A phospholipid-stimulated protein kinase from *Dictyostelium discoideum*

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A protein kinase with unusual characteristics has been found in *Dictyostelium discoideum*. This kinase can use histone H1 as exogenous substrate, and the activity is stimulated by phospholipids, but not by Ca^{2+} . This enzyme has been partially purified by using chromatography on DEAE-cellulose DE-52, spermine-agarose and phosphatidylserine-polyacrylamide. The protein kinase activity is very labile, even in the presence of protease inhibitors, making further purification difficult. In the activity-containing fractions, an endogenous protein of 140 kDa is labelled *in vitro* with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ under conditions in which intramolecular rather than intermolecular reactions are favoured. This protein is labelled only in the presence of phospholipids, but not of Ca^{2+} . We propose that the 140 kDa phosphoprotein might be the autophosphorylated enzyme.

INTRODUCTION

Protein kinases have been implicated in the control of cell proliferation and differentiation and in the transduction of extracellular signals. In particular, Ca^{2+} - and phospholipid-dependent protein kinase C is thought to play a central role in the induction of cellular responses by a variety of ligand-receptor systems [1,2]. A family of protein kinase C-related genes has been identified in different mammalian cells [3–5]. Functionally, it would appear that several subspecies are Ca^{2+} - and phospholipid-dependent, whereas some others are not yet kinetically defined [5]. It has been reported that protein kinase C can be activated by unsaturated fatty acids independently of Ca^{2+} and phospholipids [6], or by a metabolite of arachidonic acid, in the presence of Ca^{2+} , but independently of phosphatidylserine [7]. The complexity of this family has increased with the recent description of nPKC, clearly distinguished from conventional protein kinase C, as its activity is independent of Ca^{2+} [8]. These data indicate that protein kinase C consists of a large number of subspecies whose functional roles have not yet been clarified.

There are also several reports in the literature on phospholipid- or phospholipid-and-diolen (*sn*-1,2-dioleoylglycerol)-dependent protein kinases in different biological systems [9,10]. At least one of them can be chromatographically separated from protein kinase C. It is not known at present whether or not they belong to the same family.

Dictyostelium discoideum is a social amoeba whose aggregative phase is regulated by extracellular cyclic AMP relay [11]. The precise sequence of events from the binding of the chemoattractant agent for aggregation (cyclic AMP) to its specific receptors to the final cellular responses is not known. Ca^{2+} redistribution and increase in intracellular cyclic GMP are two of the fastest receptor-mediated responses elicited by cyclic AMP [12]. It has been reported that Ca^{2+} induces cyclic GMP formation [13]. Inositol trisphosphate seems to mediate this process, as its addition to permeabilized cells causes a release of

Ca^{2+} from non-mitochondrial stores within 5 s [13], and also stimulates cyclic GMP production [14]. It has also been reported that under some conditions the cyclic AMP receptors show altered binding properties assumed to be caused by phosphorylation, involving protein kinase C in the process [15,16]. Moreover, it has recently been shown that ras-Thr 12 transformants have an elevated content of inositol trisphosphate compared with cells carrying the wild type *ras* gene [17]. These data suggest that the phosphatidylinositol signal transduction pathway is active in *D. discoideum* and controlled by a *ras* protein.

Searching for protein kinase C in vegetative cells of *D. discoideum*, we have found a protein kinase activity stimulated by phospholipids, but not by Ca^{2+} or diacylglycerol, the most potent activators being phosphatidylserine and phosphatidylinositol. This activity has been partially purified and characterized.

MATERIALS AND METHODS

Growth conditions

D. discoideum strain AX-2 was grown exponentially in HL-5 medium [18] with doubling times of 7–9 h up to cell densities of 8×10^6 cells/ml. Cells were harvested 20–22 h later (stationary phase). For the experiments in the early aggregative phase, cells were developed on filters. Cells were resuspended in Mes-PDF buffer [19] at a density of 1.5×10^8 cells/ml. Portions (5 ml) of the cell suspension were spread on a Whatman no. 3 filter (15 cm) supported on two filter-paper pads soaked in the same buffer, and allowed to develop for 5 h in a humid chamber at 22 °C.

