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EFFECTS OF POLYAMINES AND HISTONES ON THE PHOSPHORYLATION OF NON-HISTONE PROTEINS IN ISOLATED RAT LIVER NUCLEI*

Francoise Farron-Furstenthal and John R. Lightholder+

Molecular Biology Laboratory, The Salk Institute
Post Office Box 1809, San Diego, California 92112

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SUMMARY

Addition of polyamines to isolated nuclei increases the rate and extent of phosphate incorporation from ATP into non-histone proteins several-fold. Similar results are obtained when histones are added to phosphorylating nuclei or when nuclei are incubated with DNAase prior to the addition of ATP. Electrophoretic analysis of the reaction products in SDS polyacrylamide gels reveals that specific non-histone proteins are preferentially phosphorylated in the presence of polyamines, some of which appear to be the same as in the presence of histones or DNAase. Removal of protein-bound phosphate during prolonged incubation of nuclei occurs with the same kinetics in the presence or absence of polyamines. Our results suggest that polyamines and histones stimulate nuclear protein phosphorylation by rendering additional phosphate acceptors accessible to the kinases.

The cyclical phosphorylation and dephosphorylation of key proteins in the cytoplasm, in synaptosomes and in plasma membranes has emerged as a major regulatory mechanism whereby cells modulate the activity of metabolic pathways, membrane potentials, transport of nutrients, swiftly and reversibly.

The rapidity and the extent to which protein bound phosphate turnover in the nucleus responds to mitogens (1), hormones (2,3), and drugs (4) strongly suggest that phosphorylation-dephosphorylation of nuclear proteins is an important means whereby cells regulate nuclear functions. It seemed, therefore, of interest to examine what the proximal effectors of this process might be.

We have previously reported that partially purified nuclear protein kinases are stimulated 2- to 3.5-fold by physiological concentrations of the polyamines spermidine and spermine (5). In order to assess the possible physiological significance of this finding we examined the effect of polyamines on the kinetics, extent and pattern of non-histone phosphorylation in

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+ Present address: Tufts New England Medical Center, Division of Radiobiology, Boston, MA 02111.

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isolated rat liver nuclei.

Since a marked stimulation of a nuclear protein kinase preparation by histones had been reported (6) but could not always be obtained in this laboratory, we also re-examined the effect of histones on nuclear phosphorylation. In isolated nuclei we consistently observe a significant stimulation of the rate and extent of protein phosphorylation and a marked effect on the pattern of phosphorylated non-histone proteins by polyamines and by histones. The ability of histones to stimulate non-histone protein phosphorylation in nuclear extracts appears to depend upon the purity of the protein kinase.

MATERIALS AND METHODS

Male Buffalo rats 90 days or older were used throughout. Histones (Sigma fraction II A) and individual purified histone fractions H-1, H2A, H2B, and H3, polylysine and polyarginine, spermine and spermidine were obtained from Sigma Chemical Co. [γ-^{32}P]ATP was prepared according to Glynn and Chappell (7). Electrophoretically pure pancreatic DNAase I was obtained from Worthington. Nuclei were isolated by the method of Reeder (8). *In vivo* labeling was by injecting 5 mCi of ^{32}P intraperitoneally 2 hours before sacrifice. *In vitro* phosphorylation was carried out by incubating 250 µg nuclear protein in 50 mM Tris-acetate pH 7.5, 0.15 M NaCl, 10 mM Mg acetate, 0.3 mM EDTA and 5 µM [γ-^{32}]ATP (containing 10^6 cpm) in a total volume of 200 µl. Addition of histones or polyamines and preincubation with DNAase I were as described in the text. The reaction was stopped by addition of a 1000-fold excess of unlabeled ATP followed by 2 ml 25% ice-cold TCA. The samples were filtered on GF/A glass fiber filters, the filters dried and counted in toluene-Liquifluor by scintillation spectrometry. For gel electrophoresis samples were precipitated with 5% TCA, centrifuged and the pellets dissolved in sample buffer. Gel electrophoresis was carried out in 1 mm slab gels containing 10% polyacrylamide and 0.1% SDS in the buffer system described by Laemmli (9).

