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REGULATION OF DIACYLGLYCEROL KINASE IN THE TRANSITION FROM QUIESCENCE TO PROLIFERATION IN **Dictyostelium discoideum**

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SUMMARY : Diacylglycerol kinase and phosphatidylinositol kinase were examined in stationary phase D.discoideum amoeba induced to synchronously proliferate by dilution into fresh medium. Membrane bound diacylglycerol kinase activity showed a rapid and transitory 3-5 fold increase in the preproliferative interphase while phosphatidylinositol kinase activity was kept quite constant during the same period. The changes in diacylglycerol kinase activity seem to be due to a translocation of the enzyme from the soluble to the particulate cell compartments.

The metabolism of phosphoinositides is thought to play an important role in transmembrane signalling in eucaryotic cells. Berrigde has proposed (1) a "bifurcating signal pathway" in which the receptor mediated hydrolysis of poly phosphoinositides coupled the activation of protein kinase C by diacylglycerol and the mobilization of Ca^{2+} by inositol polyphosphates. Diacylglycerol is converted to phosphatidic acid by a diacylglycerol kinase which competes with protein kinase C for the newly formed diacylglycerol by receptor activation. The CTP activated phosphatidic acid is then coupled with inositol to regenerate phosphatidylinositol and polyphosphoinositides by the sequential action of specific kinases. Besides its role in the phosphatidylinositol turnover, phosphatidic might be also directly acting like a growth factor, as shown in Rat-1 cells (2) and quiescent 3T3 fibroblasts (3,4).

Abbreviations: DG: 1,2 -diacylglycerol; PI: phosphatidyl inositol; PIP: phosphatidylinositol 4-phosphate; PA: phosphatidic acid.

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In Dictyostelium discoideum it has been recently reported that inositol1,4,5-triphosphate is transiently produced in response to extracelular cAMP (5), which in this organism controls chemotaxis and differentiation (6). Furthermore, a change in phosphatidylinositol kinase activity has been described to take place during Dictyostelium differentiation(7). These results suggest that phosphatidylinositol turnover could be coupled to cAMP receptors in this organism. Here we report the occurrence of a transitory increase in the particulate diacylglycerol kinase activity in the transition from quiescence to proliferation in Dictyostelium. This change is apparently due to a translocation from the soluble to the particulate compartements.

MATERIAL AND METHODS

Growth conditions . D.discoideum strain AX2 was grown exponentially in HL-5 medium (8) with doubling times of 7-9 hours up to cell densities of $8x10^6$ cell/ml. At cell densities above $1x10^7$ cells/ml growth is arrested (stationary phase). Synchronous cultures were obtained (9) by resuspending into fresh medium cells which were kept in the stationary phase for 10-16 h. Cell number was monitored by haemocytometer counting.

Membrane preparation . Crude membrane were prepared from frozen alliquots ($1-3x10^8$ cells) exactly as described (9).

DG kinase and PI kinase assays The assay mixture contained in a final volume of 0.25 ml: 36 mM Tris-HCl pH 7.5, 3 mM $MgCl_2$, 20 mM KF, 20 mM KCl, 0.1-0.2 mM ATP, 1.5 μCi of γ^{32}P ATP and 0.03-0.05 mg of protein. When crude membranes were used as the source of enzyme, the assay was performed without the addition of lipid substrate. With the 30.000xg supernatant or solubilized membranes, the reaction mixture was suplemented with liposomes of phosphatidylserine (0.15 mg/ml) with 1,2 diacylglycerol (0.3 mg/ml). Incubations were carried out at 30° C for 2 min and stopped by the addition of 0.9 ml of $CHCl_3$/ CH_3OH/ 12N HCl (50/100/1 V/V). Lipids were extracted and analyzed in TLC as described (7).

Protein was measured in triplicate aliquots using the Bradford procedure(10). γ^{32}P ATP was from Amersham. ATP and phospholipids were from Sigma. Silica Gel 60 plates were obtained from Merck.

RESULTS

In preliminar experiments directed to establish the optimal assay conditions (not shown) it was observed that the incubation of D.discoideum membranes with γ^{32}P ATP results in an increase in the radiactivity associated with PA and PIP markers in the chromatograms of the lipid extracts. This increase was linear with the time of incu-

