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ACTIVATION BY A CYTOKININ-BINDING PROTEIN OF THE PHOSPHORYLATION REACTION; SYNTHESIS OF RNA AND PROTEIN IN COTTONPLANT CHROMATIN

Zh. A. Abdurakhmanova, O. N. Veshkurova, V. V. Kim, Sh. I. Salikhov, and A. A. Takanaev

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The dependence of protein kinase and RNA polymerase activities, and also of the synthesis of protein in nuclear chromatin, on the addition of benzylaminopurine and a cytokinin-binding protein to the incubation mixture has been investigated. It has been shown that all three processes are hormone-dependent. It has been found that the activity of protein kinase C is regulated by a cytokinin.

On the basis of the common evolutionary and structural features of animal and plant cells, it may be assumed that the nature of the realization of a hormonal signal in them should also be subject to common laws and take place with the participation of the basic messengers of the hormonal regulation of eucaryotes: specific receptors, RNA polymerases, protein kinases, etc.

At the present time the fact that chromatin is an extremely dynamic structure in the functional respect is not a matter of doubt. A close relationship exists between the structural rearrangements of chromatin and the activation of replication and transcription. The results of numerous investigations witness that an important role in the realization of such rearrangements is played by modifications of the structure of the chromatin proteins.

It has been shown that some of the nonhistone proteins associated with enzymes are capable of being phosphorylated. There are statements that one of the phosphorylated acid proteins is a DNA-dependent RNA polymerase, the phosphorylation of which raises the catalytic activity of the enzyme six- to ninefold [1].

It is known from the literature that phytohormones, especially cytokinins, exert an influence on the activity of protein kinases and also on the RNA polymerase activity and template activity of chromatin in plant cells [2]. We have therefore investigated the regulation of the activity of protein kinase C (PKC) and also of RNA polymerase and of protein synthesis in a system containing isolated chromatin under the action of a cytokinin. Since the action of the phytohormone is realized through its receptor, we studied the action of cytokinin itself and of its complex with a receptor — a cytokinin-binding protein (CBP).

In the experiments we used a synthetic cytokinin analog — benzylaminopurine (BAP). As a model system we chose an incubation mixture used previously for investigating transcription processes [3, 4], which contained chromatin isolated from three-day cottonplant seedlings and a standard set of nucleotides.

The results obtained are presented in the form of a histogram (Fig. 1). As can be seen from the histogram, the addition of BAP to the model system increased the activity of the PKC by 17%, that of the RNA polymerase by 13%, and the synthesis of protein (inclusion of 35-S-Met) by 14%. The addition of BAP together with CBP to the reaction mixture increased these activities by 23, 29, and 24%, respectively. This indicates that PKC also plays an important role in the transmission of the hormonal signal in a plant cell. It may be assumed that the enzyme, like the PKC of animal cells, takes part in the phosphorylation of the unbound (free) receptor, converting it into an active form that binds the corresponding phytohormone.

S. Yu. Selivankina et al. [5] studied the influence of a cytokinin on the activity of protein kinase bound to the chromatin and RNA polymerase of barley leaves and put forward the hypothesis that RNA polymerase is a substrate of protein kinase phosphorylation. The results that we obtained and information in the literature permit the conclusion that in the action

A. S. Sadykov Institute of Bioorganic Chemistry, Academy of Sciences of the Republic of Uzbekistan, Tashkent, fax (3712) 62 70 71. Translated from Khimiya Prirodnykh Soedinenii, No. 5, pp. 745-749, September-October, 1997. Original article submitted June 17, 1996; revision submitted April 28, 1997.

