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Expression of protein phosphatase 1 during the asexual development of *Neurospora crassa*[☆]

Tamás Zeke^{a,1}, Endre Kókai^a, Balázs Szóó^{a,2}, Einat Yatzkan^b, Oded Yarden^b,
Krisztina Szirácz, Zsigmond Fehér^c, Péter Bagossi^d, Pál Gergely^a, Viktor Dombrádi^{a,*}

^aDepartment of Medical Chemistry, Medical and Health Sciences Center, University of Debrecen, H-4026 Debrecen, Hungary

^bDepartment of Plant Pathology and Microbiology, Faculty of Agricultural Food and Environmental Quality Sciences, Hebrew University of Jerusalem, Rehovot 76100, Israel

^cDepartment of Human Genetics, Medical and Health Sciences Center, University of Debrecen, H-4012 Debrecen, Hungary

^dDepartment of Biochemistry and Molecular Biology, Medical and Health Sciences Center, University of Debrecen, H-4012 Debrecen, Hungary

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Abstract

We cloned and sequenced the cDNA and the gene encoding the catalytic subunit of protein phosphatase 1 from the filamentous fungus *Neurospora crassa*. The gene, designated *ppp-1* (phosphoprotein phosphatase 1), was mapped by restriction fragment length polymorphism to linkage group III, in the vicinity of *con-7* and *trp-1*. The expression of the gene was monitored by reverse transcriptase and polymerase chain reactions, by Western blotting, and by protein phosphatase activity assays in synchronized cultures. Transcripts of *ppp-1* were detected in the dormant conidia. The abundance of *ppp-1* mRNA, Ppp-1 protein, and the activity of protein phosphatase 1 increased during germination and subsequent hyphal elongation as well as during the early stages of aerial mycelium formation.

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1. Introduction

Protein phosphorylation and dephosphorylation reactions are essential elements of signal transduc-

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*Corresponding author. Tel.: +36-52-412345; fax: +36-52-412566.

E-mail address: dombradi@jaguar.dote.hu (V. Dombrádi).

¹ Present address: MRC Protein Phosphorylation Unit, School of Life Sciences, University of Dundee, Dundee DD1 5EH, UK.

² Present address: Department of Zoology, University of Oxford, Oxford OX1 3PS, UK.

tion pathways in eukaryotic cells. The dynamic balance between the activities of protein kinases and phosphatases determines the level of phosphorylation of a given protein. Approximately 1% of the active genes are dedicated to code for the members of the protein kinase superfamily (Plowman et al., 1999). Protein phosphatases are less numerous and more diverse. Distinct protein tyrosine phosphatase (PTP), phosphoprotein phosphatase (PPP), and metal ion dependent protein phosphatase (PPM) enzyme families were identified on the bases of the primary structures of the catalytic subunits/domains (Wera and Hemmings,

Table 1
Members of the PPP family in *N. crassa*

Name	Code		Size ^a	Localization		Classification	Reference
	Gene ^b	Protein		Supercontig	Chromosome		
<i>pzl-1</i>	AF071751 2.523	AAD09995	531	136	1	PPZ	1
<i>ppp-1</i>	AF124149 2.630	AAD47567	308	178	3	PP1	Present communication
<i>ppe-1</i>	AL390091 2.160	CAB98214	334	107	6	PP6	<i>Neurospora</i> database
–	12802356 2.455	AAK07839	281	52	4	PPG-like	2
–	2.588	–	287	56	–	PP4-like	<i>Neurospora</i> database
<i>pph-1</i>	X83593 2.451	CAA58573	310	52	–	PP2A	3
<i>ppt-1</i>	2290382 2.49	AAB65138	479	213	5	PP5	4
<i>cna-1</i>	M73032 2.202	AAA33565* CAC18243*	518 517	23	2	PP2B	5

References: 1, Szőőr et al. (1998); 2, Bean et al. (2001); 3, Yatzkan and Yarden (1995); 4, Yatzkan and Yarden (1997); 5 Higuchi et al. (1991).

^a Number of amino acid residues.

^b NCBI code is above the *Neurospora* genome project code; –, missing information; *, splice variants.

