BBA 35483

PHOSPHORYLATION OF NUCLEAR PROTEINS IN AVIAN ERYTHROCYTES

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(Received July 3rd, 1969)

SUMMARY

Changes in nuclear phosphoproteins have been followed during the course of development of the avian erythrocyte. Both young reticulocytes and mature erythrocytes are capable of incorporating $^{32}P_i$ into nuclear proteins, the bulk of the activity occurring in the nonhistone protein fraction. Both phosphorylation and dephosphorylation of these proteins occur at faster rates in the younger cells. As maturation proceeds, nuclear levels of both phosphoprotein kinase and protein-bound phosphorus fall several-fold. Thus changes in the metabolism and composition of these nuclear phosphoproteins correlate with changes in the structure and activity of the nucleus.

INTRODUCTION

It is now known that phosphorylated proteins are a major component of the cell nucleus, accounting for a substantial portion of the nuclear dry weight in many cell types[1,2]. Although histones can be phosphorylated to a small extent, the major portion of the nuclear phosphoprotein is found in the acid-insoluble (nonhistone) protein fraction[3]. The phosphoprotein component of this nonhistone protein fraction has been partially purified and found to consist of mildly-acidic, chromatin-associated protein(s) which contain about 1.1–1.3% phosphorus by weight[1,2]. This phosphoprotein fraction is very actively phosphorylated and dephosphorylated in the absence of protein synthesis or breakdown, and it has been postulated that these reactions serve to modify the structure of the phosphoprotein and thereby alter the structure and metabolic activity of the chromatin with which it is associated[3,4].

Since a connection has thus been postulated to exist between this phosphoprotein fraction and nuclear activity, a system has been sought with which to test this hypothesis. The developmental sequence of avian erythrocytes represents an advantageous situation in which to observe the events associated with drastic changes in nuclear activity. Unlike the case in mammalian erythropoiesis, the nucleus is not extruded during formation of the mature avian erythrocyte. Although all mature avian erythrocytes are nucleated, these nuclei appear to be quite inert; their chromatin is compact and condensed, and they are inactive in both RNA and DNA synthesis[5,6].

In contrast, cells in the earlier stages of maturation, such as the avian reticulocyte, contain nuclei which are very active in nucleic acid synthesis and whose chromatin is highly dispersed. Therefore, comparison of the metabolism of nuclear phosphoproteins in the mature erythrocyte with that in the young reticulocyte might give some insight into the mechanisms involved in the regulation of chromatin structure and activity.

In the present paper, it will be shown that avian erythrocytes are capable of incorporating $^{32}P_i$ into nuclear proteins, the bulk of the activity occurring in the non-histone protein fraction. The rate of the phosphorylation and dephosphorylation reactions is considerably higher in young reticulocytes than it is in mature erythrocytes. As maturation proceeds, nuclear levels of both phosphoprotein kinase and protein-bound phosphorus decrease several-fold.

METHODS

Preparation of red blood cells

Experiments were performed on red blood cell suspensions obtained from 3-month-old Pekin ducks. In order to obtain cell populations which were enriched in cells at early developmental stages, ducks were made anemic by intraperitoneal injection of 2.5 ml of 2% phenylhydrazine on two consecutive days prior to bleeding. Blood was collected *via* the jugular vein, and heparin was added (1 mg/ml) to minimize clotting. Differential cell counts were made on blood smears which had been stained by a modified Giemsa technique[7]. In control ducks (without phenylhydrazine), mature erythrocytes accounted for close to 100% of the red cell population, while in anemic animals young reticulocytes accounted for up to 45% of the total.

Incubation procedure

The collected blood (400 ml from anemic birds and 300 ml from normal birds) was centrifuged at $600 \times g$ for 10 min and the serum removed. The sedimented cells were washed twice with 2.5 vol. of Eagle's minimum essential medium, and were then resuspended in 7.5 vol. of minimum essential medium to which glucose had been added to a final concentration of 0.2%. Aliquots containing approx. $1.8 \cdot 10^{11}$ cells were incubated in a final volume of 125 ml in the presence of 0.6 mC $Na_2H^{32}PO_4$ (100 mC/mmole) and 250 μg/ml puromycin. Incubations were carried out at 37° in a shaker water bath.

