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# A search for myosin in elongating hyphae of *Neurospora crassa*

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## Abstract

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Myosin light-chain from smooth muscle has been phosphorylated by a $Ca^{2+}$, calmodulin-dependent protein kinase from *N. crassa*. Evidence for a myosin-like protein based on the $Ca^{2+}$-ATPase activity in the absence of $Mg^{2+}$ has been obtained in growing mycelia.

## Introduction

Myosin-like proteins are still poorly known in plants (Jackson 1982). In Fungi, they have only been isolated and characterized, but in much smaller amounts than the other major contractile protein actin, from slime molds (Adelman and Taylor 1969) and, recently, from budding yeast cells (Watts et al. 1985). These proteins have several properties in common with muscle myosin as they not only bind to actin into actomyosin but present $Ca^{2+}$ ions activated ATPase activity (Korn 1978).

We have recently proposed a model of polar exocytosis of vesicles in outgrowing germ tubes and elongating hyphae of *Neurospora crassa* implicating the actomyosin system. Actin microfilaments were cytochemically evidenced, but the presence of myosin was only indirectly inferred from the myosin-kinase activity of our $Ca^{2+}$-calmodulin extracts of *N. crassa* hyphae (van Tuinen 1985). We now present such results corroborated by the first evidence for a myosin-like protein in *N. crassa* founded on the activity of a $Ca^{2+}$-ATPase in extracts of its elongating hyphae.

## Materials and methods

*Neurospora crassa* strain STA4 (FGSC) has been grown in Vogel's medium (1956) supplemented with 2% sucrose as previously described (Ortega Perez et al. 1981) and the harvested mycelia were frozen in liquid nitrogen and stored at −80 °C until use.

Frozen mycelia were disrupted in a blender (Moulinex, type 320) for 2 min. The dry powder was resuspended in 2 vol. extraction buffer: 20 mM imidazole-HCl pH 7.5, 370 mM sucrose,

10 mM β-mercaptoethanol, 10 mM benzamidine, 0.25 µg/ml pepstatin, and retreated as described above.

The homogenate was filtered through a single layer of nylon cloth and ultracentrifuged at 160,000 g for 90 min in a TFT. 7038 rotor (Prepspin, Kontron). The pellet was resuspended in the extraction buffer supplemented with 1% Triton X 100 and recentrifuged for 60 min as described above. The supernatant obtained was dialysed against the extraction buffer, in which the protease inhibitors were omitted. This dialysate was used for the ATPases assays. All the operations were performed at 4 °C.

The $Ca^{2+}$, calmodulin-dependent protein kinase has been partially purified by affinity chromatography on a calmodulin-Sepharose column (van Tuinen et al. 1984). The myosin light chain (MLC) of chicken geaser was a gift of Dr. F. Martin, University of Montpellier. The phosphorylation assays were performed at 37 °C in Tris-HCl buffer pH 7.5, containing 5 mM $MgCl_2$, 10 mM NaF, 1 mM β-mercaptoethanol and either 3 mM EGTA alone or 3 mM EGTA and 3.5 mM $CaCl_2$. Assays of ATPase activities were performed at 37 °C in 250 µl aliquots containing 50 mM imidazole pH 7.5, 1 mM ATP and when stated 5 mM $CaCl_2$ or 5 mM $MgCl_2$ or 2 mM EGTA, 2 mM EDTA and 500 mM KCl. The enzymatic reactions were initiated by adding ATP and stopped by boiling. The Pi was measured as according to Chen et al. (1956).

The phosphorylative reactions were initiated by adding 1 µCi [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol, Amersham Radiochemical Center) and stopped by boiling and then analyzed by elelctrophoresis on SDS-polyacrylamide gels (Laemmli 1970) followed by autoradiography. The relative incorporations were determined by densitometric analysis of the autoradiograms.

Protein concentrations were determined as described by Spector (1978), using bovin serum albumin as standard.

