Phosphorylation of Nuclear Protein Early in the Course of Gene Activation in Lymphocytes

Abstract. *Human lymphocytes treated with phytohemagglutinin undergo extensive gene activation, as evidenced by augmented synthesis of ribonucleic acids. This activation is preceded by an early stimulation in the rate of phosphorylation and dephosphorylation of nuclear proteins. This finding is consistent with a hypothesized role of phosphoproteins in the modification of chromatin structure and in modulation of the template activity of DNA in vivo.*

The recent discovery that phosphoproteins are localized and actively metabolized in cell nuclei (*1, 2*) has led to speculations on the possible role of these proteins in nuclear function. One suggested possibility has been that the negatively charged phosphate groups of the phosphoprotein might interact with the positively charged histones, thereby displacing the inhibitory histones from the DNA-histone complex and thus allowing the DNA to become active as a template for RNA synthesis (*2a*). Langan (*1*) has provided two lines of evidence to support this view: (i) phosphoproteins seem to be concentrated in chromatin fractions active in RNA synthesis, and (ii) partially purified phosphoproteins can form complexes with histones in vitro, thereby decreasing the ability of the histones to inhibit a DNA-dependent RNA polymerase system.

If phosphoproteins are actually involved in the regulation of RNA synthesis in vivo, then one would expect to see an increase in the metabolic activity of nuclear phosphoproteins during periods of intense gene activity. The present experiments on gene activation in lymphocytes stimulated by phytohemagglutinin (PHA) confirm this prediction.

Human lymphocytes treated with PHA, a protein extract of the red kidney bean, are known to undergo a striking transformation which includes a marked stimulation of metabolic activity, an increase in cell size, and other changes which eventually lead to cell division (*3*). It is now clear that an early step in this transformation is an increased capacity for RNA and protein synthesis (*4*), and the process may be regarded as a case of extensive gene activation. Although cell division does not occur for several days, an increase in RNA synthesis can be detected within minutes after the addition of PHA (*5*). Even before the increase in RNA synthesis, there is an increase in the rate of histone acetylation (*5*). We therefore decided to study the behavior of nuclear phosphoproteins during this critical time period, the first few minutes after the addition of PHA.

Human lymphocytes were prepared as described elsewhere (*5*) and cultured for 18 hours at 37°C. At this time the cultures consisted of about 90 percent lymphocytes, with the contaminating cells being mainly monocytes. We have assumed that our results represent changes in the lymphocytes, but we have no way of evaluating the possible contributions of the contaminating cells. After 18 hours of culture, 0.1 ml of PHA (Difco preparation P) was added to 5 ml of cell suspension containing 10 to 20 $\times$ 10^6 cells/ml, and the cells were reincubated at 37°C. At various times during incubation either $Na_2HP^{32}O_4$ (100 mc/mmole, 25 μc/ml) or uridine-2-C^{14} (26.7 mc/mmole, 0.5 μc/ml) was added for a 15-minute pulse. After the pulse the tubes were chilled in ice and the lymphocyte nuclei isolated by homogenization and centrifugation in 0.01*M* citric acid (*5*). Protein phosphorylation (*2*) and RNA synthesis (*5*) were determined as previously described.

Table 1. Nuclear protein phosphorylation and RNA synthesis in human lymphocytes treated with PHA. One hour after the addition of PHA, the desired isotopic precursor (P^{32}-orthophosphate for phosphoprotein, 2-C^{14}-uridine for RNA) was added for a 15-minute pulse. Abbreviation: cpm, counts per minute.

