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A calcium and calmodulin-dependent protein kinase present in differentiating *Dictyostelium discoideum*

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Abstract: A protein kinase from *Dictyostelium discoideum* which phosphorylates the synthetic peptide, calmodulin-dependent protein kinase substrate (CDPKS, amino acid sequence: PLRRTLSVAA) and is stimulated by Ca^{2+}/calmodulin is described. This is the first report of a protein kinase with these characteristics in *D. discoideum*. The enzyme was partially purified by Q-Sepharose chromatography. The protein kinase is very labile, and rapidly loses Ca^{2+}/calmodulin-dependence upon standing at 4°C, even in the presence of protease inhibitors, making further purification and characterisation difficult. In the active fractions, a 55 kDa polypeptide is labelled with [γ-^{32}P]ATP in vitro under conditions in which intramolecular rather than intermolecular reactions are favoured. The phosphorylation of this peptide is stimulated in the presence of Ca^{2+} and calmodulin but not Ca^{2+} alone. Ca^{2+}/calmodulin-dependent stimulation is inhibited in the presence of the calmodulin antagonist, trifluoperazine (TFP). It is proposed that the 55 kDa polypeptide may represent the autophosphorylated form of the enzyme.

Key words: Ca^{2+}; Calmodulin; Kinase; *Dictyostelium discoideum*; Autophosphorylation

Introduction

The emergence of specialised cell types during differentiation is a complex process involving a network of signals, receptors, and transducing elements. Due to the complexity of the process in higher organisms, simple eukaryotes are often used as model systems to study differentiation and its regulation. The cellular slime mould, *Dictyostelium discoideum* is one of the simplest eukaryotic organisms to undergo true multicellular differentiation and represents an ideal system in which to study developmental processes.

Dictyostelium initially grow as single celled amoebae which phagocytose and feed on bacteria. Upon depletion of the bacterial food source, starved amoebae undergo an aggregation process thus commencing the multicellular phase of the life cycle, acquiring the ability to synthesize and secrete cAMP, which serves as a chemotactic agent for their aggregation [1]. cAMP has been shown to induce several cellular events during differentiation. One of these is a rapid increase in the intracellular concentration of calcium [2,3] which is thought to act as a second messenger in signal transduction during the chemotactic response of *Dictyostelium* cells [4].

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In most eukaryotic cells, the processing of intracellular Ca^{2+} signals is mediated by the ubiquitous calcium receptor, calmodulin. In *Dictyostelium* calmodulin has been identified and characterised extensively [5], however, the role(s) of calmodulin as an intracelluar mediator of calcium signals is not well understood. Recent evidence suggests however that calmodulin may act to regulate biomembrane fusion events during the sexual cycle of *Dictyostelium* [6–8] and further evidence suggests that this process may involve Ca^{2+} and calmodulin-dependent protein phosphorylation [9]. It was therefore of interest to investigate if any Ca^{2+} and calmodulin-dependent enzymes exist in *Dictyostelium*. Here we demonstrate the presence of a soluble Ca^{2+} and calmodulin-dependent protein kinase in this organism during differentiation.

Materials and Methods

Materials

ATP, trifluoperazine, Kemptide and benzamidine were from Sigma Chemical Co. CDPKS was from AUSPEP. Bovine testis calmodulin, calmodulin-Sepharose 4B, Q-Sepharose and low range molecular mass markers were from Pharmacia, [γ-^{32}P]ATP was from Bresatec Ltd. and leupeptin from Peptide Institute Inc. Media components were purchased from Oxoid Ltd. All other materials used were of the highest quality available.

Routine cell culture

Dictyostelium discoideum strain V12 was grown in association with *Enterobacter aerogenes* on plates of standard medium (SM) agar (1% w/v) at 22°C. Vegetative cells were collected by centrifugation (200 × *g* for 5 min at 4°C) then washed three times in KK2. Culmination stage cells were obtained by plating washed vegetative amoebae on SM agar at a density of $3–5 \times 10^6$ cells cm^{-2} then allowing development to proceed for 15 h. *D. discoideum* strain AX3 was grown in HL5 medium as previously described [10] and culmination stage cells obtained as described above.

