CELL CYCLE VARIATION IN CYCLIC ADENOSINE 3', 5'-MONOPHOSPHATE-DEPENDENT INHIBITION OF A PROTEIN KINASE FROM PHYSARUM POLYCEPHALUM

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SUMMARY

A protein kinase present in the acellular slime mold, Physarum polycephalum, has been found to exhibit cell cycle dependence with respect to inhibition by cyclic adenosine 3',5'-monophosphate (cyclic AMP). The capacity of 1×10^{-5} M cyclic AMP to inhibit the kinase was maximal (~80%) during G2 phase. At the onset of mitosis, a sharp rate of decrease occurred in the inhibitory response to cyclic AMP which continued for approximately one hour to mid-S phase. For approximately one hour during mid-S phase, the kinase activity was independent of cyclic AMP concentration. Thereafter, the capacity of cyclic AMP to inhibit the protein kinase was restored over a two-hour interval. Complete restoration coincided with the termination of S phase. Protein kinase levels measured in these same preparations in the absence of cyclic AMP remained invariant throughout the cell cycle. Thus, control of the protein kinase is apparently achieved through coordinate action of cyclic AMP plus other unidentified factors rather than by differential synthesis and destruction of a single kinase enzyme.

INTRODUCTION

Previous studies on changes in the specific activity of protein kinases in eukaryotic cells during the cell cycle have shown that only slight variations occur in both the cyclic adenosine 3',5'-monophosphate (cyclic AMP)-activated and the cyclic AMP-independent activities (1-3). These results indicate that if the protein kinases play a significant role in regulating cell functions, they must do so via the modulation of their activity rather than by differential enzyme synthesis and/or destruction. That cyclic AMP is one regulatory parameter affecting the level of protein kinase activity has been firmly established (4). In addition, changes in the availability of substrates for protein kinases may also modulate their activity as has been suggested for histone F2b from mammalian tissue cultures (5).

In an earlier communication, we reported an additional control element with the

discovery of a cyclic AMP-inhibited protein kinase activity in the slime mold Physarum polycephalum (6). However, we observed that cyclic AMP caused variable degrees of inhibition of partially purified preparations of protein kinase which were isolated from plasmodia grown in non-synchronized shake-cultures. These observations led us to conclude that expression of the cyclic AMP-inhibitable capacity might be dependent upon a specific interval of the cell cycle. This report presents preliminary evidence for this contention, with the finding that cyclic AMP-inhibition of protein kinase occurs only during the G2 phase of Physarum's cell cycle.

EXPERIMENTAL

Culturing and Sampling Methods. Axenic cultures of P. polycephalum were maintained as microplasmodia on a glucose-tryptone, semidefined medium as described by Chin and Bernstein (7). Fusions of microplasmodia were carried out on paper filters (Whatman No. 50, 9.0 cm diameter) supported by a layer of 3mm glass beads in a Petri-dish at 27° C (8). Under these conditions, the first mitosis occurred approximately 6-7 hours after the addition of growth medium to the fused macroplasmodium. The average interdivision time thereafter was about 11 hours. Mitoses were determined by observations of wet-mounts under a phase contrast microscope. Experiments involving timing and sample collection during the mitotic cycle were carried out at approximately equal intervals before and after the second mitosis. Synchronous macroplasmodia from surface cultures were collected by blotting the filter paper supports on absorbent paper to remove excess growth medium, then transferring the plasmodial mass to an aluminum foil envelope which was immediately immersed in liquid nitrogen. These samples were subsequently stored at -20° C.

Preparation of Cell Extracts and Assays. To prepare the partially purified cyclic AMP-inhibited protein kinase, frozen plasmodia were suspended in an equal volume of buffer and fractionated as previously reported (6) with the following modification. After pH adjustment of the acidified crude extract to pH 7.0 with 1 M imidazole, solid ammonium sulfate was added to 50% saturation. The precipitated protein was collected by centrifugation and dissolved in approximately five times its volume of

5 mM potassium phosphate buffer (pH 7.0), containing 2 mM EDTA. Final dialysis for 8 hr against the same buffer (4 changes of buffer medium) yielded the enzyme preparation utilized in the assays described below.

Protein kinase was assayed at pH 6.0 in the presence or absence of cyclic AMP by the method of Wastila et. al. (9). Vitamin-free casein was used as the protein substrate. Other details were as previously reported (6).

At hourly intervals during the cell cycle, one plasmodium was harvested for determination of deoxyribonucleic acid (DNA) content. Each plasmodium was suspended in 4 ml of water and was passed once through a French pressure cell at approximately 20,000 psi. The crude soluble fraction resulting after centrifugation for 10 min at 32,000 x g, was used to assay for DNA content by a modification of the procedure of Giles and Myers (10) as described by Hames (11).

RESULTS AND DISCUSSION

The results of one representative experiment are shown in Fig. 1. A total of four such experiments have been performed with similar results. In synchronized plasmodia, the capacity of 1×10^{-5} M cyclic AMP to diminish protein kinase activity was maintained at a basal level throughout the G2 phase of the cell cycle (Fig. 1, circles). However, from the onset of mitosis to approximately mid-S phase this inhibition decreased until the kinase attained nearly complete independence from the inhibitory effect of cyclic AMP. (P. polycephalum has no G1 phase in its cell cycle; thus DNA synthesis commences immediately after mitosis.) Thereafter, the cyclic AMP inhibitable capacity was restored during the succeeding two hours. The point of essentially complete restoration of a basal inhibitory level coincided approximately with the termination of new DNA synthesis (Fig. 1, triangles) and the onset of a new G2 phase. The same partially purified extracts which demonstrated inhibition of protein kinase by cyclic AMP, also exhibited greater kinase activity in the absence of cyclic AMP, and this latter activity remained invariant throughout the entire cell cycle (Fig. 1, squares). These results suggested that control of the kinase is achieved through the coordinate action of cyclic AMP plus other unidentified factors