Phosphorylation assays

Assay mixtures (50 μl) contained 20 mM-Hepes/NaOH, pH 7.5, 10 mM- MgCl_2 , 100 μg of phospholipid/ml, 0.5–1 mg of histone H1/ml, 20 μM -ATP, 1 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 5–10 μl of the fraction. Assays were incubated for 5 min at 30 °C and terminated by addition

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of Laemmli buffer; after boiling, the samples were applied to 10–12% polyacrylamide slab gels containing SDS and electrophoresed in the Laemmli system [20]. After staining and drying, autoradiographs were obtained by exposing to Kodak X-AR films at -70°C , Cronex H₁ Plus intensifying screens (Du Pont) being used for the exposure. The reaction mixture for autophosphorylation was as described above, except that the ATP concentration was lowered to 10 nM and the radioactivity in the assay to 0.2 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, as in the conditions described by Rangel-Aldao and colleagues [21] for the autophosphorylation of the cyclic AMP-dependent protein kinase. The assays were incubated for 10 min at 4°C and terminated and processed as above. Protein was measured by the Bradford procedure [22].

Preparation of cell extracts and chromatography conditions

Cells $[(5\text{--}10) \times 10^8]$ were lysed as in ref. [23] in a lysis buffer containing 50 mM-Tris/HCl, pH 8.5, 5 mM-EDTA, 2 mM-dithiothreitol, 6% (w/v) saccharose, 10 mM-EGTA, 50 μM -tosyl-lysylchloromethane ('TLCK') and 20 μg of leupeptin/ml. The homogenates were centrifuged at 1500 g for 10 min, and the supernatant was re-centrifuged at 100 000 g for 30 min. The final supernatant (25–50 mg) was applied to a DEAE-cellulose DE-52 column (4 cm $\times$ 1.3 cm) equilibrated with 20 mM-Tris/HCl (pH 7.5)/20% (v/v) glycerol/1 mM-EDTA/1 mM-EGTA (buffer C). After washing with 5 vol. of buffer C, elution was performed with a 0–0.3 M-NaCl linear gradient in buffer C; 1 ml fractions were collected.

The fractions showing activity were pooled and applied to a spermine-agarose column (5 cm $\times$ 0.3 cm) equilibrated in buffer C with 0.1 M-NaCl. After washing with the same buffer, stepwise elution was performed with 1 M-NaCl in buffer C.

The active fractions from spermine-agarose chromatography were pooled, dialysed and applied to a phosphatidylserine column (1.5 cm $\times$ 0.9 cm) prepared as

described by Uchida & Filburn [24], equilibrated in buffer C. After extensive washing with the same buffer, two stepwise elutions were performed, with 1 M-NaCl and then with 1 M-NaCl plus 0.1% Nonidet P-40. Eluted fractions were dialysed against buffer C before assay and stored at -80°C in 20% (v/v) dimethyl sulphoxide.

RESULTS

In the search for protein kinase C we used stationary cells as starting material, since in these cells most of the enzyme was expected to be in the soluble fraction [25]. As preliminary attempts to assay protein kinase C in *Dictyostelium discoideum* using a standard assay on P-81 phosphocellulose paper to evaluate results of DE-52 chromatography gave low histone phosphorylation, we used polyacrylamide-gel electrophoresis and autoradiography to monitor the activity.

Since most protein kinases, including protein kinase C, are known to autophosphorylate by an intramolecular mechanism, a 100 000 g supernatant of stationary cells was chromatographed on a DE-52 column, and the eluate was evaluated by polyacrylamide-gel electrophoresis and autoradiography, under assay conditions that favoured intramolecular phosphorylation reactions (autophosphorylation). Fig. 1 shows the result of this fractionation. The fractions were assayed with EGTA (odd numbers) or with phosphatidylserine, Ca^{2+} and diolein (even numbers). As shown in Fig. 1(a), several proteins are labelled under these conditions, including a major band of 67 kDa, but none respond to activators except a 140 kDa phosphoprotein (pp140), which has a peak around 0.1 M-NaCl. Fig. 1(b) shows the autoradiogram of fraction 11 assayed in the presence of histone and under conditions which favour enzyme-substrate interactions (20 μM -ATP). The same fractions which contain pp140 can phosphorylate histone, and this phosphorylation is stimulated by the same activators as pp140.

