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The developmental regulation of phosphatidylinositol kinase in *Dictyostelium discoideum*

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Phosphatidylinositol kinase was examined in *Dictyostelium discoideum* since this organism offers molecular and genetic advantages to study the role of phosphatidylinositol metabolism during cell growth and development. *D. discoideum* homogenates phosphorylated phosphatidylinositol to form phosphatidylinositol 4-phosphate in a reaction which was found to be linear with time and cell concentration. Optimal activity was obtained in the presence of 1 mM $MgCl_2$ and pH 7.6 and has an apparent K_m for ATP of about 250 μM. Changes in phosphatidylinositol kinase were examined during *D. discoideum* development. Activity increased about 2-fold, 4 h after removal of the food source, to decline to almost no activity at late aggregation. During slug formation the activity increased about 15-fold and remained constant during further development. These results suggest a role for *D. discoideum* phosphatidylinositol kinase during development.

Phosphatidylinositol kinase; Differentiation; *(Dictyostelium discoideum)*

1. INTRODUCTION

In higher organisms, a wide variety of signals stimulate the cellular inositol phospholipid turnover [1]. This leads to the formation of diacylglycerol, which can activate protein kinase C [2] and inositol 1,4,5-trisphosphate (IP_3), which presumably triggers calcium release from non-mitochondrial intracellular stores [3]. There is a good correlation between rapid inositol phospholipid turnover and rapid cell proliferation and differentiation [1]. Inositol phospholipid turnover can be accelerated by either increasing lipid inositol kinase activities or phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis. The discovery that at least two tyrosine kinase oncogenes, *src* and *ros*, stimulate lipid inositol synthesis [4] either by encoding for the inositol lipid kinases or by controlling these cellular kinases, supports the concept that lipid kinases play an important role in controlling cell growth and differentiation. The mechanism by which lipid inositol kinases are regulated remain unknown.

In the cellular slime mould *Dictyostelium discoideum*, cell proliferation and development are two well separated events in its life cycle [5]. Free living, vegetative amoeba, which feeds on bacteria, enters a program of development and differentiation upon starvation (reviews [6,7]); cells aggregate by means of chemotaxis to form a multicellular structure, which ultimately transforms into a fruiting body with two cell types: spores and stalk cells. Little is known about the intracellular transduction pathways by which morphogens induce the expression of genes necessary for this

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development and differentiation. In the light of this we have attempted to see whether inositol phospholipid turnover is a possible component of this transduction pathway in *D. discoideum*. We studied this problem by examining the phosphorylation of phosphatidylinositol (PI) by phosphatidylinositol kinase (PI kinase; EC 2.7.1.67) of *D. discoideum*.

Here we demonstrate the existence of this kinase and that it is developmentally regulated, which suggests a role for inositol phospholipid turnover during the development and differentiation of this organism.

2. MATERIALS AND METHODS

[γ-^{32}P]ATP (3000 Ci/mmol) was from The Radiochemical Centre, Amersham, England. Phospholipid standards and ATP were from Sigma, St. Louis, USA. Silica gel 60 thin-layer plates were obtained from Merck, Darmstadt, FRG.

2.1. *Growth and development*

D. discoideum strain NC-4(H) was grown at 22°C in association with *Escherichia coli* 281 on a solid medium containing 5 g peptone, 5 g glucose, 4.5 g KH_2PO_4, 1.5 g $Na_2HPO_4 \cdot 2H_2O$, and 15 g agar/l. Cells were harvested in 10 mM phosphate buffer (pH 6.0) and freed from bacteria by repeated centrifugation. Cells were then either resuspended in the same buffer to a density of 10^7 cells/ml and shaken for the time required on a rotary shaker at 150 rpm, or plated on non-nutrient agar plates (10 cm diameter) at a density of 2×10^8 cells/plate and were allowed to develop at 22°C. At the times required cells were harvested, washed twice in phosphate buffer and then a second time in 136 mM Tris-HCl (pH 7.6) and finally resuspended to 10^8 cells/ml in the same buffer. Homogenates were prepared by rapidly freezing the cells in liquid nitrogen and subsequently thawing them, just before use, under continuous agitation. Homogenates from starved cells (non-nutrient agar) were briefly sonicated on ice (twice 5 s with a Branson sonifier fitted with a microtip) just before use in the assay to ensure complete homogenization of the cells in the later stages of development.

