Calcium, calmodulin-dependent protein phosphorylation in *Neurospora crassa*

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A calcium, calmodulin-dependent protein kinase activity has been partially purified by calmodulin–Sepharose affinity chromatography from the soluble fraction of *Neurospora crassa*. The phosphorylated peptide has an apparent molecular mass on SDS–polyacrylamide gel of 47 kDa. The apparent half maximal phosphorylation is obtained after 1.5 min at 30°C in the presence of calcium and calmodulin. The apparent half maximal activation of the phosphorylation is obtained at 1 μM calcium, and 0.1 or 0.2 μM calmodulin from bovine brain or *Neurospora*, respectively. The ^{32}P incorporation is enhanced about 10-fold by calmodulin.

Protein kinase *Calmodulin* *Calcium* Neurospora crassa

1. INTRODUCTION

Protein phosphorylation has been shown to be a powerful tool in the regulation of biochemical and physiological processes [1]. In higher plants, a protein kinase activity has been shown to be regulated by calcium and calmodulin [2].

In *Neurospora crassa* a cyclic AMP-dependent protein kinase has been described [3–5]. Protein phosphorylation so far seemed to be unaffected by calcium [4]. However, the presence of calmodulin has been demonstrated in this fungus [6], and a cyclic nucleotide phosphodiesterase has been shown to be calcium, calmodulin-dependent [7]. Therefore it was of interest to investigate if other enzymatic systems were calcium, calmodulin-dependent. We demonstrate the presence of a calcium, calmodulin-dependent protein kinase and describe some of its biochemical properties.

2. MATERIALS AND METHODS

The wild-type strain STA4 (FGSC no.262) of *N. crassa* was obtained from the Fungal Genetic Stock Center, Humboldt State University (Arcata, CA, USA). Growth conditions were as described in [6]. Frozen mycelia were disrupted in a Moulinex blender (type 320) for 2 min. The dry powder was resuspended in 2 ml per g of mycelial wet weight buffer A: 20 mM Tris–HCl (pH 7.5), 3 mM $MgCl_2$, 1 mM EGTA, 1 mM β-mercaptoethanol, 10 mM benzamidine, 0.25 μg/ml pepstatin and retreated again for 2 min, as described above. All further manipulations were carried out at 4°C.

The homogenate was filtered through a single layer of nylon-cloth and centrifuged at 30000 × *g* for 30 min. Soluble proteins were precipitated at 65% saturation of $(NH_4)_2SO_4$. After dialysis against buffer A containing 0.5 M NaCl, 2 mM $CaCl_2$ was added and then recentrifuged at 129000 × *g* for 3 h in an MSE Prepspin ultracentrifuge.

Endogenous calmodulin was removed from this latter supernatant by chlorpromazine–Sepharose chromatography [8]. The effluent was loaded on a calmodulin–Sepharose column [9] equilibrated with buffer B: 20 mM Tris–HCl (pH 7.5), 3 mM $MgCl_2$, 1 mM EGTA, 2 mM $CaCl_2$, 0.5 M NaCl, 1 mM β-mercaptoethanol supplemented with 0.3% (w/v) BSA. Column were washed with the

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same buffer. Before elution with 3 mM EGTA (in buffer A containing 0.5 M NaCl) BSA was removed by washing with buffer B.

Calmodulin from bovine brain and *N. crassa* were prepared as described in [8] and [6], respectively. Phosphorylation assays contained in a final volume of 45 μl 20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM NaF, 1 mM β-mercaptoethanol, and either 3 mM EGTA alone or 3 mM EGTA and an appropriate amount of calcium to give a free calcium concentration between 10^{-8} and 10^{-5} M. The free calcium concentration was calculated by a computer program kindly provided by Professor B. Gottwald, Freiburg, FRG. The amount of protein in the assay condition was between 10 and 30 μg. The calmodulin concentration was 1 μM. Proteins were determined as in [10] using BSA or calmodulin as standards.

The reactions were initiated by adding 1 μCi [γ-^{32}P]ATP (3000 Ci/mmol, from Amersham Radiochemical Center) at 30°C and terminated after 7 min by addition of 25 μl of three times concentrated electrophoresis-sample buffer containing 187 mM Tris–HCl (pH 7.8), 6% SDS, 6% β-mercaptoethanol, 45% glycerol and 0.006% bromophenol blue. The samples were then boiled for 1 min. Phosphorylated proteins were separated by electrophoresis on a 12.5% SDS–polyacrylamide gel prepared as described [11]. After electrophoresis, the gels were stained with Coomassie brilliant blue, dried and exposed on a Kodak X-Omat SO 282 film. The autoradiograms were scanned on a Varian densitometer to determine the relative P_i incorporation in the peptides.

