Phosphorylation of Histidine in Proteins by a Nuclear Extract of Physarum polycephalum Plasmodia*

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A high salt nuclear extract from the true slime mold Physarum polycephalum was used as a source of kinase activity for the incubation of calf thymus histones with [γ-32P]ATP. A major proportion of the 32P incorporated into histones was acid-labile and alkali-stable. The nature of the alkali-stable phosphorylated component was analyzed by subjecting the phosphorylated protein to total alkaline hydrolysis and separating the resultant phosphoamino acids by anion exchange chromatography. The 32P-labeled material co-chromatographed with phosphohistidine standards and did not co-chromatograph with phosphoserine, phosphothreonine, or phosphotyrosine standards. In similar experiments using reversed phase high-performance liquid chromatography to separate the phosphoamino acids, the 32P-labeled phosphoamino acid behaved like the L-isomer of phosphohistidine, in not being retained by the column, and unlike 3-phosphohistidine, phosphoserine, phosphothreonine, phosphotyrosine, and phosphoarginine, which were all retained on the column. Histone H4 was a good substrate for the histidine kinase activity and the location of the phosphorylated histidine residue was probed by peptide mapping using chymotrypsin or VS protease. Both maps were consistent with labeling of histidine 75 and inconsistent with labeling of histidine 18. The data show that Physarum nuclei contain a major kinase activity which produces phosphohistidine. The methods we have developed for studying this kinase activity provide the basis for a complete characterization of the structure and function of the Physarum enzyme and can be applied to the study of similar kinase activities in other systems.

Phosphorylation of serine, threonine, or tyrosine residues in proteins is an important regulatory mechanism. Phosphorylation of histidine and other basic amino acid residues has the potential to be of similar importance, but has been studied to a much lesser extent, due to the acid lability of the phosphoramidate linkage (reviewed by Matthews and Huebner, 1984). Since most methods of protein purification, analysis, and kinase assay employ acidic pH, which will completely hydrolyze the phosphoramidate linkages (Hulquist et al., 1966; Hulquist, 1968), different methods must be used for the analysis of these types of phosphoamino acids. We have extended the range of methods available and describe them and their application to the finding of a histidine kinase in Physarum polycephalum nuclei.

Phosphohistidine occurs in various prokaryotic and eukaryotic systems. Its presence was first described by Boyer and co-workers (Boyer et al., 1962), who isolated [32P]phosphohistidine from bovine liver mitochondria which had been incubated with [32P]phosphate (DeLuca et al., 1963). The mitochondrial phosphohistidine was identified on the basis of its acid lability and its co-migration with synthetic phosphohistidine on an ion exchange column, paper chromatography, and paper electrophoresis. Various chemical tests eliminated the possibility of the phosphate being a quinol phosphate, an acyl phosphate or a sulfur phosphate. The Pauly test for histidine showed positive results only after hydrolysis with acid. Phosphohistidine was later located in the enzyme succinyl-CoA synthetase from liver mitochondria (Mitchell et al., 1964). Zetterqvist and co-workers found significant amounts of phosphohistidine in various other cellular compartments (Zetterqvist and Engstrom, 1966), and identified nucleoside diphosphate kinase from bovine liver and erythrocytes as a protein containing both the 1- and 3-isomers of phosphohistidine as well as phosphoserine (Zetterqvist, 1967; Wålander et al., 1968; Wålander, 1968). Phosphohistidine occurs as an intermediate in the phosphoglycerate mutase reaction in rabbit skeletal muscle (Rose, 1970; Rose et al., 1975). Bovine adrenocortical cyclic AMP-independent protein kinase can autophosphorylate at a histidine residue (Kuroda and Sharma, 1982; 1983). In Escherichia coli and Staphylococcus typhimurium both the 1- and 3-isomers of phosphohistidine have been implicated as intermediates in the phosphoenolpyruvate sugar phosphotransferase system (Anderson et al., 1971; Weigel et al., 1982).