In isotope retention experiments, the cells were chilled after incubation for 30 min and centrifuged at $600 \times g$ for 10 min. They were then washed 3 times in cold minimum essential medium containing a 10-fold excess of unlabeled phosphate in order to remove the radioactive precursor. Cells were then resuspended in radioisotope-free incubation medium at the original concentration and reincubated at 37°.

Isolation of nuclei

Following incubation, the cell suspensions were chilled to 4° and the cells collected by centrifugation at $600 \times g$ for 10 min. The cells were washed 3 times in minimum essential medium and then lysed in a 0.3% saponin solution made up in 0.9% NaCl. The nuclei were isolated by differential centrifugation and washed in 0.9% NaCl (ref. 8).

Chemical analyses

Nuclei were extracted with 0.2 M HCl to remove the acid-soluble proteins. The extract was precipitated with 10 vol. of acetone, and the precipitate washed with diethyl ether and dried. Incorporation of $^{32}P_i$ into nuclear proteins was determined by measuring the formation of acid-stable, alkali-labile $^{32}P_i$ as previously described[3]. DNA analyses were performed by the Dische diphenylamine reaction as modified by GILES AND MYERS[9].

Assay of phosphoprotein kinase

Nuclei isolated as described above were broken by freezing and thawing followed by blending at 15 000 rev./min in a Sorvall Omnimixer. The nuclear fragments were dialyzed extensively against 0.05 M Tris buffer (pH 7.5) and were then incubated in the presence of [γ-^{32}P]ATP and 25 mM $MgCl_2$. Formation of alkali-labile $^{32}P_i$ in this system was determined as described elsewhere[2].

RESULTS

Phosphorylation and dephosphorylation of nuclear proteins

When erythrocytes from normal ducks are incubated in the presence of $^{32}P_i$, active incorporation of the isotope into nuclear proteins takes place (Fig. 1). Since this reaction occurs even when protein synthesis is being blocked by puromycin, the observed incorporation represents phosphorylation of previously synthesized proteins rather than *de novo* formation of phosphoproteins. The bulk of the incorporation (over 80%) occurs in proteins which are not extractable in 0.2 M HCl, indicating that as in other cell types, the major portion of nuclear phosphoprotein is nonhistone in origin[1,3].

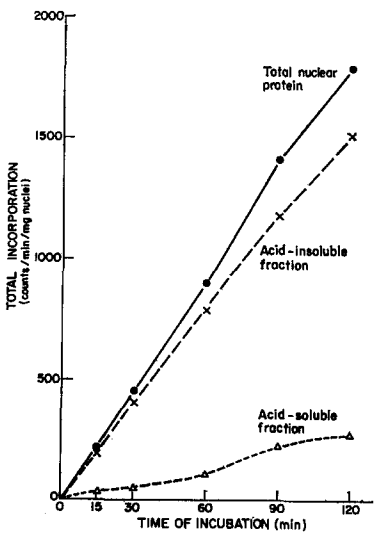

Fig. 1. Time-course of $^{32}P_i$ incorporation into the nuclear proteins of mature avian erythrocytes. The total incorporation into each fraction is plotted against the time of incubation at 37°. Note that the bulk of the incorporation is into the acid-insoluble nuclear proteins.

When the rate of phosphorylation of this protein fraction is compared in cell suspensions obtained from anemic and normal ducks, a striking difference is seen. In the anemic ducks, containing a large percentage of immature cells, the specific activity of the acid-insoluble nuclear proteins labeled with $^{32}P_i$ is found to be more than twice as high as that found in the cells from normal birds (Fig. 2). Furthermore, since the total amount of acid-insoluble protein per nucleus is also increased more than 2-fold in the cells from anemic birds (see Table II), the total incorporation of $^{32}P_i$ into protein per nucleus must be more than 4-fold higher in the immature cells than it is in the mature cells.

