ACTIVATION OF HISTONE KINASE IN G2 PHASE OF THE CELL CYCLE IN *PHYSARUM POLYCEPHALUM*

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1. Introduction

Phosphorylation of histone H1 is one of the major chemical changes associated with growth in normal and cancer cells of eukaryotic organisms [1,2]. The mycomycete *Physarum polycephalum* is an excellent model system for studying cell cycle events and it has been used to establish the time course of histone phosphorylation in the cell cycle [1]. In mid G2 phase nuclear histone kinase activity rises sharply, preceding an equally sharp rise in H1 histone phosphate content in prophase which may initiate chromosome condensation and hence mitosis [3]. Dephosphorylation of H1 occurs late in mitosis. These changes are regarded as an important part of the sequences of events which determine the progress of the nucleus through G2 phase [3–5]. Recently, studies with synchronised CHO cells have shown that the main changes in H1 phosphate content also occur in these mammalian cells [6]. The growth-associated histone kinase, kinase-HKG, has been extracted from *Physarum* nuclei and partly purified and characterised ([7], unpublished data). A similar enzyme, that apparently crossreacts immunologically with the *Physarum* kinase-HKG ([8], unpublished data), has been isolated from Ehrlich ascites cells and purified to homogeneity [9,10]. The Ehrlich ascites kinase-HKG phosphorylates H1 in vitro at sites including threonine-16, -136, -153 and serine-180 which are the sites phosphorylated in vivo during growth [11]. The magnitude of the increase in kinase-HKG activity in the *Physarum* growth cycle is 15-fold [3]. The question arises whether this increase is due to synthesis of new enzyme or activation of pre-existing enzyme. ('Activation' is used to include transport of kinase-HKG from cytoplasm to nucleus if transport occurs.) We have answered this question using the techniques of deuterium labelling and isopycnic centrifugation in metrizamide gradients [12] and found that kinase-HKG is activated.

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2. Materials and methods

Plasmodia were grown in the semi-defined medium described in [13] containing peptone, glucose, yeast extract, salts and haematin. For deuterium labelling the peptone was omitted and 10 $g.l^{-1}$ deuterated amino acid mixture (Prochem Ltd.) was added. For starvation, medium containing neither tryptone nor deuterated amino acids was used.

Nuclei were prepared by a modification of the method in [14]. Plasmodia were rinsed in 100 ml ice cold 1 mM EGTA, pH 7, to remove growth medium, drained and scraped into a Waring blender where they were homogenised in 5 mM $MgCl_2$, 10 mM Tris–HCl pH 7.4, 0.1% Triton X-100, 0.25 M sucrose at 70 V for 30 s. The homogenate was centrifuged (5 min at 70 × *g*), filtered through a milk filter and centrifuged again (10 min at 1300 × *g*). The resulting supernatant was completely removed and the nuclear pellet washed once with 0.5 M NaCl, 1 mM EGTA, 10 mM Tris–DCl, pH 7.5, and resuspended in the same solution. The nuclear suspension was sonicated (30 s at amplitude 16 μm) and then centrifuged (30 min at 10 000 × *g*). The supernatant contained the kinase-HKG activity.

The metrizamide gradient was prepared as 5 equal steps of metrizamide concentration from 40% w/w to 20% w/w in D_2O in a 5 ml centrifuge tube. The enzyme extract was incorporated into the 20% step. The gradient also contained 0.5 M NaCl, 1 mM DTT, 1 mM EGTA, 10 mM Tris–DCl pH 7.5. Centrifugation was in the MSE 3 X 5 ml swinging bucket rotor (21 h at 300 000 X *g*) at 5°C. The gradient was fractionated by pumping out from the bottom. Densities were determined from refractive index measurements as in [12] corrected for the presence of 0.5 M NaCl. A sample of 50 μl from each fraction was incubated in final vol. 250 μl containing 1 mg/ml calf thymus histone H1, 16 mM $MgCl_2$, 1 mM [γ-^{32}P]-ATP(50–150 Ci/mol), 80 mM Tris, pH 7.5 [7]. After incubation at 30°C for 1 h or 2 h the reaction was stopped by the addition of 50% trichloroacetic acid to give final conc. 10% trichloroacetic acid. The mixture was mixed and centrifuged and 250 μl supernatant spotted onto washed P81 phosphocellulose paper. The spots were washed 6 times in 5 mM sodium pyrophosphate, 2 times in water and 2 times in acetone before drying and counting [15].

