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CHANGES IN CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITY IN *TETRAHYMENA PYRIFORMIS* DURING THE GROWTH CYCLE

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Summary

An adenosine 3′:5′-monophosphate-dependent protein kinase II (ATP: protein phosphotransferase, EC 2.7.1.37) was partially purified from the cytosol fraction of an exponentially growing culture of *Tetrahymena pyriformis*. Protein kinase II represented approximately 90% of the cytosolic protein kinase activity. The enzyme had a high degree of substrate specificity for calf thymus and *Tetrahymena* histones as compared to casein, protamine and phosvitin. The enzyme incorporated the terminal phosphate of ATP into serine and threonine residues of all the histone fractions. The apparent K_m of the enzyme for adenosine 3′:5′-monophosphate (cyclic AMP) was $1 \cdot 10^{-8}$ M. Protein kinase II was also activated by other cyclic nucleotides with apparent K_m values in the range $2.5 \cdot 10^{-6}$—$5.0 \cdot 10^{-6}$ M.

The specific activity of the cyclic AMP-dependent protein kinase of *Tetrahymena* decreases markedly from initial high values during the transition from the lag to early log phase of growth. This is followed by a sharp increase in the activity of the enzyme as the log phase of growth progresses. The specific activity of the enzyme increased rapidly during the heat-induced synchronization of *Tetrahymena* cells. The capacity for rapid phosphorylation of multiple classes of organelle-specific phosphoproteins and the level of cyclic AMP were maximal in *Tetrahymena* during the earliest phase of growth. These results demonstrate that the cell cycle of *Tetrahymena* may be coordinated by marked variations in the level of cyclic AMP which in turn regulate the cyclic AMP-dependent protein kinase.

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Abbreviations: Cyclic AMP, adenosine 3′:5′-monophosphate; cyclic GMP, guanosine 3′:5′-monophosphate; cyclic UMP, uridine 3′:5′-monophosphate; cyclic CMP, cytidine 3:5′-monophosphate.

Introduction

There is ample evidence to support the postulation that the action of cyclic AMP may be propagated and amplified by activating protein kinases which in turn cause the phosphorylation of multiple classes of phosphoproteins in eukaryotic cells [1—6]. The unicellular eukaryotic organism *Tetrahymena pyriformis* offers a model system for the study of the evolutionary development of cyclic AMP as a regulatory agent in the intermediary metabolism. Previous studies from this laboratory demonstrated that the concentration of cyclic AMP in *Tetrahymena pyriformis* varies markedly during the growth cycle [7]. The level of cyclic AMP is maximal during the early log phase of growth and declines sharply during the late log to early stationary phase of growth. Recently a cyclic AMP-independent protein kinase has been partially purified from axonemes of *Tetrahymena* cilia and the enzyme causes phosphorylation of ciliary tubulin in vitro [8]. The present studies characterize for the first time a cytosol protein kinase from *Tetrahymena pyriformis* cells which is activated specifically by cyclic AMP. The level of the cyclic AMP-dependent protein kinase fluctuates markedly during the growth cycle. The results are consistent with the postulation that the protein kinase as well as cyclic AMP play a key regulatory role in the process of transition of cells from the G1 to S phase of cell cycle.

Materials and Methods

Chemicals

Adenosine 3′:5′-monophosphate, *O*-phosphoserine, *O*-phosphothreonine and phosvitin were purchased from Calbiochem. [γ-^{32}P] ATP (spec. act. 23.2—38.2 Ci/mmol) and $^{32}P_i$ (carrier-free) were products of International Chemical and Nuclear Corporation. [8-^{3}H] Adenosine 3′:5′-monophosphate (16.3 Ci/mmol) was obtained from Schwarz BioResearch. Histones (calf thymus) were from Worthington and cyclic GMP, cyclic CMP and cyclic UMP were obtained from Boehringer Mannheim. DEAE-cellulose (cellex-D) was a product of Bio-Rad and cycloheximide was from Nutritional Biochemicals. Protamine was obtained from Sigma. All other chemicals were of the highest grade commercially available.

Growth of organisms

Tetrahymena pyriformis, Strain E, was grown at 24° C with gentle shaking in Erlenmeyer flasks filled to 20% of capacity with a medium composed of 2% proteose-peptone [9]. Unless otherwise indicated, growth was initiated by adding an inoculum (1%) from an early stationary phase culture. Synchronous cell division was induced in an exponentially growing population (a 24 h culture was diluted to $1 \cdot 10^4$ cells/ml) by the method of Scherbaum et al. [10]. The culture was heated with shaking for six 30 min periods at 34° C alternating with five 30 min periods at 24° C and restored to the optimum 24° C incubation after the last heat treatment. The degree of synchronization in the first division was approximately 89%. Growth of the cultures was followed by counting cells with a Coulter counter.

Isolation of protein kinase

Cells were harvested by centrifugation at 600 × *g* and the pellet was washed twice with 0.25 M sucrose. The cells were ruptured by freezing and thawing (2×) in 0.25 M sucrose and the lysed cells were centrifuged at 5000 × *g* for 15 min. The supernate was dialyzed against 5 mM sodium glycerophosphate/HCl buffer, pH 6.0, and 0.2 mM dithiothreitol. Protein kinase activity was measured in the dialyzed preparations.

