Volume 239, number 1, 151–154 FEB 06426 October 1988

Phosphohistidine is found in basic nuclear proteins of *Physarum polycephalum*

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Received 19 July 1988

Nuclear extracts of the true slime mold, *Physarum polycephalum*, show protein histidine kinase activity towards exogenous histones [(1985) J. Biol. Chem. 260, 16106–16113]. *Physarum* microplasmodia were labeled with [^{32}P]phosphate in vivo and two basic proteins containing alkali-stable phosphate were detected. The labeled proteins comigrated with *Physarum* histones H1 (approximately) and H2A and phosphoamino acid analysis showed that each protein contained [^{32}P]-phosphohistidine. The H2A-like protein was also labeled in isolated nuclei incubated with [^{35}S]thio-ATP. We conclude that some *Physarum* nuclear proteins contain phosphohistidine.

Protein kinase; Phosphohistidine; Alkali-stable protein phosphorylation; Histone; (*Physarum polycephalum*)

1. INTRODUCTION

Protein histidine kinase has recently been described in the true slime mold, *Physarum polycephalum* [1]. The question arose whether nuclear protein histidine phosphorylation occurs in vivo or whether the observed in vitro nuclear protein histidine kinase activity is an artefactual reaction of an enzyme with a different activity in nuclei.

2. MATERIALS AND METHODS

Physarum plasmodia were grown and labeled as described [2,3]. A 40% GuCl extract of *Physarum* nuclei [3,4] was prepared and added to 10 ml of hydroxyapatite resin (2.2 g) (Biogel HTP, BioRad), which had been equilibrated with 50 mM potassium phosphate, pH 8.0. This step removes all the nucleic acid that can be detected spectrophotometrically [5]. Basic nuclear proteins were purified from the hydroxyapatite supernatant using BioRex-70 ion-exchange resin [3] and subjected to gel electrophoresis or fractionated by chromatography on a Superose-12 column (10 mm × 30 cm) eluted with 40% GuCl, 50 mM potassium phosphate, pH 8.0, 10 mM dithiothreitol at 0.5 ml/min. SDS gel electrophoresis of the labeled crude nuclear proteins was modified so that fixing, staining, autoradiography and fluorography were done under neutral or alkaline conditions [5,6].

Protein samples were digested with KOH and analyzed by ion-exchange chromatography [1,6,7]. Recently, a fast ion-exchange method has been used, involving a MonoQ column (Pharmacia) eluted with a linear 20 ml gradient of 0.10–1.4 M *N*-methyldiethanolamine, pH 9.0 [6].

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Abbreviations: thioATP, adenosine-5′-thiotriphosphate; GuCl, guanidinium hydrochloride

3. RESULTS

Physarum microplasmodia were labeled with [^{32}P]phosphate in vivo and basic nuclear proteins were extracted and analyzed by polyacrylamide gel electrophoresis. Several labeled bands were observed, two of which were stable to mild alkaline hydrolysis. One alkali-stable band comigrated with *Physarum* histone H2A and the other migrated slightly faster than *Physarum* histone H1 (fig.1). The band that migrates close to histone H1 may not be H1 since the major H1 variants are degraded by the conditions used to remove serine and threonine phosphates [1]. Nevertheless, the data do not rule out the possibility that this band may