3. RESULTS

A soluble fraction of the *N. crassa* mycelia was tested for endogenous calcium, calmodulin-dependent phosphorylation: in the crude soluble fraction partially purified by ammonium sulfate precipitation, a peptide of 63 kDa was endogenously phosphorylated in the presence of calcium alone and in the presence of calcium and exogenous calmodulin (fig.1, lane A). In the absence of calcium the 63-kDa peptide is not phosphorylated, but a peptide of 70 kDa is highly phosphorylated. This latter peptide was only a little phosphorylated in the presence of calcium, alone, or calcium and calmodulin. In this fraction,

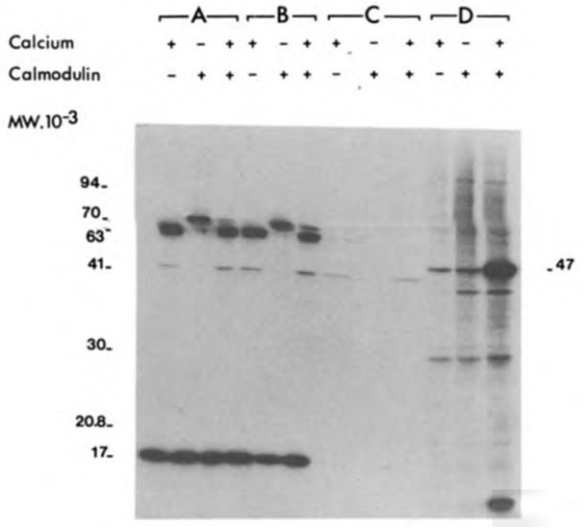

Fig.1. Autoradiograph of phosphorylated peptides separated on a 12.5% SDS–polyacrylamide gel. (A) Ammonium sulfate fraction; (B) pass through fraction of the chlorpromazine and calmodulin–Sepharose columns; (C) EGTA eluate fraction of the calmodulin affinity column obtained in the presence of low ionic strength, and (D) high ionic strength. The phosphorylation assays were performed in the presence of 3 mM EGTA (minus calcium), 3 mM EGTA and 3.5 mM $CaCl_2$ (plus calcium), and where stated 1 μg bovine brain calmodulin.

two other peptides incorporated ^{32}P: one, a 41-kDa peptide, showed calcium-dependent phosphorylation while the other, a 17-kDa peptide, did not exhibit calcium dependence. No difference in the phosphorylation pattern was observed after passage of the ammonium sulfate precipitate through a chlorpromazine–Sepharose column to remove endogenous calmodulin, and through a calmodulin–Sepharose column to retain calmodulin-binding proteins (fig.1, lane B). When the calmodulin–Sepharose column was eluted with EGTA in the absence of sodium chloride (fig.1, lane C), no endogenous calcium, calmodulin-dependent phosphorylation could be detected in the eluate; only the 41-kDa peptide was present in this fraction. The elution fraction of the calmodulin-affinity column obtained with EGTA and high ionic strength (0.5 M NaCl) revealed a polypeptide of 47 kDa which could be phosphorylated in a calcium, calmodulin-dependent manner (fig.1, lane D). This 47-kDa

peptide, which was phosphorylated to some extent in the presence of calcium or calmodulin, could not be detected in the crude homogenate. It is therefore not possible to determine the purification factor. When the extraction was performed in the absence of protease inhibitors (i.e., 10 mM benzamidine and 0.25 μg/ml pepstatin) partial hydrolysis of the 47-kDa peptide was observed. These proteolytic fragments, with molecular masses of 30 to 14 kDa could also be phosphorylated in a calcium, calmodulin-dependent manner (see fig.1, lane D).

3.1. *Calcium dependence*

The calcium dependence of the phosphorylation of the 47-kDa peptide was investigated in the presence of saturating concentrations of calmodulin (fig.2). The phosphorylation activity was insensitive to concentration of calcium below 0.5 μM. Maximal phosphorylation activity was obtained at a free calcium concentration of 10 μM. The apparent half-maximal phosphorylation was obtained with 1 μM calcium.

3.2. *Calmodulin dependence*

The ^{32}P incorporation of the 47-kDa peptide was assayed in the presence of various amounts of calmodulin from different sources at saturating calcium concentrations (fig.3). The apparent half-maximal activation was obtained at 0.1 μM calmodulin from bovine brain or 0.2 μM calmodulin from *N. crassa*. The phosphorylation activity was enhanced about 10-fold by calmodulin. The magnitude of the kinase activity amplification, as the slope of the dose response curve, were similar for bovine brain and *N. crassa* calmodulin. At saturating concentrations of calcium and calmodulin, the phosphorylation activity was inhibited 20 and 54% by the presence of, respectively, 56 and 112 μM chlorpromazine, a calmodulin antagonist (not shown).

