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RECONSTITUTION OF A CYCLIC AMP-DEPENDENT PROTEIN KINASE FROM *DICTYOSTELIUM DISCOIDEUM*

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***Dictyostelium discoideum* has a cyclic AMP-dependent protein kinase activity which can be assayed after DEAE-ion exchange chromatography. Mixing cAMP binding protein (free of kinase activity) from the DEAE column eluate with the protein kinase catalytic activity from a chromatofocusing column leads to a restoration of the cyclic AMP-activatable protein kinase activity.**

The study of protein kinase in *Dictyostelium discoideum* has a tortuous history. The cyclic AMP-dependent protein kinase was first reported by Sampson [1]. Upon DEAE chromatography of cells starved for 2 h he found two cAMP-dependent and two cAMP-independent activities. Shortly thereafter reports by Veron and Patte [2] and Rahmsdorf and Gerisch [3] described protein kinase activity but no cAMP activation was reported. Leichtling et al. [4] demonstrated that bovine protein kinase catalytic subunit could be inhibited by the purified *Dictyostelium* cAMP binding protein. Recently Leichtling et al. [5] reported that DEAE chromatography can be used to demonstrate cAMP-dependent protein kinase activity when the substrate used is the synthetic heptapeptide, kemptide.

Cyclic AMP is a central molecule in the developmental cycle of *Dictyostelium*. During aggregation it is the molecule which mediates the chemotactic response, and there is evidence that cAMP is also involved in the processes of cell differentiation [6–9]. Our efforts have been directed toward analyzing the components of the phosphorylation-dephosphorylation system. Here we demonstrate that by a judicious use of different chromatographic separations one can eliminate the interfering effects of an endogenous phosphatase and reconstitute a cAMP-dependent protein kinase from its constituents.

Adenosine [γ-^{32}P]triphosphate was purchased from Amersham, and [^{3}H]cAMP from ICN. Casein, histone VII-S and kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) were purchased from Sigma. P81 paper was from Whatman.

Dictyostelium discoideum AX3 was grown at 21°C axenically in HL5 broth [10] with shaking to a density of $(2–5)\cdot 10^6$ cells/ml. The cells were harvested by centrifugation, washed twice with buffer 1 (20 mM KH_2PO_4/K_2HPO_4 (pH 6.1)/2 mM $MgSO_4$), suspended in buffer 1 at a concentration of 10^7 cells/ml, and shaken at not over 90 rpm in a New Brunswick rotary shaker. After 17–18 h aggregates were harvested by centrifugation and used immediately.

When DEAE chromatography (or gel exclusion chromatography) was performed Mes/dithiothreitol buffer (0.005 M Mes (pH 7.0)/1.0 mM EDTA/2 mM dithiothreitol/10% glycerol) was used, and when the extracts were chromatofocused

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Abbreviation: Mes, 4-morpholineethanesulfonic acid.

imidazole buffer (0.025 M imidazole (pH 7.4)/2 mM dithiothreitol) was used. During the sonication (but not the chromatography) both buffers contained 1 mM Tos-Lys-CH_2Cl, an irreversible inhibitor of *Dictyostelium* Proteinase I [11]. The cells were lysed for 30 s at 0°C with a Branson sonicator with a microtip. The sonicates were immediately centrifuged twice for 2 min in a Microfuge and the supernatants were used directly for chromatography.

Ion-exchange chromatography was performed using DEAE-Trisacryl (Pharmacia) (or, in early experiments, DEAE-cellulose) as a matrix. Protein was eluted with an 80 ml linear gradient of NaCl from 0.0 M to 0.2 M. Chromatofocusing was performed with a Pharmacia matrix (PBE94) which is eluted with polybuffer (74) containing 2 mM dithiothreitol adjusted to pH 4.0. At the start of chromatography the eluate is pH 7.4 and with elution the pH decreases linearly to 4.0. The size of both columns was 0.9×12.0 cm, and the flow rate was 1.0 ml/min. All chromatography and extract preparation was performed at 0°C.

The final reaction mixture contained 50 mM Mes buffer, pH 6.5, 0.4 mM EGTA, 2.0 mM $MgCl_2$, 2.0 mM dithiothreitol, 10.0 mM NaF, 20 μM kemptide and 0.2 mM ATP. Radioactive ATP was added to make the final specific activity 300–3000 cpm/pmol. cAMP, when used, was added to a final concentration of 0.10 mM. After adding the reagents the reaction was incubated for 5 min at 30°C and terminated by adding acetic acid to a concentration of 33%. Then 60 μl of the acidified reaction were added to a square of P81 paper and the papers were washed with two large washes of 30% acetic acid and two washes with 15% acetic acid [12]. The papers were washed with a final rinse of acetone, dried with air and counted in a toluene-based fluor.