1995). The members of the latter two families are specific for the dephosphorylation of phosphoester bonds of Ser and Thr residues. Protein phosphatase 1 (PP1) is a prominent representative of the PPP family. It was one of the first protein phosphatases identified by biochemical methods and by molecular cloning (Bollen and Stalmans, 1992). PP1 is present in all of the eukaryotes from yeast to human. The comparison of the amino acid sequences of 44 different PP1 catalytic subunits revealed the extremely high level of conservation of the protein's structure, indicating the fundamental roles and the large number of vital interactions of these proteins (Lin et al., 1999). Genetic analysis of several mutant organisms suggests that PP1 is involved in the regulation of cell division cycle, glycogen metabolism, muscle contraction, cellular morphology, and the deposition of memory traces (Dombrádi, 1997).

Neurospora crassa is a well-characterized model organism of fungal genetics. Its genome sequence has been recently determined. Several protein phosphatases were identified in this filamentous fungus by biochemical and genetic approaches even before the completion of the genome project. Protein phosphatase activity was first detected by Tellez de Inon and Torres (1973) in *N. crassa*. Subsequently, four phosphatases were identified and classified according to biochemical criteria

(Szőőr et al., 1995; Zapella et al., 1996; Szőőr et al., 1997). They were termed PP1, PP2A, PP2B and PP2C. The first three enzymes belong to the PPP family, while PP2C is a representative of the PPM family. The catalytic subunits of PP2A (Szőőr et al., 1995) and PP1 (Szőőr et al., 1997) were also purified and characterized. Molecular cloning substantiated the above findings (Higuchi et al., 1991; Yatzkan and Yarden, 1995, and present communication) and extended the PPP family by three novel members including Ppt-1 (Yatzkan and Yarden, 1997), Pzl-1 (Szőőr et al., 1998), and a PPG-like partial sequence (Bean et al., 2001). In addition, sequences for a homologue of PP6 and a putative PP4 can also be found in the *Neurospora* database <http://www-genome.wi.mit.edu/annotation/fungi/neurospora> (Table 1). In the present communication we have analyzed the expression of the *ppp-1* gene that codes for the catalytic subunit of PP1 in *N. crassa* and we suggest that this enzyme may be involved in the regulation of the asexual phases of fungal life cycle.

2. Materials and methods

2.1. Materials

Oligonucleotides were purchased from Amer-sham Pharmacia Biotech. ³²P-labeled rabbit muscle

phosphorylase *a* was prepared according to Cohen et al. (1989). [α - 32 P]dCTP and [γ - 32 P]ATP were obtained from the Institute of Isotopes Ltd. (Hungary). High DNA mass-ladder molecular mass standard of GIBCO-BRL was used in agarose gel electrophoresis. Low range molecular mass protein markers (Sigma) were used in SDS polyacrylamide gel electrophoresis. The PP1 γ N-19 (sc-6109) anti-peptide antibody and the corresponding competitor peptide (sc-6109P) were purchased from Santa Cruz Biotechnology Inc. Peroxidase conjugated anti-goat secondary antibody was obtained from Sigma–Aldrich.

2.2. Screening of cDNA and genomic DNA libraries

An *N. crassa* λ ZAP cDNA library obtained from the Fungal Genetics Stock Center (FGSC) was screened with a 1.9 kb *Xba*I-*Eco*RI fragment of the *pzl-1* gene (Szöör et al., 1998) under stringent conditions (Sambrook et al., 1989). Two positive clones were found among 50 000 plaque forming units. One of them represented the cDNA of *pzl-1* (Szöör et al., 1998), whereas the other coded for a protein similar to the known PP1 catalytic subunits. The Orbach/Sachs *N. crassa* genomic cosmid library (FGSC) was screened with the 1.7 kb insert of the cDNA clone to facilitate the isolation of the corresponding gene termed *ppp-1*. Two identical cosmids were isolated this way. A 3 kb DNA fragment containing the *ppp-1* gene was excised from one of the genomic clones with the restriction enzymes *Xba*I and *Eco*RI and was subcloned in the Bluescript pKS vector (Stratagene).

2.3. DNA sequencing

The nucleotide sequences of the *ppp-1* cDNA and gene were determined by the chain termination method (Sanger et al., 1977) on both strands using Sequenase Version 2.0 DNA sequencing kit (United States Biochemical) with universal (T3 and T7) as well as specific (NPP11-NPP17) oligonucleotide primers.

