DISTRIBUTION OF POLY(ADP-RIBOSE) IN HISTONES OF HELA CELL NUCLEI

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Summary: The reaction product obtained from HeLa cell nuclei incubated with [^{3}H]NAD was specifically hydrolyzed with snake venom phosphodiesterase. Analysis of the hydrolyzed product revealed that it is a homopolymer consisting of 4-5 repetition of ADP-ribose units. The [^{3}H]poly ADP-ribosylated histone fraction was analyzed by urea-acetic acid polyacrylamide gel electrophoresis. The radioactive peak was clearly separated from the stained histone H1 band, while a slight overlap was observed. When chromatographed on a SP-Sephadex C-50 column, more than 90% of the radioactivity of [^{3}H]poly(ADP-ribose) was eluted in accordance with histones but not with nonhistone contaminants. On a sodium dodecyl sulfate polyacrylamide gel electrophoresis, a major radioactive peak appeared at a position very close to the histone H1 band, which disappeared by the treatment with alkali prior to electrophoresis. A selective extraction of histone H1 with 5% perchloric acid showed that histone H1 contained about 85% of the radioactivity incorporated into whole histones.

Introduction

Poly(ADP-ribose) is known to be synthesized from NAD by chromatin bound poly(ADP-ribose) polymerase in mammalian cell nuclei (1-6). The homopolymer is distributed among various nuclear protein fractions obtained from nuclei which have been incubated in vitro with NAD (7-9). Concerning the acceptor proteins and the chemical nature of the linkage between poly(ADP-ribose) and the acceptors, several investigations have suggested that poly(ADP-ribose) is covalently associated with various types of histones and perhaps with nonhistone proteins as well (10-13).

In order to clarify these problems further, we have analyzed, in the present report, the nature of poly ADP-ribosylated histones by several techniques, especially by polyacrylamide gel electrophoresis.

Materials and Methods

Chemicals and Enzymes: [^{3}H]NAD (3.28 Ci/mmol) was purchased from New England Nuclear, Boston, Massachusetts. Snake venom phosphodiesterase was obtained from Boehringer Mannheim. Bovine pancreatic DNase and yeast RNase were obtained from Worthington Biochemical Corporation.

Cell culture: HeLa S3 cells were maintained in monolayer culture in Eagle's minimal essential medium supplemented with 10% calf serum.

Isolation of nuclei: HeLa S3 cells were suspended in a buffer containing 10 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$ (2.5 x 10^7 cells/ml), and homogenized with 10 strokes in a Potter-Elvehjem type glass homogenizer with a Teflon pestle. An equal volume of a buffer containing 160 mM NaCl, 22 mM glucose, 1 mM EDTA, 2 mM 2-mercaptoethanol, 120 mM Tris-HCl (pH 8.5), and 0.1% Triton X-100 was added to the homogenate. After rehomogenization as described above, the homogenate was centrifuged at 800 x g for 5 min to pellet nuclei. At this point, the nuclear preparation was free of intact cell or cytoplasmic contamination as judged by electron microscopy.

Poly ADP-ribosylation of nuclear proteins: Isolated nuclei were suspended in a reaction buffer containing 15 mM $MgCl_2$, 50 mM KCl, 3.3 mM 2-mercaptoethanol, 82.5 mM Tris-HCl (pH 7.5), and sonicated for 15 sec. The nuclear suspension was incubated with [^{3}H]NAD (0.1 mM, 0.1 mCi/μmol) for 40 min at 25^0.

Analysis of reaction product: The reaction product thus obtained was treated with various enzymes. Enzyme digestion was performed for 1 hour at 37^0 under the condition favourable for the respective enzyme. The snake venom phosphodiesterase digest was layered on a polyethyleneimine-cellulose thin-layer plate and run, using as solvents first 0.9 N acetic acid until the front reached 1 cm ahead of the origin, following by 0.9 N acetic acid-0.3 M LiCl. AMP, ADP, ADP-ribose were added as markers. After running the chromatogram, the thin-layer plate was scraped off 1 cm in width and the material was solubilized with 0.5 ml of Soluene-350 (Packard). The radioactivity was counted in 10 ml of toluene scintillation fluid containing 50 mg of PPO and 3 mg of dimethyl POPOP with a scintillation spectrometer.

