probed with a hexamer-labeled 750-bp *Eco*RV *pph-1* gene fragment isolated from pEY98. The native (4.5 kb) and the homologously disrupted (6.9 kb) *pph-1* fragments are marked by *arrows*

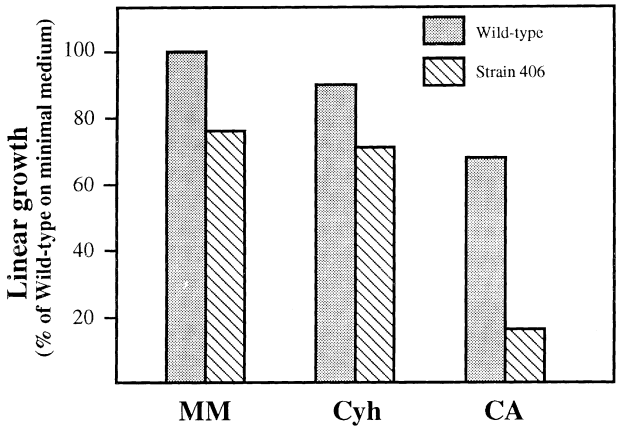

Fig. 6 Effects of two inhibitors on growth of wild-type and 406 strains. Cycloheximide (Cyh) or cantharidin (CA) was added to minimal medium at 70 nM or 100 μM, respectively. Linear growth was measured 70 h after applying a conidial suspension at the end of a 30 cm race tube

greater inhibitory effect on PP2A activity in extracts from strain 406 than on those prepared from the wild-type strain. This was expected, as PP2A activity in the mutant strain was significantly lower to begin with. However, the relative activity (determined on the basis of total activity in each strain) was similar in extracts from both strains tested (Fig. 7). The PP2A IC_{50} value calculated from the wild-type and 406 strains inhibition curves was between 75 and 95 nM cantharidin, which is in close agreement with the previously published value (Szoor et al. 1995). The similar inhibition curves support the conclusion that the effect of the drug is due to quantitative and not qualitative (e.g. altered PPH1 structure) changes in *pph-1* expression.

During the course of this study, we demonstrated that cantharidin inhibited wild-type hyphal growth in a temperature-dependent manner (Fig. 2). To analyze this phenomenon further and to determine if PP2A expression levels can influence it, we performed hyphal growth assays with wild-type and 406 strains grown on solid medium on large petri dishes, supplemented with 100 μM cantharidin, at 25° C or 34° C. Both wild-type and 406 strains grew in a temperature-dependent manner and 63 h after germination, the growth rate at 25° C was higher than at 34° C. Raising the temperature had a more pronounced effect on the growth rate of 406 than on the wild-type strain. At 34° C, the wild-type strain grew to 80% of the colony area measured at 25° C. In contrast, when grown at 34° C, the 406 strain colony area was only 54.5% of that measured at 25° C.

Phenotypic consequences of *pph-1* antisense expression

An additional method of altering PPH1 activity was based on antisense expression (Takayama and Inouye 1990; Prokisch et al. 1997). A construct (pEY36) harboring the *pph-1* gene cloned in an antisense orientation downstream of the inducible isocitrate lyase (*acu-3*) prometer (Gainey et al. 1991), was constructed. *N. crassa* was transformed with pEY36 and selection for positive transformants was performed by comparing growth rates on solid medium containing acetate to that on solid medium with glucose. Among 16 transformants which were passed through four serial homokaryon purification cycles, strain 36-4, in which the *acu-3*::*pph-1* chimera had integrated in an intact form (as verified by Southern analysis), was chosen for further experiments. This strain grew at a slower rate (75%) than wild-type on glucose, yet exhibited significantly restricted growth (approximately 10% of wild-type) when transferred to acetate medium.