3. Results and discussion

The normal *Physarum* growth medium was modified by replacing the peptone with a deuterated amino acid mixture. *Physarum* will not grow continuously on this medium but in experiments with synchronous plasmodia we found that plasmodia transferred at mitosis-2 (the second mitosis after fusion) from normal to deuterated medium grew with the normal cycle time of 9 h at least to mitosis-3. We conclude that the first cycle of growth after transfer is not affected by the presence of deuterated amino acids although they do appear to be incorporated, as shown below.

The normal banding pattern of kinase-HKG activity was established by harvesting normal plasmodia 6.5 h after mitosis-2, at the peak of kinase-HKG activity [3,7], and preparing the nuclear extract containing kinase-HKG. The extract was loaded on a metrizamide–D_2O gradient and centrifuged for 18 h, 21 h or 40 h [12]. In each case a smooth density gradient was formed containing 3 bands of kinase activity, similar to the solid line in fig.1. The positions and relative sizes of the bands were similar in all 3 gradients although a very slight increase in apparent density of all 3 bands was observed in the 40 h gradient. We conclude that the kinase(s) have banded at their approximate isopycnic positions after 18 h centrifugation. In subsequent experiments 21 h centrifugation was used.

Table 1 shows the mean densities observed for the 3 bands in 8 separate experiments with normal plasmodia. The standard deviations of these means show the high degree of reproducibility of density as expected for isopycnic centrifugation. Small variations in the relative sizes of the bands were observed from experiment to experiment.

Table 1
Densities of kinase-HKG bands

Band no.	Density (g/ml) of kinase-HKG from plasmodia transferred to		Density difference
	Normal medium	Deuterated medium	
1	1.2638 ± 0.0015	1.2638 ± 0.0003	0.0046
1[a]	–	1.2684 ± 0.0009	
2	1.2730 ± 0.0003	1.2730 ± 0.0010	0.0046
2[a]	–	1.2776 ± 0.0009	
3	1.2840 ± 0.0009	1.2849 ± 0.0013	0.0043
3[a]	–	1.2886 ± 0.0012	

[a] Band containing deuterated amino acids

Values given as mean ± SEM; 8 experiments with normal medium; 6 experiments with deuterated medium

Physarum kinase-HKG can be separated into 3 components by ion-exchange chromatography or gel filtration in 0.5 M NaCl. At least 2 of these components can be distinguished by their substrate specificity since one phosphorylates protamine 5-times more effectively than H1 whereas the other is specific for H1 [7]. A gradient similar to the solid line in fig.1 was assayed with H1 and with protamine substrate. All 3 bands were highly active with protamine substrate and the ratio 'activity towards H1: activity towards protamine' was constant across the gradient. We concluded, to our surprise, that the 3 bands appeared to be identical with respect to the presence of different kinase-HKG activities. The point was checked by separating a preparation of kinase-HKG from microplasmodial nuclei by DEAE-cellulose chromatography into the run through peak and peaks A and B eluting at 0.04 M NaCl and 0.15 M NaCl, respectively. The peaks were pooled separately, precipitated with ammonium sulphate, and applied to separate metrizamide gradients. After centrifugation each gradient showed a similar profile, namely one major band at a density of 1.264 $g.ml^{-1}$ similar to the density of the major bands observed in gradients of unfractionated kinase-HKG. Clear bands at higher densities were not observed, either due to lack of sensitivity or due to the fact that the protein concentrations in the gradients of fractionated enzyme were relatively low. We conclude that the band at density 1.264 $g.ml^{-1}$ includes all kinase-HKG enzymes and that the bands at densities 1.273 $g.ml^{-1}$ and 1.284 $g.ml^{-1}$ probably also contain all kinase-HKG enzymes. The origin of the two bands at higher densities has not been definitely established but, following [16–18] we consider that they arise from different levels of hydration of the kinase-HKG, perhaps associated with aggregation. For the purpose of the present experiment it is sufficient to establish a reproducible isopycnic banding pattern for normal enyzme and this has been achieved.

Plasmodia were grown in normal medium to mitosis-2 and then transferred to deuterated medium. After 6.5 h they were harvested and the kinase-HKG analysed on metrizamide density gradients as before. Control plasmodia were transferred from normal medium to normal medium and harvested at the same times. Kinase-HKG from control plasmodia gave 3 bands as in the example shown in fig.1, solid line.

