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MONO(ADP-RIBOSYL)ATION OF HEN LIVER NUCLEAR PROTEINS SUPPRESSES PHOSPHORYLATION[1]

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SUMMARY: The phosphorylation of nuclear proteins from hen liver nuclei was suppressed under conditions of incubation with NAD. The reconstituted protein kinase assay system containing heat-treated and subsequently ADP-ribosylated nuclei and NI type protein kinase revealed that the ADP-ribosylated nuclear proteins are poor acceptors for the phosphorylation reaction. Therefore, mono(ADP-ribosyl)ation may contribute to the regulation of phosphorylation reaction in nuclei.

ADP-ribosyltransferase catalyzes the transfer of ADP-ribose[2] from NAD to acceptors such as arginine and histones, forming the mono(ADP-ribose)-acceptor conjugate (1-4). In eukaryotes, this enzyme activity is present in erythrocytes, in the thyroid cell membrane and in the 27,000 x g supernatant of the rat liver (3-5). The physiological role of mono(ADP-ribosyl)ation has yet to be determined, however, it is known that the ADP-ribosyltransferase modifies several endogenous proteins so that the adenylate cyclase system is activated (2,5,6).

Phosphorylation-dephosphorylation reactions, as a form of regulatory control, are assuming increasing importance in understanding of cellular processes (7,8). Peptide hormones, prostanoids, and amines promote the phosphorylation via

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2. Abbreviations: ADP-ribose, adenosine diphosphate ribose; cAMP, cyclic adenosine monophosphate.

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cAMP-dependent protein kinase, however, the regulation of the covalent modification by cAMP-independent protein kinase such as nuclear protein kinases is poory understood.

We now report evidence that mono(ADP-ribosyl)ation of hen liver nuclei *in vitro* suppresses the phosphorylation of endogenous nuclear proteins.

MATERIALS AND METHODS

Rhode Island Red hens were obtained from Yamasaki Farms, Shimane. [γ-^{32}P]ATP and [adenylate-^{32}P]NAD were obtained from New England Nuclear.

Preincubation of nuclei with NAD - Nuclei prepared from hen liver nuclei (9) were preincubated with 1 mM NAD at 25°C for 1 h in a total vol of 2 ml of a medium containing 50 mM Tris-Cl buffer (pH 9.0), 1 mM EDTA and 1 mM dithiothreitol. The mixture was then centrifuged at 10,000 x g for 20 min, and the pellet washed twice with 8 ml of the medium which contained 30% glycerol, 50 mM Tris-Cl buffer (pH 8.0), 1 mM EDTA and 1 mM 2-mercaptoethanol. This pellet was then resuspended in 1 ml of the same medium and used for the phosphorylation assay.

Enzyme preparations - ADP-ribosyltransferase from hen liver nuclei was purified about 3,000-fold by salt extraction, hydroxyapatite, Sephadex G-200, phenyl-Sepharose, CM-cellulose and DNA-Sepharose column chromatography[3]. NI type protein kinase was purified from hen liver nuclei, according to the methods of Thornburg *et al.* (10). The active fractions from the casein-Sepharose column were used for the experiments.

Protein kinase assay - The standard incubation mixture containing an appropriate concentration of nuclei, 4 μmoles $MgCl_2$, 2.5 μmoles Tris-Cl buffer (pH 7.5), 2.5 nmoles [γ-^{32}P]-ATP (1×10^5 cpm per tube), in a total vol of 0.25 ml was incubated for 10 min at 30°C. The reaction was terminated by adding 2.5 ml of cold 25% trichloroacetic acid. Acid-insoluble material was collected on a glass filter and washed with a total of 15 ml of 25% trichloroacetic acid. The radioactivity of the ^{32}P-samples was determined using a Packard liquid scintillation spectrometer.

ADP-ribosyltransferase assay - The assay mixture containing 1 mM [adenylate-^{32}P]NAD (1×10^5 cpm per tube), 50 mM Tris-Cl buffer (pH 9.0), 1 mM EDTA, 0.1 μg of purified ADP-ribosyltransferase and an appropriate concentration of the heat-treated nuclei in a total vol of 0.2 ml was incubated at 25°C for 30 min. The radioactivity of acid-insoluble fraction was measured as described above.

Preparations of acid- and heat-treated nuclei - Nuclei containing 6.3 mg protein were dialyzed against 2 liters of glycine buffer (pH 3.5) containing 1 mM EDTA and 1 mM 2-mercaptoethanol for 8 h at 4°C. The nuclei were continually dialyzed against 2 liters of 50 mM Tris-Cl buffer (pH 8.0) containing 1 mM EDTA and 1 mM 2-mercaptoethanol for 8 h at 4°C. The so prepared nuclei were used as acid-treated nuclei and contained no detectable poly(ADP-ribose) synthetase activity, under the standard assay conditions. In some experiments, nuclei were exposed to 75°C heat for 5 min and used as acceptor proteins for both ADP-ribosylation and phosphorylation.