Reports by R. A. Smith's group of the presence of two histidine kinase activities in the nucleus of Walker-256 carcinoma and other cells and tissues led us to initiate the studies described here. The two kinase activities observed had pH optima of 9.5 and 6.5. Histone H1 appeared to be the best substrate for the pH 6.5 kinase activity and histone H4 for the pH 9.5 kinase activity (Smith et al., 1973). These kinases were separated and partially characterized (Smith et al., 1974). A similar kinase in regenerating rat liver increased in activity during the period of DNA synthesis following partial hepatectomy (Chen et al., 1974). Regenerating rat liver was labeled in vivo with [32P]phosphate and acid-labile radioactive activity was found on histones H1 and H4 and on acidic nuclear proteins. Proteolytic digestion and paper chromatography of the resulting phosphoamino acids were interpreted as identifying the phosphoamino acid in H1 as phosphohistidine and in H4 as phosphohistidine. Phosphorus-31 nuclear magnetic resonance studies confirmed the presence of 3-phosphohistidine in H4 histone that had been incubated with ATP and unpurified kinase from Walker-256 carcinoma cells (Fujitaki et al., 1981).

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Phosphorylation of Calf Thymus Histone H4—

Calf thymus histone H4 was purified from commercial calf thymus histones (Sigma IIS) by chromatography on Sephadex G-75 superfine (1.5 × 100 cm column eluted with 5% acetic acid at room temperature). The reaction mixture and incubation were as described above, except for a larger volume of 3–5 mL. The reaction was stopped by mixing the reaction mixture with an equal volume of cold 0.5 M sodium phosphate, pH 8.0, and then treated with 20% trichloroacetic acid for 30 min. The sample was dialyzed against 20 mM sodium phosphate, pH 8.0, and then against 20 mM ammonium bicarbonate and lyophilized.

Base Hydrolysis and Chromatography of Phosphoamino Acids—Lysophosphorylated protein was taken up in 3 N KOH and incubated at 105°C for 4 h. The hydrolysate was diluted 100-fold, loaded onto a 1 × 30 cm column of AG 1-X8 (Bio-Rad) in the KClO₄ form, and eluted with a linear gradient of KClO₄ (400 mL for a 0–1 M gradient or 180 mL for a 0.2–0.5 M gradient). Phosphoserine, phosphothreonine, and phosphotyrosine standards were obtained from Sigma and histidine was obtained from Kodak. They were detected by reaction with fluorescamine (Lai, 1977). A phosphohistidine standard was synthesized from the potassium salt of phosphomonoamide and [³²P]histidine as described by Hultquist et al. (1966) using phosphomonoamide synthesized from POCl₃ as described by Sheridan et al. (1971). Acid treatment of fractions from the column was accomplished by lyophilizing the sample, dissolving it in 50% acetic acid and incubating at room temperature for 4 h. The sample was diluted 35-fold before rechromatography.

Separation of phosphoamino acids was carried out by high-performance liquid chromatography on a reverse phase column (Hamilton PRP-1). The apparatus included two pumps (Waters model 600A and Waters model M-49), a Waters system controller, a UK-6 injector and fluorimeter (LDC/Milton Roy Fluoromonitor III). The elution buffers and orthophthalaldehyde derivatization methods described by Fleury and Ashley (1983) were used. The sample was derivatized by mixing it with an equal volume of derivatizing solution made fresh daily by mixing 10 mg of orthophthaldehyde, 14.9 mL of methanol, 5.3 mL of saturated boric acid, pH 9.5, and 25 μL of ethanol and loaded onto the column 3 min later. The column was eluted isocratically at 1.0 mL/min with 14.3 mM sodium phosphate, pH 7.2, 1.1% tetrahydrofuran, 6.6% acetonitrile (Carlomago et al., 1986). For the identification of the phosphohistidine isoforms, the elution program was modified. Two elution buffers, 25 mM triethylammonium phosphate, pH 7.2, 1.25% tetrahydrofuran (buffer A) and 25 mM triethylammonium phosphate, pH 7.2, 75% acetonitrile (buffer B), were used with an elution program of 95% buffer A, 5% buffer B for 10 min followed by a gradient to 30% buffer A, 70% buffer B over a further 10 min.