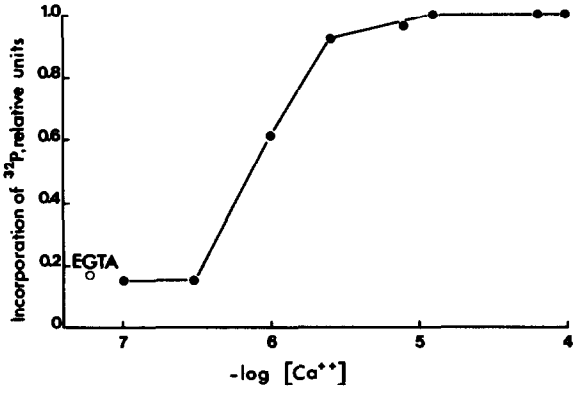

Fig.2. Calcium dependence of the phosphorylation of the 47-kDa peptide was tested in the presence of saturating concentrations of calmodulin.

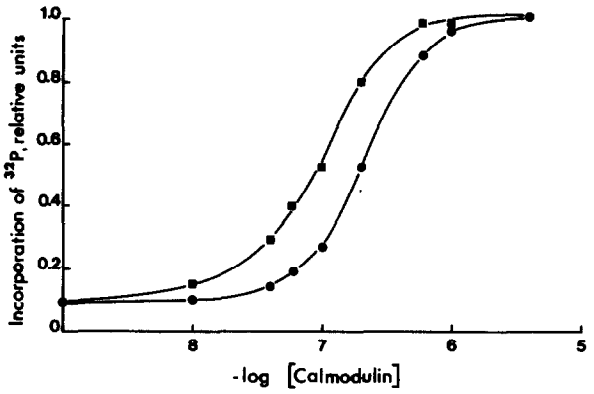

Fig.3. The calmodulin dependence of the 47-kDa peptide phosphorylation was tested with various amounts of bovine brain (■—■) or *N. crassa* (●—●) calmodulin, in the presence of saturating concentrations of calcium.

4. DISCUSSION

The data described clearly demonstrate the presence of a soluble calcium, calmodulin-dependent protein kinase activity in *N. crassa* mycelia. This activity could only be detected after chromatography on a calmodulin–Sepharose column. We were not able to detect this calcium, calmodulin-dependent phosphorylated peptide in the partially purified soluble fraction; this is probably due to the low concentration of this peptide in this fraction or to the presence of an inhibitor. The kinase activity is strongly enhanced by calmodulin. As compared to the basal activity, i.e., activity in the presence of EGTA and calmodulin, or calcium alone, the incorporation of ^{32}P in the 47-kDa peptide is increased about 10-fold. The concentration of calmodulin needed to obtain the apparent half-maximal activation of the protein kinase activity is relatively high. Such

a concentration has already been reported for the cyclic nucleotide phosphodiesterase of *N. crassa* [7] and for zucchini protein kinase [2]. As the calmodulin-dependent phosphorylation assays were performed in the calmodulin–Sepharose column eluate, all the other calmodulin-binding proteins were potential calmodulin competitors. The calmodulin-dependent phosphorylation demonstrated in the EGTA eluate of the calmodulin–Sepharose column implicates the presence of the enzyme and its substrate. Several hypotheses can be suggested in order to explain this result: (i) the substrate can be a contamination of the protein kinase preparation; (ii) the protein kinase–substrate complex has a high calcium-dependent affinity for calmodulin; (iii) the protein kinase and the substrate could each have a calcium-dependent affinity for calmodulin; (iv) the protein kinase might be autophosphorylated in the presence of calcium and calmodulin. Attempts to separate the protein kinase and the substrate, and to demonstrate their molecular identity are being pursued.

The data reported bring new insights in the regulation of protein phosphorylation in lower eukaryotic organisms. At present, the physiological relevance of the calcium, calmodulin-dependent protein phosphorylation in *N. crassa* is unknown.

REFERENCES

[1] Krebs, E.G. and Beavo, J.A. (1979) Annu. Rev. Biochem. 48, 923–959.
[2] Salimath, B.P. and Marmé, D. (1983) Planta 158, 560–568.
[3] Powers, P.A. and Pall, M.L. (1980) Biochem. Biophys. Res. Commun. 95, 701–706.
[4] Judewicz, N.D., Glikin, G.C. and Torres, H.N. (1981) Arch. Biochem. Biophys. 206, 87–92.
[5] Trevillyan, J.M. and Pall, M.L. (1982) J. Biol. Chem. 257, 3978–3986.
[6] Ortega Perez, R., Van Tuinen, D., Marmé, D., Cox, J.A. and Turian, G. (1981) FEBS Lett. 133, 205–208.
[7] Ortega Perez, R., Van Tuinen, D., Marmé, D. and Turian, G. (1983) Biochim. Biophys. Acta 758, 84–87.
[8] Jamieson, G.A. jr and Vanaman, T.C. (1979) Biochem. Biophys. Res. Commun. 90, 1048–1056.
[9] Watterson, D.M. and Vanaman, T.C. (1976) Biochem. Biophys. Res. Commun. 73, 40–46.
[10] Spector, T. (1978) Anal. Biochem. 86, 142–146.
[11] Laemmli, U.K. (1970) Nature 227, 680–685.