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Detection of a protein phosphatase 2A holoenzyme in Neurospora crassa.

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Abstract

A high molecular mass (about 280 kDa) protein phosphatase 2A holoenzyme was detected in a crude *N. crassa* extract by gel filtration coupled with phosphatase activity assays. According to Western blot analysis the holoenzyme consists of a 36-kDa catalytic subunit complexed with an additional, yet unidentified, regulatory subunit(s).

Detection of a protein phosphatase 2A holoenzyme in *Neurospora crassa*.

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A high molecular mass (about 280 kDa) protein phosphatase 2A holoenzyme was detected in a crude *N. crassa* extract by gel filtration coupled with phosphatase activity assays. According to Western blot analysis the holoenzyme consists of a 36-kDa catalytic subunit complexed with an additional, yet unidentified, regulatory subunit(s).

Protein phosphorylation is one of the major reversible post-translational mechanisms regulating almost all cellular functions. Protein phosphatase 2A (PP2A), a member of the serine/threonine protein phosphatase subfamily, plays an essential role in the regulation of a wide range of metabolic and cellular processes including motility, cell division, growth signaling, and gene expression (Mumby and Walter 1993 Physiol. Rev. 73: 673-699).

In higher eukaryotes, the PP2A holoenzyme exists as a heterotrimer. The heterotrimer is comprised of a core complex consisting a 36-kDa catalytic subunit (PP2Ac) tightly associated with a 65-kDa regulatory subunit (Reg A). This dimeric core binds a third, variable, regulatory subunit (Reg B). The Reg B subunit has been shown to control enzyme activity and specificity (Mayer-Jaekel and Hemmings 1994 Trends Cell Biol. 4: 287-291). Previously, we have cloned *pph-1*, the gene encoding the PP2Ac, and demonstrated its essential role in *N. crassa* (Yatzkan and Yarden 1995 Curr. Genet. **28:** 458-466). We have shown that lower levels of *pph-1* transcript and lower PP2A activity conferred increased sensitivity to phosphatase inhibitors and reduction in hyphal growth (Yatzkan *et al.*, 1998 Mol. Gen. Genet. in press). Though the *N. crassa* PP2Ac has been purified and biochemically characterized (Szoor *et al.*, 1995 Comp. Biochem. Physiol. **112B:** 515-522), the quaternary structure of PP2A has not been studied in a filamentous fungus. In the present communication we describe the fractionation and partial purification of the *N. crassa* PP2A holoenzyme as well as the immuno-detection of the PP2Ac in the fractionated enzyme complex.

Protein extraction from *N. crassa* wild-type strain 74-OR23-1A (FGSC987) and phosphatase assays were performed as described earlier (Szoor *et al.*, 1995 Comp. Biochem. Physiol. **112B**: 515-522), with a minor modification. Namely, the extraction buffer was amended with 0.3 M sucrose in order to stabilize the PP2A holoenzyme. The 15,000g supernatant of the mycelial extract (1 ml) , was loaded on a Superdex 200HR 10/30 (Pharmacia) column. The sample was fractionated on a Pharmacia FPLC syster using an imidazole buffer (50 mM, pH 7.4) containing 5 mM EDTA, 10 mM β-mercapthoethanol and 100 mM NaCl, in the cold a a flow rate of 500 µl/min. Void volume was determined with Blue Dextran 2000 (Pharmacia). Fractions were assayed for protein phospbatase activity in the absence or in the presence of specific phosphatase inhibitors. Fractions 19-25 (0.5 ml each) were precipitated with 1 ml acetone and the precipitate was subsequently resolved in a 10% SDS polyacrylamide gel. Anti-PP2Ac antibodies raised against a synthetic peptide corresponding to residues 296-309 of the C-terminal region of human PP2A (Upstate Biotechnology) were diluted 5,000-fold and were used in Western blot analysis as described by Szoor *et al.*, (1995 Comp. Biochem. Physiol. **112B**: 515-522).

The size of the *N. crassa* PP2A holoenzyme was calculated to be approximately 280 kDa based on the elution profile of the okadaic acid-sensitive phosphatase active fractions (Figure 1A). The highest PP2A activity was measured in fractions 21-25 with peak activity in fractions 23-24. These results correlate well with the immuno-detection of PP2Ac in the same fractions (Figure 1B). Thus practically all of the PP2A activity and the PP2Ac protein is present in a high molecular mass holoenzyme. Since the molecular mass of PP2Ac is 36 kDa, the holoenzyme must exist as a complex between the catalytic subunit and other, yet to be identified regulatory submit(s). The phosphatase activity sensitive to the inhibitor-2 protein can be attributed to protein phosphatase 1 (PP1). Even though it is evident that a portion of PP1 is eluted in the high molecular mass range, most of the PP1 activity appears in the lower molecular mass fractions (Figure 1A), as expected under these purification conditions, which do not favor the maintenance of a stable PP1 complex.

Further characterization of the putative Reg A and Reg B subunits of the *N. crassa* PP2A holoenzyme along with dissection of the actual components of the functional complex(es) described here will serve to complement the genetic data which is accumulating concerning this protein phosphatase.