Proteolytic Digestion and Peptide Separation—Histone H4 was phosphorylated by the nuclear extract as described above and digested with chymotrypsin that had been pretreated with a-p-tosyl-L-lysine chloromethyl ketone. The reaction mixture contained 1 mg/ml histone H4, 0.5% sodium chloride, 25 mM phosphate buffer, pH 8.0, and it was incubated at room temperature for 30 min. The reaction was stopped by the addition of a molar excess of N-tosyl-L-phenylalanine chloromethyl ketone. The digest was diluted 5-fold and urea was added to a final concentration of 6 M. The solution was loaded onto a 1 × 20 cm column of Cm-cellulose and eluted with a linear

1 The abbreviation used is: SDS, sodium dodecyl sulfate.
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**RESULTS**

Initial Screening for Histidine Kinase—Physarum nuclei were extracted with high salt, and enzyme was precipitated with ammonium sulfate as described under "Methods" and the extract was incubated with [γ-32P]ATP and calf thymus core histones. After incubation, the extract was divided into two aliquots and treated with either 0.3 M NaOH, 0.1% SDS (basic treatment), or 10 mM Tris, 0.1% SDS (neutral treatment), and incubated at 60°C for 10 min. The aliquots were analyzed by SDS-gel electrophoresis. A parallel experiment was carried out using the catalytic subunit of cyclic AMP-dependent protein kinase in place of the nuclear extract in order to provide a control for the efficiency of the base treatment in removing phosphate from serine and threonine residues. Fig. 1 shows scans of autoradiographs of the gels. About half (50-60%) of the phosphate incorporated into histones by the nuclear extract was alkali-stable and 10% was alkali-stable. Protein staining of the gels showed that the difference in phosphate label was not due to differences in protein loading, and that the alkali treatment had not affected the proteins to any significant extent, except that the small amount of histone H1 present was destroyed. The incubation was carried out at two different pH values, 7.0 and 9.5, to determine whether there was any analogy with the mammalian system, which contains two kinases with different pH optima and substrate specificities (Smith et al., 1973). Comparison of phosphate incorporation at these two pH values shows that there is a difference in substrate specificity for alkali-stable phosphorylation of histones. While histone H4 is the best core histone substrate at pH 7.0, the histones H2A and H2B are the best core histone substrates at pH 9.5.

In a separate experiment, purified calf thymus histone H4 was phosphorylated with the extract at pH 7.0 and assayed by subjecting it to an acid treatment as well as a neutral and base treatment. The amount of phosphate incorporated into histone H4 was quantitated by scanning the autoradiographs and integrating the peaks obtained (Matthews, 1984). Relative to the neutral treatment, 64% of the phosphate incorporated was acid-labile and 50% was alkali-labile. The phosphorylated H4 thus contains both alkali-labile and acid-labile phosphates. The experiment suggests, but does not show, that the alkali stable phosphate is acid-labile and vice versa. Losses in H4 could account for the minor proportion (14%) of the phosphate that is apparently both acid- and alkali-labile, although Coomassie staining showed that major losses of H4 protein did not occur.

Earlier experiments using whole nuclei as the enzyme source did not produce significant amounts of alkali-stable phosphorylation although some alkali-labile phosphorylation occurred. It therefore appears that histidine kinase must be released from the nucleus before it can interact with the exogenous substrate.

A number of other methods for determining acid-labile
phosphorylation was investigated including the use of ion exchange columns (Smith et al., 1974) and gel filtration columns to separate product and substrate. The most sensitive and convenient method used phosphocellulose filter papers, and Table I shows an example of the assay of histidine kinase in the Physarum nuclear extract using this method. The validity of the procedure in specifically detecting acid-labile phosphates is demonstrated by using the catalytic subunit of the cyclic AMP-dependent protein kinase as the source of kinase activity. After the differences in background were accounted for, the identical amount of phosphate incorporation was detected using the acidic and neutral wash procedures. When the nuclear extract from Physarum was used, however, 55% of the $^{32}$P incorporated was acid-labile, in reasonable agreement with the results from gel electrophoresis (64% acid-labile as noted above).