Fig. 1. Autoradiography after DE-52 chromatography of the soluble fraction of *D. discoideum*

Cells were harvested during the stationary phase of growth and were lysed and processed as indicated in the Materials and methods section. Fractions 7, 9, 11, 13 and 15, eluted around 0.1 M-NaCl (part c), were assayed under autophosphorylation conditions (see the Materials and methods section) and electrophoresed (part a, lanes 1–2, 3–4, 5–6, 7–8, 9–10 respectively). Part (b) shows the autoradiography of an assay of fraction 11 with histone H1 as exogenous substrate. Odd-numbered lanes represent fractions assayed with 1 mM-EGTA; even-numbered lanes represent fractions assayed with 100 μg of phosphatidylserine/ml, 10 μg of diolein/ml and 1 mM- CaCl_2 . Positions of molecular-mass markers are shown on the left of part (a). The arrow in part (a) indicates the position of pp140, and that in part (b) indicates the position of histone H1 in the gel.

Fig. 2. Autoradiography after spermine-agarose chromatography of the protein kinase activity eluted from DE-52

The DE-52 fractions containing pp140 were pooled and applied to a spermine-agarose chromatography column. Fractions were eluted stepwise as described in the Materials and methods section. Part (a) shows the assay under autophosphorylation conditions. Part (b) shows the assay with histone H1 as exogenous substrate. Lanes: 1–2, flow-through; 3–4, fraction 3; 5–6, fraction 4; lanes 7–8, fraction 5. Odd-numbered lanes represent assays in the presence of 1 mM-EGTA, and even-numbered lanes represent assays in the presence of 100 μ g of phosphatidylserine/ml, 10 μ g of diolelin/ml and 1 mM- CaCl_2 . Part (c) shows the A_{280} profile of the stepwise elution from the spermine-agarose column. Positions of molecular-mass markers are shown on the left of part (a). The arrow in part (a) indicates the position of pp140, and that in part (b) indicates the position of histone H1 in the gel.

These results suggest that pp140 could be associated with a protein kinase C-like activity. To explore this possibility further, activity-containing fractions from DE-52 were pooled and applied to a spermine-agarose column. As shown in Fig. 2, pp140 is retained in the column, and the fractions showing phospholipid activation of pp140 phosphorylation (Fig. 2a, lanes 3–8) also support phospholipid-stimulated histone phosphorylation (Fig. 2b, lanes 3–8). However, the flow-through, which is devoid of the pp140 phosphoprotein (Fig. 2a, lanes 1–2) shows a basal histone phosphorylation not activated by phosphatidylserine (Fig. 2b, lanes 1–2).

The fractions containing the phospholipid-stimulated activity were pooled, dialysed and used to study the activation requirements shown in Fig. 3. Fig. 3(a) shows the assay under autophosphorylation conditions, with EGTA as control (lane 1). Phosphatidylserine in the presence of EGTA supports the phosphorylation of p140 (lane 2), but addition of either diolelin (lane 3) or diolelin and Ca^{2+} (lane 4) promotes no further increase in phosphorylation. Neither Ca^{2+} alone (0.5–1 mM) nor Ca^{2+} plus 60 nM-12-*O*-tetradecanoylphorbol 13-acetate ('TPA'; a known analogue of diacylglycerol) had any effect on the phosphorylation of this protein (results not shown). Fig. 3(b) shows the protein kinase activity of the same fractions assayed with histone as substrate and under conditions for substrate phosphorylation. As with pp140, phosphatidylserine in the presence of EGTA (lane 2) activates histone phosphorylation markedly over the control (lane 1), but addition of either diolelin (lane 3) or diolelin and Ca^{2+} promotes no further stimulation (lane 4).