Protein assay - Protein was determined as described by Lowry *et al.* (11).

RESULTS AND DISCUSSION

As shown in Fig. 1A, preincubation of hen liver nuclei with 1 mM NAD produced about 60% suppression of ^{32}P-incorporation from [γ-^{32}P]ATP into the nuclear proteins, compared with that of preincubation without NAD. We already found that when the hen liver nuclei were incubated with NAD, poly- and mono-(ADP-ribose)-protein adducts were formed by poly(ADP-ribose) synthetase and ADP-ribosyltransferase, respectively (12). Therefore, the results shown in Fig. 1A indicate that poly(ADP-ribosyl)-ation, mono(ADP-ribosyl)ation, or both may be related to the NAD-dependent suppression of phosphorylation of these proteins. Actually, the addition of nicotinamide, a potent inhibitor of both poly(ADP-ribose) synthetase and ADP-ribosyltransferase,

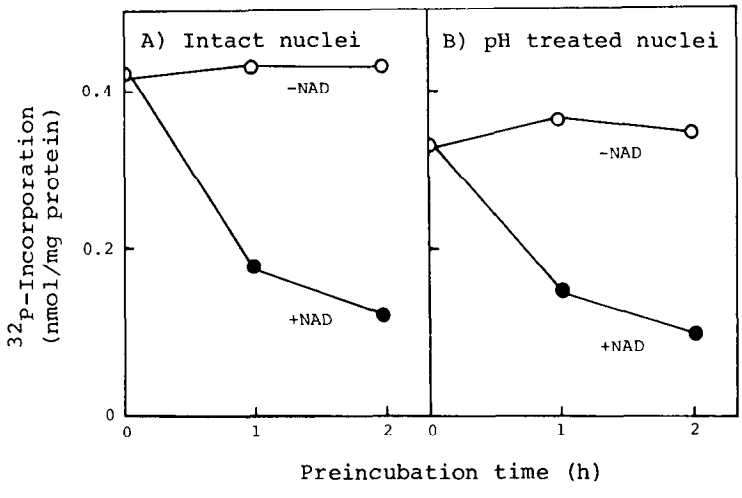

Fig. 1. Protein kinase activity of hen liver nuclei treated with or without NAD. A: The hen liver nuclei (8 mg protein) were preincubated with or without NAD at 25°C for the indicated time, and used for protein kinase assay, respectively. Details were described in the Methods. The protein contents of the control and NAD treated nuclei were 86.4 µg and 89.1 µg, respectively. o; control and ●; NAD-treated nuclei. B: The acid-treated nuclei prepared as described in the Methods, were used for protein kinase assay. Other experimental conditions were the same as Fig. 1A experiment. The protein contents of the control and NAD-treated nuclei were 73.4 µg and 69.8 µg, respectively. o; control and ●; NAD-treated nuclei.

reduced the NAD-dependent suppression of the phosphorylation (data not shown).

The effect of cAMP on the phosphorylation of nuclei treated with or without NAD was also investigated. There was no increase in the phosphorylation by addition of cAMP to either the NAD-treated or non-treated nuclei (data not shown).

When the hen liver nuclei were treated at pH 3.5 for 8 h, the ADP-ribosyltransferase retained its activity, while poly-(ADP-ribose) synthetase completely lost the activity[3]. These acid-treated nuclei retained more than 78% of the protein kinase activity in the non-treated nuclei. Using acid-treated nuclei, we studied the effect of preincubation with NAD on the phosphorylation activity. As shown in Fig. 1B, the incorporation of the terminal phosphate from [γ-^{32}P]ATP into nuclear proteins was suppressed to an extent similar to that shown in Fig. 1A. These results indicate that at least mono(ADP-ribosyl)ation suppresses the phosphorylation of nuclear proteins. Concerning these reciprocal changes in the ADP-ribosylation and phosphorylation, we speculate that the suppression of phosphorylation may relate to the inactivation of either protein kinase, acceptor proteins for phosphorylation or both. For clarification, acid-treated and subsequently NAD-treated nuclei were extracted with 50 mM Tris-Cl$^-$ buffer (pH 8.0) containing 0.35 M NaCl and other compounds. The extract was then dialyzed to remove the unreacted NAD, and the preparation was incubated with [^{32}P]ATP in the presence and absence of casein, an acceptor protein for the phosphorylation (10), and with or without purified nuclear protein kinase NI. The results of ^{32}P-incorporation into the acid-insoluble fraction are shown in Table I. In extract prepared from NAD-treated nuclei, there was approximately 65%

3. Tanigawa, Y., Tsuchiya, M. and Shimoyama, M.: submitted for publication.

Table I

Effect of NAD treatment of acid-treated nuclei on the phosphorylation in the presence and in the absence of casein or NI type protein kinase