Column fractions (0.2 ml) were incubated with 1.2 μM [^{3}H]cAMP (spec. act. 15 Ci/mmol) and 200 μM 5'-AMP (added to reduce nonspecific binding) at 0°C for 30 min. The incubation was terminated by adding 2.0 ml of wash buffer (0.2 M KH_2PO_4/K_2HPO_4 (pH 6.4)) and fractions were immediately filtered through a nitrocellulose filter. The filter was washed with an additional 10 ml of wash buffer, dried, and counted in a scintillation counter.

Protein concentration was determined by using the Bio-Rad dye-binding assay (measured at 595 nm).

In all of our work we have found a correspondence between the cAMP-binding protein assayed by the millipore filtration method using tritiated cAMP, or assayed using photoaffinity labeling with a radioactive analogue of cAMP [13]. When fresh extracts are prepared in the presence of Tos-Lys-CH_2Cl (an inhibitor of the major Proteinase I of *Dictyostelium*) only one major band is found after gel electrophoresis and autoradiography (unpublished data). The molecular weight determined by SDS-polyacrylamide gel electrophoresis is 40 000 [14,15]. Thus, unlike the mammalian systems, which exhibit two different cAMP-binding proteins [17], there appears to be only one soluble binding protein in *Dictyostelium* [5].

Leichtling et al. [5] have reported that chromatography of extracts on DEAE matrices can be used to demonstrate a cAMP-dependent protein kinase activity in *Dictyostelium*. Fig. 1 illustrates the cAMP-dependent protein kinase activity in such a DEAE eluate. The kinase activity using

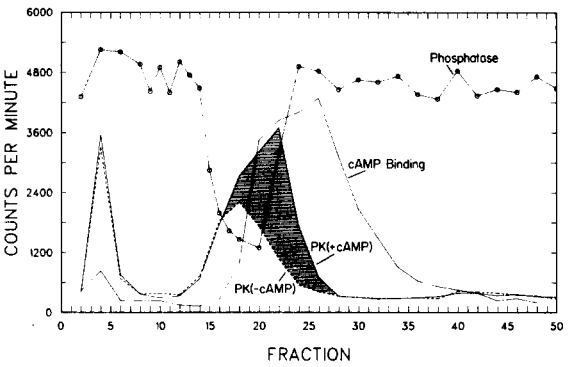

Fig. 1. DEAE-Trisacryl chromatography of a soluble extract from *Dictyostelium* prepared after development for 18 h. The soluble proteins were prepared as described using the Mes/dithiothreitol buffer with Tos-Lys-CH_2Cl. The extract was applied to the column in a volume of 3.0 ml (26.3 mg/ml) and after washing with the buffer the proteins were eluted with a gradient of NaCl from 0.0 to 0.2 M. 2-ml fractions were collected. The cAMP-binding activity, protein kinase (PK) (with and without cAMP), phosphatase (without added cation) and the protein were assayed in each fraction. The peak protein fraction 4 had a protein concentration of 2.5 mg/ml. The dip in the phosphatase assay indicates the least amount of remaining kemptide phosphate and the highest phosphatase activity.

kemptide as a substrate elutes between fractions 15 and 25, and the cAMP activation is confined mainly to the later fractions, as indicated by the shaded area. cAMP-binding activity overlaps the protein kinase activity and the overlap area coincides with the fractions which have a demonstrable activation by cAMP. This result is reproducible. There is no evidence that there are multiple peaks of kemptide-phosphorylating activity. Note that in fractions 26–36 there is cAMP-binding activity which is separated from the phosphatase activity (see below) and which has a very low protein kinase activity.

Chromatofocusing elutes proteins from a column according to their isoelectric point. Proteins having a p*I* greater than 7.4 do not bind to the column under our conditions, and as elution proceeds proteins are eluted which have succesively lower isoelectric points. With chromatofocusing the protein kinase activity elutes prior to the cAMP-binding activity, and there is no overlap (Fig. 2). There is no significant activation by cAMP of the protein kinase. Thus it is possible to separate the protein kinase activity and the cAMP-binding activity, and have a catalytic activity devoid of sensitivity to cAMP. The protein kinase