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00145793/88/$3.50

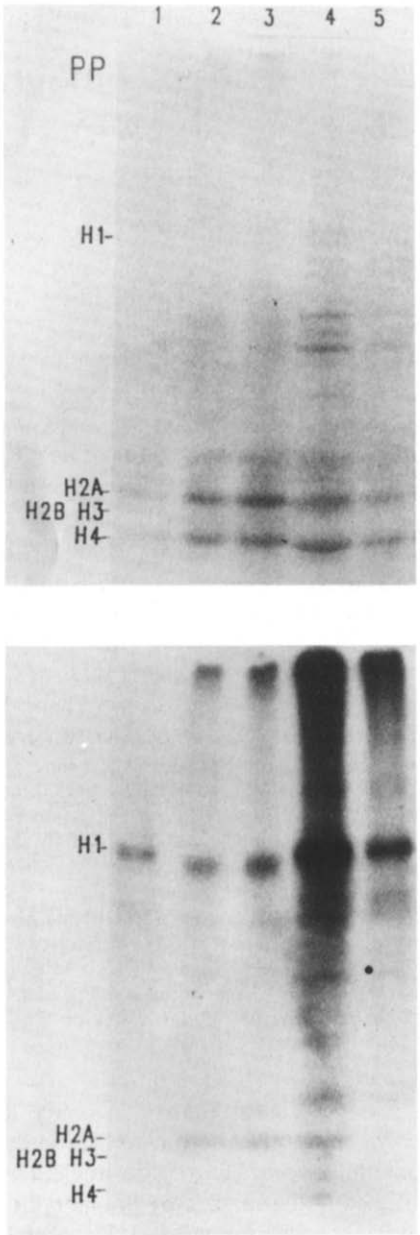

Fig.1. Alkali-stable phosphorylation of nuclear basic proteins in vivo. (Upper panel) Coomassie blue stain. (Lower panel) Autoradiograph. Lanes: 1, 40 μg of sample pretreated with 0.5 M HCl (60°C, 30 min); 2,3, 40 μg and 80 μg, respectively, pre-treated with 0.5 M KOH (60°C, 30 min); 4,5, 200 μg and 40 μg, respectively, without pretreatment. H1, H2A, H2B, H3 and H4, positions of *Physarum* histones. *Physarum* H2B and H3 show a low recovery in the high pH protocol used for this experiment.

be a minor, alkali-stable, H1 variant. Similarly, the data do not rule out the possibility that one band may be a proteolytic product of the other, although there is no indication of enzymatic proteolysis in these preparations. The hydrolysis conditions are sufficient to remove the serine phosphorylation generated in histone H4 by the catalytic subunit of cyclic AMP-dependent protein kinase [8]. However, the stability of serine or threonine phosphate is dependent on the local sequence surrounding the phosphoamino acid [9], and phosphotyrosine is resistant to alkaline

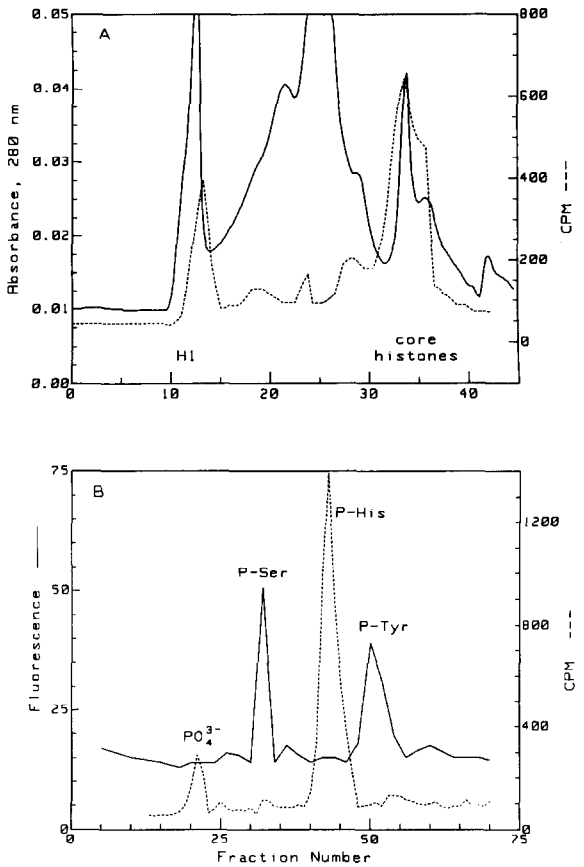

Fig.2. Preparative fractionation and phosphoamino acid analysis of proteins labeled in vivo. (A) Solid line, absorbance at 280 nm; broken line, radioactivity; 'H1' and 'core histones', positions of histone standards. (B) Phosphoamino acid chromatography (Dowex 1 × 8-50). Data are shown for the fraction that eluted in the core histone region of the Superose column. Broken line, radioactivity; solid line, fluorescence of standards. The order of elution is different from that found with the Mono Q column (fig.3).

hydrolysis. Hence, alkali stability is necessary but not sufficient to show the presence of phosphohistidine.