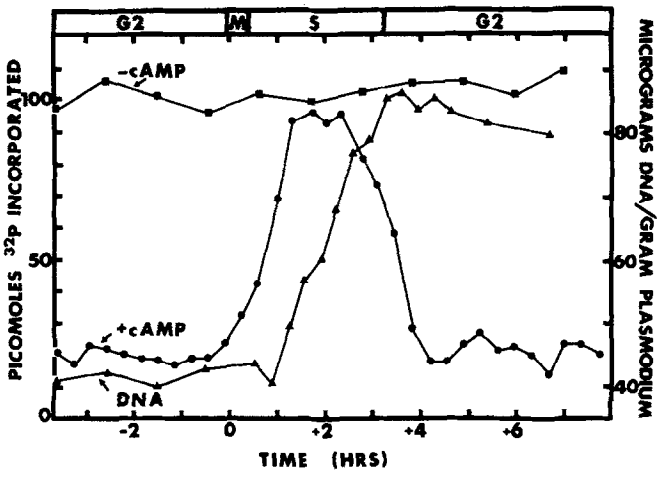

Fig. 1. Cell cycle variability in cyclic AMP-dependent inhibition of a protein kinase from Physarum polycephalum. Assays were conducted in the presence (circles) or absence (squares) of 1 x 10^{-5} M cyclic AMP as described in the text. Each assay contained 50 µgm of enzyme preparation. The specific activity of the γ-^{32}P-ATP substrate used was 8.22 x 10^{7} cpm per µmole. The increase in DNA content during S phase (triangles) was determined from analyses of single macroplasmodia harvested at the indicated times in the cell cycle. Time intervals on the abcissa are oriented with repect to the second mitosis after addition of growth medium to fused microplasmodia.

rather than by differential kinase synthesis and destruction alone. These findings also provided an apparent explanation for the variability observed earlier in cyclic AMP inhibition of protein kinase activity in non-synchronized, shake-cultures (6).

To examine the possibility that cyclic AMP, acting in concert with an inhibitor, might be responsible for inhibition of the protein kinase during G2 phase, the extent of production of a putative inhibitor was investigated. Thus, extracts derived from mid-S phase plasmodia were incubated with varying amounts of protein from extracts prepared from plasmodia in G2 phase. Plasmodia from the former phase would presumably contain a minimum concentration of an inhibitory factor, whereas plasmodia from the latter phase would contain the highest concentration of inhibitor. Data in Table 1 show that the kinase activity in extracts prepared from mid-S phase plasmodia was preserved when combined with extracts derived from G2 periods of the cell cycle. Thus, any factor which might have conferred the cyclic AMP inhibitory capacity on a protein kinase in P. polycephalum was apparently not produced in excess during the G2 phase. Moreover, the effect of cyclic AMP on protein kinase was reversible since kinase pre-

TABLE I

TEST FOR INHIBITOR PRODUCTION

Assay No.	Extracts Derived from Stage of Cell Cycle[a]	Presence of 10 μM cyclic AMP	Protein Kinase Activity	
			Observed cpm[b]	Expected cpm[c]
1	G2 phase (50)	yes	2,102	--
2	G2 phase (50)	no	7,313	--
3	mid-S phase (50)	yes	6,451	--
4	mid-S phase (50)	no	6,947	--
5	G2 phase (50) + mid-S phase (50)	yes	8,851	8,553
6	G2 phase (50) + mid-S phase (50)	no	13,888	14,260
7	G2 phase (100) + mid-S phase (50)	yes	11,413	10,655
8	G2 phase (100) + mis-S phase (50)	no	20,063	21,573

[a]Each assay contained the micrograms of protein given in parentheses derived from extracts prepared from plasmodia at the indicated stage of the cell cycle. Assays 5-8 represent trials in which protein samples derived from different cell cycle phases were preincubated together for 5 min at 30°C prior to assay for kinase activity.

[b]Activities are expressed as the acid-stable cpm of ^{32}P from γ-^{32}P-ATP which were transferred to casein in 20 min of reaction time (6). The specific activity of the γ-^{32}P-ATP substrate used was 1.14×10^8 cpm per μmole.

[c]The *Expected* values listed are derived from appropriate combinations of the *Observed* activities from assays 1-4, assuming that there was no further inhibition by cyclic AMP than was observed in assays 1 and 3.

parations which were inhibitable by cyclic AMP were fully reactivated after dialysis or Sephadex G-25 chromatography (data not shown). Desalted fractions also retained the kinase inhibitory response to cyclic AMP. While these observations support the notion that some factor(s) in conjunction with cyclic AMP cooperates to effect inhibition of the protein kinase, other explanations are equally plausible. For example, coincident inverse oscillations in the expression and destruction, respectively, of two kinases, one inhibited and the other unaffected by cyclic AMP, would yield the same observations depicted in Fig. 1. However, evaluation of these proposals must await isolation of the protein kinase and the cyclic AMP-dependent inhibitory system.

The physiological significance of the variation in a protein kinase catalytic response to cyclic AMP during the S phase of the cell cycle in *P. polycephalum* re-

mains to be determined. To propose that this variation may account for increased histone phosphorylation during S phase, as has been reported in synchronized mammalian cells (2), seems unlikely, since histones have yet to be demonstrated to function as substrates for the protein kinase involved here (6). This report, however, does demonstrate an example of marked protein kinase regulation by a specific effector or process which is restricted to a defined state of cell development. It will be of interest to know whether this dependence extends to other systems in which cyclic AMP inhibition of protein kinases have recently been reported (12).

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