The specificity of phospholipid activation for histone phosphorylation, as well as for p140 phosphorylation, was studied with the same preparation. As shown in Fig. 4, under both conditions phosphatidylinositol (lanes 3, 10) was the only phospholipid tested that was as effective as phosphatidylserine (lanes 2, 8); neither phosphatidylethanolamine (lanes 5, 9) nor phosphatidylcholine (lanes 4, 11) had any effect. In all assays when Ca^{2+} is absent, EGTA is present at a concentration of 1 mM.

Sphingosine, which has been reported as an inhibitor of protein kinase C activity [26], has no effect on this activity (lane 6).

Fig. 3. Effect of phosphatidylserine, diolelin and Ca^{2+} in the protein kinase activity eluted from the spermine-agarose chromatography column

Part (a) of the autoradiograph shows the assays of the activity that interacts specifically with the column under autophosphorylation conditions; part (b) shows assays of the same fractions with histone H1 as exogenous substrate. Lanes: 1, 1 mM-EGTA; 2, 100 μ g of phosphatidylserine/ml, 1 mM-EGTA; 3, 100 μ g of phosphatidylserine/ml, 1 mM-EGTA 10 μ g of diolelin/ml; 4, 100 μ g of phosphatidylserine/ml, 10 μ g of diolelin/ml and 1 mM- CaCl_2 . Positions of molecular-mass markers are shown on the left of part (a). The arrow in part (a) indicates the position of pp140, and that in part (b) indicates the position of histone H1 in the gel.

Fig. 4. Effect of different phospholipids on the protein kinase activity eluted from the spermine-agarose chromatography column

The protein kinase activity eluted from the spermine-agarose chromatography column was assayed in the presence of 100 μ g of different phospholipids/ml, and 1 mM-EGTA when Ca^{2+} is absent. Lanes 1–6 show the activity with histone H1 as exogenous substrate. Lanes 7–11 show the same fraction assayed under autophosphorylation conditions. Lanes: 1 and 7, 1 mM-EGTA; 2, phosphatidylserine, 10 μ g of diolein/ml and 1 mM- CaCl_2 ; 3 and 10, phosphatidylinositol; 4 and 11, phosphatidylcholine; 5 and 9, phosphatidylethanolamine; 8, phosphatidylserine; 6, phosphatidylserine plus 0.1 mM-sphingosine. The arrow in panel (a) indicates the position of histone H1 in the gel; that in panel (b) indicates the position of pp140. Positions of molecular-mass markers are indicated beside each panel.

Phospholipid activation was not observed with substrates such as casein and histone H2A (results not shown).

Although protease inhibitors are present throughout the process, the phospholipid-stimulated kinase is extremely labile and the activity on histone is rapidly lost. However, phosphorylation of the 140 kDa protein can be followed for longer periods of time, probably because of the sensitivity of the method. The phosphatidylserine activation of p140 phosphorylation suggests the possibility for a polyacrylamide-immobilized phosphatidylserine column as a further step for purification. Fractions from the spermine-agarose column were pooled and applied to a polyacrylamide-immobilized-phosphatidylserine column. Fig. 5 shows that the pp140 kDa is retained by this affinity chromatography. Lane 3 shows the silver stain of the 1 M-NaCl eluate and lane 6 the autoradiography of the same fraction assayed under autophosphorylation conditions in the presence of phosphatidylserine and EGTA. The phospholipid requirements for the phosphorylation of this band (results not shown) were the same as those shown for spermine-agarose chromatography (Fig. 4).

The pp140 phosphoprotein is distributed among soluble and particulate fractions (results not shown). The precise distribution among these fractions should be explored more carefully.