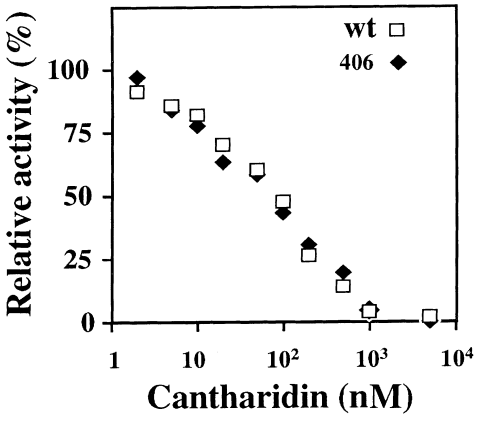

Fig. 7 In vitro inhibition of PP2A activity by cantharidin. PP2A specific activity was measured in cell-free extracts in the presence of various cantharidin concentrations (upto 1 mM). A result representation of five independent assays is shown. Relative activity was determined separately in each of the two strains

The wild-type and 36-4 strains were grown on solid medium in large petri dishes with glucose or acetate as a sole carbon source, with or without 50 μM cantharidin. When grown on glucose medium, similar degrees of inhibition (approximately 25%) were observed in the growth rates of both wild-type and 36-4 strains. On cantharidin-containing acetate medium, no significant increase on wild-type growth inhibition was observed, suggesting the acetate medium does not influence the sensitivity to the drug. However, since the growth of the 36-4 strain on acetate medium was extremely slow, it was not possible to quantitate the effect of the drug on the fungus when grown on this medium. Strain 36-4 conidiated earlier than wild type when grown on acetate medium. At 20 h post germination, minor constrictions were visible in the 36-4 strain, in contrast to the wild type in which, at this time point, no evidence for initiation of conidiation was detectable. When grown on glucose medium, the differences in conidiation were not significant. When strain 36-4 was grown on acetate medium conidiation occurred earlier than in the wild type. Twenty-four hours after germination on the acetate medium, the number of conidia per colony area produced by strain 36-4 was 6 to 7-fold higher than that of wild type. The morphological and developmental differences observed in the 36-4 strain grown under antisense induction conditions may be stress-responsive consequences of a reduction in steady-state levels of PPH1, resulting from the presence of *pph-1* antisense transcripts. To verify this, we performed RT-PCR analysis in order to determine the relative abundance of *pph-1* sense transcripts. A significantly lower abundance of *pph-1* sense RT-PCR product was amplified from the RNA isolated from the 36-4 strain grown under *acu-3*-inducing conditions, when compared to the product abundance in the various controls. *ppt-1* RT-PCR reactions were performed to verify that sufficient amounts of RNA were used in the various reactions (Fig. 8)

PP2A enzyme activity in the 36-4 strain was monitored in order to establish linkage between the reduced growth rates and antisense expression in that mutant. Wild-type and strain 36-4 mycelia were grown in Vogel's liquid medium supplemented with appropriate carbon sources. The PP2A enzyme activity of strain 36-4 on the inducing medium (acetate) was found to be 40% of that

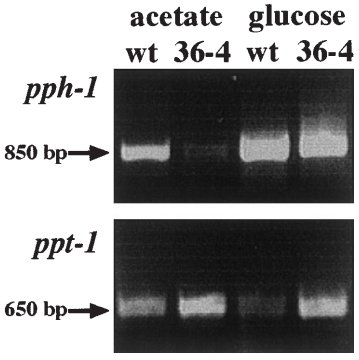

Fig. 8 Products of RT-PCR analysis in which the *pph-1* sense transcript was amplified from wild-type and 36-4 strains. Total RNA template was isolated from strains grown on liquid medium with either 1% glucose or 50 mM sodium acetate as sole carbon sources. For each strain and medium, *ppt-1* products were used to verify the presence of sufficient quantities of RNA template in the reaction

Table 1 PP2A specific activity of wild-type and 36-4 strains

Strain	Medium	PP2A specific activity ($\pm$SE)[a]
Wild-type	Glucose	0.51 ± 0.04
	Acetate	0.59 ± 0.06
36-4	Glucose	0.50 ± 0.04
	Acetate	0.20 ± 0.02

[a] Activity is expressed in units, where one unit of activity is defined as that releasing 1 μmol of P_i from the ^{32}P-phophorylase *a* substrate in 1 min under the described reaction conditions. Specific PP2A activity was measured in the presence of 100 U inhibitor-2. The difference between the specific activities of PP2A measured in strain 36-4 on inducible and non-inducible medium is significant ($P < 0.0001$)

measured in extracts prepared from cultures grown on non-inducing medium (glucose) ($P < 0.0001$) (Table 1), while the difference in PP2A activity between extracts prepared from wild type on each medium was not significant ($p \gg 0.05$). PP1 activity in the 36-4 strain was not significantly affected by the growth medium. However, an unexpected increase in PP1 activity was measured in the wild-type strain grown on acetate, when compared to glucose medium (data not shown). Further analysis is required to elucidate the biological significance of this observation; however, this may well be an indication of the complex changes in phosphatase activity which are most likely to be induced by adverse growth conditions.