## Results

### *a) Phosphorylation of myosin light chain by $Ca^{2+}$, calmodulin-dependent kinase*

Following our isolation of a protein kinase from hyphae of *N. crassa,* we have searched for its protein targets (van Tuinen et al. 1984). After detection of an endogenous peptide determined to have molecular weight of 47 kDa, we have tested some other exogenous proteins for their ability to be phosphorylated in a calcium-calmodulin depending manner. Casein, phosphorylase b, histone, microtubule-associated

Table 1. Phosphorylation of MLC by $Ca^{2+}$, calmodulin-dependent protein kinase from *Neurospora crassa*

| | $Ca^{2+}$ CaM | $Ca^{2+}$ | EGTA CaM | $Ca^{2+}$ CaM | $Ca^{2+}$ CaM |
|---|---|---|---|---|---|
| Protein kinase | – | + | + | + | + |
| MLC | + | + | + | + | – |
| Relative Pi incorporation in the peptide: | | | | | |
| 47 kDa | – | 32 | 20 | 100 | 176 |
| MLC | 0 | 35 | 26 | 45 | – |

The phosphorylation assays were performed at 37 °C in 45 µl aliquots containing 20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 3 mM EGTA (minus calcium) and 3.5 mM $CaCl_2$ (plus calcium), 0.3 M KCl, 1 µCi ATP and were stated 1 µg calmodulin (CaM). The EGTA eluate of the calmodulin-Sepharose column contained the protein kinase and the 47 kDa peptide.

Table 2. ATPase activity in the Triton X 100 soluble, supernatant preparation of *Neurospora crassa*

| | No addition | $K^+$/EDTA | $CaCl_2$ | $MgCl_2$ |
|---|---|---|---|---|
| Activity nmol/min · mg | ≦ 5 | 36 | 399 | 210 |

The ATPase assays were performed at 37 °C in 250 µl aliquots containing 50 mM imidazole/HCl pH 7.5, 1 mM ATP and one of the following (1) 0.5 M KCl, 2 mM EDTA, 2 mM EGTA; (2) 5 mM $CaCl_2$, 2 mM EDTA; (3) 5 mM $MgCl_2$, 2 mM EGTA.

protein, glycogen synthase were not phosphorylated by the $Ca^{2+}$, calmodulin-dependent protein kinase. In contrast, the light chain myosin (MLC) from chicken geaser could efficiently by phosphorylated by a protein kinase eluted from calmodulin-Sepharose affinity column (Table 1). The phosphate incorporation in the MLC was stimulated by a factor of 1.3 by calcium alone and 1.7 by the presence of both calcium and calmodulin with the activity increased in the presence of calmodulin but in the absence of calcium (EGTA).

*b) $Ca^{2+}$-ATPase activity*

The $Ca^{2+}$ requirement is known to be characteristic for one of the myosin ATPase activities. Therefore we analyzed ion requirements for the ATPase activity present in the ultracentrifuged supernatant, obtained as described above, of *N. crassa* supernatant.

The ATPase activity in the presence of $CaCl_2$ is about twice as high as the activity observed in the presence of $MgCl_2$ alone (Table 2). On the other hand, the activity in the presence of $K^+$/EDTA is relatively low. A low activity in the presence of $K^+$/EDTA has also been observed in *Saccharomyces cerevisiae* (Watts et al. 1985), but in opposition to *N. crassa,* the myosin ATPase activity in yeast was higher in the presence of $MgCl_2$ than in the presence of $CaCl_2$.

## Discussion

Our ATPasic myosin-like activity will need further characterization to be related to a true fungal myosin, presumably microfilamentous and therefore able to interact with actin microfilaments productive of the shearing force required for the polarized vesicular traffic in the hyphal tips (Turian et al. 1985).

Such interaction could be related to the $Ca^{2+}$-calmodulin dependent myosin-kinase activity detected in elongating hyphae of *N. crassa,* in contrast to higher plants, where MLC from smooth muscle was not phosphorylated by protein kinase (Polya et al. 1983).

In order to exert a mechanical force, the contractile assembly – actin and myosin filaments – must someway be anchored to other cellular components such as cell membranes (Alberts et al. 1983). The association of our myosin-like protein with the particulate fraction could therefore be explained by the binding of myosin to membranes.

Myosin is the only actin-associated protein that can generate mechanical force derived from ATP hydrolysis (Fulton 1984). Anticipating on its detection in outgrowing germ tubes and elongating hyphal tips of *N. crassa,* we have proposed a model for such an ATP-derived mechanical force for the directional driving of vesicles or exocytosis to the acidified polarly expanding tips (Turian et al. 1985). The first evidence of myosin-like protein in *N. crassa* growing hyphae reenforces our proposal.

**Résumé**

La chaîne légère da la myosine de muscle lisse a été phosphorylée par une protéine $Ca^{2+}$, calmoduline-dépendante de *N. crassa.* Dans le mycélium en croissance, la présence d'une protéine de type myosine a été suggérée par l'activité $Ca^{2+}$-ATPasique obtenue en l'absence d'ions Mg.

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