In studies on the subcellular distribution of protein kinase and properties of the cytosol enzyme, the cells from 24-h cultures were harvested by centrifugation at 600 × *g* and washed twice with 0.25 M sucrose. The cell pellet was homogenized in a glass, motor-driven Potter-Elvehjem homogenizer and the homogenate was centrifuged at 110 000 × *g* for 60 min. To the resulting cytosol fraction, solid ammonium sulfate was added with stirring to bring the ammonium sulfate concentration to 50% saturation. After 30 min, the precipitate was collected by centrifugation at 27 000 × *g* for 10 min and the residue was discarded. The supernate was dialyzed extensively against 0.2 mM potassium phosphate buffer, pH 7.0. The dialyzed enzyme preparation was applied to a DEAE-cellulose column (0.9 × 16 cm) previously equilibrated with 0.2 mM potassium phosphate buffer, pH 7.0. The column was washed with 30 ml of the equilibrating buffer prior to elution with a linear gradient of potassium phosphate buffer (0.2—67 mM) in a total volume of 150 ml of the buffer. The flow rate was 7.3 ml/h, and the volume in each fraction was 2.2 ml. All steps were carried out at 0—4° C.

Assay of protein kinase

The activity of protein kinase in the dialyzed samples was measured by a slight modification of the method described previously [11]. The standard assay medium contained 10 μmol of sodium glycerophosphate/HCl (pH 6.0), 1.0 mg of calf thymus histones, 1 nmol of [γ-^{32}P] ATP containing $1 \cdot 10^5$—$5 \cdot 10^5$ cpm, 2 μmol of sodium fluoride, 0.4 μmol of theophylline, 0.06 μmol of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid, 2 μmol of cobalt chloride and protein kinase with or without 200 pmol of cyclic AMP in a total volume of 0.2 ml. The incubation was carried out at 30° C for 30 min, and the reaction was stopped with the addition of 5 ml of cold 10% trichloroacetic acid. Casein (2.5 mg), non-isotopic ATP (1.0 μmol), and disodium phosphate (10 μmol) were then added and the mixture was allowed to stand at 0° C for at least 30 min. The precipitate was collected on Whatman GF/C glass fiber discs and washed successively with 20 ml of 10% trichloroacetic acid, ethanol, ethanol/ether (3 : 1, v/v) and finally with ether. The filter discs were placed in vials containing toluene/0.4% 2,5-diphenyloxazole/0.005% 1,4-bis(5-phenyloxazole)benzene scintillation fluid and the amounts of ^{32}P incorporated into histone were determined by liquid scintillation spectrometry. Systems lacking the enzyme preparation served as blanks. The incorporation of ^{32}P into the endogenous proteins of the enzyme preparations was insignificant. A unit of enzyme activity was defined as the amount of enzyme which catalyzed the transfer of 1 pmol of ^{32}P from [γ-^{32}P] ATP to the recovered protein during 30 min under the standard assay conditions. The specific activity of the protein kinase is expressed as units of activity per μg of protein.

Incorporation of ^{32}P into phosphoproteins of Tetrahymena cells and various subcellular fractions

Tetrahymena pyriformis, strain E, was grown with gentle shaking at 24° C in a 2% proteose-peptone medium [9]. 1% inoculum was used from a stationary phase culture for the growth of this organism. [^{32}P] Orthophosphate was added to the media (17 μCi/ml) at various stages of growth cycle. Following a 4 h incubation with ^{32}P, a large excess of non-isotopic inorganic phosphate (5 μmol/ml) was added and the culture chilled immediately. To insure the recovery of the ^{32}P-labeled phosphoproteins, a large excess of non-labeled cells (approx. $1.8 \cdot 10^5$ /ml) obtained from a stationary phase culture was added to the flask. The cells were harvested by centrifugation at 600 × *g* for 10 min and washed twice with ice-cold 0.25 M sucrose. The cell pellet was homogenized in a Potter-Elvehjem homogenizer and the homogenate centrifuged at 1000 × *g* for 10 min to sediment nuclei. Mitochondria were obtained from the supernate by centrifugation at 12 000 × *g* for 10 min and the resulting supernate was centrifuged at 110 000 × *g* for 60 min to sediment microsomes. Ribosomes were isolated from the crude microsomal pellet by the method of Moldave and Skogerson [12]. All of the particle fractions were washed twice with 0.25 M sucrose and finally suspended in 0.25 M sucrose.

Nuclear protein fraction was fractionated into histones and nuclear acidic proteins [13]. For the measurement of radioactivity in ^{32}P-labeled phosphoproteins, an aliquot of the preparation of subcellular fractions was treated with ice-cold 5% trichloroacetic acid and the suspension was heated at 90° C for 30 min and centrifuged. The process of hot trichloroacetic acid extraction was repeated and the precipitate was washed three times in 5% trichloroacetic acid, three times in ether/ethanol (1 : 3, v/v) and finally in ether [1]. The precipitate was dissolved in soluene (Packard) and counted in toluene scintillation solvent.