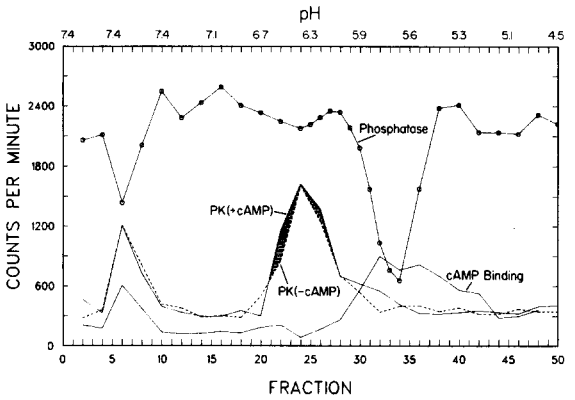

Fig. 2. Chromatofocusing of an extract from *Dictyostelium* which had been starved for 18 h. The same cells as in Fig. 1 were used. The extract of soluble proteins was prepared as described using imidazole/dithiothreitol buffer with Tos-Lys-CH_2Cl. The extract was applied to the column in a volume of 3.0 ml (33.9 mg/ml) and after passing 6.0 ml of imidazole buffer through the column elution was started with a 1/8 dilution of the polybuffer (74) (Pharmacia). 2-ml fractions were collected and assayed as in Fig. 1. The protein peak (fraction 7) contained 2.34 mg/ml.

elutes from the column at a pH of 6.4 and the binding protein at a pH of 5.9–5.2.

In crude extracts of *Dictyostelium* there is a phosphatase which is active on kemptide phosphate (unpublished data). Because of this phosphatase the kinase assay in crude extracts is not linear with time, and generally stops after 5 min. When column chromatography is performed and the phosphatase is assayed using kemptide phosphate as a substrate as indicated in Figs. 1 and 2, it can be seen that the phosphatase can be separated from the kemptide kinase by chromatofocusing but not by DEAE-chromatography. This fortuitous situation allows the reconstitution of the binding protein and the catalytic activity.

As described above it is possible to use chromatofocusing to obtain a cAMP-independent protein kinase activity, and DEAE chromatography to isolate cAMP-binding protein devoid of protein kinase activity, both of which are devoid of phosphatase activity. We have been able to reconstitute a cAMP-dependent protein kinase from its presumed constituents (Table I). When the protein kinase from the chromatofocusing column is mixed in the cold for 10 min with a DEAE fraction which contains binding protein but no protein kinase activity, a cAMP-dependent protein kinase activity is obtained.

The phosphorylation and dephosphorylation of proteins is widely recognized as a regulatory mechanism in cellular metabolism [16,17]. It is the interplay between the two enzymatic activities which determines the activity of a large number of proteins. Here we describe the reconstitution of a cAMP-dependent protein kinase of *D. discoideum* from its separated constituents.

By including Tos-Lys-CH_2Cl as an inhibitor of proteolytic activity, and using DEAE chromatography, we have been able to achieve a reproducible cAMP activation of protein kinase activity eluted from the column. Using chromatofocusing we have been able to resolve the protein kinase catalytic activity from the cAMP-binding activity and an endogenous phosphatase. In the absence of phosphatase we have been able to achieve extensive phosphorylation over longer periods than have been achieved before. It is possible to reconstitute a cAMP-dependent protein kinase activity from the separated binding protein (devoid of kinase)

TABLE I

RECONSTITUTION OF cAMP-DEPENDENT PROTEIN KINASE

The results have been corrected for a background of 250 cpm. The reaction was allowed to proceed for 60 min at 30°C before stopping with acetic acid and determining the remaining phospho-kemptide using P81 paper as described in the text. The protein kinase was taken from the protein kinase peak of a chromatofocusing column (CF). It did not exhibit any significant cAMP-dependence and was separated from the phosphatase. The binding protein was taken from the fractions of a DEAE column which had cAMP-binding activity but no protein kinase activity. The protein concentrations were 0.284 mg/ml for the kinase and 0.724 mg/ml for the binding protein, with the concentrations halved for each in the mixture.

Fraction(s)	cpm		Activation ratios and differences	
	+cAMP (A)	−cAMP (B)	A−B	A/B
Protein kinase (CF)	3403	3460	−57	0.98
Binding protein (DEAE)	857	573	284	1.46
Protein kinase (CF)+binding protein (DEAE)	3389	990	2399	3.42

and a protein kinase catalytic activity (devoid of binding activity of phosphatase activity). Although others have also reported cAMP-activated protein kinase activity in *Dictyostelium* [18,19], this is the first reported reconstitution of the activity from the separate *Dictyostelium* constituents.

The specific activity of the protein kinase in the absence of phosphatase may be similar to that observed with mammalian protein kinases. The cAMP-binding protein is present in crude extracts at a concentration of 3 pmol/mg protein based on binding activity. For a protein of M_r 40000, this is a concentration of 0.1% by weight. If the protein kinase is present at the same relative concentration, then the measured activity of the protein kinase in *Dictyostelium* is very similar to that determined using a purified cAMP-dependent bovine protein kinase and synthetic peptides [20–22]. That is, a measured activity of 17000 pmol/min per mg protein for the impure *Dictyostelium* protein would translate into an activity of 17 μmol/min per mg protein if the enzyme was purified 1000-fold. This is similar to the activities found for the purified mammalian protein kinase.

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