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ADP-RIBOSYLATION OF NUCLEAR PROTEIN A24*

Hiroto Okayama and Osamu Hayaishi

Department of Medical Chemistry, Kyoto University
Faculty of Medicine, Kyoto 606, Japan

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SUMMARY: Nuclear protein A24, which is composed of histone H2A and ubiquitin, a nonhistone protein, joined by an isopeptide linkage [Goldknopf and Busch (1977) Proc. Natl. Acad. Sci. USA 74, 864-868], is found to be ADP-ribosylated in isolated rat liver nuclei.

ADP-ribosylation is one of the post-translational covalent modifications of nuclear proteins in eukaryotes (1) and has been suggested to be involved in the regulation of various nuclear functions, such as DNA synthesis, histone function, and cell differentiation (1-6). Histones H2B and H1 are the major acceptors (7-12). Some nonhistone proteins are also reported to be ADP-ribosylated (8, 12-14).

During the analysis of nonhistone acceptor proteins by using covalent chromatography recently developed in our laboratory (12), we found that a unique protein, A24, is ADP-ribosylated. A24 is a basic chromosomal protein that consists of histone H2A and ubiquitin spliced by an isopeptide linkage (15-17). This protein was found in nucleoli by Orrick et al. (18) in 1973 and has been suggested to play a role in the regulation of nucleolar gene activity (19-21).

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Abbreviation: SDS, sodium dodecyl sulfate.

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In this communication we provide evidence for ADP-ribosylation of this protein in rat liver nuclei.

MATERIALS AND METHODS

Chemicals. [Adenine-U-^{14}C]NAD (237 Ci/mol) was purchased from the Radiochemical Centre, Amersham; urea (ultra pure), from Schwarz/Mann; CM-cellulose (CM 23), from Whatman; m-aminophenyl boronic acid, from Sigma; Bio-Gel P-60, from Bio-Rad Laboratories. Dihydroxyboryl-substituted Bio-Gel P-60 was prepared by the method of Inman and Dintzis (22).

Preparation of Rat Liver Nuclei. Nuclei were prepared from livers of Wistar rats weighing 300-400 g by the method of Chauveau et al. (23) and stored at -60° until use. In order to prevent proteolysis by serine proteases, 0.4 mM phenylmethylsulfonyl fluoride was used in all buffers (15, 24).

ADP-ribosylation of Isolated Nuclei and Extraction of A24. The reaction mixture contained 0.1 M Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 0.1 mM [adenine-^{14}C]NAD (3 Ci/mol), and rat liver nuclei (17 g wet weight) from 40 rats in a total volume of 200 ml. The reaction was carried out at 15° for 10 min and terminated by immediately cooling the mixture to 0°. The ADP-ribosylated nuclei were collected by centrifugation at 4,000 X g for 10 min and washed sequentially with 75 mM NaCl/25 mM Na_2EDTA (pH 7.5), 0.35 M NaCl and 5% perchloric acid (200 ml each). A24 was then extracted with 200 ml of 0.2 M H_2SO_4 together with histones H2A, H2B, H3 and H4 (15). The extracted proteins were precipitated by 20% CCl_3COOH. The precipitate was washed with ethyl ether and dissolved in 15 ml of 0.1 M potassium phosphate (pH 6.0)/6 M guanidine-HCl, followed by dialysis against 10 mM acetic acid and lyophilization.

Dihydroxyboryl Bio-Gel P-60 Column Chromatography of 0.2 M H_2SO_4 Extract. The lyophilized H_2SO_4 extract was dissolved in 15 ml of 50 mM morpholine-HCl (pH 8.2)/6 M guanidine-HCl. After the pH was adjusted to 8.2, the solution was applied to a dihydroxyboryl Bio-Gel column (1.8 X 20 cm) equilibrated with 50 mM morpholine-HCl (pH 8.2)/6 M guanidine-HCl. Chromatography was carried out as described previously (12). The column was washed with one bed volume of the same buffer and then eluted with 150 mM potassium phosphate (pH 6.0)/6 M guanidine-HCl. The flow rate was maintained at 40 ml/hr. Fractions of 4.2 ml were collected. The fractions containing ADP-ribosylated proteins (Fig. 1) were pooled, extensively dialyzed against 10 mM acetic acid, and lyophilized.

