ADP-ribosylation regulates the phosphorylation of histones by the catalytic subunit of cyclic AMP-dependent protein kinase

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Phosphorylation of whole histones from calf thymus by the catalytic subunit of cyclic AMP-dependent protein kinase was markedly reduced when the histones were ADP-ribosylated. NAD, nicotinamide or free ADP-ribose molecule did not suppress the phosphorylation. Urea gel electrophoretic analyses of the phosphorylated histones which had already been ADP-ribosylated revealed that the suppression of phosphorylation occurred in both H1 and core histones. Therefore, the possibility that ADP-ribosylation may regulate the phosphorylation of histones phosphorylation in nuclei warrants further investigation.

Mono(ADP-ribosyl)ation *ADP-ribosyltransferase* *NAD* *cAMP-dependent protein kinase* *Phosphorylation* *Histone*

1. INTRODUCTION

ADP-ribosyltransferase catalyzes the transfer of ADP-ribose moiety of NAD to acceptors such as arginine, other guanidino-compounds and proteins, forming mono(ADP-ribose)–acceptor conjugates [1–5]. This enzyme was found in membranes from turkey and human erythrocytes [3], in thyroid cells [4] and in 27000 × *g* supernatant of the rat liver [5]. We found that in hen liver nuclei there was a high ADP-ribosyltransferase activity which utilizes chromatin proteins as acceptors for the ADP-ribosylation reaction [6]. The biological functions of mono(ADP-ribosyl)ation have yet to be determined, however, it has been proposed that ADP-ribosylation activates adenyl cyclase by catalyzing the ADP-ribosylation of a specific protein component of the cyclase system [1,7–9].

As histones are acceptors for both ADP-ribosylation [1–5] and cAMP-dependent phosphorylation reactions [10], the question arises as to whether ADP-ribosylation influences cAMP-dependent phosphorylation of the same protein.

Abbreviations: ADP-ribosyltransferase, adenosine diphosphate ribosyltransferase; SDS, sodium dodecylsulfate

We have obtained evidence that ADP-ribosylated histones are poor acceptors for phosphorylation by the catalytic subunit of cAMP-dependent protein kinase.

2. MATERIALS AND METHODS

2.1. *Materials*

Hens of Rhode Island Red species were obtained from the Hara Farms (Shimane). [γ-^{32}P]ATP (3000 Ci/mmol) and [*adenylate*-^{32}P]NAD (960 Ci/mmol) were obtained from New England Nuclear. Calf thymus whole histones (type II), cAMP, ATP and NAD were obtained from Sigma (St Louis MO). All other reagents were purchased from Miyata (Shimane) and were used without further purification.

2.2. *Preparation of ADP-ribosylated whole histones*

The calf thymus whole histones (400 μg) were incubated in a total volume of 0.2 ml of a medium containing 50 mM Tris–Cl$^-$ (pH 9.0), 1 mM dithiothreitol, 0.1 mM EDTA, and an appropriate amount of purified ADP-ribosyltransferase with 1 mM NAD. After incubation at 25°C for 60 min,

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the reaction was terminated by the addition of 0.2 ml of 0.8 N H_2SO_4. The preparation was then centrifuged at 5000 × *g* for 15 min and the precipitate discarded. The histones were collected by centrifugation at 10000 × *g* for 15 min, in a final concentration of 25% trichloroacetic acid. The pellet was washed twice with 2.5 ml 25% trichloroacetic acid and then twice with 2.5 ml acetone. The washed pellet was desiccated and then resuspended in 0.2 ml 10 mM Tris–Cl^- buffer (pH 7.5). For preparation of control (not ADP-ribosylated) histones, whole histones were treated in much the same manner as the ADP-ribosylated histones, except that NAD was omitted from the reaction mixture.

2.3. *Enzyme purifications*

The ADP-ribosyltransferase was purified from hen liver nuclei, prepared as in [11], with the use of salt extraction, gel filtration, hydroxyapatite, phenyl–Sepharose, CM-cellulose and DNA–Sepharose column chromatography. The enzyme was purified to homogeneity as evidenced by the presence of a single protein band upon 10% polyacrylamide gel electrophoresis in the presence of 0.1% SDS (submitted). The catalytic subunit of cAMP-dependent protein kinase was purified from hen liver as in [12].