Discussion

The fact that *N. crassa* has, apparently, only a single-copy gene encoding PP2Ac makes this organism convenient for the study of the cellular functions of PP2A. Interference with type 2A protein phosphatase activity has deleterious effects on *N. crassa*. Our results show that okadaic acid, the compound of choice for drug-mediated inhibition studies of PP2A, had no obvious effect on *N. crassa* in vivo, even though it is a potent inhibitor of the fungal enzyme in vitro (Szoor et al. 1995). In mammalian cells it has been demonstrated that okadaic acid must be 100-fold more concentrated than calyculin A (a less potent in vitro inhibitor of PP1 and PP2A) in order to cross the membrane at similar rates (Favre et al. 1997). It is possible that okadaic acid is either poorly taken up or perhaps quickly metabolized by *N. crassa*. Cantharidin and calyculin A, two structurally unrelated inhibitors of PP1 and PP2A, affected hyphal morphology of *N. crassa*, particularly at hyphal tips, in a similar fashion. Flaishman et al. (1995) observed that hyphal abnormalities occurred in *Colletotrichum gloeosporiodes* when the phytopathogenic fungus was grown in the presence of calyculin A, suggesting that the effects we have observed may be common to other fungal species as well. Both intracellular organization, as well as cell wall biosynthesis or assembly, could be affected by the drugs in a way that would give rise to the observed phenotype. Nuclear staining was carried out in an attempt to determine if major changes in nuclear division or distribution occurred in the presence of the drugs. DAPI nuclear staining patterns indicate that cantharidin and calyculin A do not bring about a change in the abundance or spatial arrangement of nuclei (Fig 3A, C–D).

Liauw and Steinberg (1996) have suggested that PP2A (or a closely related enzyme) dephosphorylates the catalytic subunit of cAMP-dependent protein kinase (PKA). Recently, Bruno et al. (1996) demonstrated that disruption of the *N. crassa* PKA regulatory subunit affected hyphal growth polarity in a manner which was mainly characterized by the mislocalization of septa. They suggested a possible role for this enzyme in the organization of actin patches at the cell cortex. Calcofluor staining of *N. crassa* mycelia grown in the presence of cantharidin (Fig 3B) indicates that normal septa (in terms of localization and morphology) are produced under the growth conditions described. Thus, an effect on PKA function via PP2A probably did not occur under the conditions tested here.

It is possible that the cantharidin and calyculin A-induced leakage of cytoplasm was due to the impairment of one of the cell wall components. When the deposition of such components is disrupted hyphal tip abnormalities can result (Yarden and Yanofsky 1991; Specht et al. 1996). One of the main components of the filamentous fungal cell wall is chitin. Borgia (1992) showed that cells of the *bimG11* strain of *A. nidulans*, a temperature-sensitive protein phosphatase 1 mutant, are deficient in chitin. He proposed that chitin biosynthesis is partially regulated by PP1. We have found that the characteristically intense Calcofluor staining at hyphal tips – indicative of an abundance of exposed $\beta\ 1 \rightarrow 4$ polysaccharides – is substantially diminished in hyphae grown in the presence of cantharidin (Fig. 3B). Similarly, under the same conditions, regenerating protoplast cell walls stain with lower intensity. Moreover, when one

compares the phenotype of the hyphal tips of the *A. nidulans chsD* chitin synthase-defficient mutant (Specht et al. 1996) with the phenotype of *N. crassa* grown on 320 μM cantharidin, the cytoplasmic leakages from regions slightly distal to the hyphal tips are extremely similar. The fact that both cantharidin and calyculin A can inhibit a variety of Ser/Thr PPs reduces our ability to determine which specific phosphatase is involved in controlling cell wall synthesis. Nonetheless, additional evidence for the possible involvement of PP2A in cell wall integrity has been provided by Lin and Arndt (1995), who have shown that in *S. cerevisiae*, a temperature-sensitive PP2A mutant shows abnormal chitin deposition. They suggested that defects in actin organization might be involved in these defects.