Since these experiments measured only the rate of appearance of ^{32}P in nuclear proteins, it is conceivable that factors other than an increased rate of protein phosphorylation could be responsible for the results. For example, an increase in the rate of transport of $^{32}P_i$ into the cells, or an increase in the specific activity of the intranuclear phosphate pool, might also result in an increased rate of appearance of ^{32}P in nuclear proteins.

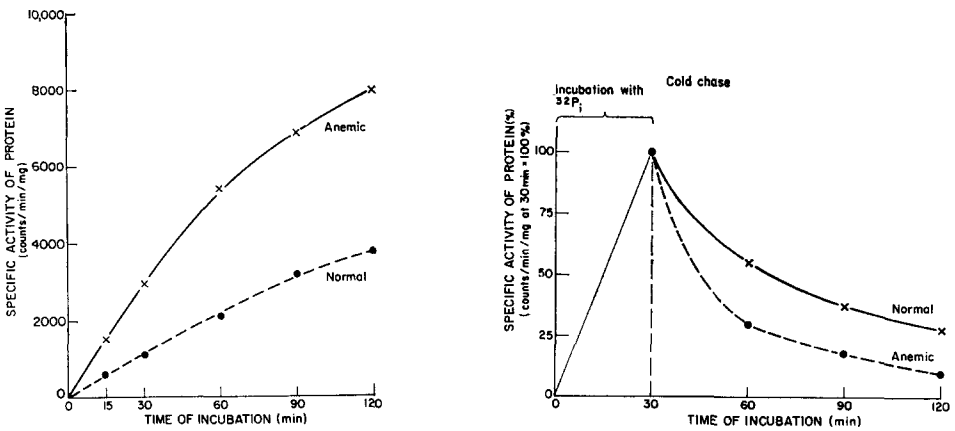

Fig. 2. Comparison of incorporation of $^{32}P_i$ into the acid-insoluble nuclear proteins of red cell suspensions obtained from normal and anemic ducks. The specific activity of the nuclear protein is plotted against the time of incubation at 37°. Note that the rate of labeling is considerably higher in the cells obtained from the anemic animals.

Fig. 3. Comparison of the turnover of previously incorporated $^{32}P_i$ in nuclear proteins of red cells obtained from normal and anemic ducks. Cells were incubated for 30 min with $^{32}P_i$, after which they were washed and resuspended in radioisotope-free media. The retention of isotope is plotted as a function of time of incubation at 37°.

One approach to this problem is to study the rate of turnover of ^{32}P from previously labeled proteins. In one such experiment, cells from normal and anemic ducks were first exposed to $^{32}P_i$ for 30 min to label the nuclear proteins. They were then washed extensively to remove the radioactive precursor and were reincubated in radioisotope-free media. Samples were withdrawn at 30-min intervals, and the retention of the radioactivity was determined. The results are summarized in Fig. 3, which shows that the rate of turnover of previously incorporated phosphate groups is considerably faster in the immature cells from anemic animals.

TABLE I

PHOSPHOPROTEIN KINASE ASSAYS OF NUCLEAR FRAGMENTS

Nuclear fragments containing 500 μg DNA were incubated in a final volume of 1.0 ml containing 10 nmoles [γ-^{32}P]ATP, 25 μmoles $MgCl_2$, and 40 μmoles Tris buffer (pH 7.5). 1 mg phosvitin was added where indicated. After incubation at 37° for 20 min, incorporation of ^{32}P into alkali-labile phosphate was determined as described elsewhere[3]. Results from two separate experiments are combined.

	Alkali-labile $^{32}P_i$ formed (pmoles)
Nuclei from normal ducks	2.27
Nuclei from anemic ducks	4.62
Normal *plus* phosvitin	4.73
Anemic *plus* phosvitin	10.19
Normal *minus* $MgCl_2$	0.04
Normal, zero time	0.01

Nuclear levels of phosphoprotein kinase

Another approach for determining whether the increased appearance of ^{32}P in nuclear proteins really represents an increased rate of protein phosphorylation is to perform a direct assay for the enzyme catalyzing this reaction, phosphoprotein kinase. When nuclear fragments are incubated in the presence of [γ-^{32}P]ATP and Mg^{2+}, incorporation of ^{32}P into alkali-labile phosphate occurs (Table I). The incorporation is more than 2-fold higher in preparations from anemic birds than it is in control preparations. Since the rate of this reaction *in vitro* depends on the concentration of substrate as well as phosphoprotein kinase in the nuclear fragments, excess exogenous substrate (phosvitin) was added in some incubations to give a direct measure of the phosphoprotein kinase activity of the nuclear fragments. In such cases, the kinase activity was again found to be more than twice as high in the nuclear fragments from the immature cells.