Expt.	Condition	Specific activity of phosphoprotein		Specific activity of RNA	
		(cpm/mg)	(% control)	(cpm/μg RNA-P)	(% control)
1	Control	18		16	
	PHA	38	211	23	144
2	Control	65		77	
	PHA	99	152	114	148
3	Control	43		67	
	PHA	90	209	131	196
4	Control	402			
	PHA	1014	252		
5	Control	538		16	
	PHA	655	122	40	250
6	Control	3495			
	PHA	4517	129		

Although a certain variability was encountered owing to the need for employing different blood donors, all lymphocytes tested displayed a stimulation of nuclear protein phosphorylation following the addition of PHA (Table 1). An examination of the early kinetics of the process indicates that this increase in protein phosphorylation can be detected within the first 15 minutes after the addition of PHA (Fig. 1A). Furthermore, comparison of the kinetics of the stimulation of protein phosphorylation with those of RNA synthesis indicates that protein phosphorylation does not exhibit the early lag in stimulation characteristic of RNA synthesis under the same conditions (*5*); these kinetics suggest the possibility that the stimulation in protein phosphorylation precedes the major stimulation in RNA synthesis. In this respect, the results are similar to those indicating that histones are acetylated before lymphocyte nuclei increase their capacity for RNA synthesis (*5*).

Although it is clear that the rate of P^{32} uptake into nuclear proteins is increased in PHA-treated cells, a possible objection is that since adenosine triphosphate (ATP) is known to be the immediate phosphate donor (*1*), an increased specific activity of the ATP pool rather than an increased rate of protein phosphorylation could equally well explain the present findings. Therefore we decided to examine the uptake of P^{32} into the ATP pool after the addition of PHA.

In the first experimental approach, the incorporation of P^{32} into charcoal-adsorbable, easily hydrolyzable nucleotide phosphate (*6*) was measured as an approximation of the ATP pool. Although some increase in the specific activity of this phosphate pool was observed, the increase was not sufficient to account for the increase in specific activity of the phosphoprotein fraction (Fig. 1B). In the second approach, ATP was isolated directly by chromatography on Dowex-1-formate (*7*). In this experiment a 15-minute pulse of P^{32}-orthophosphate was given 1 hour after the addition of PHA. The specific activity of the phosphoprotein fraction

of the PHA-treated cells was 129 percent that of the controls, while the specific activity of the ATP in these PHA-treated cells was actually depressed to 86 percent of the control values. Thus in both types of experiment it does not appear that the increase in specific activity of the phosphoprotein fraction can be explained solely in terms of an increased specific activity of the ATP pool.

Since the phosphorylation of nuclear protein appears to involve a dynamic equilibrium in which previously incorporated phosphate groups are "turned over" (*2*), a comparison was made of the retention of P^{32}-phosphate in the phosphoproteins of PHA-treated and control cells. Lymphocytes were incubated for 15 minutes in the presence of P^{32}-orthophosphate to label the phosphoproteins, and the cells were subsequently washed and resuspended in radioisotope-free media; PHA was added to one group of cells after the washing procedure. Samples were withdrawn at later times for measurement of the specific activity of the nuclear phosphoproteins. The results are shown in Fig. 2. There is a striking difference in the extent of loss of previously incorporated phosphate groups between control and PHA-treated lymphocytes. Within 2 hours the PHA-treated cells have lost 75 percent of the P^{32} label, while the controls, after an initial period in which P^{32}-uptake continues, lose only about 40 percent of their maximal specific activity. It is not yet known whether these differences primarily reflect a difference in phosphate "turnover" or whether the situation is complicated by a degradation of the protein moieties concerned. The finding that the cold chase is effective more quickly in the PHA-treated cells also suggests the possibility that PHA might be affecting the transport or permeability of phosphate into the cells.

Since we have previously shown that phosphorylation of nuclear proteins in thymus nuclei proceeds independently of protein synthesis (*2*), the effects of puromycin were tested in the human lymphocyte system in an attempt to determine whether the increase in protein phosphorylation following PHA is entirely independent of new protein synthesis. When 10 μg of puromycin per milliliter were added (enough to inhibit protein synthesis by 86.4 percent) and PHA-treated cells examined 1 hour later with a 15-minute pulse of P^{32}-phosphate, it was found that protein phosphorylation was stimulated 166.9 percent in the PHA-treated cells in the absence of puromycin, while in the presence of puromycin the stimulation was only 127.4 percent. Thus, although most of the protein phosphorylation continues in the absence of protein synthesis, the slight decrease caused by puromycin in the PHA-stimulated cells is consistent with some new synthesis of phosphoprotein. However, possible indirect effects of puromycin cannot be overlooked as an alternative explanation.