CM-cellulose Column Chromatography of Borate Column Eluate. The lyophilized material from the borate column was dissolved in 4 ml of 20 mM potassium phosphate (pH 6.0)/7 M urea and applied to a CM-cellulose column (1.2 X 15 cm) equilibrated with the same buffer. The column was washed with the equilibration buffer and

eluted with a linear gradient of 0-0.3 M NaCl contained in the equilibration buffer (total volume, 200 ml). Fractions of 3 ml were collected. Fractions 31-39 (Fig. 2), which represented the early one third portion of the main protein peak, were pooled and dialyzed against 10 mM acetic acid. After lyophilization, this material was dissolved in 1 ml of 10 mM acetic acid.

Two-dimensional Polyacrylamide Gel Electrophoreses. Samples were incubated at 37° for 30 min in 1 M NH_4OH in order to remove the bound ADP-ribose and its oligomer. After lyophilization, the samples were dissolved in 10% 2-mercaptoethanol and incubated for 2 min in a boiling water bath. After being dried up, the samples were dissolved in 30 µl of 0.9 M acetic acid/6 M urea and electrophoresed. Electrophoreses were performed by the method of Orrick et al. (18) on 10% acrylamide/4.5 M urea/0.9 M acetic acid first dimension and 12% acrylamide/6 M urea/0.1% SDS/0.1 M sodium phosphate (pH 7.1) second dimension gels. After electrophoreses, the gels were stained with Coomassie blue.

SDS-Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out by the method of Weber and Osborn (25) with a slight modification of solubilization conditions; a sample was dissolved in 30 µl of 10 mM sodium phosphate (pH 7.1)/1% SDS/50% glycerol/ 150 mM 2-mercaptoethanol and incubated for 3 hr at room temperature.

RESULTS AND DISCUSSION

Purification of ADP-ribosylated A24. Liver nuclei isolated from 40 rats were ADP-ribosylated with 0.1 mM [^{14}C]NAD. Under the reaction conditions used, 5.6 µmoles of ADP-ribose were incorporated into 20% CCl_3COOH-insoluble material. After washing the nuclei sequentially with 75 mM NaCl/25 mM Na_2EDTA, 0.35 M NaCl and 5% perchloric acid, protein A24 was extracted with 0.2 M H_2SO_4 together with histones (15). Approximately 12% of the total ADP-ribose incorporated into the 20% CCl_3COOH-insoluble material was recovered in this extract.

The ADP-ribosylated proteins were then isolated from the extract by covalent chromatography on a dihydroxyboryl Bio-Gel column (12). Fig. 1 shows the elution profiles of unmodified proteins and ADP-ribosylated proteins. Approximately 95% of the protein passed through the column at pH 8.2, whereas 90% of the