A similar preparation of labeled basic nuclear proteins was separated by gel filtration (fig.2A), giving two major labeled peaks, one near the position of histone H1 and one in the core histone position. Phosphoamino acid analysis showed that each peak contained phosphohistidine (fig.2B,C). Hence, *Physarum* nuclei contain at least two proteins with phosphohistidine residues. Phosphotyrosine was not detected.

Phosphoamino acid analysis of the proteins labeled with [^{35}S]thiophosphate [12] in isolated nuclei showed that most of the label was in a single peak that shows extreme alkali-stability, acid-lability and elution on ion-exchange chromatography close to the position of 1-phosphohistidine. We conclude that the major labeled amino acid is thio-phosphohistidine. The remainder of the radioactivity co-eluted with phosphotyrosine and was acid stable. Phospholysine almost co-elutes with phosphotyrosine in this system but would be acid-labile. Hence, some tyrosine thio-phosphorylation may occur in these isolated nuclei. This experiment shows that the protein histidine kinase will use thio-ATP as a phosphate donor. Gel electrophoresis (not shown) showed that the major band labeled in nuclei comigrated with histone H2A. Label was absent from the positions of H1 or H4.

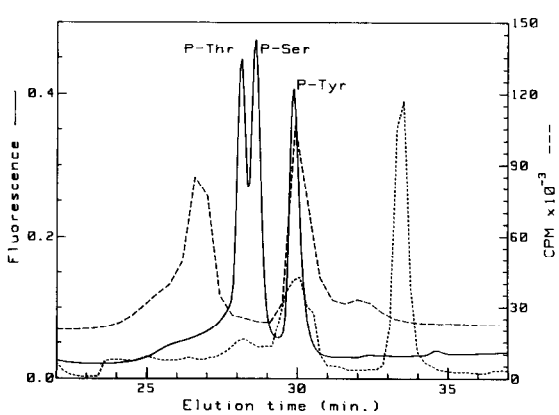

Fig.3. Phosphoamino acid analysis of ^{35}S-labeled proteins on a MonoQ column. (Solid line) Fluorescence of standards. (Dotted lines) Radioactivity (lower, alkali-stable phosphoamino acids; upper, alkali- and acid-stable phosphoamino acids displaced upwards for clarity).

4. DISCUSSION

Phosphohistidine in nuclear proteins was first reported by Smith's group in the mid 1970s, in rat tissue and cultured cells [10]. We have extended this result to a lower eukaryote, *P. polycephalum*. In rats the major protein containing phosphohistidine was identified as histone H4 unlike *Physarum* macro- and microplasmodia where the major amounts of phosphohistidine are seen in bands that comigrate with histone H2A and histone H1 (approximately). Phosphorylation of the rat H4 occurred long after its synthesis [10]. There are minor differences between rat and *Physarum* histone H4 in the region of the histidine phosphorylation site (Lys-77 $\longrightarrow$ Arg; partial methylation of Lys-77) [11]. Neither the rat nor the *Physarum* kinase has been fully characterized, but both phosphorylate histones including histone H4 in vitro. The *Physarum* enzyme does not phosphorylate histone H4 in nucleosome core particles [8] nor does a histidine kinase from yeast (*S. cerevisiae*) [8]; the rat enzyme has not been tested on nucleosome core particles. The masking of the histone H4 phosphorylation site in chromatin suggests either: (i) that histidine phosphorylation is associated with chromatin replication (when 'old' H4 is transiently separated from DNA) in rats but not in *Physarum*; or (ii) that the protein identified as H4 in the rat system may actually be an H4-like non-histone protein, possibly analogous to the H2A-like protein in the *Physarum* system. Further work is needed in both systems to identify the in vivo substrates unequivocally.

The results support the notion that nuclear protein histidine kinase is active in phosphorylating histidine in nuclear proteins in vivo. The function of histidine phosphorylation is unknown, but we have suggested previously that it may be used to modulate the affinity of DNA-binding proteins for DNA [8].

Acknowledgements: We thank Mr C. Holman for the use of his data on phospholysine and Mr J. Morgan and other colleagues for their comments on the manuscript. This research was supported in part by a grant (NP 431) from the American Cancer Society.

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