2.4. Molecular modeling

A homologous model of *Neurospora crassa* protein phosphatase 1 catalytic subunit was built based on the crystal structure of rabbit muscle PP1 α (amino acid residues 7–300; Goldberg et

al., 1995; PDB accession number: 1FJM) using the Modeller3 program (Sali and Blundell, 1993). Sequence alignment was performed with ClustalW software (Thompson et al., 1994). The amino acid identity was 88% between the two sequences and the root mean square deviation was 0.135 Å for the C α atoms of the two structures.

2.5. Culturing of *N. crassa* strains

Wild type strain 74A (FGSC 2489) of *N. crassa* was grown on solid Vogel's sucrose medium (Davis and de Serres, 1970). Conidia were washed off from the surface, were collected by sieving through cheesecloth and then were resuspended in Vogel's medium. The conidial concentration was determined by counting in a hemocytometer. Erlenmeyer flasks containing fluid Vogel's medium were inoculated to a density of 3×10^7 conidia/ml and were incubated overnight at 4 °C in order to initiate synchronized cultures. Next day they were transferred to an orbital shaker and were shaken at 34 °C and 120–150 rev./min for the indicated time periods. The germinating conidia (0, 1, 3, 7 h) were collected by centrifugation, whereas vegetative mycelia (11, 24 h) were filtered on a filter paper in a Büchner funnel. To obtain aerial mycelia the 24-h liquid cultures were filtered as mentioned before then the mycelia collected on the membranes were transferred to Petri dishes containing a monolayer of sterile glass beads (0.5 cm), which were just about covered with Vogel's sucrose medium. These samples were further grown at 34 °C for 2, 4, 8, 24 h and were harvested (at 26, 28, 32, 48 h of the total culturing time) by peeling off the mycelium from the filter paper. After measuring their mass the samples were immediately frozen in liquid N₂ and were stored at –70 °C until use.

2.6. Southern blotting and gene localization by restriction fragment length polymorphism

Genomic DNA was prepared from *N. crassa* (FGSC 2489) and digested with different restriction endonucleases. Southern blots were made and probed by the 1.7 kb *ppp-1* cDNA fragment labeling with the Rediprime II kit (Amersham Pharmacia Biotech) and [α - 32 P]dCTP (Sambrook et al., 1989). To localize the *ppp-1* genomic DNA was isolated from the progeny of the 'small cross' of the parental Mauriceville-1C-A and Oak Ridge strains (Metzenberg and Grotelueschen, 1989).

The DNA were digested with *Bgl*III, separated by agarose gel electrophoresis and transferred to a Hybond-N+ (Amersham Pharmacia Biotech) membrane. These blots were analyzed with the full-length *ppp-1* genomic insert of the cosmid clone in a high-stringency Southern blotting experiment. Hybridizations were carried out in the presence of 50% formamide at 42 °C and the positive bands were visualized by autoradiography (Sambrook et al., 1989).

2.7. Reverse transcriptase and polymerase chain reactions

Total RNA was isolated from *N. crassa* (Chomczynski and Sacchi, 1987). RNA concentration was measured spectrophotometrically in 1 mM Na₂HPO₄ (pH 7.5). A sample of 0.3 µg of RNA was reverse transcribed at 48 °C for 45 min, denatured at 94 °C for 2 min and amplified in 30 cycles (94 °C for 0.5 min, 57 °C for 1 min, 68 °C for 2 min) with the Access RT-PCR kit of Promega. A final extension step at 68 °C was performed for 7 min. Oligonucleotides NPP12: CATGACTCGGCGGATCTGC and NPP13: GCTTCTTGCTGGGATGCT were used in the direct reaction, while NPP14: AAA-CATGGCGGCGGTAGAC and NPPP15: ATGCACGGTGGTTGAGCC were applied in the competitive set-up (Gilland et al., 1990). The quality of the RNA preparations was monitored by control amplifications of the *rgb-1* transcript (Yatzkan and Yarden, 1999). According to our preliminary tests the RNA preparation was devoid of genomic DNA contamination. It was also shown that RT-PCR yielded in a linear response under the assay conditions. The optimal ratio of the competitor *ppp-1* genomic DNA fragment (the 3 kb *Xba*I-*Eco*RI fragment subcloned into pKS+ vector) to total RNA was determined. As a result of this calibration 5 pg of the *ppp-1* genomic DNA was added to the samples in the competitive reactions. The amplified products were separated by agarose gel electrophoresis and were quantitated by scanning the ethidium bromide stained bands with a Pharmacia LKB ImageMaster DTS densitometer.