Assay of Alkali-stable Phosphorylation by an Extract from Beef Heart—In a survey experiment to determine whether a kinase activity such as was found in Physarum is also present in mammalian systems, we assayed the fractions from a DEAE-cellulose column upon which a beef heart extract had been loaded. This particular preparation contained both the cyclic AMP-dependent protein kinase and the Ca$^{2+}$-phospholipid-dependent protein kinase (Bechet et al., 1977). Fractions were assayed using the gel electrophoresis procedure. The efficiency of the alkali treatment in removing alkali-labile phosphates is confirmed by the neutral and alkali treated phosphorylated in vitro with the nuclear extract and $[^{32}$P]ATP and subjected to complete alkaline hydrolysis. The hydrolysate was analyzed by anion exchange chromatography on an AG 1-X8 column (Fig. 3). Marker amino acids, histidine, phosphoserine, phosphothreonine, and phosphotyrosine were included in the run. Two peaks of radioactivity were evident, one eluting very early in the gradient, the other eluting at approximately 0.6 mM KHCO$_3$. The first radioactive peak was determined to be inorganic phosphate after an inorganic phosphate standard was run on the same column under the same conditions. The second radioactive peak did not elute with any of the phosphomono acid standards, ruling out the possibility that the alkali-stable phosphorylation is due to phosphorylation of hydroxy groups of serine, threonine, or tyrosine. The position of this peak also did not correspond to the position reported for 1-phosphohistidine (Wilhelm et al., 1966). Fig. 3C shows that the synthesized $[^{14}$C]phosphohistidine co-chromatographs with the $[^{32}$P]phosphoamino acid from histone H4.

High-performance Liquid Chromatography of the $[^{32}$P] Phosphoamino Acid—The peak shapes for the chemically synthesized $[^{14}$C]phosphohistidine and the $[^{32}$P]phosphoamino acid were analyzed by high-performance liquid chromatography on a DEAE-cellulose column (Fig. 2). Marker amino acids, histidine, phosphoserine, phosphothreonine, and phosphotyrosine were included in the run. Two peaks of radioactivity were evident, one eluting very early in the gradient, the other eluting at approximately 0.6 mM KHCO$_3$. The first radioactive peak was determined to be inorganic phosphate after an inorganic phosphate standard was run on the same column under the same conditions. The second radioactive peak did not elute with any of the phosphomono acid standards, ruling out the possibility that the alkali-stable phosphorylation is due to phosphorylation of hydroxy groups of serine, threonine, or tyrosine. The position of this peak also did not correspond with the position reported for 1-phosphohistidine (Wilhelm et al., 1966). The material in this peak was treated with acid under conditions where hydroxyl phosphates are stable and then rerun on the AG 1-X8 column (Fig. 3B). The radioactivity was quantitatively converted to inorganic phosphate, showing that the radioactive material is both alkali-stable and acid-labile.

In order to investigate further whether this peak was actually phosphohistidine, we synthesized phosphohistidine using $[^{14}$C]histidine and phosphoramidate (Hultquist et al., 1966). Fig. 3C shows that the synthesized $[^{14}$C]phosphohistidine co-chromatographs with the $[^{32}$P]phosphoamino acid from histone H4.
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A polymer-based column was used, to allow the use of eluents with pH values at or above neutrality. Samples were derivatized with orthopthaladehyde and eluted as described under "Methods" (Fleury and Ashley, 1983; Carlomagno et al., 1985). Standard phosphoamino acids eluted from the column in this order: phosphoserine, phosphothreonine, phosphotyrosine, phosphohistidine (Fig. 4A). When a phosphohistidine standard was run, two peaks were observed, one which was not

![Graphs and charts showing chromatography results](image)

**FIG. 4.** Reversed phase high-performance liquid chromatography of phosphoamino acids. A, standards. Retention times were: 1-phosphohistidine, 2.4 min; phosphoserine, 5.7 min; 3-phosphohistidine, 8.1 min; phosphothreonine, 12.1 min; phosphotyrosine, 16.2 min; and phosphoarginine, 18.1 min. B, [32P]phosphoamino acid from AG 1-X8 chromatography of alkaline hydrolysate of phosphorylated histone H4. Fractions of 0.45 ml were collected and radioactivity was determined by Cerenkov counts.