Preparation of ^{32}P-labeled histones

Calf thymus histones were phosphorylated enzymatically by the isolated protein kinase II. The reaction mixture contained 40 μmol of sodium glycerophosphate/HCl (pH 6.0), 6.0 mg of calf thymus histones, 4 nmol of [γ-^{32}P]-ATP containing $1 \cdot 10^7$ cpm, 2 μmol of cobalt chloride, 8 μmol of NaF, 0.24 μmoles of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid, 25 μg of protein kinase II with or without 1.0 nmol of cyclic AMP in a total volume of 0.8 ml. The incubation was carried out at 30° C for 30 min. The reaction was arrested by chilling and the reaction mixture was rapidly dialyzed against deionized water at 4° C with several changes of water. The ^{32}P-labeled histone preparations were used for amino acid analysis and polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis

^{32}P-labeled histones were fractionated by polyacrylamide gel electrophoresis [13]. The upper gel contained 15% acrylamide, 0.1% ethylene diacrylamide, 0.3 M acetate buffer (pH 4.4) and 0.125% potassium persulfate. The lower gel contained 5% acrylamide, 2.5% bisacrylamide, 0.43 M acetate buffer (pH 6.7) and 0.001% riboflavin. Both the gels contained 6 M urea and the buffer for both reservoirs was 0.1 M β-alanine-acetate buffer, pH 4.2. The elec-

trophoresis was carried out at a constant current of 4 mA per tube. After the electrophoresis, the gels were stained with amido black, fixed in 7.5% acetic acid, frozen and finally sectioned with a manual slicing device [14], the thickness of each gel section being 1 mm. The gel sections were counted directly in Bray's solution [15].

Other methods

The cyclic AMP content of *Tetrahymena pyriformis* at various stages of the growth cycle was determined as previously described [7]. Protein concentration in the samples was measured by the method of Lowry et al. [16] using bovine serum albumin as the standard.

^{32}P-labeled phosphoproteins were hydrolysed in 2 M HCl at 100°C for 16 h and the hydrolysate was subjected to paper electrophoresis to separate *O*-phosphoserine and *O*-phosphothreonine [1,17]. The amount of radioactivity in [^{32}P]serine and [^{32}P]threonine spots was counted in toluene scintillation solvent.

Results

Enzyme purification

The cytosol protein kinase preparation from *Tetrahymena pyriformis* was resolved by DEAE-cellulose chromatography into two distinct peaks of enzyme activity (Fig. 1). The activity of protein kinase II was greatly stimulated (7—9-fold) by cyclic AMP whereas protein kinase I was activated to a much smaller extent (1.5-fold). Approximately 90% of the cytosol protein kinase activity was associated with protein kinase II. Rechromatography on DEAE-cellulose yielded a single peak of activity with each enzyme preparation. Protein kinase II (fractions 24 to 34) was purified to 80-fold as compared to the homogenate of *Tetrahymena* cells, and in the subsequent studies this preparation was used to evaluate certain properties of the enzyme.

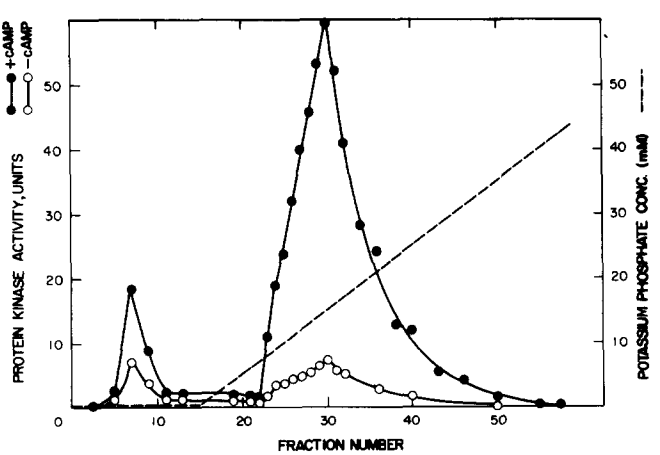

Fig. 1. Separation of two cytosol protein kinases (I and II) of exponentially growing (24 h culture) *Tetrahymena pyriformis* by chromatography on DEAE-cellulose. An aliquot of 25 µl of each fraction was assayed for protein kinase activity in the presence and absence of cyclic AMP by the standard assay method.

Enzyme properties

The amount of histones phosphorylated by protein kinase II increased linearly with time for a period of at least 40 min in the presence or absence of cyclic AMP. The rate of phosphorylation of calf thymus histones also increased linearly as the amount of the enzyme in the assay mixture was increased to 330 units.

The effect of varying concentrations of histones on the activity of *Tetrahymena* protein kinase II was examined. The K_m value for histones of kinase II was 0.6 mg per ml when assayed in presence or absence of cyclic AMP. The V value was increased from 13.3 to 147.1 units by the addition of cyclic AMP. The K_m value for ATP of the *Tetrahymena* protein kinase II was 3.1 μM, when assayed in presence or absence of cyclic AMP. V increased from 16.6 to 200.0 units with the addition of cyclic AMP. The enzyme had high activity in the acidic pH range and maximal activity was observed between pH 5.5 and 6.0. The pH vs activity curves of the enzyme in the presence and absence of cyclic AMP were nearly identical. The enzyme was stable to heat treatment at 40°C for 15 min. A further increase in temperature rapidly inactivated the enzyme and the enzyme lost its activity when heated at 60°C for 15 min. The heat sensitivity of the enzyme was similar in the presence and absence of cyclic AMP.