2.8. Western blotting

A 50 µg samples of protein of the crude *N. crassa* extracts were loaded into each lane of a

12% polyacrylamide gel and were separated by electrophoresis (Laemmli, 1970). Recombinant human PP1δ (10 µg) was used as positive control (Hirano et al., 1995). Protein bands were transferred to nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech) by electro-blotting. The load was checked by Ponceau-S staining of the membranes and the density of all lanes was determined with a BioRad Fluor-S MultiImager using the Quantity One version 4 software. After Western blotting protein phosphatase 1 catalytic subunits were detected with a 1:100 diluted PP1γ N-19 antibody (Santa Cruz Biotechnology Inc.) by an enzyme-coupled chemiluminescence (ECL) kit of Amersham Pharmacia Biotech according to Harlow and Lane (1988). ECL signals were recorded on CEA RP medical X-ray films after 1–3 min exposure and were quantitated by densitometry as before. Band intensities were corrected for variations in load and were normalized for the positive control band intensity in each film.

2.9. Assay of protein phosphatase activity

PP1 activity of *N. crassa* crude extracts was determined with ³²P-labeled rabbit muscle glycogen phosphorylase *a* substrate in the absence and presence of rabbit skeletal muscle inhibitor-2 protein as described earlier (Szöör et al., 1997). Protein concentration was measured by the dye-binding assay, using bovine serum albumin as a standard (Read and Northcote, 1981).

3. Results

3.1. Cloning and localization of the *ppp-1* gene

We cloned a cDNA from a *N. crassa* λZAP cDNA library on the bases of its sequence similarity to *pzl-1* (Szöör et al., 1998). The 1727-bp-long nucleotide sequence of the insert overlaps with the central portion of the 2233 bp cDNA sequence AF049853 (Zapella et al., 1996) that codes for the catalytic subunit of protein phosphatase 1. We isolated the corresponding gene, termed *ppp-1*, with the cDNA probe from the Orbach/Sachs *N. crassa* genomic cosmid library. The genomic DNA sequence is 99% identical with nucleotides 16410–14835 and 14780–14266 of contig 2.630 of the *Neurospora* genome project. The small DNA segment between nucleotides 14834 and 14781 may represent an intron that is

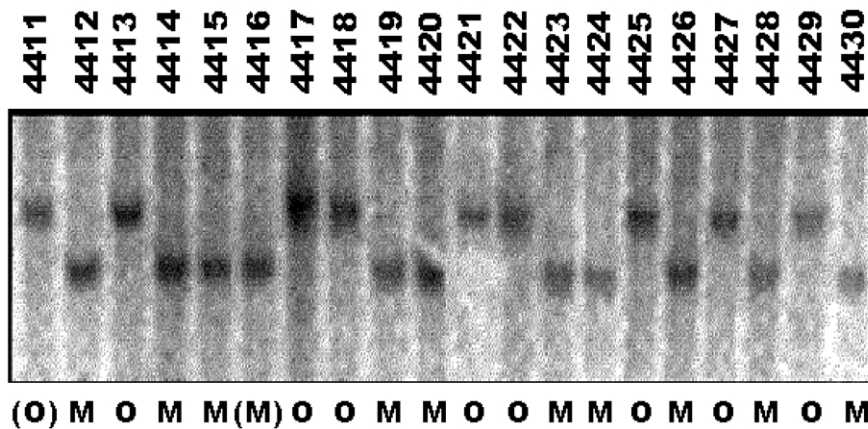


Fig. 1. Chromosomal localization of the *ppp-1* gene. Southern blot analysis was carried out after *Bgl*III digestion of the genomic DNA isolated from 18 progeny, originating from the 'small cross' as well as the parental Oak Ridge (O) and Mauriceville-1C-A (M) strains (Metzenberg and Grotelueschen, 1989). Fungal Genetics Stock Center numbers are shown above the lanes. The segregation pattern of the polymorphism is indicated in capital letters.