2.2. *Phosphatidylinositol kinase assay*

PI kinase activity in *D. discoideum* homogenates was assayed by adding 100 μl homogenate to 400 μl prewarmed assay buffer containing 136 mM Tris-HCl (pH 7.6), 1.25 mM $MgCl_2$, 125 mM KF, 32.5 mM KCl, 125 μM MgATP and 4 μCi [γ-^{32}P]ATP. Incubations were carried out at 37°C and stopped after 0, 30, 60, or 90 s by adding 1.8 ml $CHCl_3/CH_3OH$/12 N HCl (50:100:1, v/v). Lipids were extracted by the method of Garcia Gil et al. [8] and separated on thin-layer chromatography (TLC) on preactivated (1 h at 110°C) silica gel 60 plates, with a mobile phase consisting of $CHCl_3/CH_3OH$/14 N NH_4OH/H_2O (90:90:5:22, v/v) or 1-propanol/4 N NH_4OH (130:70, v/v). The plates were then autoradiographed to visualize the ^{32}P-labeled lipids. Radioactive spots, comigrating with the phosphatidylinositol 4-phosphate (PIP) standard (visualized by exposure to iodine vapour), were scratched from the plates and eluted by shaking 20 min in 1 ml of 20 mM tetrabutylammonium sulphate in methanol, and counted after addition of 5 ml scintillation fluid (Normascint cocktail 122 from Sharlau) by liquid scintillation spectrometry.

3. RESULTS AND DISCUSSION

3.1. *PI kinase activity in* D. discoideum *homogenates*

When *D. discoideum* homogenates were incubated with [γ-^{32}P]ATP and subsequently extracted with $CHCl_3/CH_3OH$/14 N HCl, analysis by TLC indicated PIP synthesis. Comigration with an authentic standard was demonstrated using two different TLC systems as described in section 2. To demonstrate further the identity of the phosphorylation product, after TLC, the ^{32}P-labeled area was scratched from the plate and extracted with $CHCl_3/CH_3OH/H_2O$/14 N HCl (87:147:69:0.3, v/v). After conversion into two phases by adding 0.8 vol. $CHCl_3$ and 0.14 vol. of 2 M KCl, the organic phase was taken, dried under N_2 and subjected to phospholipase C (Type I: from *Clostridium perfringens*) hydrolysis as in [9] in the presence of 10 μg/ml exogenous PIP. Under these conditions most of the ^{32}P-labeled material was in the water phase. When the water phase was then applied to a Dowex-1 column and eluted with different concentrations of ammonium formate [10],

most of the radioactivity eluted as inositol bisphosphate (IP_2) (not shown).

The PI kinase activity was found to be linear with time, up to 90 s, and amount of homogenate, up to at least 4×10^7 homogenized cells/assay. No change of activity was found in the presence of an ATP regenerating system containing 20 mM creatine phosphate and 150 μg/100 μl creatine kinase. The assay was carried out at 37°C because this yielded an about 5-fold higher enzyme activity than at room temperature.

In order to determine the pH optimum of the PI kinase, identical aliquots of homogenate were placed in buffered solutions encompassing a pH range of 5.0–9.0, and standard phosphorylation assays were performed. Fig.1 shows that the enzyme is optimally active at pH 7.6. This neutral pH optimum for *D. discoideum* PI kinase is similar to that of the PI kinase found in e.g. rat liver nuclear envelopes [11] or bovine brain coated vesicles [12].

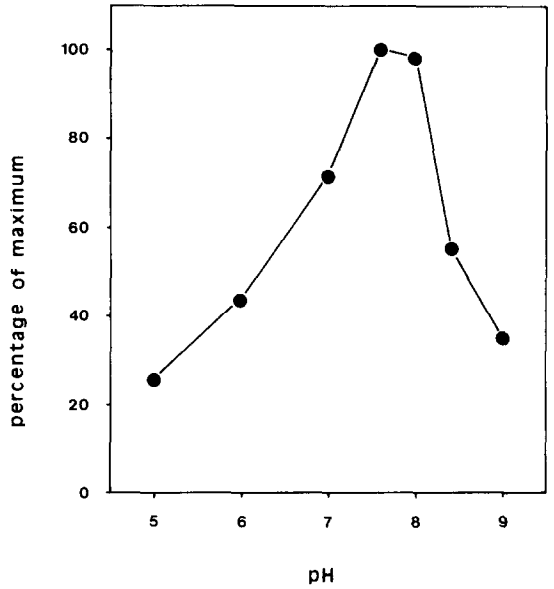

Fig.1. pH dependence of PI kinase activity. Cells starved for 5 h in a shaken suspension were collected by centrifugation and washed in either 100 mM phosphate buffer (pH range between 5 and 7) or 136 mM Tris-HCl buffer (for the pH values above 7) adjusted to required pH. After homogenization, the PI kinase activity was determined as described in section 2 in the same buffers as the homogenates were made in. The results presented are the means of three experiments in triplicate.