Isolation of histones: The reaction mixture was chilled to 0^0 and centrifuged at 10,000 x g for 10 min. The pellet was washed twice with 10 volumes of 0.15 M NaCl-50 mM Tris-HCl (pH 7.5) buffer each, to remove 0.15 M NaCl-soluble proteins. Histones were isolated by extracting three times with 5 volumes of 0.4 N H_2SO_4 each according to the method described by Panyim et al. (14).

Isolation of histone H1: Histone H1 was extracted with 5% (v/v) perchloric acid based on the method of Johns (15). After extraction of histone H1, the remaining histones were extracted with 0.4 N H_2SO_4 from the nuclear residue.

Polyacrylamide gel electrophoresis: SDS-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn (16). Urea-acetic acid polyacrylamide gel electrophoresis was carried out following the method of Panyim and Chalkley (17). The gels were stained with 0.3% Coomasie Brilliant blue in 10% acetic acid-50% methanol for 1 hour and destained in 7.5% acetic acid-5% methanol. For measurement of isotope distribution, the gels were sliced 1 mm in thickness and kept at 55^0 overnight in 0.5 ml of Soluene-350. The solubilized radioactivity was measured as described above.

SP-Sephadex C-50 column chromatography: Fractionation of isolated histones was performed by chromatography on a SP-Sephadex C-50 using stepwise elution of 0.15 M and 0.8 M NaCl containing 5 M urea-0.01 M Tris-HCl (pH 7.0). Chromatography was performed at 4^0 on a column (1 x 10 cm) at flow rate of 15 ml/hour.

Assay for acid insoluble radioactivity: Twenty per cent trichloroacetic acid precipitable materials were collected on a glass fiber filter and washed with 3 ml of cold 20% trichloroacetic acid 5 times, ethanol and acetone once successively. The radioactivity was determined as described above.

Results and Discussion

Analysis of reaction product: The reaction product of HeLa cell nuclei incubated with [^{3}H]NAD was sensitive to snake venom phosphodiesterase and was resistant to both DNase and RNase. When the product hydrolyzed with snake venom

Abbreviation: SDS, sodium dodecyl sulfate

Table 1

Average Chain Length of Poly(ADP-ribose) Produced by HeLa Cell Nuclei

Fraction	Phosphodiesterase Digest		Average Chain Length
	5'-AMP (A) (c.p.m.)	Phosphoribosyl-AMP (B) (c.p.m.)	[1 + (B)/(A)]
Whole Nuclear Proteins	1,500	4,900	4.3
Histones	750	2,800	4.7

5'-AMP and phosphoribosyl-AMP obtained from phosphodiesterase digestion were separated by polyethyleneimine-cellulose thin-layer chromatography. The radioactivity was measured as described in Materials and Methods.

phosphodiesterase was run on a polyethyleneimine-cellulose thin-layer plate, all the radioactivity was found as two spots, one running with 5'-AMP, the other at the position of phosphoribosyl-AMP (Table 1). The average chain length of the polymer obtained in this system was caluculated by the method of Fujimura, S. and Sugimura, T. (18). The result indicates that the reaction product is a homopolymer consisting of 4-5 ADP-ribose units.

Electrophoretical patterns and the chromatographic behavior of the poly ADP-ribosylated histone fraction: Synthesis of poly(ADP-ribose) requires nuclear proteins such as histones as an acceptor. In order to determine which histone incorporated [^{3}H]radioactivity, isolated histone fraction of HeLa cell nuclei incubated with [^{3}H]NAD was subjected to the high resolution urea-acetic acid polyacrylamide gel electrophoretical analysis described by Chalkley et al.(15). The electrophoretical pattern of [^{3}H]poly ADP-ribosylated histones is shown in Fig. 1. The radioactivity which appeared as a single peak was considerably separated from the stained histone H1 band, while a slight overlap was observed. No radioactivity was found in the regions of other histones.