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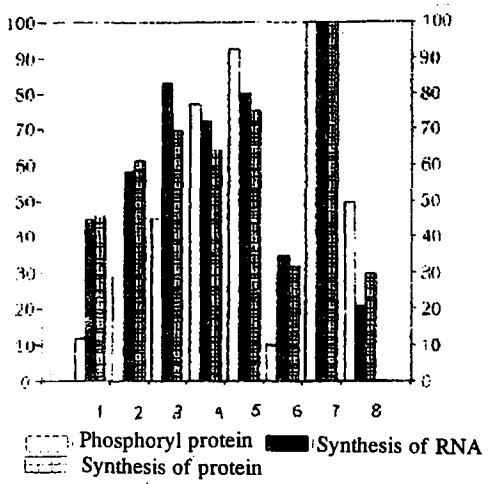

Fig. 1. Study of the transmission of a hormonal signal in an *in vitro* system: *1*) Chromatin (Xr) — control; *2*) Xr + BAP; *3*) Xr + BAP + CBP; *4*) Xr + PKC; *5*) Xr + BAP + CBP + PKC; *6*) Xr + BAP + rifampicin; *7*) Xr + BAP + CBP + PKC spermidine; *8*) Xr + BAP + CBP + PKC + PMB.

mechanism both of phytohormones and of animal hormones the modification of proteins with the aid of protein kinases may play an important role in the transmission of a hormonal signal.

Thus, induction either by a protein kinase or by a complex of a phytohormone and a receptor is sufficient for the activation of chromatin. We observed an even greater increase in the template activity of chromatin on the combined addition of BAP, CBP, and PKC, although the effects were not additive.

It may be concluded that the activation that occurs on the addition of BAP and CBP and the activation on the addition of protein kinase C alone are almost the same and are fairly high, but activation is not additive on the combined employment of all three of the components investigated and exceeds them only slightly. It is possible to judge the correlation of these activities by inhibiting one of these enzymes, and we therefore tested the action of the following inhibitors on all three activities: rifampicin — an inhibitor of RNA polymerase; spermidine, which is reported in the literature as an inhibitor for animal cells and an activator for the protein kinase C of plant cells [6], and also permexin B — an inhibitor of protein kinase C. On the addition of BAP, CBP, protein kinase C, and rifampicin to the reaction mixture, we observed suppression of not only the total protein kinase C activity resulting from the addition of exogenous BAP, CBP, and protein kinase C, but also almost complete suppression of its endogenous activity. In the case with permeksin B the RNA polymerase activity also fell by 20% below the control; i.e., permeksin B inhibits RNA polymerase more strongly by an order of magnitude than its characteristic inhibitor rifampicin. The protein kinase C activity was 42% lower than in the case where exogenous BAP, CBP, and protein kinase C acted without inhibitors. Under these conditions protein synthesis was 16% lower than in the control.

It is interesting to note that a specific inhibitor of protein kinase C — permeksin B — caused a fall in the activity of RNA polymerase. And, conversely, a specific inhibitor of RNA polymerase — rifampicin — retarded the phosphorylation process. This shows a common link in the transmission of a hormonal signal in plant and animal cells and the fact that phosphorylation of the proteins of transcription and translation complexes is one of the major steps in this process. This was confirmed by a pronounced activation of the observed processes by spermidine, which, as is known, participates in the regulation of protein kinase activity in animals [7].

It is known that polyamines take part in the structuration of chromatin and, consequently, in processes changing its template activity. Therefore, in investigating the processes occurring in chromatin under the action of a phytohormone and its receptor, we studied the influence of spermidine on protein kinase C activity. When spermidine was added to the reaction mixture, an increase in all three activities to a maximum was observed. It is desirable to mention that all three activities reacted in almost the same way to the addition of all the components investigated. This suggests that these processes are interdependent

and are linked with one another in virtue of a functional or structural bond in the native plant cell and that there is possibly a chain of enzyme reactions in which all three enzymes are involved.

On the basis of what has been reported above, it may be assumed that phosphorylation determines not only RNA synthesis but also the nonribosomal *de novo* synthesis of protein that takes place in isolated chromatin.