**TABLE II**

Percentages of phosphohistidine isomers as a function of reaction times for the synthesis of phosphohistidine

<table>
<thead>
<tr>
<th>Reaction time</th>
<th>Peak Areas</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>1 2 3 4</td>
<td>0.0 0.0 1.6 98.4</td>
</tr>
<tr>
<td>60 min</td>
<td>15.9 2.7 33.4 48.0</td>
<td></td>
</tr>
<tr>
<td>5 h</td>
<td>37.2 3.3 46.9 12.7</td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>7.4 45.9 46.7</td>
<td></td>
</tr>
</tbody>
</table>

Assigned identities: Hist(1-P) His(1,3-diP) Hist(3-P) His.
physohistidine. The isomers were identified by performing a time course of the phosphohistidine synthesis reaction with samples taken at 0 time, 60 min, 6 h, and 5 days and analyzed by high-performance liquid chromatography. The relative amounts of the two phosphohistidine isomers are shown as a function of time in Table II. The two peaks were assigned on the basis of the rates of appearance and disappearance of product during the course of the reaction and the kinetics of the reaction as described by Hultquist and co-workers (Hultquist, 1968, Hultquist et al., 1986). The peak which was not retained by the column appeared rather early in the reaction, yet seemed to decrease in amount over time, corresponding to the 1-isomer of phosphohistidine. The retained peak appeared to be the predominant product after 3 days, and was therefore the 3-isomer, the more stable reaction product.

When the [32P]phosphoamino acid was analyzed by high-performance liquid chromatography the radioactivity was not retained (Fig. 4B). The possibility that the [32P]phosphoamino acid had been completely hydrolyzed to inorganic phosphate, which would also not be retained on the column, was ruled out, since rerunning the same sample on the AG 1-X8 column showed that no hydrolysis had occurred. Therefore the [32P]phosphoamino acid is 1-phosphohistidine.

The difference in peak shapes observed on the AG 1-X8 column can now be explained, since this preparation of synthesized phosphohistidine was a mixture of 1- and 3-phosphohistidine containing predominantly 3-phosphohistidine, and the [32P]phosphoamino acid is 1-phosphohistidine. Wältinder et al. (1968) showed that 1-phosphohistidine elutes slightly before 3-phosphohistidine on the AG 1-X8 column.

Analysis of Phosphorylated Peptides—Histone H4 contains 2 histidine residues (DeLange et al., 1969). Peptide analysis was used to determine the site(s) of phosphohistidine formation as follows. Calf thymus histone H4 was phosphorylated as described above, treated with dilute alkali to remove alkalilabile phosphorylation, and digested with either chymotrypsin or Staphylococcus V8 protease. The chymotryptic digest was fractionated by ion exchange chromatography on Cm-cellulose (Fig. 5). The radioactivity eluted in a single peak early in the chromatogram. Gel electrophoresis (not shown) revealed substantially a single peptide eluting at this point and amino acid analysis showed the presence of histidine. Analysis of the peptide by acid-urea gel electrophoresis resulted in complete loss of radioactivity, confirming the acid-lability of the phosphopeptide. The early elution position of the peptide and the absence of high lysine in the amino acid analysis showed that the peptide was not from the highly basic amino-terminal region of histone H4, where histidine 18 occurs. However, the sample was not pure enough to identify unambiguously the labeled peptide.

The phosphopeptides from the V8 digest eluted in the included volume of a Sephadex G-50 column. Further purification on a column of Sephadex G-25 showed the radioactivity to be present as a doublet (Fig. 6). Amino acid analysis of both peaks showed that the peptides from both peaks contained histidine and did not contain high amounts of basic residues characteristic of the amino terminus of histone H4. Again, the peptide obtained was not pure enough to give a perfect match of amino acid composition to that expected for the peptides. However, the composition obtained together with the sizes of the peptides deduced from elution from Sephadex G-25 and the predicted pattern of cleavage by V8 protease strongly implies that the peptides are residues 75–93 and 75–85. Hence, the phosphorylated residue is histidine 75. The data are inconsistent with phosphorylation of histidine 18, the only other histidine in histone H4. The purified V8 protease phosphopeptides were subjected to total base hydrolysis and the identity of the phosphoamino acid as phosphohistidine was confirmed by co-migration with a phosphohistidine standard on an AG 1-X8 column.