RESULTS AND DISCUSSION

When isolated rat liver nuclei are incubated under phosphorylating conditions, incorporation of phosphate from γ-labeled ATP into TCA precipitable material proceeds for no more than 10 to 15 min (10). Parameters such as NaCl or sucrose concentrations, or addition of sulfhydryl compounds, do not materially alter the kinetics of phosphate incorporation into protein (11,12). However, when the polyamines spermine and spermidine or histones are added at 200 µg per 250 µg nuclear protein, phosphate incorporation proceeds for at least 90 min to yield a 3- to 3.5-fold total phosphate incorporation into nuclear proteins. Similar results are obtained when nuclei are incubated with pancreatic DNAase I for 10 min at 30^{o} prior to the addition of [γ-^{32}P]ATP as shown in Fig. 1.

Several mechanisms could account for this observation: a) a direct stimulation of the nuclear protein kinases by histones or polyamines; b) stimulation of phosphoprotein phosphatases, if protein-bound phosphate turnover were required for the *in vitro* labeling of a protein fraction that is already phosphorylated in the nuclei as they are isolated; c) inhibition

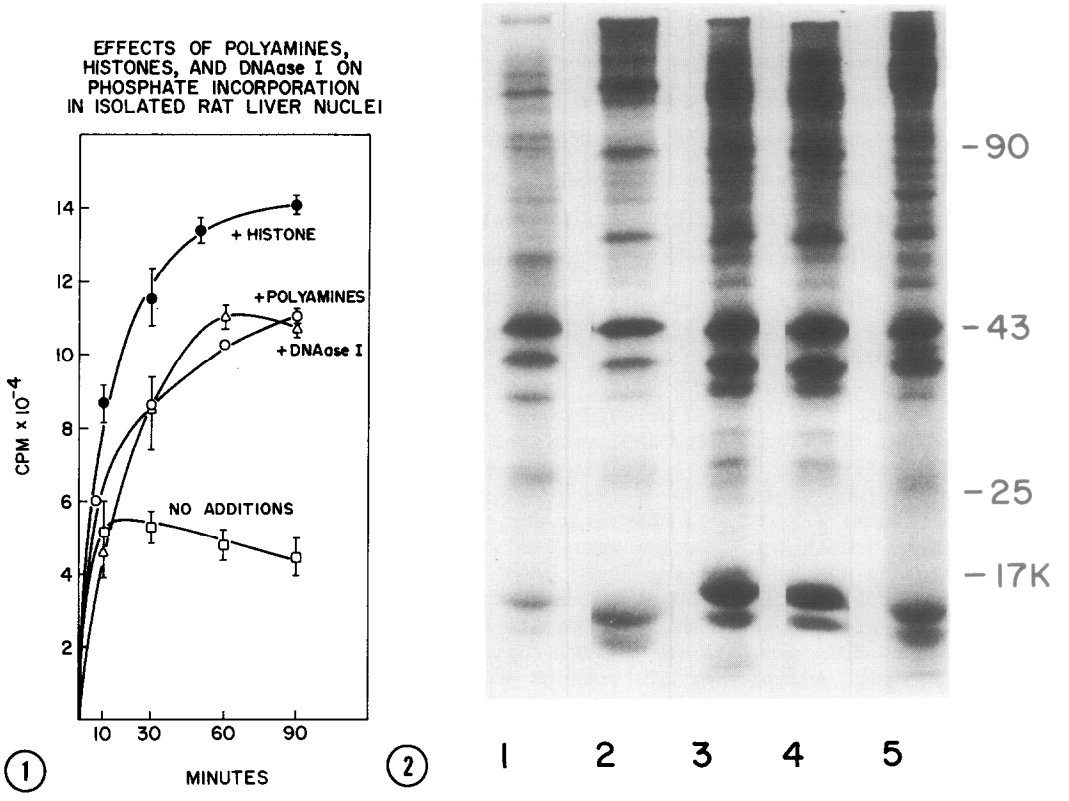

Fig. 1. Nuclei were isolated as described in Methods. Aliquots containing 250 µg nuclear protein were incubated in the standard reaction mixture at 30°: a) without additions; b) in the presence of 100 µg each spermidine and spermine; c) with 200 µg histones (Sigma IIA); and d) after 10 min at 30° in the presence of 5 µg pancreatic DNAase I.