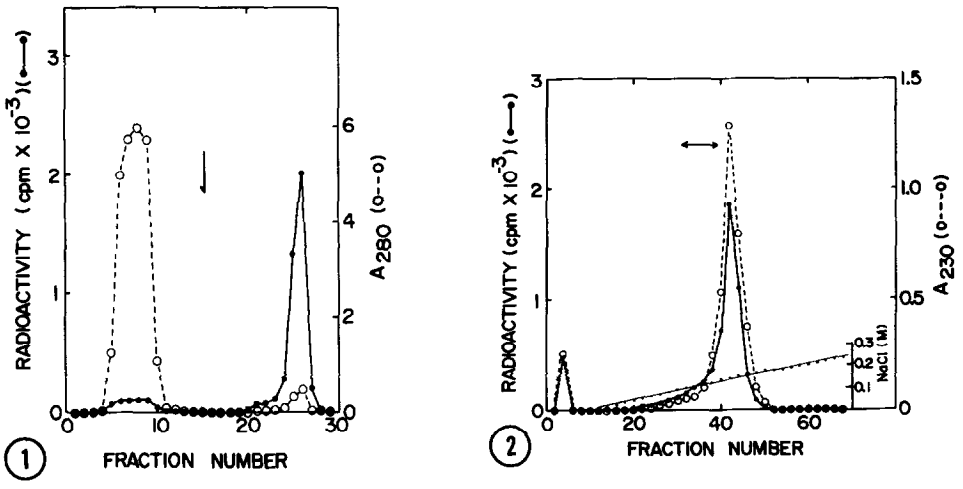

Fig. 1. Dihydroxyboryl Bio-Gel P-60 column chromatography of 0.2 M H_2SO_4 extract. Chromatography was carried out as described under "Methods". A 10 µl aliquot of each fraction was examined for radioactivity. The arrow represents the time of the buffer change.

Fig. 2. CM-cellulose column chromatography of the borate column eluate. A 20 µl aliquot was taken from each fraction and examined for radioactivity. ———, NaCl concentration.

ADP-ribosylated proteins was adsorbed and eluted from the column upon shifting the pH to 6.0.

The ADP-ribosylated proteins thus isolated were further purified by CM-cellulose column chromatography in the presence of 7 M urea (Fig. 2). The major peak of protein was eluted at about 0.15 M NaCl in association with radioactivity. The major protein in the peak fraction was identified as ADP-ribosylated histone H2B as reported previously (12). Small amounts of modified histones H2A, H3 and H4 were also contained. Fractions just before the peak of protein (shown by the arrow in Fig. 2), which contained A24, were pooled and subjected for analyses.

Identification of ADP-ribosylated A24. The CM-cellulose fraction was first analyzed by two-dimensional polyacrylamide gel electrophoresis that had been used for identification of A24 (15).

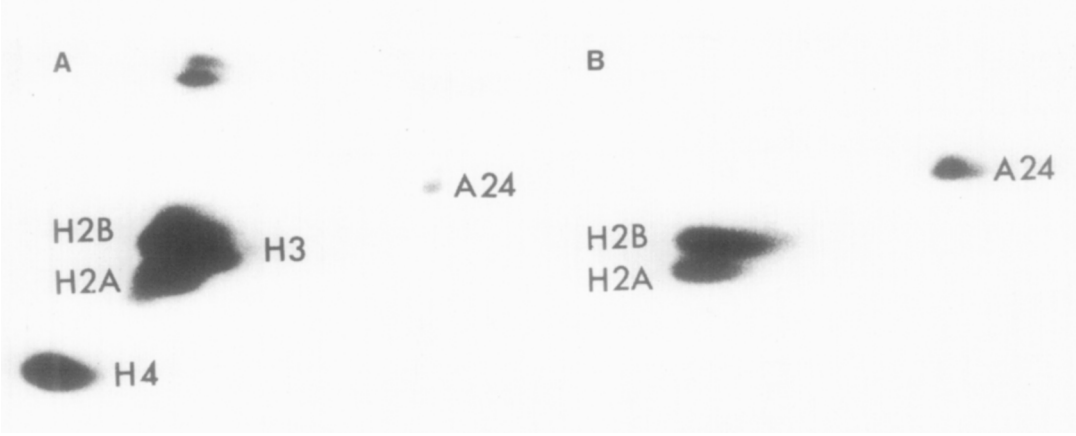

Fig. 3. Two-dimensional polyacrylamide gel electrophoreses of (A) 0.2 M H_2SO_4 extract (90 µg of protein) and (B) CM-cellulose fraction (66 µg of protein). Electrophoresis was from right to left in the first dimension and from top to bottom in the second.