DISCUSSION

Although the presence of phosphohistidine in enzyme intermediates is well established, reports of the existence of a histidine kinase from Smith's group (e.g. Smith et al., 1973; Fujitaki et al., 1981) have not been confirmed. We have demonstrated the transfer of phosphate from [γ-32P]ATP to 1-phosphohistidine on histidine 75 of histone H4 by a nuclear extract, in vitro. The primary system of study is the true slime mold, P. polycephalum, so the results do not strictly confirm or deny previous reports of a histidine kinase in mammalian tissues. However, the results do show the presence of histidine kinase in Physarum and, taken together with earlier reports...
of a mammalian histidine kinase, suggest that such a kinase may be widespread, at least in eukaryotes. This appears to be confirmed by the preliminary results with beef heart, although in this case further characterization of the phosphoamino acid is necessary.

This paper has not addressed the question of whether phosphohistidine is found in vivo or whether histone H4 is the in vivo substrate of the histidine kinase activity, but preliminary results suggest that alkali-stable phosphorylation of histones does occur in Physarum in vivo (Huebner, 1985). Earlier work in this field in mammalian cells estimated that acid-labile phosphates are quantitatively equal to acid-stable phosphates in vivo (Zetterqvist and Engstrom, 1966; Chen et al., 1977), and a more recent report also found substantial acid labile protein phosphorylation in mammalian cells (Mattoo et al., 1984). These experiments measured phosphohistidine using the indirect method of assaying for acid-lability or base-stability. Neither of these techniques, however, is entirely definitive for the presence of phosphohistidine. We have developed a sensitive analytical technique to determine the phosphoamino acid composition of a protein using high-performance liquid chromatography (Fig. 4; Carlomagno et al., 1985) and will use this technique to probe directly for the presence of phosphohistidine in vivo. The new techniques are complementary to the use of NMR (Fujitaki et al., 1981) or high-performance ion exchange chromatography (Steiner et al., 1980) which are slower, more complex, and less sensitive than the procedures described here.

Physarum plasmodia grow on a medium at pH 4.6 but the internal pH is approximately 7.2 (Gerson and Burton, 1977; Morisawa and Steinhardt, 1982). At this pH, 1-phosphohistidine has a relatively short half-life (Wälinder et al., 1968; Wälinder 1968), although the microenvironment in a protein may extend it. It thus seems likely that the function of phosphohistidine may be in a rapidly turning over process. Such a rapid turnover is already familiar in the case of acetylsine in core histones (e.g. Jackson et al., 1975; Waterborg and Matthews, 1983, 1984). In Physarum, the transcript-associated acetylsines on histones H3 and H4 turn over with a half-life of about 3 min (Waterborg and Matthews, 1983).

Our peptide studies show that the modified histidine in histone H4 is histidine 75 and not histidine 18. This is in contrast to the observations by Bruegger (1976) who reported that both histidines appeared to be phosphorylated. The previously known post-synthetic modifications of Physarum H4 occur in the amino-terminal region (acetylation of lysines 5, 8, 12, and 16 and methylation of lysine 20) (Waterborg et al., 1983) as in mammalian cells. However, we recently discovered that Physarum histone H4 has methyllysine at residue 79 (Waterborg et al., 1983) and the sequence in this region is very hydrophilic, -Thr-Glu-His-Ala-Arg-Arg-Lys-Thr-. These facts suggest that histidine 75 is probably on the exterior of the globular portion of H4 and thus accessible to modifying enzymes. It is also possible that -Arg-Arg-Lys- sequence binds DNA in the nucleosome. If this is the case, then a phosphate group on histidine 75 could interact with the -Arg-Arg-Lys- and thus have a major effect on its interaction with DNA. This could serve to relax the nucleosome. This discussion serves to indicate the potential importance of phosphohistidine in histone H4, but more experimental data is required before we can deduce its function.

It is important to note that most methods of kinase assay and phosphoamino acid analysis utilize acidic pH, so that any phosphohistidine which may be present would be completely hydrolyzed. In the course of this project, we have been forced to develop alternate methods of analysis of phosphorylated proteins which may prove useful in other systems. These methods, particularly the SDS-gel analysis of the products of the kinase reaction, the phosphocellulose filter assay for kinase activity, and the high-performance liquid chromatography identification of phosphohistidine, provide the tools that were previously missing and which should now allow rapid progress in analyzing the role of phosphohistidine in the cell nucleus.

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