missing from our genomic clone. The full-length genomic insert of approximately 36 kb was used for the localization of the gene by RFLP. Out of 31 restriction endonucleases tested only *Bgl*III was suitable for the detection of polymorphism between the parental Oak Ridge and Mauriceville-1C-A strains with this probe (Fig. 1). The restriction fragment pattern coincided with that of the *lze-4*, *con-7* and *trp-1* genes (Metzenberg and Grotelueschen, 1989). Thus, *ppp-1* was mapped to linkage group III in the vicinity of *con-7* and *trp-1*. Southern blots obtained after digestion of genomic DNA with several restriction enzymes indicated that there was no close homologue of the *ppp-1* gene in the *N. crassa* genome (data not documented).

3.2. Expression of the *ppp-1* gene during the asexual development of *N. crassa*

In order to analyze the expression of *ppp-1* gene in synchronized *N. crassa* cultures an RT-PCR strategy was designed. Two oligonucleotide pairs were selected in such a way that the binding sites of the forward and reverse primers bracket two introns in the gene (Fig. 2a). Consequently, the sizes of the PCR fragments amplified from mRNA were smaller than those obtained from genomic DNA in both cases. The strategy resulted in an unambiguous detection of the mRNA related bands. This arrangement also allowed the construction of a competitive RT-PCR scheme where pre-

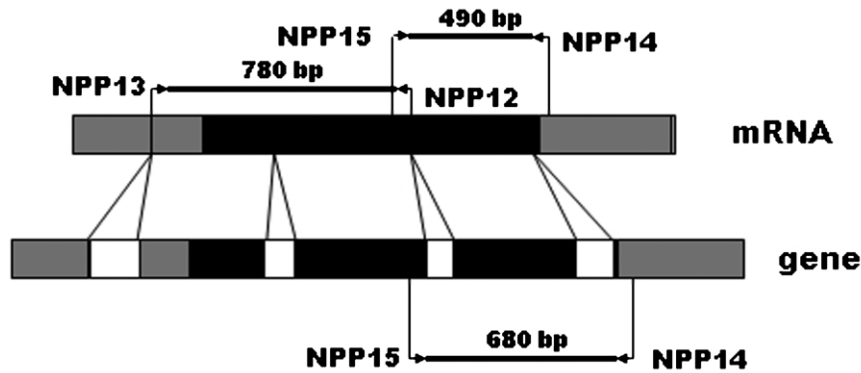
determined amounts of externally added genomic DNA competed with the reverse-transcribed cDNA in the PCR cycles.

Densitometric scanning of the 780 bp band obtained with the primer pair NPP13-NPP12 at different stages of the asexual development suggested that the *ppp-1* transcript is present in the dormant conidia and its level increases during germination and at the beginning of aerial mycelium formation (Fig. 2b). This conclusion was substantiated by quantitative RT-PCR with the primer pair NPP15-NPP14, where the ratio of the 490 bp band (mRNA) to the 680 bp band (genomic DNA) was determined (Fig. 2c and Fig. 3a).

3.3. Translation of the Ppp-1 protein during the asexual development of *N. crassa*

From a panel of PP1 antibodies we found a goat IgG, raised against the N-terminal 19 amino acids of the human PP1 γ isoform that cross-reacted with the *N. crassa* Ppp-1 protein in Western blotting (Fig. 2d). The antibody detected a single band of 35 kDa, in accordance with the predicted molecular mass of the Ppp-1 polypeptide. The specificity of the reaction was proven by the fact that a 10-fold molar excess of the PP1 γ competitor peptide completely eliminated the signal (data not documented). The amount of Ppp-1 protein increased significantly in the first 3 h of cultivation (Fig. 2d and Fig. 3b). A smaller rise was observed 4 h after the initiation of aerial mycelium formation