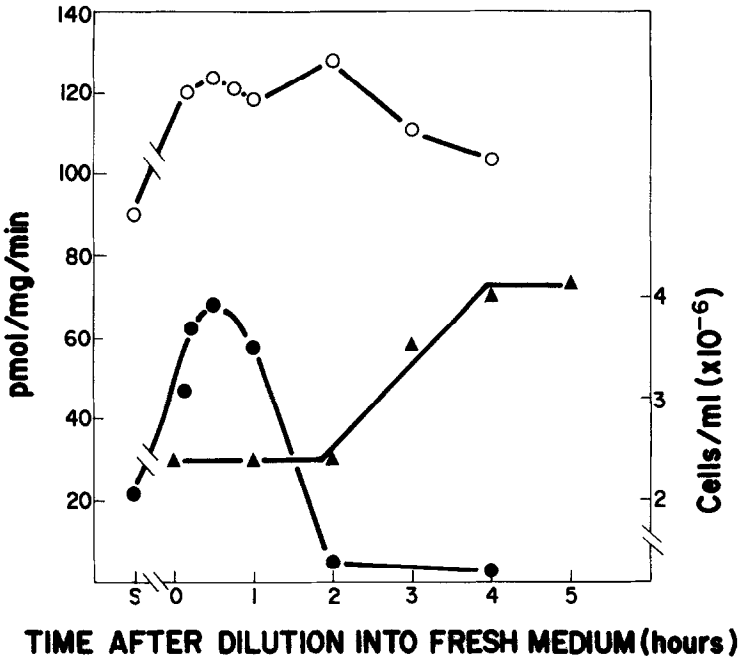

Fig 1. Changes in particulated diacylglycerol kinase and phosphatidyl kinase activities in the transition from quiescence to proliferation. Stationary cells (S) were diluted into fresh medium and assayed for membrane-bound DG kinase (●) PI kinase (○) and cell number (▲) as indicated in Methods. The data shown correspond to the result of a single experiment in triplicate. Similar results were obtained in three separate experiments.

bation for up to two minutes at 30°C and with the amount of membrane protein up to 50 ug per assay. The activities PI kinase and DG kinase were dependent on added Mg^{+2} ions for optimal activity, with a maximum at 2-4 mM and inhibitory effects above 10 mM. Similar Mg^{+2} requirements had been reported for the microsomal DG kinase from rat liver (11) and the PI kinase from D.discoideum (7). The radiolabel associated to PA and PIP did not increase when the assays were carried out in the presence of added lipid substrate indicating that the endogenous phospholipids support the activity of these membrane-bound enzymes in our assay conditions.

In order to study the changes in DG kinase and PI kinase along the transition from quiescence to proliferation in Dictyostelium, stationary phase amoebae were diluted into fresh medium as described in Methods. Under this condition they synchronously initiate a new cell division cycle after a lag period of 2-3 hours (see cell number in fig 1). PI

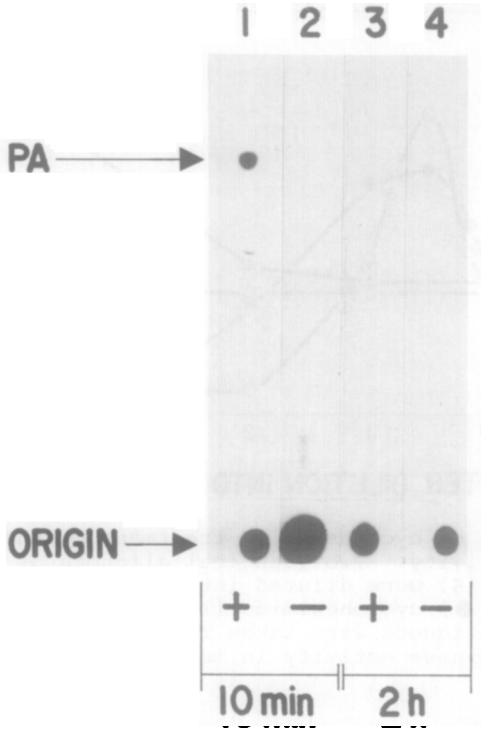

Fig 2. Membrane solubilized DG kinase. Membranes prepared from cells collected 10 min and 2 hours after dilution into fresh medium (fig 1) were solubilized with 1% NP-40 and the 100.000xg supernatant assayed with (+) and without (-) diacylglycerol in phosphatidylserine liposomes. The figure represents the autoradiography of the ^{32}P labelled PA in the TL chromatograms of the lipids extracted from each assay.

kinase activity fluctuated along the first 4 hours without any defined pattern or significant change. On the contrary, the membrane-bound DG kinase activity showed a rapid and transitory 3-5 fold increase in the interphase preceeding the onset of cell division (fig 1). Peak values were always observed within the first 10 - 30 min period after the change of medium; after that DG kinase activity rapidly decayed to near undetectable values.