Fig. 2. Purified nuclei (250 µg nuclear protein) were incubated for 60 min at 30° under standard conditions as described in Methods. Channel 2: 80 µg protein, no additions; channel 3: 40 µg protein, in the presence of 200 µg histones; channel 4: 40 µg proteins, preincubated with DNAase I; channel 5: 40 µg proteins in the presence of 100 µg spermine and 100 µg spermidine. Channel 1: nuclei were isolated from livers of rats that had been injected with 5 mCi ^{32}P two hours prior to sacrifice, 80 µg protein.

of phosphoprotein phosphatases, if net phosphate incorporation ceased as a result of rapid dephosphorylation in vitro; d) a structural rearrangement of the chromatin upon addition of histones or polyamines, or preincubation of nuclei with DNAase, that would render certain non-histone proteins more accessible to the action of the nuclear protein kinases. Although a direct stimulation of partially purified nuclear protein kinases by physiological concentration of polyamines has been previously reported (5), higher

Table I. Effect of polaymines and individual histone fractions on phosphate incorporation in isolated nuclei

Additions		^{32}P incorporated* (cpm)
None		25,000
Spermidine and spermine	100 µg each	58,600
Histone (Sigma II A)	200 µg	91,500
H1	200 µg	71,500
H2A	200 µg	69,000
H2B	200 µg	73,400
H3	200 µg	28,200
Polylysine	200 µg	74,800
Polyarginine	200 µg	29,100

* Values are the mean of 4 determinations. SD did not exceed $\pm$ 8%.

Aliquots of 250 µg nuclear protein were incubated under the standard conditions described in Methods. Incubations were for 60 min at 30°.

concentrations of polyamines are required in order to produce the effects observed in isolated nuclei. Data summarized in Table I show that polyamines and all histone fractions, as well as polylysine and (marginally) polyarginine stimulate phosphate incorporation in intact nuclei albeit to varying degrees.

The ability of histones to stimulate protein kinase activity in nuclear extracts appears to depend at least on two factors: the nature of the substrates and the presence of nuclei acids in the extracts, as shown by the data summarized in Table II. When a 0.14 M NaCl extract was fractionated on phosphocellulose, pooled active fractions of each peak incorporated a modest amount of ^{32}P in the absence of added substrate, hence they contained some phosphate acceptor proteins. This basal activity could be stimulated approximately 2-fold by addition of histones. The source of the enzymes used in experiment 2 was a 0.5 M NaCl extract that had been treated with DNAase and RNAase, and had been fractionated on casein-Sepharose prior to chromatography on phosphocellulose, as previously described (13). The active fractions obtained after phosphocellulose chromatography were substrate-free and 10 µg of enzyme-free substrates were added to assess the basal activity. It can be seen that in the presence of these substrates histones do not stimulate protein-phosphorylation. We conclude, therefore, that histones do not interact with nuclear protein kinases directly, a conclusion Kaplowitz et al. (6) have also reached based on different lines of evidence.

Lastly, the observed increased phosphorylation in nuclei preincubated with DNAase cannot readily be explained as a direct stimulation of protein kinase activity.

Since in vitro phosphorylation of nuclear proteins might conceivably require active turnover of protein-bound phosphate, the possibility that histones or polyamines exert their effects on net phosphorylation by increasing phosphate turnover was investigated in vitro and in vivo. Isolated nuclei were labeled in vitro with [γ-^{32}P]ATP in the presence or absence of histones or polyamines or preincubated with DNAase I for 10 min at 30°. Phosphorylation was stopped by the addition of EDTA in 25 molar excess over Mg^{++} present and the incubation was continued for 90 min at 30°. Samples were withdrawn at different time intervals and the remaining TCA precipitable ^{32}P determined by scintillation spectrometry as described in Methods. The kinetics of phosphate removal were nearly identical under all conditions, except that histones slowed the loss of phosphate slightly in experiments using freshly isolated nuclei. Storage of nuclei at 0° as a "scurose pellet" for 4-5 days completely abolished phosphatase activity without diminishing the capacity of the nuclei to phosphorylate proteins and without abolishing the stimulation by histones or polyamines.