0.35 M Nacl extracts prepared from:	^{32}P-incorporation		
	No addition	Casein	Protein kinase NI
	pmol/reaction mixture		
Control nuclei	13.6	42.1 (28.5)	23.6
NAD treated nuclei	5.0	37.1 (32.1)	5.8

Acid-treated nuclei were preincubated with or without NAD, washed as described in the Methods and the nuclei treated with 1 ml of 0.35 M NaCl containing 30% glycerol, 50 mM Tris-Cl$^-$ buffer (pH 8.0), 1 mM EDTA and 2-mercaptoethanol, homogenized and centrifuged at 10,000 x g for 20 min. Using these extracts, the protein kinase assay was carried out. The reaction mixture contained 20 µl of extract (10.8 µg protein) prepared from control nuclei or those (11.1 µg protein) from NAD-treated nuclei and 50 µg of casein, 0.5 µg of NI type protein kinase or none. Other conditions are described in the Methods. The net increases in phosphorylation by casein are shown in parenthesis.

suppression of the endogenous protein kinase activity. When casein was added to the incubation mixture, the rate of ^{32}P-incorporation increased 3.1-fold in the control and 7.4-fold in NAD-treated extract. The net increases in the phosphorylation were 28.5 pmol in the controls and 32.1 pmol in the NAD-treated preparation. This means that protein kinase activity in the extract prepared from NAD-treated nuclei is comparable to the activity in the control nuclei. To further extend our investigation, NI type protein kinase partially purified from hen liver nuclei was added to the protein kinase assay systems containing the extracts from control and NAD-treated nuclei, respectively. As shown in Table I, there was a 2-fold increase in ^{32}P-incorporation in the control but only a slight increase in the NAD-treated preparations. Therefore, NAD treatment seems to bring about inactivation of acceptor proteins for phosphorylation, but not protein kinase.

The reconstituted system was then used for the protein kinase assay. The nuclei were heated for 15 min at 75°C to

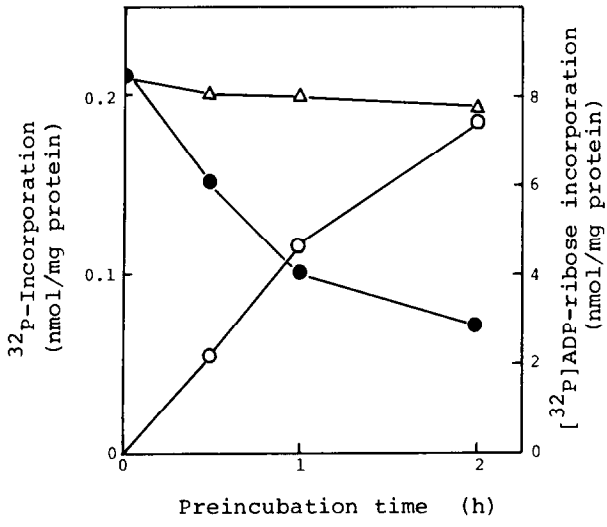

Fig. 2. Reciprocal relationship between ADP-ribosylation and phosphorylation. The heat-treated nuclei (6.3 mg of protein) prepared as described in the Methods, were preincubated with 1 μg of purified ADP-ribosyltransferase in the presence and absence of NAD, for indicated times. The protein kinase assay system contained NAD-treated nuclei 59.1 μg) or control nuclei (62.0 μg) as acceptor proteins for phosphorylation and 1.3 ug of NI type protein kinase. Other assay conditions are described in the legend to Fig. 1. ○ ; ADP-ribose formation, ●; protein kinase activity with NAD-treated nuclei, and △; protein kinase activity with untreated nuclei.

inactivate both the ADP-ribosyltransferase and protein kinase activities. The mixture of heat-treated nuclei and purified ADP-ribosyltransferase was preincubated with unlabelled NAD, the NAD removed and the nuclei incubated with NI type protein kinase in the presence of [^{32}P]ATP. As shown in Fig, 2, decrease in the phosphorylation occured when the preincubation time for ADP-ribosylation was increased. These results are in good agreement with the data in Fig. 1A and B.

We found that the mono(ADP-ribosyl)ation of hen liver nuclei suppressed the phosphorylation of the endogenous nuclear proteins. The enzymes associated with nucleic acids synthesis, such as DNA-dependent RNA polymerase (13) and poly(adenylic acid) polymerase (14), are among the many enzymes which undergo reversible regulation of phosphorylation-dephosphorylation. The

existence of numerous nuclear phosphoproteins led to the conclusion that phosphorylation is involved in the regulation of gene activity (15).

We are now attempting to identify the ADP-ribosylated nuclear proteins which are poor acceptors for phosphorylation.

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