The intranuclear polymerization of amino acids was first observed in thymocyte nuclei by Allfrey [8]. In later investigations [9-11] it was shown that an analogous synthesis takes place in chromatin isolated from plant and animal cells, that this synthesis is DNA-dependent and that, in plant chromatin, it is controlled by phytohormones [12]. But, on the whole, the main principles and the functional significance of this process have not so far been elucidated.

The results reported in the present paper show that the hormonal regulation of the nonribosomal synthesis of protein is closely linked with phosphorylation processes, and the coupling of the synthesis of RNA and of protein is apparently explained by the participation in this process of a specific protein possessing both protein kinase and RNA polymerase activities.

The results that we have obtained and an analysis of the literature has enabled us to detect in an *in vitro* system the regulation of protein kinase C by a cytokinin, which shows a probability of the participation of this protein kinase in the reception, transformation, and realization of hormonal signals in plant cells.

The action on the cell not only of phytohormones but also of other factors is obviously realized through a phosphorylation–dephosphorylation process. Although the question remains open as to what extent the pathways for the realization of the action of hormones and other factors coincide for animal and plant cells, there is no doubt about their at least partial common nature.

EXPERIMENTAL

The protein kinase was isolated by a method described previously [13].

Isolation of Nuclear Chromatin. The seedlings were weighed and, after the addition of five volumes of a buffer containing 0.1 M Tris-HCl, pH 7.8, 0.25 M sucrose, 0.1 M $MgCl_2$, and 0.02 M β-mercaptoethanol, were homogenized at 14,000 rpm for 1.5 min. The homogenate was filtered through layers of calico and gauze and was centrifuged at 1300 *g* for 10 min. The deposit was suspended in a buffer containing 0.01 M Tris-HCl, pH 7.8, 0.35 M sucrose, 0.02 M 2-mercaptoethanol, and 2% of Triton X-100, and was centrifuged at 7000 *g* for 2 min. This procedure was repeated three times, after which the deposit was washed with the same buffer but without the Triton. The final chromatin deposit was suspended in a buffer containing 0.05 M tris-HCl, pH 7.8, 0.002 M $MgCl_2$, 0.001 M β-mercaptoethanol, and 10% of glycerol, and was used for the determination of its protein kinase, RNA polymerase, and protein-synthesizing activities. The concentration of DNA in the chromatin suspension was 1 μg/0.1 ml.

Protein kinase activity was determined in a medium containing 50 mM tris-HCl (pH 7.5), 0.5 mM $MgCl_2$, 10 mM NaF, 0.5 mM $CaCl_2$, and 40 μg of ATP(γ-^{32}P) with a total activity of 1 MBq, and 0.1 ml of chromatin suspension. After incubation for 15 min at 24°C, 10-μl aliquots were taken and were deposited on P-11 phosphocellulose filters with dimensions of 1 × 1 cm (Whatman, England). The filters were washed four times with 10 ml of 96% ethanol and dried, and radioactivities were measured in a Beta-1 counter.

RNA polymerase activity was determined in a reaction mixture containing 50 mM Tris-HCl, pH 7.8, 8 μM $MgCl_2$, and 0.1 μM each of ATP, UTP, GTP, and CTP. As a labeled precursor we used [^{33}P]-UTP (OP Radiopreparat, Tashkent) in an amount of 25 μl with a total activity of 1 MBq. To 0.25 ml of reaction mixture was added 0.1 ml of a chromatin suspension containing 1.0 μg of DNA. The samples were incubated at 30°C for 25 min. The reaction was stopped by rapid cooling on ice and the addition of 1 ml of 10% TCA. After 30 min, the samples were deposited on Synpor filters and were washed 3 times with cold TCA and 5 times with 96% ethanol. After this, the radioactivity of the acid-insoluble residue was determined in ZhS scintillation liquid in a Beta-1 counter.

Protein-synthesizing activity was determined under the same conditions as RNA polymerase activity. As the labeled precursor we added to the incubation mixture 50 μl of [^{35}S]-methionine (OP [Experimental Factory] Radiopreparat, Tashkent) with a total activity of 1 MBq.

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