To obtain nuclei labeled in vivo, rats were injected with 5 mCi ^{32}P two hours prior to sacrifice and their liver nuclei isolated by our standard procedure. These nuclei were incubated in 50 mM Tris acetate pH 7.5 containing 0.15 M NaCl for 90 min at 30° in the presence or absence of added histones or polyamines and with and without unlabeled ATP in order to test protein-bound phosphate turnover directly. The kinetics of loss of phosphate were identical to those found with in vitro labeled nuclei indicating, parenthetically, that these nuclei do not appear to contain active nucleases. Again, it was observed that histones slowed the loss of phosphate slightly in freshly prepared nuclei and that phosphatase activity decayed with 4-5 days upon storage at 0° without lowering the protein-bound phosphate content of ^{32}P-labeled nuclei more than accountable by the decay of the isotope; i.e. phosphatases did not act during storage of the nuclei. The loss of phosphate during incubation at 30° in freshly isolated nuclei was unchanged by the addition of unlabeled ATP under all conditions, indicating that neither polyamines nor histones increased protein-bound phosphate turnover (data not shown).

These experiments suggest that the stimulation of the phosphorylation of non-histone proteins by polyamines or histones is not due to increased protein-bound phosphate turnover, nor to inhibition of phosphoprotein phosphatases. Rather they appear to alter the structure of the chromatin

Table II. Effect of histones on the activity of individual nuclear protein kinase isozymes.

Phosphocellulose fraction	^{32}P incoporated (cpm x 10^{-3}) Substrates		
	endogenous	casein (200 µg)	histones (IIA) (200 µg)
Experiment 1 (0.14 M NaCl extract)	(none added)		
1	12.4	45	27
4	5	61	14
5	5.8	61	14
6	4.5	12	10
Experiment 2 (0.5 M NaCl extract)	(10 µg)		
1	31	144	9.5
2	7.4	66	8.0
3	15	92	11.5
4	15	61	14
5	8.4	57	8
6	4.4	8.4	4.7

Protein kinases were fractionated on phosphocellulose as previously described (5). In experiment 1 the source of the enzymes was a 0.14 M extract of purified nuclei. In experiment 2 the enzymes were separated from endogenous substrates on casein-Sepharose as previously described (13), approximately 0.5 µg protein kinase protein was incubated for 10 min at 30° in the same reaction mixture as described for nuclei (see Methods).

in such a way as to render more phosphate acceptor substrates accessible to the action of the nuclear protein kinases.

Figure 2 depicts the electrophoretic separation of the phosphorylated proteins in SDS-containing polyacrylamide gels. Channels 1 and 2 contain 80 µg nuclear proteins each, labeled *in vivo* and *in vitro*, respectively. Channels 3, 4 and 5 contain only 40 µg nuclear proteins -- in order to reveal qualitative differences -- incubated in the presence of histones, DNAase I and polyamines, respectively. The analysis shows that only a relatively small fraction of non-histone proteins is phosphorylated *in vivo* since many more proteins can be phosphorylated *in vitro* under the appropriate conditions. Addition of histones and digestion of the DNA promotes the phosphorylation of the same specific proteins within the limit of resolution of one-dimensional

electrophoresis. Addition of histones to nuclei whose DNA has been digested has no further effect (data not shown). Addition of polyamines increases the preferential phosphorylation of some of the same, in addition to other, protein species.

Since increased phosphorylation of nuclear non-histone proteins has been shown to occur during late G_1 and the early part of S-phase of the cell cycle (14,15), i.e. concomitant with histone biosynthesis, the observed stimulation of phosphate incorporation by histones in isolated nuclei may represent one way in which the phosphorylation of non-histone nuclear proteins is modulated *in vivo*. The concentration of polyamines has been shown to be several-fold higher in dividing and neoplastic than in quiescent cell populations (16), which could account, at least in part, for the higher specific phosphate content of the non-histone proteins in rapidly growing cells and the higher protein kinase activity in nuclei from rapidly growing tissues (17).

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