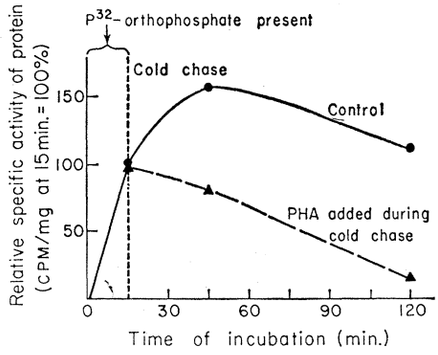

Fig. 2. Effects of PHA on turnover of previously incorporated P^{32}-phosphate in nuclear phosphoprotein. Cells were incubated for 15 minutes with P^{32}-orthophosphate, after which they were washed and resuspended in radioisotope-free media, and PHA was added. The retention of isotope was measured as a function of time. Note the rapid loss of P^{32} from the PHA-treated cells.

It is clear from the present experiments that the rate of phosphorylation and dephosphorylation of nuclear protein increases within the first few minutes of PHA-induced gene activation in human lymphocytes. Although this finding is consistent with the proposed role of phosphoproteins in the regulation of DNA template activity, alternative explanations, such as the possible role of phosphoproteins in the storage of phosphate or as an enzymatic intermediate in phosphate transfer should not be overlooked.

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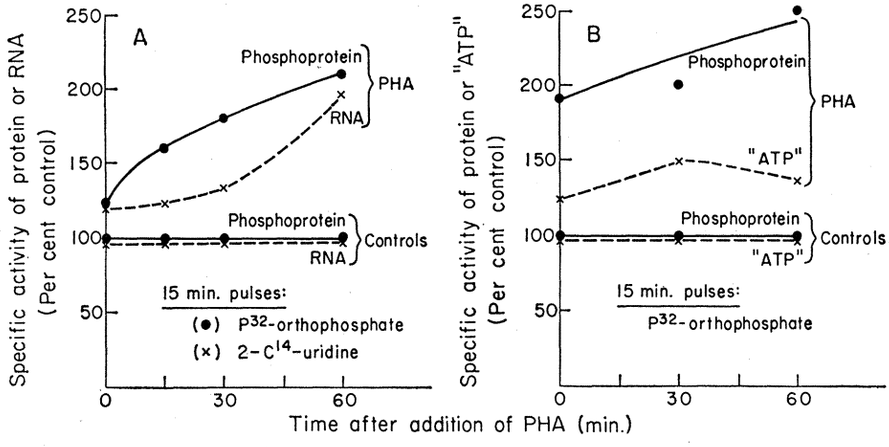

Fig. 1. (A) Comparative effects of PHA on nuclear protein phosphorylation and RNA synthesis in human lymphocytes. Cells were exposed to PHA and at the indicated times were pulse-labeled for 15 minutes in the presence of P^{32}-orthophosphate or 2-C^{14}-uridine. The specific activities of the phosphoprotein and RNA are plotted as percentages of control values. Note that the stimulation of protein phosphorylation seems to precede that of RNA synthesis. (B) Comparative effects of PHA on protein phosphorylation and ATP phosphorylation. Cells were pulse-labeled for 15 minutes with P^{32}-orthophosphate at the indicated times. The specific activities of the nuclear phosphoprotein and "ATP" (measured as easily hydrolyzable nucleotide phosphate—see *6*) are expressed as percentages of control values. Note that the stimulation in protein phosphorylation exceeds the increase in specific activity of the ATP pool.

References and Notes

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