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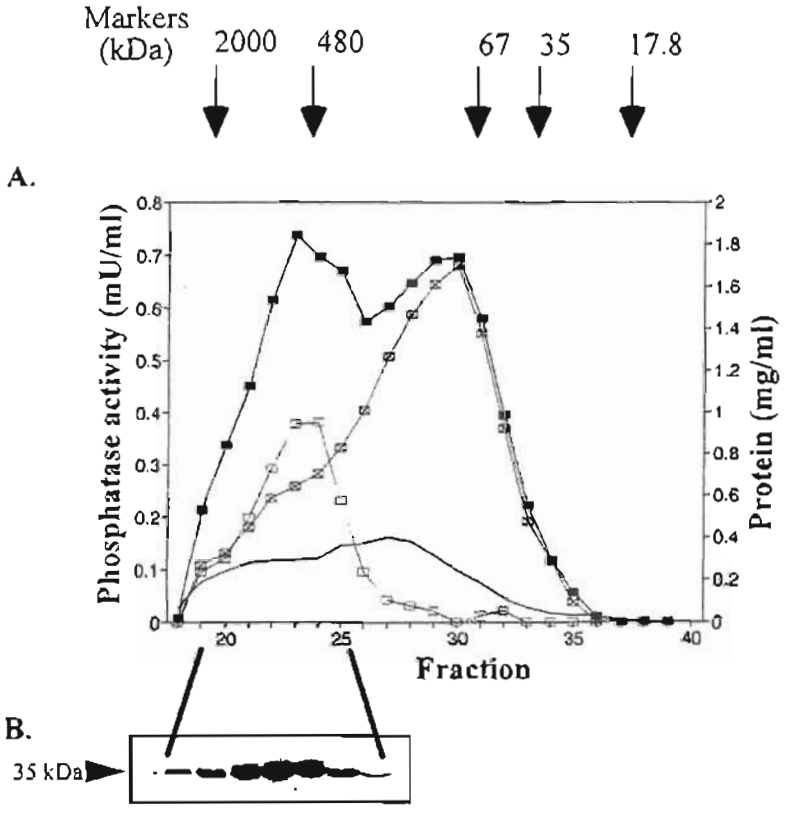

Fig. 1 - A. Gel filtration of *N. crassa* PP2A holoenzyme on Superdex 200HR. Fractions were analyzed for total protein phosphatase activity (■), and in the presence of 1 nM okadaic acid, a specific inhibitor of PP2A (X) or 100U/ml inhibitor-2, a specific inhibitor of PP1 (□). Protein concentration (mg/ml) is presented as an unmarked line (—). The elution positions of molecular mass markers used for the calibration of the column were :Blue Dextran 2000, BSA, rabbit muscle PP1c and myoglobin (indicated by arrows).
B. Western blot analysis of fractions No. 19-25 with antibodies raised against a peptide synthesized on the basis of the C-terminal region of human PP2Ac. The arrow indicates the position of the 35kDa marker.

Brief Notes

Storage of Aconidial Strains of *Neurospora crassa* by Freezing at -80°C.

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Aconidial strains of *N. crassa* can be difficult to store for extended periods of time. Methods involving heterokaryons formed with helper strains (Perkins 1986 FGN 33:35-41), amassing mycelial inocula for freezing (P. Margolis, pers. comm. 1997), continual passage, or mycelial storage on silica gel (Wilson 1986 FGN 33:47-48) have been used previously. For other fungi, freezing larger quantities of mycelia as agar plugs had been used as a convenient storage technique. Storage of aconidial strains of *Neurospora crassa* by freezing of mycelia as agar plugs and slants at -80°C and -20°C, similar to the preservation of slime strains (Selitrennikoff 1978 *Neurospora* Newsl. 25:16, Jong and Davis 1979 *Neurospora* Newsl. 26:26) was investigated.

Fluffy A (FGSC 4960), Fluffy a (FGSC 4961), and Acon-3 (FGSC 5074) were grown on plates of Vogel's Minimal Medium with 2% sucrose for 3 days at room temperature to a confluent mycelial lawn. Approximately 1 cm square plugs were cut from the agar and placed into sterile 1.5 ml Eppendorf tubes. The tubes were placed in a -80°C freezer with no flash freezing and no glycerol or DMSO added. Agar plugs were retrieved from -80°C after 7 days to 9 months and plated onto the same medium as above. They grew to form a confluent lawn in 3 days. Fluffy *A* and Fluffy *a* functioned normally in mating type tests after storage at -80°C. Whole agar slants with mycelia from the aconidial strains were frozen at -80°C. Pieces chipped from the frozen agar grew well on plates following storage of 1 month. Recovery from agar plugs and slants stored for 1 month (the only time tested) at -20°C was slower than from those stored at -80°C, with 3-7 days required for a confluent lawn of mycelia to grow. The recovery of aconidial strains after freezing under these conditions may be due to the larger amount of mycelia stored initially. Sufficient mycelia are present in the 1 cm plugs to allow recovery of the strains, providing a convenient alternative storage technique for aconidial strains.

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