The effects of various cyclic nucleotides on the activity of *Tetrahymena* protein kinase II were evaluated. At lower concentrations of cyclic nucleotides, cyclic AMP was most effective for the activation of protein kinase II (approx. 10-fold increase), as compared to the other cyclic nucleotides. However, at higher concentrations ($1 \cdot 10^{-4}$ M) all the cyclic nucleotides stimulated the activity of protein kinase II to nearly the same extent. The apparent K_m values of the enzyme for cyclic AMP, cyclic GMP, cyclic UMP and cyclic CMP were 0.01 μM, 2.5 μM, 2.5 μM and 5.0 μM, respectively.

The rates of phosphorylation of various protein substrates by *Tetrahymena* protein kinase II were compared (Table I). The results demonstrate that protein kinase II phosphorylates histones at much faster rates than those for protamine, phosvitin and casein. Histones from *Tetrahymena pyriformis* were phosphorylated at a slightly lower rate than that in calf thymus histones. Proteins in intact ribosomes of *Tetrahymena* were potential substrates for the isolated cytosol protein kinase II whereas proteins of intact nuclei and mitochondria were relatively ineffective as substrate for the cytosol enzyme. Cyclic AMP augmented the rates of phosphorylation of all the proteins by protein kinase II.

^{32}P-labeled calf thymus histones obtained by phosphorylation with protein kinase II were fractionated by polyacrylamide gel electrophoresis (Fig. 2). The major protein peaks were designated according to the notation of Johns [18] and were previously identified by comparing their electrophoretic mobilities with those of pure histone fractions obtained from rat liver and HeLa cells by the method of Johns [18]. Whereas *Tetrahymena* protein kinase II caused the phosphorylation of all the histone fractions, F2b histone was the most heavily phosphorylated fraction. Cyclic AMP coordinately stimulated the rates of phosphorylation of all the histones by protein kinase II. ^{32}P-labeled calf thymus histones were further studied by amino acid analysis. The ^{32}P was

TABLE I

SUBSTRATE SPECIFICITY OF *TETRAHYMENA* PROTEIN KINASE II

Standard assay conditions were used except for the variation in the amount and nature of protein substrate.

Substrate	Amount of protein (μg)	Relative activity (%)	
		+cyclic AMP	—cyclic AMP
Calf thymus	250	100[a]	13
	50	53	6
Casein	250	34	6
	50	17	3
Tetrahymena histones[b]	250	92	13
	50	44	6
Protamine	250	85	12
	50	40	5
Phosvitin	250	0	0
	50	0	0

[a] Enzyme activity was 110 units.

[b] *Tetrahymena* histones were extracted from the isolated nuclear pellet with 0.25 M HCl at 4°C for 4 h and the suspension was centrifuged at 27 000 X *g* for 30 min [11]. The supernate containing histones was dialyzed extensively against deionized water at 4°C and finally lyophilized.

distributed between the serine (54%) and threonine (46%) residues of histones. Cyclic AMP augmented the rates of incorporation of ^{32}P into serine and threonine residues by protein kinase II to the same magnitude.

The presence of a specific cyclic AMP-binding protein in *Tetrahymena*

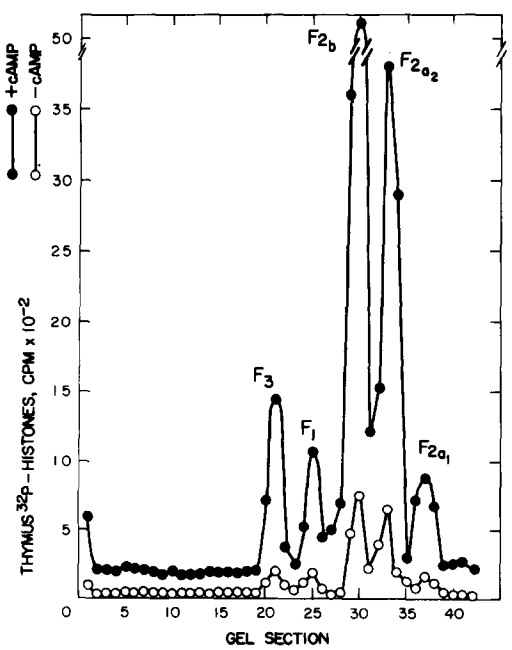

Fig. 2. Electrophoretogram radioactivity profiles of calf thymus ^{32}P-labeled histones, phosphorylated by *Tetrahymena* protein kinase II in vitro, in the presence (●———●) and absence (○———○) of cyclic AMP.

protein kinase II was demonstrated by using the assay procedure described previously [1]. The conversion of the cyclic AMP-dependent protein kinase to a cyclic AMP-independent protein kinase was demonstrated by DEAE-cellulose chromatography of the enzyme [1] in the presence of $1 \cdot 10^{-6}$ M cyclic AMP. These observations are compatible with the view that the *Tetrahymena* cyclic AMP-dependent protein kinase, like the protein kinases of many animal tissues, is an inactive complex of a regulatory subunit (R) and a catalytic subunit (C) and cyclic AMP activates the enzyme by its specific binding with the regulatory subunit thereby causing its dissociation from the active catalytic subunit [1,19–24].