(a) RT-PCR strategy



(b) RT-PCR with NPP13 and NPP12 primers



(c) Competitive RT-PCR with NPP15 and NPP14 primers

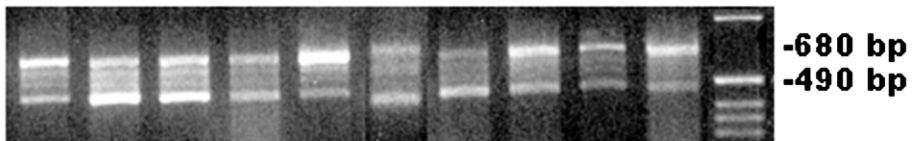
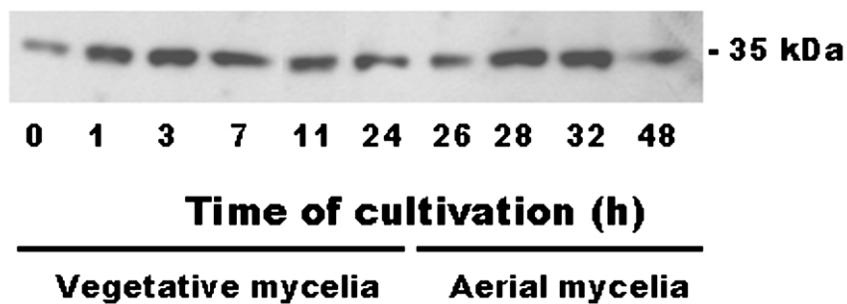
(d) Western blot with PP1 γ antibody

Fig. 2. RT-PCR and Western analysis of *ppp-1* transcript and Ppp-1 protein levels during the asexual development of *N. crassa*. The hybridization positions of synthetic oligonucleotides NPP12-NPP15 (arrowheads) are shown in a schematic representation of the *ppp-1* mRNA and gene structures (a). Coding regions are black, and non-coding regions are gray. Introns in the gene are white and the poly-A tail in the mRNA is light gray. Bold bars indicate the sizes of the expected PCR products. Synchronized *N. crassa* cultures were grown for 1 day in liquid medium (vegetative mycelia) and for another day on the surface of a filter paper (aerial mycelia). Total RNA was isolated and analyzed by RT-PCR with the NPP13-NPP12 primer pair (b) or by competitive RT-PCR with the NPP15-NPP14 primer pair (c). The PCR products were resolved in 1.6 % agarose gel and stained with ethidium-bromide. A crude protein extract of the samples was analyzed by Western blotting with PP1 γ N-19 antibody (c). The results of representative experiments are shown in panels b–d. St denotes the lane for molecular mass standards in b and d.