The *Dictyostelium* PI kinase is dependent on the presence of Mg^{2+}, with maximal activity at 1 mM $MgCl_2$. Fig.2 demonstrates that higher concentrations of Mg^{2+} inactivate the enzyme. This Mg^{2+}-dependence pattern is different from those observed in other systems, which generally require up to 100 mM $MgCl_2$ for optimal enzyme activity [11–13]. However, 0.6 mM is the approximate physiological free Mg^{2+} concentration in *Dictyostelium* as determined from the ^{31}P-NMR signals from the phosphates of the nucleotide triphosphates [14]. The PI kinase of *D. discoideum*, as those of other systems, appears to be Ca^{2+}-independent, as addition of up to 10 mM $CaCl_2$ had no effect on enzyme activity.

Fig.3 shows an Eadie-Hoffstee plot of the production of PIP as related to ATP concentration in the presence of 5 mM $MgCl_2$. These results indicate that the enzyme has an apparent V_{max} of about 33 pmol/min per mg protein and an apparent K_m of about 250 μM for ATP.

3.2. *PI kinase activity during development*

As lipid kinases (and inositol phospholipid turnover) are thought to play important roles in

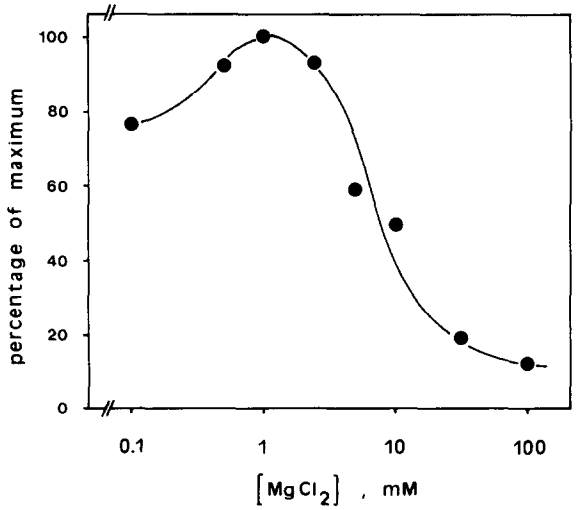

Fig.2. Effect of $MgCl_2$ on PI kinase activity. Vegetative cells, starved for 5 h in a shaken suspension, were harvested, homogenized and assayed for PI kinase activity, as described in section 2, with 100 μM [γ-^{32}P]ATP and various concentrations of $MgCl_2$ for 90 s. Results shown are the means of three experiments in triplicate.

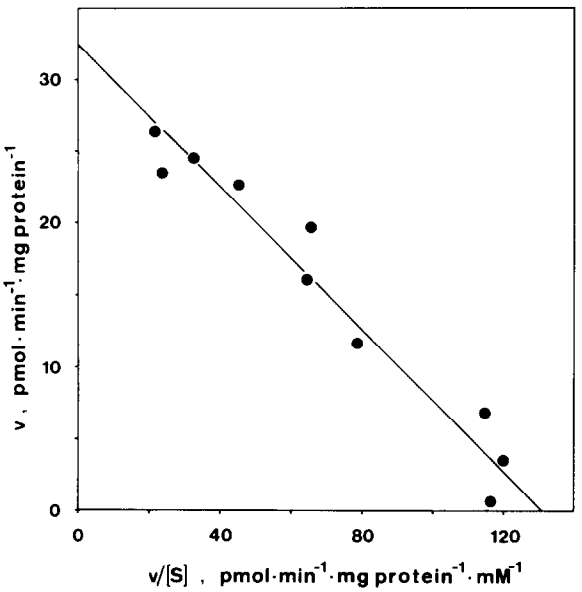

Fig.3. Eadie-Hoffstee plot of the ATP dependency of PIP production. Cell homogenates were prepared as in fig.1, and assayed for PI kinase activity in the presence of different concentrations of [γ-^{32}P]ATP. Regression analysis of the data shown yields a correlation coefficient of 0.973, an apparent K_m of 250 μM, and a V_{max} of 32.5 pmol/min per mg protein. Results shown are the means of three experiments in triplicate.