Subcellular distribution of protein kinase

The enzyme activity was measured in subcellular fractions of *Tetrahymena pyriformis* isolated by differential sedimentation. Table II shows that approximately 75% of the total protein kinase activity of the cell was recovered in the cytosol fraction although smaller amounts of enzyme were associated with nuclei, mitochondria and microsomes. For the phosphorylation of histones, organelle-bound protein kinases were activated by cyclic AMP to a much smaller extent (20–70% increase) than the cytosol enzyme (130% increase). Results similar to those in Table II were also obtained when the purified organelles were isolated from *Tetrahymena* by zonal centrifugation through a linear sucrose gradient [25]. It was further observed that intrinsic protein kinase activities in the purified nuclei, mitochondria and ribosomes can cause the phosphorylation of the endogenous phosphoproteins and these reactions were not influenced by cyclic AMP.

Protein kinase activity during the growth cycle of Tetrahymena

Protein kinase measured during a normal unsynchronized growth cycle of the organism shows a striking decrease (approx. 4-fold) in the specific activities

TABLE II

SUBCELLULAR LOCALIZATION OF PROTEIN KINASE ACTIVITY IN *TETRAHYMENA PYRIFORMIS*

Cells from an exponentially growing culture (24-h) of *Tetrahymena* were harvested and homogenized in 0.25 M sucrose containing 0.4 mM Tris · HCl buffer, pH 7.4 and 0.1 mM EDTA in a motor-driven Potter-Elvehjem homogenizer. The homogenate was centrifuged at 1000 × *g* for 10 min to sediment nuclei and the resulting supernate was centrifuged at 8000 × *g* for 10 min to isolate mitochondria. Microsomes were isolated from the supernate by centrifugation at 110 000 × *g* for 60 min. Ribosomes were isolated from the crude microsomal pellet by treatment with sodium deoxycholate and purified further by treatments with varying concentrations of $MgCl_2$ and NH_4 Cl [12]. All the particulate fractions were washed twice in the homogenizing buffer. The activity of protein kinase was measured by the standard assay method.

Subcellular fractions	Protein kinase activity (% distribution)		Spec. act. (units/μg protein)	
	–cyclic AMP	+cyclic AMP	–cyclic AMP	+cyclic AMP
Homogenate	100	100	5.3	10.8
Nuclei	5.7	4.9	3.8	6.7
Mitochondria	17.8	13.0	3.0	4.5
Microsomes	4.1	2.5	1.2	1.5
Supernate	72.4	79.6	13.0	29.5

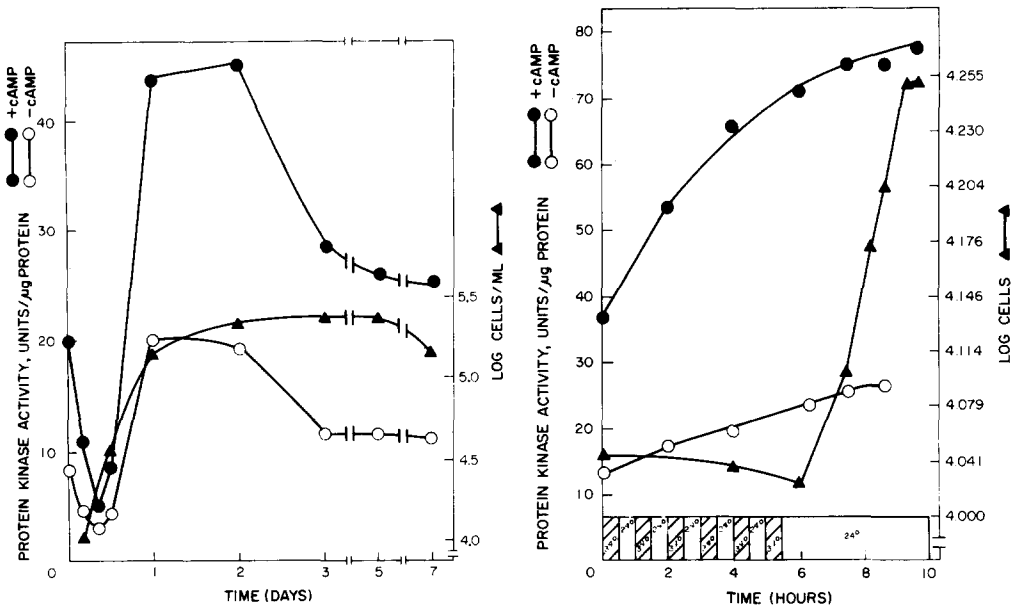

Fig. 3. Specific activities of protein kinase in *Tetrahymena pyriformis* during a normal growth cycle. Protein kinase activity was measured by the standard assay method in presence (●———●) and absence (○———○) of cyclic AMP.

Fig. 4. Effect of synchronization of *Tetrahymena pyriformis* on the specific activity of protein kinase. The synchronous cell division was induced in *Tetrahymena* cells by the procedure described in Materials and Methods and protein kinase activity was measured by the standard assay procedure.

of both the cyclic AMP-dependent protein kinases within the first 8 h of growth (Fig. 3). Subsequently during the log phase of growth a dramatic increase occurs and the maximal specific activity of the enzyme is attained at 24—48 h of growth. During the late log and early stationary phases of growth there is a slow decrease in the specific activity of the enzyme. Both cyclic AMP-independent and -dependent protein kinases show these changes.