Preparation of enzyme extracts

All the operations were performed at or below 4°C. Cells were resuspended in buffer A (10 mM benzamidine, 1 mM dithiothreitol (DTT), 0.1% Triton X-100, 10 mg leupeptin ml^{-1} in 30 mM Tris · HCl, pH 7.4) and disrupted by sonication (3 × 15-s bursts) using a Sony sonicator probe at the maximum energy setting (7 amps). The homogenate was centrifuged at 40 000 × *g* for 15 min to remove particulate material. The resulting supernatant was subsequently used for all experiments.

Q-Sepharose chromatography

High speed supernatant was applied to a Q-Sepharose column (1 × 15 cm) at a flow rate of 0.7 ml min^{-1}. The column was then washed with 4 bed volumes of buffer A until OD 280 nm < 0.01 and proteins specifically bound to the resin were eluted in a 200 ml linear salt gradient (0–0.7 M NaCl in buffer A) and collected in 3 ml fractions.

Calmodulin-Sepharose 4B chromatography

Q-Sepharose fractions containing Ca^{2+}/calmodulin-dependent protein kinase activity were pooled and adjusted to 2 mM Ca^{2+} and 0.2 M NaCl. Sample was applied to calmodulin-Sepharose at a flow rate of 0.5 ml min^{-1}. Following washing with 4 column volumes of buffer B (10 mM benzamidine, 1 mM DTT, 2 mM Ca^{2+}, 0.2 M NaCl, 0.1% Triton X-100 in 30 mM Tris · HCl, pH 7.4) proteins specifically binding to the resin were eluted in 20 ml of buffer C (10 mM benzamidine, 1 mM DTT, 2 mM EGTA, 0.2 M NaCl, 0.1% Triton X-100 in 30 mM Tris · HCl, pH 7.4) and 1 ml fractions collected.

Estimation of protein kinase activity

For experiments involving the analysis of phosphorylated endogenous proteins by SDS-PAGE the assay system contained, in a final volume of 50 μl, 30 mM Tris · HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM sodium (ortho)vanadate (NaV), 10 μM [γ-^{32}P]ATP (sp. act. 14 Bq $pmol^{-1}$) and either 1 mM Ca^{2+} and 50 mg/ml calmodulin or 5 mM EGTA. Kinase reactions were initiated upon the addition of enzyme fraction (20 μl) and incubated at 30°C for 5 min. Reactions were terminated with 25 μl

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of SDS sample buffer followed by immediate boiling for 2 min. Radiolabelled proteins were subject to SDS-PAGE [11] and visualised upon exposure of Coomassie blue (R-250) stained gels to Kodak X-Omat film at −70°C.

To monitor kinase activity using exogenous substrates, the standard assay system contained, in a final volume of 60 μl, 30 mM Tris · HCl (pH 7.4), 5 mM $MgCl_2$, 1 mg/ml BSA, 1 mM NaV, 40 μM [γ-^{32}P]ATP (sp. act. 3 Bq pmol^{-1}), CDPKS or Kemptide (50 μM) and either 500 μM Ca^{2+} and 50 μg/ml calmodulin or 5 mM EGTA. Reactions were initiated with 20 μl enzyme fraction, incubated for 10 min at 30°C and terminated by spotting a 50 μl aliquot onto a square of Whatman P81 paper (2 × 2 cm). The paper squares were then washed in 75 mM orthophosphoric acid (4 × 5 min) dried and counted. Kinase activity in the absence of exogenous substrate was used as the control and subtracted from all counts.

Protein determinations

Protein assays were carried out as previously described [12].

Results

Ca^{2+}/calmodulin-dependent protein kinase activity could not be assayed directly in the high speed supernatant due to the presence of endogenous phosphatases and fractionation on Q-Sepharose was required to detect the enzyme. Preliminary experiments using histone II-A as substrate gave low and variable levels of phosphorylation in the presence of Ca^{2+} and calmodulin (data not shown). Subsequently the synthetic peptide CDPKS (camodulin-dependent protein kinase substrate) was used.