All the morphological changes at colony edges and the multiple branching occurred at high inhibitor concentrations, while at the lower concentrations the phenotype that was observed was limited to a reduction in linear hyphal extension. Cantharidin and calyculin A are both specific in vitro inhibitors of PP1 and PP2A (the latter being more sensitive to both compounds). In mammalian systems, these inhibitors have been shown to promote tumor formation, suppress cell transformation and induce apoptosis. However, limited information has accumulated concerning the specific in vivo inhibitory effect of these drugs in fungi. Our observations concerning the pleiotropic in vivo effects of the PP inhibitors on *N. crassa* growth and morphology are in agreement with the concerns raised by MacKintosh and MacKintosh (1994) regarding the comparison of effects of phosphorylative enzyme inhibitors on cell extracts versus intact cells. Thus, the presence of multiple inhibitor targets at different concentrations and subcellular compartments makes assessing the specific inhibitor-enzyme interaction in intact cells a difficult task. As it is apparent that in fungi PP1 can only partially compensate for the lack of PP2A, and vice versa (Hughes et al. 1996), we assume that the different PP1- and PP2A-regulated cellular processes which are affected by the drugs are probably independent of each other. Drug-mediated inhibition of phosphatase activity can not provide a comprehensive picture of the role of PP2A in *N. crassa* growth, while the genetic approach has probably provided a basis for more accurate analysis. In this study we used two different approaches (ectopic integration and antisense) to achieve deregulation of the *pph-1* gene. In the first approach the expression level of the recombinant gene was probably influenced by the site of integration in the genome (Wu 1997). In the second approach, a reduction in PP2A levels was achieved by antisense expression. The mechanism of antisense RNA-mediated mutations is not well understood, and no single model can encompass all the results obtained in different systems. RNA hybrids are usually not detected in antisense-transformed cells and are therefore believed to be rapidly degraded (Nellen and Lichtenstein, 1993). This model may provide an explanation for why RT-PCR antisense *pph-1* products were not detectable but the level of *pph-1* sense RT-PCR products was reduced in strain 36-4 when grown on induction medium.

The reduction in PP2A activity that was consistently measured in both the 406 and 36-4 strains does not confer the abnormal hyphal tip and branching phenotypes observed when the wild type was grown in the presence of phosphatase inhibitors. Similarly, when stained with Calcofluor, staining intensity at hyphal tips was comparable with that observed in the wild type (data not shown). However, we do not know if a more significant reduction in PP2A activity in the 406 and 36-4 strains would lead to abnormalities in hyphal morphology. Both the 406 and 36-4 strains, when grown under appropriate conditions (on cantharidin and with the inducer, respectively), show extremely slow growth rates, and both strains produced more conidia per colony area than wild type. These two phenomena were also observed in the wild type grown on solid medium containing cantharidin (Fig. 2). The inhibition of growth was temperature dependent. We suggest that these two phenomena and the temperature effect can be explained as processes which involve PP2A activity. The temperature-dependent effect of phosphatase inhibitors described here is not unique to the cantharidin-*N. crassa* interaction, as it has been shown that PP2B inhibitors (cyclosporin A and FK506) inhibit *Cryptococcus neoformans* growth at 37° C but not at 24° C (Odem et al. 1997).

Based on the PP2A holoenzyme model of Mayer-Yaekel and Hemmings (1994), which postulates a trimmeric complex of a catalytic subunit and two regulatory subunits, and based on the fact that *N. crassa* has a single PP2Ac-encoding gene, all PP2A activity in this organism is apparently dependent on *pph-1* expression. we assume that multiple variable regulatory subunits are present in *N. crassa* (as has been determined in other organisms) and are involved in determining substrate specificity. Our current study provides evidence that even though *pph-1* is an essential gene, suboptimal levels of PP2Ac expression are sufficient to support hyphal growth and conidiation. Identification of the variable regulatory subunits, target substrates and analyzing their involvement in various aspects of fungal growth and development may assist us in further understanding the different roles of PP2A in fungal proliferation.

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