Since marked changes in the specific activity of protein kinase occurred during the log growth phase of *Tetrahymena*, it was of interest to measure the activity of the enzyme in a synchronized cell culture and relate the level of protein kinase to the cell cycle. There was a rapid increase in the specific activity of protein kinase (assayed in presence and/or absence of cyclic AMP) during the periods of heat-shocks which are prerequisites for induction of synchronous division in *Tetrahymena pyriformis* (Fig. 4). The specific activity of the protein kinase increased by approximately 40% and 75%, respectively, at the end of 2nd and 4th heat-shocks. Subsequently the activity of the enzyme increased slowly and by the end of first synchronous cell division the activity of protein kinase had increased approximately 100%.

In order to evaluate whether the increase in specific activity of protein kinase in *Tetrahymena* during heat-induced synchronization represents merely an activation of the existing protein kinase molecules or is due to new protein synthesis, the synchronization of the organism was carried out in presence or absence of cycloheximide, a potent inhibitor of protein synthesis. The addition of cycloheximide to the culture medium completely prevented the induction of

protein kinase in the heat-induced synchronized culture. This indicates that the increase of protein kinase activity in the synchronized cells requires concomitant synthesis of protein.

Relationship of protein kinase activity to cyclic AMP levels and phosphorylation of proteins

The results on the incorporation of $^{32}P_i$ into phosphoproteins of *Tetrahymena* during the growth cycle is shown in Fig. 5. A striking feature of these data is that cells during lag phase of the growth cycle (0—4 h) showed the highest rate of incorporation of ^{32}P into phosphoproteins of histones, nuclear acidic proteins, mitochondria, ribosomes and cytosol.

The ability of these cells to incorporate ^{32}P into proteins decreased progressively with the advance of the growth cycle. Such decrease in the amount of ^{32}P incorporated was most dramatic (4—15-fold) during the early log phase of the growth cycle. Of all the phosphoproteins tested, mitochondrial proteins were most heavily phosphorylated and histones were least phosphorylated during the entire growth cycle. Amino acid analysis of the ^{32}P-labeled organelle-bound proteins demonstrated that ^{32}P was incorporated into the serine and threonine residues of these proteins and the ratios of the radioactivity in [^{32}P]serine and [^{32}P]threonine were 1.1, 0.3, 1.0, 0.9 and 0.4 for histones and nuclear acidic, mitochondrial, ribosomal and cytosol proteins, respectively. These results indicate that the observed variation in the net rate of incorporation of ^{32}P into proteins during the growth cycle of *Tetrahymena* reflects differential rate of phosphorylation of phosphoproteins.

The temporal relationship of protein kinase activity in *Tetrahymena*

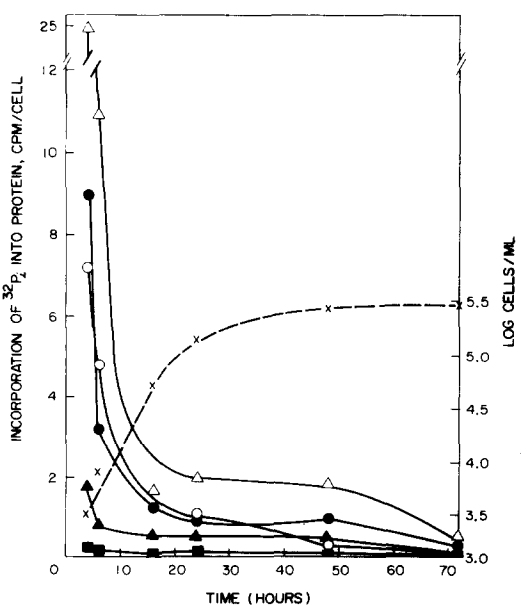

Fig. 5. Rate of incorporation of $^{32}P_i$ into multiple classes of phosphoproteins by intact *Tetrahymena pyriformis* during growth cycle. Each point in the incorporation curve represents the amount of isotope incorporated during the preceding 4 h period.

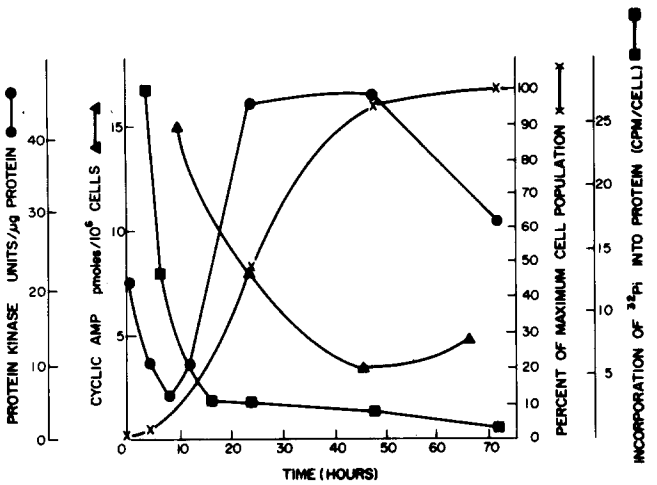

Fig. 6. Specific activities of protein kinase (assayed in presence of cyclic AMP, ●———●) in relation to levels of cyclic AMP (▲———▲), and the rates of phosphorylation of total phosphoproteins (□———□) in *Tetrahymena pyriformis* during growth cycle. Activity of protein kinase was measured by the standard assay method. Each point in protein phosphorylation represents the incorporation of ^{32}P during the preceding 4 h period. Total ^{32}P-labeled phosphoproteins were isolated from whole homogenates of ^{32}P-labeled cells by the procedure described in Materials and Methods.