2.4. *Gel electrophoresis*

Acid–urea polyacrylamide gel electrophoresis was performed with a 15% gel column (0.6 × 7 cm) containing 0.9 M acetic acid and 2.5 M urea as in [13]. After electrophoresis, the gel was stained with 0.2% Coomassie brilliant blue in 9.2% acetic acid and 45.4% methanol (v/v), destained, then dried.

2.5. *Assays*

cAMP-dependent protein kinase assay mixture containing an approximate concentration of the purified catalytic subunit of cAMP-dependent protein kinase, 5 mM $MgCl_2$, 10 mM Tris–Cl^- (pH 7.5), 10 μM [γ-^{32}P]ATP (1 × 10^5 cpm/tube), and 100 μg ADP-ribosylated histones in a final volume of 0.25 ml was incubated for 5 min at 30°C. The reaction was terminated by adding 2.5 ml cold 25% trichloroacetic acid. Acid-insoluble materials were 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 was carried out as in [14]. The standard assay mixture containing 1 mM [*adenylate*-^{32}P]NAD (1 × 10^5 cpm/tube), 50 mM Tris–Cl^- buffer (pH 9.0), 1 mM EDTA, 1.6 μg purified ADP-ribosyltransferase and 400 μg histones in a total volume of 0.2 ml was incubated at 25°C for 60 min. The radioactivity of the acid-insoluble fraction was measured as above. Protein was determined as in [15].

3. RESULTS AND DISCUSSION

When ADP-ribosylated histones were used as acceptor proteins for phosphorylation catalyzed by the catalytic subunit of cAMP-dependent protein kinase, suppression of phosphorylation was indicated by an increase in the ADP-ribosylation of the histones (fig.1A–C). These experiments were performed with ADP-ribosylated histones prepared with increasing concentrations of NAD (fig.1A), increasing incubation time (fig.1B) and increasing amounts of ADP-ribosyltransferase (fig.1C) in the ADP-ribosylation system containing whole histones as acceptor and unlabeled NAD as substrate. The increasing ADP-ribosylation of the histones, as a function of the respective increase in the NAD concentrations, incubation time, and the amounts of the ADP-ribosyltransferase were confirmed when [^{32}P]NAD was used as substrate (fig.1A–C). Autoradiographic analysis of acid–urea electrophoretically separative products formed in the ADP-ribosylation reaction clearly showed that ADP-ribosylation occurred in both H1 and core histones and was dependent on the incubation time (fig.2a,b). We also found that with either ADP-ribosylated or non-ADP-ribosylated histones as acceptors, the phosphorylation was in proportion to the incubation time (up to 15 min), the protein kinase concentrations (up to 20 μg) and the amounts of acceptor proteins (up to 100 μg), respectively. In addition, we confirmed that NAD, nicotinamide, or free ADP-ribose molecule added to the phosphorylation assay system, at 10–400 μM of each compound, did not inhibit the protein kinase activity (not shown).

We attempted to analyze the ADP-ribosylated and subsequently phosphorylated histones by