Chromatography on Q-Sepharose of *D. discoideum* enzyme extract, resolved two sharp peaks of protein kinase activity that phosphorylate CDPKS in the presence of Ca^{2+} and calmodulin (Fig. 1). These activity peaks, eluting at approximately 0.125 and 0.365 M NaCl, were designated PKI and PKII respectively. When fractions were re-assayed in the presence of EGTA, PKI activity

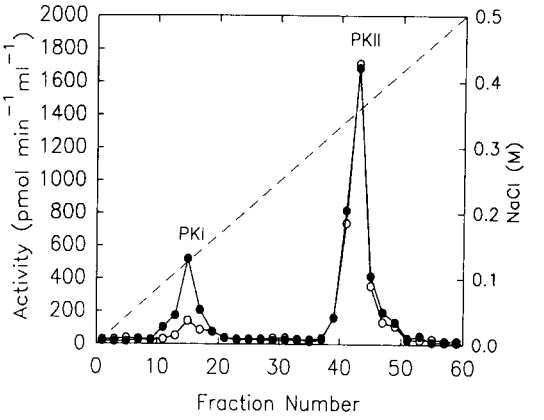

Fig. 1. Elution pattern of protein kinase activity from a Q-Sepharose column. Protein kinase activity assayed with EGTA (○), or with Ca^{2+} and calmodulin (●). NaCl concentration (------). Conditions were described in Materials and Methods. Kinase activity is expressed as the pmol of [^{32}P] incorporated min^{-1} ml^{-1} of fraction collected. Each point on the graph represents the mean of triplicate determinations.

was dramatically reduced, but PKII was unaffected.

To characterise the nature of PKI and PKII further, fractions 10–20 (PKI) and fractions 38–50 (PKII) were pooled and the action of various potential effector molecules on these protein kinase peaks studied. The activity of PKI was stimulated three-fold above basal levels (no additions) in the presence of Ca^{2+} and calmodulin (Table 1). This activity was dependent upon the formation of a Ca^{2+}/calmodulin complex since Ca^{2+}

Table 1

Effect of Ca^{2+}, calmodulin, cAMP, EGTA and TFP on PKI and PKII protein kinase activity peaks

Treatment	Protein kinase activity (pmol min^{-1} ml^{-1}) ±S.E.M.	
	PKI	PKII
None	110± 8	873±23
Ca^{2+}	113± 7	895±11
Ca^{2+}/calmodulin	362±11	854±17
Ca^{2+}/calmodulin/EGTA	94± 8	867± 8
Ca^{2+}/calmodulin/TFP	101± 9	895±19
cAMP	99± 6	2435±43

Concentrations are as follows: Ca^{2+}, 500 μM; calmodulin, 50 μg/ml; EGTA, 5 mM; TFP, 100 μM; cAMP, 10 μM. All values presented are the means of triplicate determinations.

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alone had little effect. Furthermore this activation was blocked by EGTA and the potent calmodulin antagonist, trifluoperazine (TFP). The activity of PKII was unaffected by Ca^{2+} and calmodulin, but was stimulated in the presence of cAMP. PKII also phosphorylated Kemptide in a cAMP dependent manner (data not shown) and is likely to represent the cAMP-dependent protein kinase.

Although no Ca^{2+}/calmodulin-dependent protein kinases have been reported in prokaryotes [13], to confirm the kinase activity was not of bacterial origin the enzyme assays were performed using axenically grown *Dictyostelium discoideum* AX3 cells (culmination stage) and similar results were obtained. Vegetative AX3 cells gave no kinase activity when tested in the same way (data not shown).