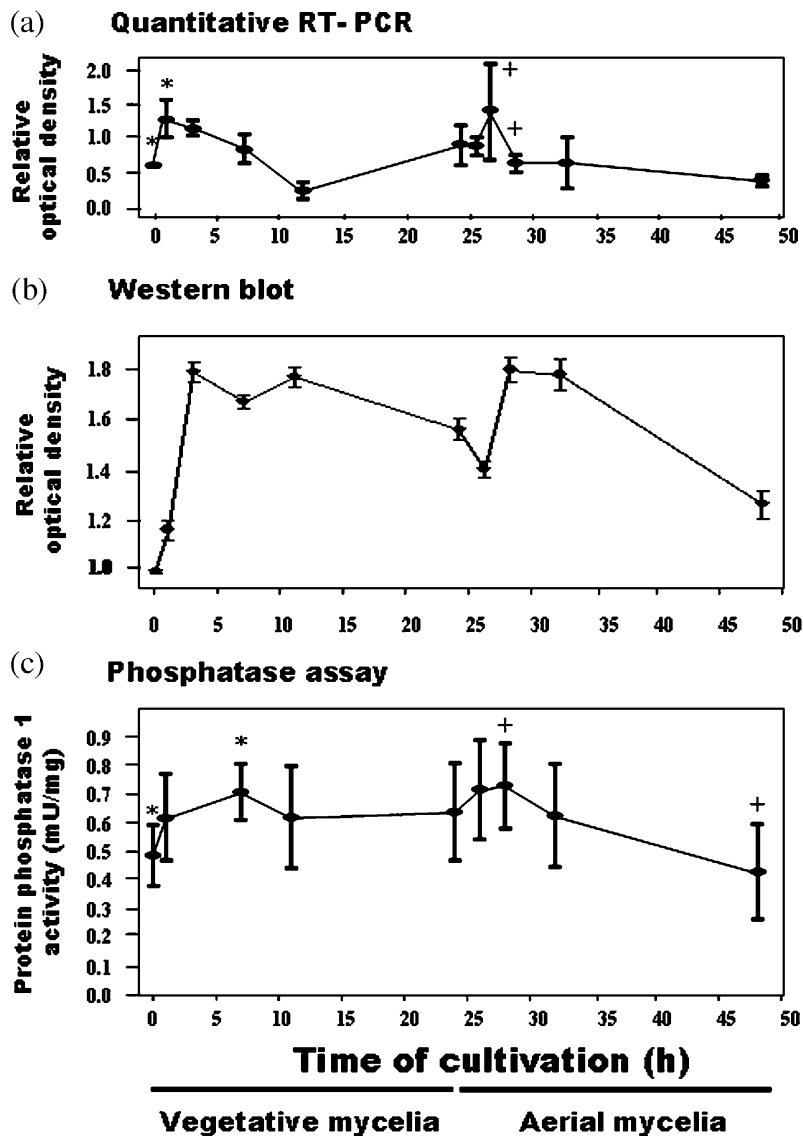


Fig. 3. Quantitative evaluation of *ppp-1* gene expression. Synchronized *N. crassa* cultures were prepared and analyzed as described in Fig. 2. The band intensities were estimated by densitometry after RT-PCR (a) and Western blotting (b). The ratio of the mRNA (cDNA) to the competitor genomic DNA is given in relative units in panel a. The Ppp-1 protein levels relative to the starting value (0 h) are shown in panel b. In parallel experiments the protein concentration and PP1 activity were assayed in the crude extracts and the specific activity of PP1 was determined (c). Error bars indicate the standard deviation of three independent experiments. Asterisks and crosses label significant changes ($P < 0.05$ for RT-PCR and Western, while $P < 0.25$ for the phosphatase assays) in the vegetative and aerial mycelia, respectively.

that was followed by a gradual decrease in the protein level during prolonged cultivation. Since *ppp-1* is a single copy gene coding for the catalytic subunit of PP1 the assay of PP1 activity can also be used to monitor the gene expression at the protein level. The specific activity of PP1 elevated at the early phases of the vegetative growth and aerial mycelium formation (Fig. 3c). The peaks of

the activity appeared at 7 and 28 h, i.e. 7 h after the onset of culturing in liquid medium and 4 h after the transfer to solid support and exposure to open air.

4. Discussion

In most organisms several PP1 catalytic subunit isoforms have been detected (Dombrádi, 1997).