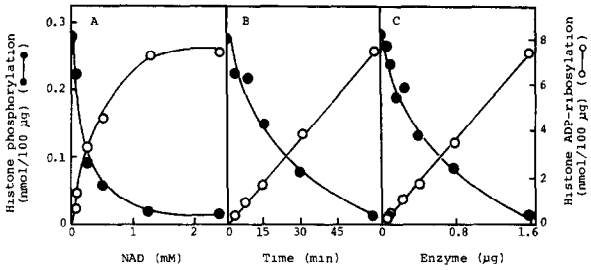

Fig.1. Decrease in phosphorylation of histones with increase in self ADP-ribosylation. (A) Decrease in phosphorylation of ADP-ribosylated histones with increase in NAD concentrations for ADP-ribosylation. Calf thymus whole histones (400 μg) were incubated with 1.6 μg of purified ADP-ribosyltransferase in the presence of various concentrations of [^{32}P]NAD. After incubation at 25°C for 60 min, the incorporation of radioactivity into the acid-insoluble fraction was measured. For the phosphorylation reaction, the ADP-ribosylated histones used as acceptors were prepared under conditions similar to those described above, except for the substitution of unlabeled for labeled NAD. The phosphorylation was carried out using these histones (100 μg) and the purified catalytic subunit (20 μg) cAMP-dependent protein kinase. Incubation was carried out at 30°C for 5 min. (B) Decrease in phosphorylation of histones with increase in the incubation time for ADP-ribosylation. Assays for labeled ADP-ribose incorporation from [^{32}P]NAD into calf thymus histones and the preparation of unlabeled ADP-ribosylated histones were carried out as in A except that 1 mM NAD was used for ADP-ribosylation and the reaction mixture was incubated for the time indicated. Phosphorylation conditions were the same as in (A). (C) Decrease in phosphorylation of histones with increase in ADP-ribosyltransferase concentrations. Assays for [^{32}P]ADP-ribosylation and preparation of unlabeled ADP-ribosylated histones were carried out as in A except that NAD was added at 1 mM and ADP-ribosyltransferase added at the indicated concentrations. Phosphorylation assays were the same as in A.

acid–urea gel electrophoresis. For this purpose, the whole histones, previously ADP-ribosylated or not ADP-ribosylated, were subjected to a phosphorylation reaction with the catalytic subunit of cAMP-dependent protein kinase. The acid-insoluble products were then separated by electrophoresis and autoradiography. Suppression of the phosphorylation was detected with both ADP-ribosylated H1 and core histones, with a prolonged incubation time for ADP-ribosylation (fig.2c–e).

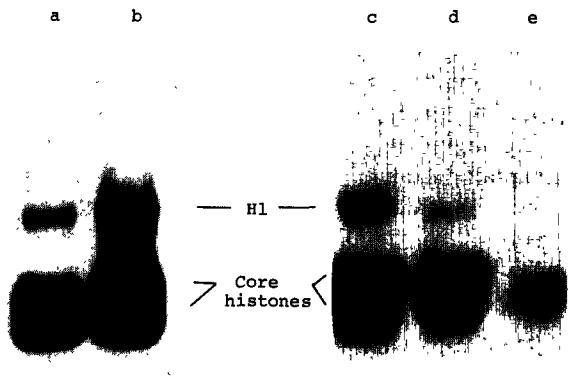

Fig.2. Acid–urea polyacrylamide gel electrophoresis of ADP-ribosylated and/or phosphorylated whole histones. Whole histones (400 μg) from calf thymus were incubated with 1 mM [^{32}P]NAD and 1.6 μg purified ADP-ribosyltransferase at 25°C for 30 min and 60 min, respectively. The ADP-ribose–histone adducts formed were isolated, and 25 μg of the sample was analyzed by acid–urea polyacrylamide gel electrophoresis (a,b). The ADP-ribosylated histones were prepared as above except that unlabeled NAD was used. Control and ADP-ribosylated histones (100 μg each) were incubated with [^{32}P]ATP and 20 μg of the purified catalytic subunit of cAMP-dependent protein kinase at 30°C for 15 min. The phosphorylated histones (25 μg) were subjected to electrophoresis. Dried gels were exposed to Kodak film for 3 days. The other experimental conditions were described in section 2. Lanes: (a,b) histones incubated with [^{32}P]NAD for 30 and 60 min, respectively; (c–e) histones incubated with unlabeled NAD for 0, 30 and 60 min, respectively, and then incubated with [^{32}P]ATP.

Therefore, the ADP-ribosylated histones apparently can be phosphorylated to a lesser extent, compared with the rate of phosporylation of non-ADP-ribosylated histones.

We then looked at the effect of phosphorylation of whole histones on the ADP-ribosylation reaction. Here, whole histones were phosphorylated and then ADP-ribosylated with the catalytic subunit of cAMP-dependent protein kinase and ADP-ribosyltransferase, respectively. Significant changes in the rate of ADP-ribosylation were not detected (not shown).

The physiological significance of the ADP-ribosylation-induced reduction in the phosphorylation remains to be determined. As ADP-ribosyltransferase catalyzes the transfer of an ADP-ribose moiety from NAD to the arginine

residue of acceptor proteins [6] and because this arginine is on the NH_2-terminal side of the phosphoserine in almost all of the cAMP-dependent protein kinase phosphorylation sites in which arginine is responsible for the enhanced phosphorylation of acceptor proteins [15,16], the possible regulation of the phosphorylation of histones by ADP-ribosylation has to be considered.

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