We have checked for the presence of the pp140 phosphoprotein in the early aggregation period (cells differentiated for 5 h). Using the same experimental

Fig. 5. Autoradiography after phosphatidylserine/polyacrylamide chromatography of the protein kinase activity eluted from the spermine-agarose chromatography column

Fractions from the spermine-agarose column containing the phospholipid-stimulated protein kinase were pooled and applied to a phosphatidylserine/polyacrylamide column as described in the Materials and methods section. The silver-stained gel of the proteins separated by this chromatography is shown in lanes 2–4. Lane 1, high-molecular-mass markers; lane 2, flow-through; lane 3, fraction eluted with 1 M-NaCl; lane 4, fraction eluted with 1 M-NaCl plus 0.1% Nonidet P40. Once dialysed, the fractions were assayed, under autophosphorylation conditions in the presence of 100 μ g of phosphatidylserine/ml plus 1 mM-EGTA, and the respective autoradiographs are shown in lanes 5, 6 and 7. The arrow in the right panel indicates the position of pp140; that in the left panel indicates the protein band that migrates at 140 kDa.

approach, we have found in the fractions eluted around 0.1 M-NaCl from the DE-52 a phosphoprotein with the same characteristics as those described for pp140 in vegetative cells (results not shown).

DISCUSSION

We describe a protein kinase activity that phosphorylates histone H1 and is activated by phospholipids, with phosphatidylserine and phosphatidylinositol as the most effect activators. This is the first report of a protein kinase with these characteristics in *Dictyostelium discoideum*. This protein kinase differs from protein kinase C, as neither diolein nor Ca^{2+} is required for activation; in most instances, an inhibitory effect of Ca^{2+} is observed.

Several lines of evidence suggest that transmembrane signalling in *D. discoideum* could be mediated by a protein kinase C-like activity [15,16]. This protein kinase, however, has not yet been found. There are several possible explanations for this: the activity is extremely labile, or is present in small amounts, or the requirements for optimal activity are different from those of its higher-eukaryote counterpart. In this respect, we mention that cDNA clones from rabbit brain have been isolated which encode a protein related to protein kinase C (nPKC), whose kinase activity is regulated by phospholipid,

diacylglycerol and phorbol ester, but is independent of Ca^{2+} [8].

In addition to protein kinase C, the literature reports protein kinases which can be activated by phospholipids alone [9,27] or by phospholipids/diolein [10]. The latter was chromatographically resolved from protein kinase C. It is not yet known whether or not they belong to the same family.

The phospholipid-stimulated protein kinases described in malignant murine haemopoietic cells [9] and in human leukaemic cells [27] have not been purified, but the phospholipid preference of these kinases differs from the pattern described for protein kinase C [28].

Phospholipid activation has been described for the *src* tyrosine kinase, but there the activation observed depends on the substrate used. For instance, the phosphorylation of vinculin is highly enhanced by phospholipids, whereas that of casein is not affected [29] and that of p36 (tyrosine kinase substrate) requires both phospholipids and Ca^{2+} [30].

The phospholipid-stimulated protein kinase from *Dictyostelium discoideum* described here appears to differ from the mouse and human protein kinases, as in those systems phosphatidylserine has very little activating effect. We think that the *Dictyostelium* enzyme is more related to nPKC [8], whose activity and autophosphorylation are phospholipid-dependent and Ca^{2+} -independent. Nevertheless, a higher degree of purification would allow better comparison.

We propose that the phospholipid-stimulated protein kinase from *D. discoideum* might correspond to the 140 kDa phosphoprotein. Our main arguments are that the activation requirements and phospholipid activation sequence are the same for the 140 kDa protein in autophosphorylation conditions and for the phosphorylation of histone, through several chromatographic steps, and the fact that the pp140 is retained in an affinity chromatography in which the ligand (phosphatidylserine) is the activator of the protein kinase.

It is not clear whether the requirements for the autophosphorylation reaction of kinases are identical with those for substrate phosphorylation. For brain protein kinase C, maximal autocatalytic reaction and phosphorylation of exogenous substrates are observed in the presence of Ca^{2+} , phosphatidylserine and diolein [31], although some other alternative activators have been reported for enzyme-substrate interactions [6,7]. For the phospholipid-stimulated protein kinase from *D. discoideum*, the requirements for autocatalytic and exogenous phosphorylation are the same.

Since this enzyme is present in vegetative cells as well as in the early aggregation period, it may play a role in different physiological situations. In a preliminary experiment, it has been found that stationary-phase cells have more phospholipid-stimulated pp140 (in the autophosphorylation assay) than do proliferative cells. Conditions for the stabilization of this activity must be established in order to pursue this observation.

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