Nuclear levels of protein-bound phosphorus

Since the rates of phosphorylation and dephosphorylation of nuclear proteins are both increased in immature red cells, the question arises as to whether any net change in the total concentration of phosphoprotein phosphate occurs during develop-

TABLE II

PHOSPHORUS CONTENT OF NUCLEAR PROTEINS OF AVIAN ERYTHROCYTES

Data for young reticulocytes were obtained from analysis of red cell suspensions from anemic ducks which contained about 45% young cell forms (mainly reticulocytes) and 55% mature erythrocytes. Cell suspensions containing essentially 100% mature erythrocytes were obtained from normal ducks. Results are summarized from four different experiments.

	Young reticulocytes	*Mature erythrocytes*
Total nuclear protein (% P)	0.12	0.09
Acid-insoluble protein/DNA ratio	3.4	1.2
Protein-P/DNA-P ratio	0.074	0.023

ment. Chemical determinations of alkali-labile phosphorus showed that the immature cells contained more than 3 times as much protein-bound phosphorus per nucleus as the mature cells (Table II). Thus the overall effect of the changes in the rates of phosphoprotein metabolism which occur during red cell maturation is to markedly decrease the intranuclear concentration of protein-bound phosphate groups.

DISCUSSION

The results indicate that as maturation of the avian red blood cell proceeds, the rate of phosphorylation and dephosphorylation of nuclear proteins decreases several-fold. This decrease in reaction rates is accompanied by a decreased level of nuclear phosphoprotein kinase activity and a decreased content of protein-bound phosphorus. It should be pointed out that the values given for "young cells" are actually based on cell populations which contain only about 45% immature forms, with the rest being mature erythrocytes. Thus it is likely that the reported differences between the two cell types are minimal estimates and that the differences would be considerably greater if a pure population of young reticulocytes were available.

Since the younger red cell forms contain nuclei with highly dispersed chromatin which are very active in nucleic acid synthesis, the present findings represent another example of the relationship which exists between phosphoproteins and nuclear activity. A similar situation exists in mammalian lymphocyte nuclei where the stimulation of gene activity induced by phytohemagglutinin is known to be correlated with an increased metabolic activity of nuclear phosphoproteins[10]. The fact that the nuclei of immature erythrocytes are metabolically active and contain 3 times as much phosphoprotein phosphate per nucleus as the mature inactive erythrocytes is strikingly paralleled by the situation previously described for calf-thymus *versus* rat-liver nuclei[2]. The liver nuclei, which are very active in RNA synthesis, contain more than 3 times as much phosphoprotein phosphate as the thymus nuclei, which are quiescent and relatively inactive in RNA synthesis.

Thus the present studies on avian erythrocytes provide another correlation between nuclear phosphoprotein content and metabolism on the one hand, and capacity for nuclear synthetic activity, such as nucleic acid synthesis, on the other. These studies further suggest that as in mammalian cell nuclei, the nuclear phosphoproteins of avian cells may play an integral role in chromatin structure and function.

ACKNOWLEDGMENTS

Supported in part by grants GB-8123 from the National Science Foundation, Institutional Research Grant No. IN-401 to The University of Michigan from the American Cancer Society, and a grant from the Faculty Research Fund of the Horace H. Rackham School of Graduate Studies of The University of Michigan. We wish to thank Dr. James Neelin of the National Research Council, Ottawa, Canada, for his advice on several of the procedures employed. We are also greatly indebted to Dr. Vincent G. Allfrey of the Rockefeller University, New York, in whose laboratory some of these experiments were performed, for his many helpful suggestions and comments.

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