during the growth cycle to levels of cyclic AMP and rates of phosphorylation of proteins was evaluated (Fig. 6). The concentration of cyclic AMP was highest in 10-h cultures (earliest period examined). Subsequently with the advance of the log phase there was a marked decrease in the level of cyclic AMP and at the end of 48 h of culture the level of cyclic AMP declined by approx. 5-fold. The pattern of change in the concentration of cyclic AMP in *Tetrahymena* during growth cycle strongly suggests that the level of cyclic AMP may be near maximal during the lag phase of growth. Thus the lag phase of the growth cycle which is characterized by a high rate of phosphorylation of multiple classes of organelle-specific phosphoproteins is temporally associated with a high level of cyclic AMP and a rapidly declining level of cyclic AMP-dependent protein kinase activity which may be undergoing rapid turnover. Subsequently with the advance of the log phase there was a marked decrease in the level of cyclic AMP and the amount of phosphorylation of multiple classes of proteins in contrast to a sharp increase in the specific activity of cyclic AMP-dependent protein kinase.

Discussion

The present studies demonstrate the presence of a cytosol protein kinase II in *Tetrahymena pyriformis* which is specifically activated by cyclic AMP. Protein kinase II represents approximately 90% of total cytosol enzyme activity. The apparent K_m of the enzyme for cyclic AMP is $1 \cdot 10^{-8}$ M. The enzyme has a high degree of specificity for the phosphorylation of histones as compared to protamine, phosvitin and casein. The enzyme (RC) consists of a regulatory (R) and a catalytic subunit (C) and causes the phosphorylation of serine and threonine residues of all the major histone fractions. The properties of the

cytosol cyclic AMP-dependent protein kinase of *Tetrahymena* cells are strikingly different from those of the cyclic AMP-independent kinase extracted from the *Tetrahymena* ciliary axonemes in relation to substrate specificity, pH optimum and K_m for ATP [8].

An inoculum from stationary phase culture was used for the growth of *Tetrahymena* in these studies. During stationary phase of culture the cells are likely to be mostly in the G1 phase of cell cycle. Consequently at the onset of the growth cycle (lag phase) cells are expected to be mostly in the process of transition from G1 to S phase of cell cycle and subsequently with the progress of the growth cycle, the proportion of the cells undergoing transition from G1 to S phase are likely to be reduced markedly because of the appearance of cells of the various other stages [26] of cell cycle and increase in the generation time of these cells. From the results of the present studies, it can be postulated that the cells undergoing transition from G1 to S phase of cell cycle have maximal level of cyclic AMP as compared to the cells of the other phases of the cell cycle. High level of cyclic AMP in these cells will cause a marked increase in the rate of formation of the functionally active catalytic subunit of protein kinase (C) by specific interaction of cyclic AMP with the cyclic AMP-dependent inactive protein kinase complex (RC) which is metabolically stable and the storage form of the enzyme in the cell. The nascent catalytically active protein kinase, which has a high rate of turnover, will act rapidly on protein substrates to cause the phosphorylation of multiple classes of organelle-specific phosphoproteins which may be important determinants for the progress of the cell cycle from G1 to S phase. Because of the high level of cyclic AMP, the cells towards the end of G1 phase of cell cycle will possess the highest level of functionally active protein kinase (C). On the other hand, the cells of the other phases [26] of cell cycle will possess protein kinase activity mostly in the form of the stored functionally inactive protein kinase complex (RC) because of the rate-limiting concentration of cyclic AMP and hence the rate of phosphorylation of proteins will be minimal in these cells despite the presence of high concentration of the cyclic AMP-dependent protein kinase complex.

The above postulation provides an explanation for the observed decrease in the activity of protein kinase during the early phase of the growth (Fig. 3, 0—8 h). The subsequent sharp increase in the specific activity of the protein kinase during the log to early stationary phase of growth can be attributed to the presence of much lower proportion of cells undergoing transition from G1 to S phase because of the appearance of a large population of cells of the other phases of cell cycle which are rich in the cyclic AMP-dependent inactive protein kinase complex. The above hypothesis also explains the observed high amount of phosphorylation of specific phosphoproteins during the lag phase and the subsequent rapid decrease in the phosphorylation of these proteins with the progress of growth cycle (Fig. 5). The possible presence of a short-lived catalytically active protein kinase (C) and a relatively stable cyclic AMP-dependent inactive protein kinase (RC) in intact cells of *Tetrahymena* is supported by the finding that the dissociated catalytic subunit of the enzyme is highly unstable in vitro as compared to the cyclic AMP-dependent protein kinase. The catalytic subunit lost approximately 70% of its activity during storage at 4°C for 20 h, whereas under the same conditions of storage the cyclic AMP-dependent protein kinase complex did not lose any significant activity.