Since the histone fraction is known to be contaminated with a small amount of nonhistone proteins, the radioactive peak might be due to the poly ADP-ribosylated nonhistone contaminants. However, chromatography on a SP-Sephadex C-50

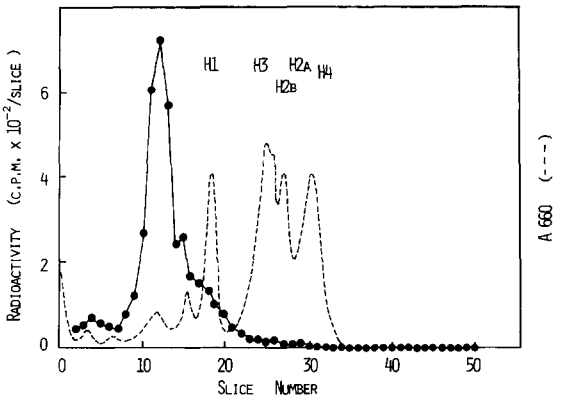

Figure 1. Urea-acetic acid polyacrylamide gel electrophoresis of [^{3}H] poly ADP-ribosylated histones. [^{3}H]poly ADP-ribosylated histones were subjected to electrophoresis at 2 mA/gel for 2.5 hours on a 2.5 M urea-15% polyacrylamide gel. Electrophoresis was carried out toward cathode (left to right).
(- - -) Absorbance at 660 nm of the stained histone bands.
(-●-) [^{3}H]-radioactivity.

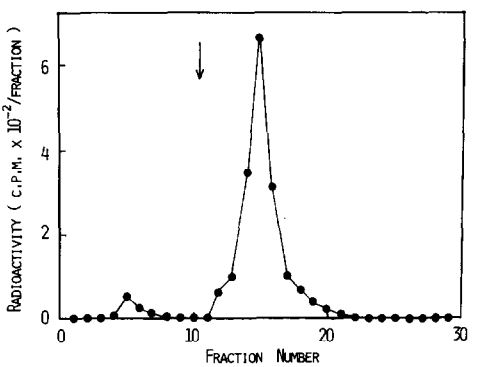

Figure 2. Elution profile of [^{3}H]poly ADP-ribosylated histones from SP-Sephadex C-50 column. [^{3}H]poly ADP-ribosylated histone fraction was applied on the column and washed with a buffer containing 0.15 M NaCl, 5 M urea, 0.01 M Tris-HCl (pH 7.0). Histones were eluted with a buffer containing 0.8 M NaCl, 5 M urea, 0.01 M Tris-HCl (pH 7.0). Acid insoluble radioactivity was measured as described in Materials and Methods. The arrow indicates the position at which the column was eluted with a buffer containing 0.8 M NaCl, 5 M urea, 0.01 M Tris-HCl (pH 7.0).

column of the histone fraction showed that more than 90% of the radioactivity was recovered associated with histones and only a small amount appeared in the flow-through fraction which contained minor nonhistone proteins (Fig. 2). The result indicates that a large amount of the radioactivity of the histone fraction