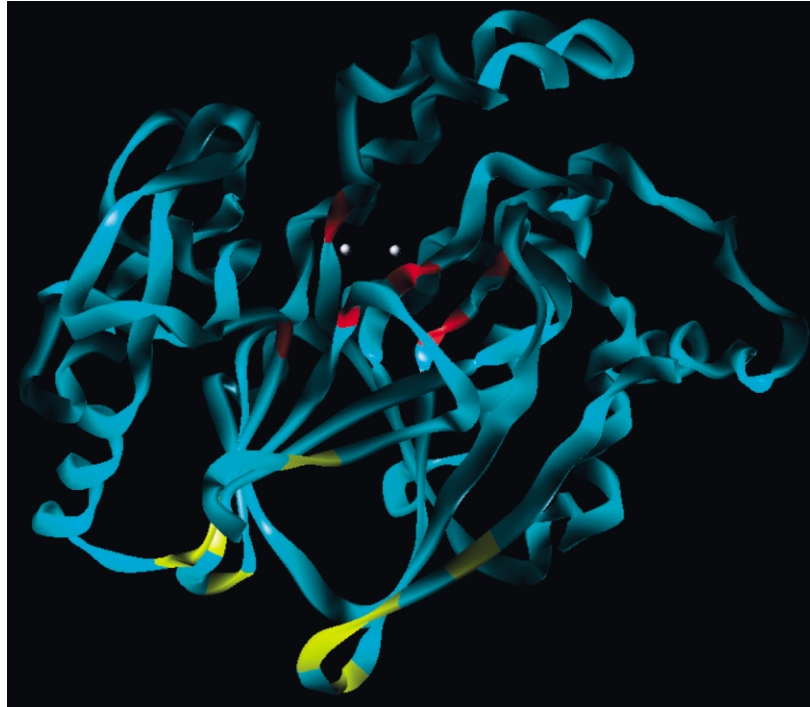


Fig. 4. Homologous molecular model of *N. crassa* protein phosphatase 1 catalytic subunit. The C α backbone of the Ppp-1 protein (amino acids 7–300) is depicted in blue. Residues essential for catalytic activity are shown in red, while the ones involved in the binding of putative regulatory subunits are highlighted in yellow. White spheres represent the metal ions in the active site.

Saccharomyces cerevisiae and *N. crassa* are the only known exceptions. In the latter case repeat induced point mutation (RIP) may have prevented the functional duplication of the gene. This assumption is supported by the fact, that all of the PPP enzyme family members are represented by a single gene in the *N. crassa* genome (Table 1). We found a significant sequence similarity between *ppp-1* and *pzl-1*, a fact that explains why the *ppp-1* cDNA was detected by a *pzl-1* probe in our screening.

The essential function of PP1 was demonstrated by gene disruption and by the genetic analysis of conditional, and hypomorphic mutants in different organisms. GLC7/DIS2S1 is indispensable in the cell cycle regulation, glycogen metabolism, cell wall integrity and morphogenesis of the baker's yeast (Ohkura et al., 1989; Andrews and Stark, 2000). A mutation of the *A. nidulans bimG* gene results in a block in mitosis, hyperphosphorylation of nuclear proteins and excessive swelling of the conidia (Doonan and Morris, 1989). Most probably a defect in chitin synthesis is in the background of the abnormal shape and osmotic instability of

the germlings (Borgia, 1992). Our results are in agreement with the above findings. Although the enzyme was present in *N. crassa* at all stages of the asexual life cycle we noticed two small but significant elevations of the *ppp-1* gene expression at well-defined points. As a general tendency, the translation of the Ppp-1 protein followed the transcription of the *ppp-1* mRNA with some delay. The first peak was observed a few hours after the beginning of cultivation, when the germination of conidia and the outgrowth of hyphae took place. The second increase coincided with the formation of the aerial mycelium. In both cases intensive chitin synthesis is required for the building up of new cell wall masses (Yarden and Yanofsky, 1991; Sietsma et al., 1996).

The structure of PP1 catalytic subunits has been well conserved during evolution. The central core (amino acids 45–301 in *N. crassa* Ppp-1) is nearly the same in all examples; most of the replacements represent conservative substitutions (Lin et al., 1999). The striking 93% amino acid identity to BimG from *A. nidulans* indicates common functions for the fungal enzymes. The structural simi-

larity to the animal counterparts was demonstrated by homologous modeling based on the atomic coordinates of rabbit PP1 α (Goldberg et al., 1995) (Fig. 4). The predicted three-dimensional structure of the α -carbon backbone of *N. crassa* Ppp-1 overlaps with that of the rabbit protein. The metal-ion binding sites within the catalytic center are all well conserved in the fungal enzyme. The conservation of amino acid residues that are responsible for the interaction with regulatory proteins in the structure of Ppp-1 strongly suggests the existence of such proteins in *N. crassa*. However, there is only limited information on the Ppp-1 regulatory subunits in this filamentous fungus. A heat- and thermostable inhibitor called INc was partially purified and characterized (Zapella et al., 1996; da-Silva et al., 1999), but none of the Ppp-1 interacting proteins have been cloned yet. The computer analysis of the *N. crassa* genome project's database revealed a number of putative Ppp-1 interacting proteins including the homologues of inhibitor-2, inhibitor-3, SHP1, ribosomal protein L5, GRP78, Sds22 and the glycogen binding subunit G_M (Csóka et al., unpublished data). All of these putative PPP-1 interacting proteins contain a typical binding motif important for complex formation with the catalytic subunit (Egloff et al., 1997). The investigation of the function of the hypothetical interacting proteins is currently in progress in our laboratory.

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