During the course of these experiments it became apparent that the Ca^{2+}/calmodulin-dependent kinase activity of PKI decreased over time; if fractions were not assayed immediately after the completion of the column (approximately 2 h) the Ca^{2+}/calmodulin-stimulated phosphorylation of CDPKS became less and less on standing at 4°C. When PKI was assayed immediately after collection of fractions from the column the addition of Ca^{2+} and calmodulin led to a 3-fold increase in the phosphorylation of CDPKS. However, when PKI was assayed 4 h later the Ca^{2+} and calmodulin-dependent phosphorylation of CDPKS was only 2-fold greater than the phosphorylation observed under basal conditions. This trend continued until, by 24 h, the Ca^{2+}/calmodulin-dependent stimulation of CDPKS phosphorylation was neglible.

Although protease inhibitors (benzamidine and leupeptin) were present throughout the process, the kinase activity appears to be extremely labile. A number of attempts to stabilise the enzyme directly after elution from Q-Sepharose were unsuccessful; the addition of protease inhibitors to fractions containing PKI activity did not maintain Ca^{2+}/calmodulin-dependent activity, and freezing the sample at either −20°C or −80°C in the presence of 30% glycerol resulted in complete loss of kinase activity (data not shown).

Most Ca^{2+}/calmodulin-dependent protein kinases are able to autophosphorylate by an intramolecular mechanism. Therefore fractions containing PKI activity were pooled, passed through a calmodulin-Sepharose column and the eluate incubated with [γ-^{32}P]ATP, under conditions favouring intramolecular phosphorylation reactions (autophosphorylation) (Fig. 2). The eluate was incubated in the presence of EGTA (lane 1), Ca^{2+} (lane 2), Ca^{2+} and calmodulin (lane 3), or Ca^{2+}, calmodulin and TFP (lane 4) and then autophosphorylation examined using SDS-PAGE and autoradiography. As shown in Fig. 2, a single

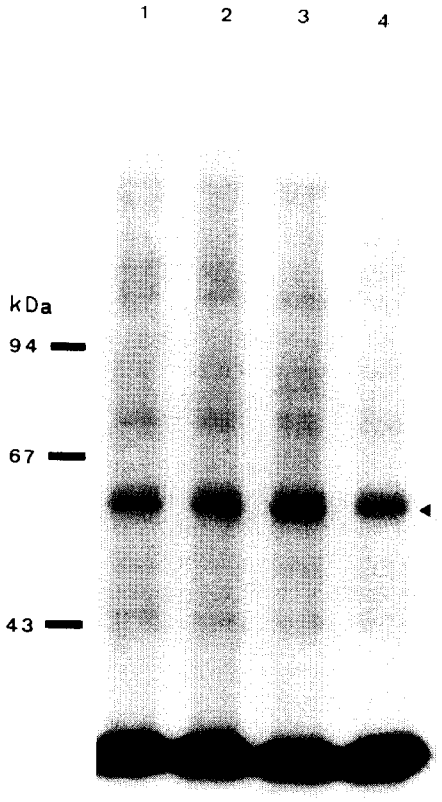

Fig. 2. Endogenous phosphorylation of proteins eluted from calmodulin Sepharose. Calmodulin-Sepharose eluate (concentrated 25-fold) was assayed under conditions favouring intramolecular phosphorylation. Lane 1, EGTA; lane 2, Ca^{2+}; lane 3, Ca^{2+} and calmodulin; lane 4, Ca^{2+}/calmodulin and TFP. Following termination of the reaction, radiolabelled proteins were subject to SDS-PAGE on a 10% polyacrylamide gel and autoradiography performed for 21 h. The migration positions of the standard molecular weight proteins are indicated in kilodaltons (—). pp55 is also indicated (◂).

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major phosphorylated polypeptide of apparent molecular mass 55 kDa was detected (designated pp55).