In order to eliminate the effect of the bound ADP-ribose on the mobilities of proteins, samples were treated with mild alkali before electrophoresis. Fig. 3A shows the electrophoretic pattern of the 0.2 M H_2SO_4 extract. As reported by Goldknopf et al. (15), this extract contained A24 in addition to histones H2A, H2B, H3 and H4. Two protein spots above H3 are probably H3 dimers produced by intermolecular disulfide bond formation during electrophoresis. The pattern of the CM-cellulose fraction is shown in Fig. 3B. As judged by the electrophoresed positions, three protein spots were detected that corresponded to A24 and histones H2A and H2B, respectively.

In order to further identify the A24 protein, its molecular weight was examined by SDS-polyacrylamide gel electrophoresis. By using Escherichia coli RNA polymerase, bovine serum albumin, soybean trypsin inhibitor and egg white lysozyme as the molecular standard, it was calculated to be 26,500 in good agreement with the value, 27,000, reported by Goldknopf et al. (15). These results taken together indicate that the nonhistone protein recovered in the CM-cellulose fraction was A24 itself.

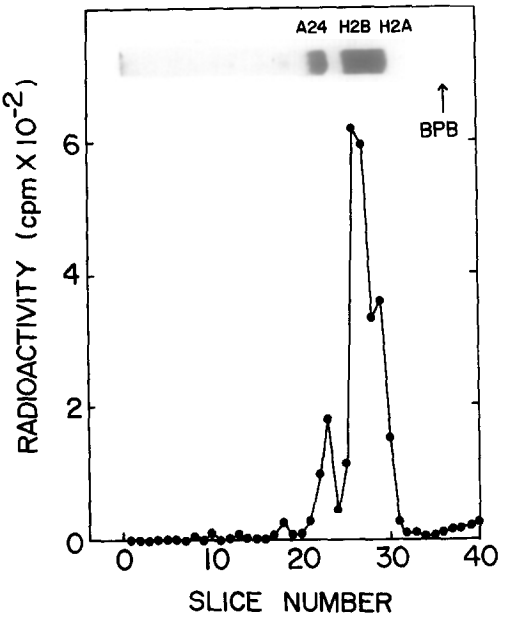

Fig. 4. SDS-polyacrylamide gel electrophoresis of the CM-cellulose fraction. CM-cellulose fraction (50 μg of protein) was electrophoresed at 6 mA for 7.5 hr. After electrophoresis, the gel was cut in half axially. One half was stained with Coomassie blue and the other half was sliced into 2-mm pieces. The radioactivity was eluted from the pieces with Soluene 100 (0.5 ml) and determined.

Evidence that ADP-ribose was associated with the A24 was obtained by SDS-polyacrylamide gel electrophoresis after mild solubilization which preserved the ADP-ribose-protein linkage intact. As shown in Fig. 4, radioactivity co-migrated with the A24 as well as histones H2A and H2B. The radioactive material associated with these proteins was ascertained to be ADP-ribose and its oligomer by paper chromatography of the material released by mild alkali treatment of the CM-cellulose fraction. Rough estimation of a molar ratio between the bound ADP-ribose and A24 indicated that approximately 0.9 mole of ADP-ribose was attached to 1 mole of A24. The amount of ADP-ribose attached to the total H2A recovered from the CM-cellulose column was approximately 10 times

as much as that of ADP-ribose attached to the total A24 recovered. Since A24 is present only in 3-4% of H2A molecules in rat liver nuclei (15), A24 appeared to be several times more efficiently modified than H2A under the conditions used.

Chromosomal protein A24 is of interest in two aspects: its unique structure in which histone H2A and ubiquitin are spliced, and its possible function as a repressor of ribosomal gene activity (19-21). The results presented here show that this protein undergoes modification by ADP-ribosylation. Although the biological meaning of this modification is unknown, it may be involved in the regulation of the repressor activity of this protein.

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