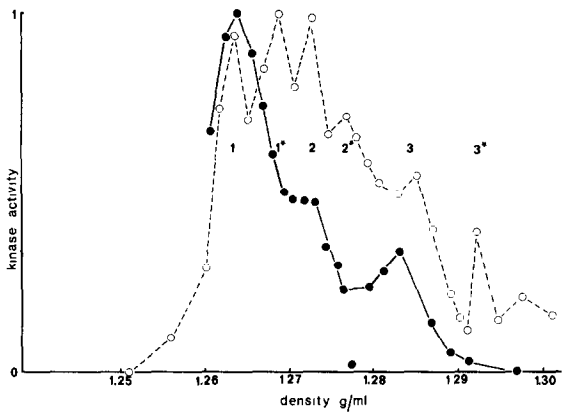

Fig.1. Density gradient centrifugation of kinase-HKG. Kinase-HKG activity as a fraction of maximum value for each gradient as a function of density determined for each fraction on the gradient from refractive index measurements. (—•—) Kinase-HKG from control plasmodia transferred from normal medium to normal medium at mitosis-2 and harvested 6.5 h later; (- -○- -) kinase-HKG from plasmodia transferred to deuterated medium at mitosis-2 and harvested 6.5 h later.

Kinase-HKG from plasmodia transferred to deuterated medium gave 6 bands as in the example shown in fig.1, broken line. Three of these bands coincided with normal kinase-HKG bands and represent kinase-HKG containing only normal amino acids. The other 3 bands were displaced ~ 4.5 $mg.ml^{-1}$ from each of the 3 normal bands. These new bands at higher densities must represent enzyme containing deuterated amino acids. Table 1 shows the mean densities obtained in 6 experiments. The presence of specific bands at higher densities argues that the amino acid pools in the plasmodium equilibrated rapidly relative to the 6.5 h labelling period since slow equilibration would have given band broadening rather than the double bands that were observed. The effect of inserting a starvation period of 30 min was checked and no change in the results was observed. We have found in other types of experiment that starvation periods used elsewhere are unnecessary under our conditions. We conclude that the enzyme activity in the new, higher density, bands represents enzyme synthesised during the deuterium labelling period, mitosis-2 to mitosis-2 plus 6.5 h. Each of the new bands is approximately equal in size to its normal density partner (fig.1) so the amount of enzyme has doubled, approximately

in parallel with total cell mass. However, during the deuterium labelling period the kinase-HKG activity increased 15-fold [3]. Therefore, most of this increase is due to activation (or transport) of inactive kinase-HKG molecules. Further work is needed to determine the mechanism of activation.

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References

[1] Bradbury, E. M., Inglis, R. J., Matthews, H. R. and Sarner, N. (1973) Eur. J. Biochem. 33, 131–139.
[2] Balhorn, R., Balhorn, M., Morris, H. P. and Chalkley, R. (1972) Cancer Res. 32, 1775–1780.
[3] Bradbury, E. M., Inglis, R. J. and Matthews, H. R. (1974) Nature 247, 257–261.
[4] Bradbury, E. M., Inglis, R. J., Matthews, H. R. and Langan, T. A. (1974) Nature 249, 553–556.
[5] Inglis, R. J., Langan, T. A., Matthews, H. R., Hardie, D. G. and Bradbury, E. M. (1976) Exp. Cell Res. 97, 418–425.
[6] Gurley, L. R., D'Anna, J. A., Barham, S. S., Deaven, L. L. and Tobey, R. A. (1978) Eur. J. Biochem. 84, 1–15.
[7] Hardie, D. G., Matthews, H. R. and Bradbury, E. M. (1976) Eur. J. Biochem. 66, 37–42.
[8] Zanker, K., Inglis, R. J., Matthews, H. R. and Bradbury E. M. (1977) Trans. Biochem. Soc. 5, 953–956.
[9] Langan, T. A. (1977) Methods Cell Biol. 18, 143–152.
[10] Schlepper, J. and Knippers, R. (1975) Eur. J. Biochem. 60, 209–220.
[11] Langan, T. A. (1977) Methods Cell Biol. 18, 127–142.
[12] Hutterman, A. (1975) in: Biological Separations (Rickwood, D. ed) Information Retrieval Ltd.
[13] Daniel, J. W. and Baldwin, H. H. (1964) Methods Cell Physiol. 1, 9–54.
[14] Mohberg, J. and Rusch, H. P. (1971) Exp. Cell Res. 66, 306–316.
[15] Witt, J. J. and Roshoski, R. (1975) Anal. Biochem. 66, 253–258.
[16] Hütterman, A. and Gunterman, U. (1975) Anal. Biochem. 64, 360–366.
[17] Hütterman, A. and Wendlberger-Schieweg, G. (1976) Biochim. Biophys. Acta 453, 176–184.
[18] Hütterman, A., Gebauer, M., Wessel, I. and Hoffman, W. (1975) Biochim. Biophys. Acta 384, 493–497.