controlling cell growth and differentiation in higher organisms [1,4], we have studied the PI kinase activity during the development of *D. discoideum* cells both in a shaking suspension (fig.4) and on non-nutrient agar plates (fig.5). In both systems of development, PI kinase activity increased up to about 2-fold, 4 h after removal of the food source. In a shaking suspension, the activity then remained high for a few hours and subsequently dropped after about 6 h of starvation (see fig.4). In cells developed on non-nutrient agar plates, the PI kinase activity dropped somewhat earlier and declined to almost zero at late aggregation during the formation of tight aggregates. Later during slug formation the level of activity increased again to about 15-fold higher than that in the tight aggregate stage and then remained high during further development (see fig.5). This developmental pattern indicates that the PI kinase activity of *Dictyostelium discoideum* is developmentally regulated. The first peak of activity seems to coincide with the expression of at

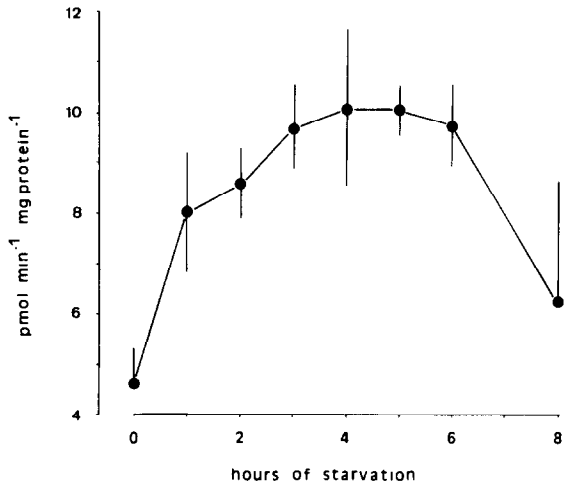

Fig.4. PI kinase activity during development in a shaking suspension. Vegetative cells were harvested and washed free from bacteria by repeated centrifugation, after which they were resuspended to a density of 10^7 cells/ml in 10 mM phosphate buffer, pH 6.0, and were kept on a rotary shaker at 150 rpm. At the times indicated, samples were taken and assayed as described in section 2. Results shown are the means ± SD of three experiments in triplicate.

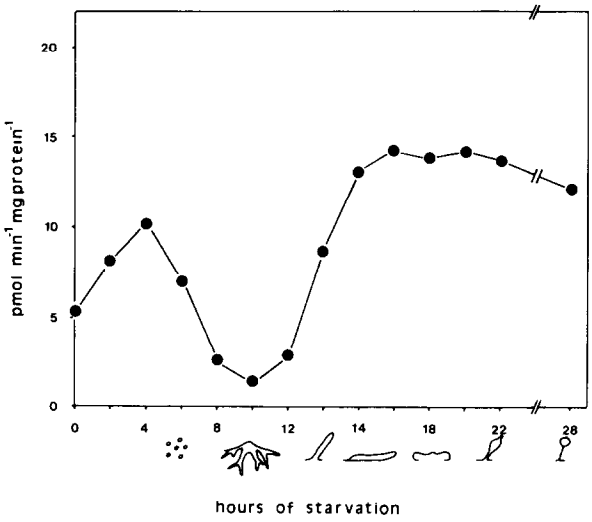

Fig.5. PI kinase activity during normal development. Vegetative cells, freed from bacteria, were placed on non-nutrient agar plates (2×10^8 cells/plate) and were allowed to develop normally. At the times indicated cells were harvested and homogenates were prepared and assayed for PI kinase activity as described in section 2. The data shown are the means of a single experiment in triplicate. Two separate experiments gave very similar results.

least one prestalk specific mRNA during early development [15], but does not coincide with the developmental patterns of components of the signal transduction pathway of *D. discoideum*, such as cyclic AMP cell-surface receptors and cyclic AMP phosphodiesterase activity [16], which occur somewhat later during aggregation (8 h starvation) and culmination (20 h starvation), and guanylate cyclase activation and adenylate cyclase activation, which peak at about 8 h [17]. The second induction of PI kinase activity coincides with the expression of genes which are required for spore formation and further differentiation, such as glycogen phosphorylase [18], ornithine decarboxylase [19] and MUD-1 prespore antigen [20].

Changes in activity of the PI kinase are probably not due to changes in substrate concentration, as it was shown that the levels of phosphatidylinositol change only slightly during development and certainly do not follow the same pattern as the PI kinase activity [21]. Thus it appears that PI kinase may play a role in the development of *D. discoideum* (which in this system is not coupled to proliferation [5]); hypothetically by modulating the production of diacylglycerol and therefore the activity of a protein kinase C, leading to the activation of gene expression.

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