To be sure that the changes in DG kinase activity were not due to endogenous substrate availability, crude membranes prepared from cells collected 10 min and 2 hours after dilution into fresh medium were treated with NP-40, centrifuged at 100.000xg and the supernatant containing the solubilized DG kinase assayed with and without added diacyl glycerol in phosfatidylserine liposomes. Under this condition DG kinase activity showed an absolute dependence for added exogenous substrate (compare lanes 1 and 2 in fig 2) but could not be detected in preparations from 2 h

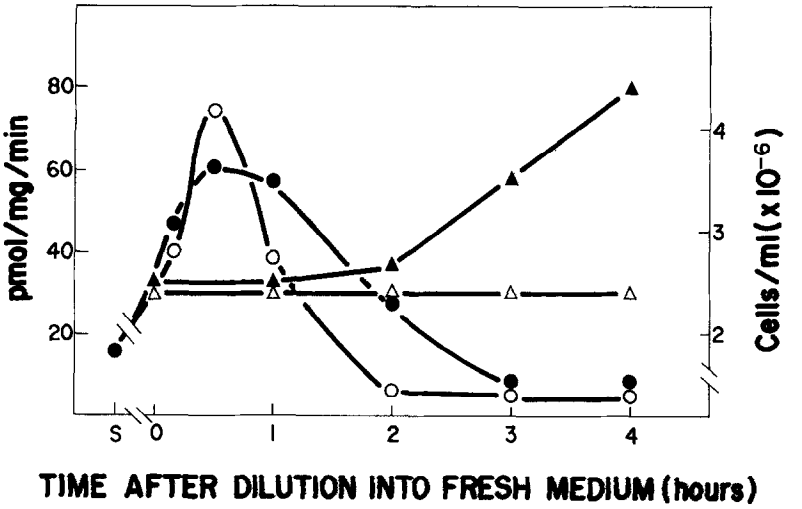

Fig 3. Effect of cycloheximide treatment on the particulate DK kinase activity. Two identical aliquots of stationary phase amoeba (S) were diluted into fresh medium with (○) and without (●) cycloheximide (1 mg/ ml). At the indicated time points aliquots were taken for cell counting (△,▲) and assay of DK kinase activity in membrane preparations.

cells (compare lanes 1 and 3 in fig 2), in which the particulate activity was very low (fig 1). This result indicates that the changes in membrane-bound DG kinase activity described in fig 1 are independent on endogenous substrate availability and therefore represent true changes in enzymatic activity.

The preproliferative changes in the particulate DG kinase activity were not blocked by cycloheximide in conditions in which cell proliferation is supressed (fig 3), indicating that protein synthesis is not involved in the modulation of this activity. This result is at contrast with our observations of a drastic inhibition by cycloheximide of the increase in plasma membrane ATPase activity of D.discoideum under similar experimental conditions (9). Moreover, these changes in particulate DG kinase did not seem to be due to differences in the Km for ATP since this parameter is kept around 0.16 mM all along the period of experimental observation. This result argues against the involvement of a postsynthetic modification mechanism as the cause of the changes in activity.

Reversible translocation from the soluble to the particulated compartements is a well characterized mechanism of protein kinase C regulation (12-14) which seems also to

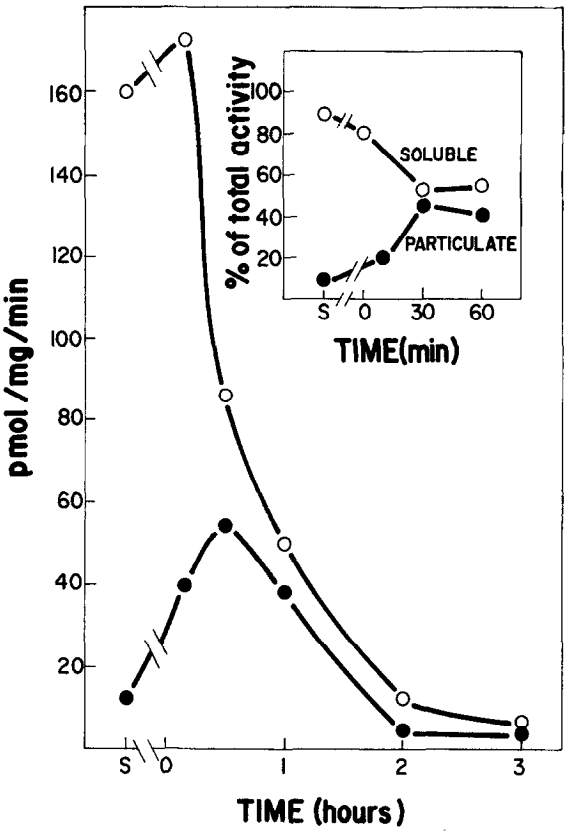

Fig 4. Evolution of the soluble and particulate DG kinase activity in the transition from quiescence to proliferation. Crude membrane preparations (●) and the 30.000 x g supernatant (○) were obtained at the indicated times and assayed for DG kinase activity as described in Methods. The insert show the relative soluble (S) and particulate (P) activities in relationship to the total DK kinase activity (S+P) for each experimental point.