The observation that the activity of cyclic AMP-dependent protein kinase is induced rapidly in *Tetrahymena* during the heat-shocks which are prerequisites for the induction of synchrony in these cells extends further support to the view that protein kinase which may be induced specifically in cells towards the end of G1 phase of cell cycle may play a pivotal regulatory role for the transition of cells from G1 to S phase of the cell cycle. It is, therefore, possible that during the normal growth cycle the activity of protein kinase is also induced in *Tetrahymena* during the lag phase of the growth although net increase in protein kinase activity was detectable only after 8 h of culture. One possible explanation for this observation is that protein kinase turns over rapidly in cells undergoing transition from G1 to S phase of the cell cycle as postulated above. The *Tetrahymena* protein kinase thus exhibits the property of a regulatory protein which mediates acute adaptation to environmental stimuli; the enzyme is induced rapidly, it is activated by cyclic AMP and the activated form undergoes rapid turnover.

According to the above hypothesis, the progress of the cell cycle is regulated by marked variations in the level of cyclic AMP which is integrated with switching off and on of the activity of cyclic AMP-dependent protein kinase. Previous observations showing that the level of cyclic AMP changes markedly during the cell cycle of mammalian cells in tissue culture [27,28] and the postulation that a decreased level of cyclic AMP at a specific point in the cell cycle, the mitotic phase [28], may trigger the cell cycle are in accordance with the above proposed role of cyclic AMP in the regulation of cell cycle. At present it is not known how this variation in the cyclic AMP level is controlled. The activities of cyclic nucleotide phosphodiesterase and adenylate cyclase show reciprocal changes during the growth cycle of *Tetrahymena* [7]. The controls of these enzymes and the functional role played by the phosphoproteins in the regulation of cell cycles require further study.

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References

1 Majumder, G.C. and Turkington, R.W. (1972) J. Biol. Chem. 247, 7207—7217
2 Kuo, J.F. and Greengard, P. (1969) Proc. Natl. Acad. Sci. U.S. 64, 1349—1355
3 Corbin, J.D., Reimann, E.M., Walsh, D.A. and Krebs, E.G. (1970) J. Biol. Chem. 245, 4849—4851
4 Huttuenen, J.K., Steinberg, D. and Mayer, S.E. (1970) Proc. Natl.,Acad. Sci. U.S. 67, 290—295
5 Laugan, T.A. (1969) J. Biol. Chem. 244, 5763—5765
6 Soderling, T.R., Hickenbottom, J.P., Reimann, E.M., Hunkeler, F.L., Walsh, D.A. and Krebs, E.G. (1970) J. Biol. Chem. 245, 6317—6328
7 Voichick, J., Elson, C., Granner, D. and Shrago, E. (1973) J. Bacteriol. 115, 68—72
8 Murofushi, H. (1973) Biochim. Biophys. Acta 327, 354—364
9 Shrago, E., Shug, A.L. and Ferguson, S.M. (1971) Int. J. Biochem. 2, 312—318
10 Scherbaum, O.H., James, T.W. and Yahn, T.L. (1959) J. Cell. Comp. Physiol. 53, 119—137
11 Majumder, G.C. and Turkington, R.W. (1971) J. Biol. Chem. 246, 2650—2657
12 Moldave, K. and Skogerson, L. (1967) Methods Enzymol. 12A, 478—481
13 Turkington, R.W. and Riddle, M. (1969) J. Biol. Chem. 244, 6040—6049

14 Chrambach, A. (1966) Anal. Biochem. 15, 544—548
15 Bray, G.A. (1960) Anal. Biochem. 1, 279—285
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
17 Schaffer, N.K., May, S.C. and Summerson, W.H. (1953) J. Biol. Chem. 202, 67—76
18 Johns, E.W. (1964)) Biochem. J. 92, 55—59
19 Reimann, E.M., Walsh, D.A. and Krebs, E.G. (1971) J. Biol. Chem. 246, 1986—1995.
20 Labrie, F., Lemaire, S. and Courte, C. (1971) J. Biol. Chem. 246, 7293—7302
21 Kumon, A., Yamamuya, H. and Nishizuka, Y. (1970) Biochem. Biophys. Res. Commun. 41, 1290—1297
22 Tao, M., Salas, M.L. and Lipmann, F. (1970) Proc. Natl. Acad. Sci. U.S. 67, 408—414
23 Erlichman, J., Hirsch, A.H. and Rosen, O.M. (1971) Proc. Natl. Acad. Sci. U.S. 68, 731—735
24 Gill, G.N. and Garren, L.D. (1971) Proc. Natl. Acad. Sci. U.S. 68, 786—790
25 Muller, M., Hogg, J.F. and deDuve, C. (1968) J. Biol. Chem. 243, 5385—5395
26 Prescot, D.M. (1968) Cancer Res. 28, 1815—1820
27 Otten, J., Johnson, G.S. and Pastan, I. (1971) Biochem. Biophys. Res. Commun. 44, 1192—1198
28 Burger, M.M., Bombik, B.M., Breckenridge, B.McL. and Sheppard, J.R. (1972) Nat. New Biol. 239, 161—163