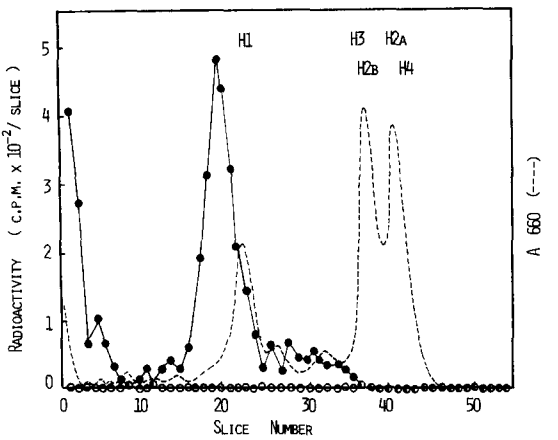

Figure 3. SDS-polyacrylamide gel electrophoresis of [^{3}H]poly ADP-ribosylated histones. [^{3}H]poly ADP-ribosylated histones were separated by electrophoresis in 10% polyacrylamide gel. Electrophoresis was carried out at 1 mA/gel for first 15 min and then at 8 mA/gel for 4 hours toward anode (left to right).
(- - -) Absorbance at 660 nm of the stained histone bands.
(-○-) [^{3}H]-radioactivity of NaOH treated poly ADP-ribosylated histones.
(-●-) [^{3}H]-radioactivity of non-treated poly ADP-ribosylated histones.

is due to the poly(ADP-ribose) bound to histones. The behavior of the poly ADP-ribosylated histones on urea-acetic acid polyacrylamide gel electrophoresis, therefore, would be explained by the covalent attachment of negatively charged poly(ADP-ribose) to histone H1, decreasing its over-all positive charge and, at the same time, increasing its molecular weight.

To confirm this, the distribution of [^{3}H]poly(ADP-ribose) among histones was examined by SDS-polyacrylamide gel electrophoresis which separates proteins solely on the basis of molecular weight (Fig. 3). This time, the major portion of [^{3}H]radioactivity was found close to the stained histone H1 band. Minor peaks appeared in the region between the H1 band and H3-H2B band, while no radioactivity was found along with the histone H3, H2B, H2A, and H4 bands. This behavior of the radioactivity indicates the increase in molecular weight of histones by poly ADP-ribosylation. When the [^{3}H]poly ADP-ribosylated histones were treated with 0.3 N NaOH prior to electrophoresis, the radioactive peaks entirely disappeared. It is strongly suggested, therefore, that the linkage between the polymer and histones is covalent in nature and not ionic. At the same time, urea-acetic acid

Table 2

Distribution of [^{3}H]poly(ADP-ribose) in Histones

Fraction	Protein (mg)	Acid Insoluble Radioactivity (c.p.m.)	(% total)	(% total histones)	(c.p.m./mg)
Whole Nuclear Proteins (4 x 10^7nuclei)	2.0	310,000	100	—	155,000
Histone H1	0.16	45,000	14.5	84.9	281,000
Other Histones (H2A, H2B, H3, H4)	0.62	8,000	2.6	15.1	13,000
Whole Histones (Histone H1 + Other Histones)	0.78	53,000	17.1	100	68,000

A selective extraction of histone H1 with 5% perchloric acid was followed by an extraction of the remaining histones with 0.4 N H_2SO_4 as described in Materials and Methods.

gel electrophoretical pattern shows that it is unlikely to be hydrogen bond. Recently, Smith et al. (19-20) proposed that 1-hydroxyl group of ADP-ribose was linked to the phosphate group of phosphoserine in histone H1.

Selective extraction of poly ADP-ribosylated histone H1: Considering these results obtained from the two different gel systems and the column chromatography, it is suggested that poly(ADP-ribose) is bound to histone H1. In order to confirm this idea, histone H1 was selectively extracted with 5% perchloric acid according to the method of Johns (15). As shown in Table 2., histone H1 contained about 85% of the radioactivity incorporated into whole histones, while other histones only 15%. It should be noted that histone H1 had the highest specific activity.

By performing the two different polyacrylamide gel electrophoreses, the column chromatography and the selective extraction of histone H1,we conclude that histone H1 is a specific acceptor molecule among histones and the linkage between the polymer and histone H1 is an alkaline labile covalent bond. The present studies can serve as a basis for more detailed investigation into the biological significance of poly ADP-ribosylation of nuclear proteins.

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