The addition of Ca^{2+} and calmodulin appeared to slightly stimulate the phosphorylation of pp55 (lane 3) whereas the calmodulin antagonist, TFP, inhibited the phosphorylation to below basal levels (lane 4). The extent of phosphorylation of pp55 under basal conditions was quite high (lane 1). The autoradiograph was scanned using a densitometer to quantify more accurately the changes in the phosphorylation of pp55. The formation of a Ca^{2+}/calmodulin complex stimulated pp55 phosphorylation 38% above basal levels whereas trifluoperazine (TFP), in the presence of Ca^{2+} and calmodulin, reduced the Ca^{2+}/calmodulin-dependent phosphorylation of pp55 10% below basal levels.

Discussion

Work with *D. discoideum*, including studies of Ca^{2+} uptake [2–4], and Ca^{2+} and calmodulin antagonists [6,14,15] indicates that Ca^{2+} and calmodulin-dependent processes are involved in the differentiation of *D. discoideum*. Accordingly, one would expect to find Ca^{2+}/calmodulin-dependent enzymes. One obvious possibility is a Ca^{2+}/calmodulin-dependent protein kinase(s).

Q-Sepharose chromatography of crude extracts prepared from *D. discoideum* resolved two distinct peaks (PKI and PKII) of kinase activity phosphorylating the synthetic peptide CDPKS. To further characterise PKI and PKII, the peaks of activity were pooled and the dependence on Ca^{2+} and calmodulin investigated. PKI was found to be stimulated three-fold above basal levels (EGTA) in the presence of Ca^{2+} and mammalian calmodulin. This activity was not stimulated by Ca^{2+} alone. In a manner analogous to Ca^{2+}/calmodulin-dependent kinases identified in other organisms, the calmodulin antagonist trifluoperazine (TFP) inhibited the calmodulin-dependent activity of this enzyme. These results suggest that PKI activity is only stimulated in the presence of a Ca^{2+}/calmodulin complex, an essential requirement of all calmodulin-dependent protein kinases identified to date. These data clearly indicate the presence of a soluble Ca^{2+}/calmodulin-dependent protein kinase activity in *Dictyostelium discoideum*. PKII was unaffected by these modulators and probably represents the cAMP-dependent protein kinase.

When the DDCPK activity-containing fractions from Q-Sepharose were pooled, passed through a calmodulin-affinity column and assayed under conditions favouring autophosphorylation reactions, a phosphoprotein of apparent molecular mass 55 kDa (pp55) was resolved. This phosphoprotein, although phosphorylated under basal conditions, was stimulated in the presence of Ca^{2+} and calmodulin and inhibited in the presence of TFP. It is proposed that the Ca^{2+} and calmodulin stimulated protein kinase may correspond to the 55 kDa phosphoprotein. The arguments for this are three-fold: firstly, both activities are stimulated upon the formation of a Ca^{2+}/calmodulin complex and both are inhibited by TFP; secondly pp55 was present in fractions pooled from Q-Sepharose in which Ca^{2+}/calmodulin-dependent phosphorylation of CDPKS was clearly demonstrated; and thirdly, pp55 was retained in an affinity column in which the ligand (calmodulin) is the activator of the protein kinase.

The Ca^{2+}/calmodulin-dependent kinase activity identified in *D. discoideum* is extremely labile. After separation on Q-Sepharose, the Ca^{2+}/calmodulin-dependent activity of the enzyme rapidly declined within approximately 4 h of cell disruption and by 24 h Ca^{2+}/calmodulin-stimulated activity was almost neglible. This instability may reflect co-purification of a proteolytic activity in the pooled enzyme fractions.

Two phosphoproteins of M_r 106 kDa and 54 kDa have recently been shown to be phosphorylated in a calmodulin-dependent manner [9] in sexually developing *Dictyostelium* cells. The 54 kDa phosphoprotein, however, does exhibit calmodulin-independent phosphorylation similar to the 55 kDa phosphoprotein reported here. However, these authors did not report any such activity in asexually differentiating cells nor did they assay the enzyme using exogenous substrates.

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The Ca^{2+}/calmodulin stimulated protein kinase identified in culmination stage cells could not be detected in vegetative amoebae. These results suggest a role for *D. discoideum* Ca^{2+}/calmodulin-dependent protein kinase during development.

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