be operative in the regulation of DG kinase (15,16). In order to explore this likely mechanism, DG kinase activity was measured simultaneously in the particulate and soluble (30.000 x g supernatant) fractions from D.dicoideum following the same experimental approach described in fig 1. As shown in fig 4, during the first 30 min. there is a reciprocal change in the soluble and particulate activities compatible with the postulated mechanism of translocation. Later on the DG kinase activity rapidly dissapear from both subcellular fractions.

DISCUSSION

In this report a membrane-bound diacylglycerol kinase activity has been characterized for the first time in

Dictyostelium discoideum. The rapid and transient increase in activity observed after dilution of stationary cells into fresh medium suggests that phosphoinositides turnover must be involved in proliferation signalling in Dictyostelium.

Diacylglycerol kinases had been described both in particulate and soluble cell fractions (11,17). The properties of these activities seem to be similar (11), but for the moment there is no complete evidence as to whether or not these correspond to the same molecular species. Polyclonal antibodies have been raised against a cytosolic pig brain DG kinase (18) that show cross-reactivity with the particulated activity in microsomes and synaptosomes. Translocation of protein kinase C from cytosol to membranes has been reported in a number of cell types (12-14) and seems to be an important process in ligand-receptor mediated cell response. Our data in fig 4 suggest that translocation could be a regulatory mechanism of DG kinase activity in D.discoideum as well as in higher eukariotyc cells. Besterman et al (15) have been able to promote translocation of DG kinase in vitro in the presence of diacylglycerol. Our preliminary studies indicated that the cytosolic activity disappears from this fraction when incubated with membranes in the presence of diacylglycerol. The rapid decay in the soluble and particulate DG kinase activities (fig 4) suggests a mechanism of proteolytic inactivation of the membrane translocated activity as has been described for protein kinase C (19,20). In this regard it is worth to mention that in our experimental conditions, the soluble DG kinase assay is linear for up to 8 min of incubation while the particulated activity is linear for only 2 min. We are currently setting up conditions to study this phenomenon as well as the presumptive translocation mechanism in more detail.

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REFERENCES

1. Berridge. MD, (1983) Biochem J, 212: 849-858
2. Moolenar WH, Kruijer W, Tilly BC, Verlaan I, Bierman AJ Laat SW (1986) Nature 323: 171-173

3. Siegaman DW (1987) Biocem Biophys Res Commun 145: 228-233
4. Murayama T, Ui M (1987) J Biol Chem 262: 5522-5529
5. Europe-Finner GN, Newell PC (1987) J Cell Sci 87: 221-229
6. Loomis WF (editor), The development of Dictyostelium discoideum, Academic Press, New York.
7. Varela I, Van Lookeren Campagne MM, Alvarez FJ, Mato JM (1987) FEBS Lett 211, 64-68
8. Watts DJ, Asworth JM (1970) Biochem J 116: 161-170
9. Jímenez B, Fernández-Renart M, Pestaña A (1986) Biochem Biophys Res Commun 141: 1092-1098
10. Bradford MM (1976) Anal Biochem 102: 344-352
11. Kanoh H, Akesson B (1978) Eur J Biochem 85, 225-232
12. Halsay DL, Girard PR, Kuo JF, Blackshear PJ (1987) J Biol Chem 262, 2234-2243
13. Melloni E, Pontremoli S, Michetti M, Sacco O, Sparatore B, Salamino F, Horecker BL (1985) Proc Natl Acad Sci USA 82, 6435-6439
14. May WS, Sahyoun N, Wolf M, Cuatrecasas P (1985) Nature 317, 549-551
15. Besterman JM, Pollenz RS, Booker EL, Cuatrecasas P (1986) Proc Natl Acad Sci USA 83, 9378-9382
16. Ishitoya J, Yamakawa A, Takenawa T (1987) Biochem Biophys Res Commun 144, 1025-1030
17. Lapetina EG, Hawthorne JN (1971) Biochem J 122, 171-179
18. Kanoh H, Iwata T, Ono T, Suzuki T (1986) J Biol Chem 261, 5597-5602
19. Kraft AS, Anderson WB (1983) Nature 301, 621-623
20. Melloni E, Pontremoli S, Michetti M, Sacco O, Sparatore B, Horecker BL (1986) J Biol Chem 261, 4101-4105