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Poly(ADP-ribose) Glycohydrolase is Present in Metaphase Chromosomes of HeLa S3 Cells

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Summary: Poly(ADP-ribose) glycohydrolase was found in metaphase chromosomes of HeLa S3 cells. Adenosine diphosphate ribose and 3', 5'-cyclic AMP inhibited the glycohydrolase activity, whereas ADP, ATP, NAD and 3',5'-cyclic GMP did not. The hydrolytic product of poly(ADP-ribose) bound to metaphase chromosomes with this enzyme was identified as adenosine diphosphate ribose.

Introduction

Poly(ADP-ribosyl)ation is a post-translational modification of chromosomal proteins (1 - 4). Its role in the function of chromatin is unknown, but it has been suggested to be related to DNA replication (5, 6), DNA repair (7 - 9) and cell differentiation (10, 11).

To obtain information on the roles of poly(ADP-ribosyl)ation of chromosomal proteins in the structure and function of chromatin, we investigated its correlation with events in the cell cycle of HeLa S3 cells. Previously we found that the level of poly(ADP-ribosyl)ation, assayed with a nuclear system, is highest in the late G2 - M phase (12, 13) and that of the histones examined only H1 histone is a specific acceptor for poly(ADP-ribose) (14). These findings suggest that the modification is involved in the mechanism of chromosomal condensation. We also showed (12) that isolated metaphase chromosomes from HeLa S3 cells contain poly(ADP-ribose) polymerase, but we did not examine whether they contain poly(ADP-ribose) glycohydrolase. Since the metabolism of poly(ADP-ribose) depends mainly on these two enzymes, it seemed important to know whether poly(ADP-ribose) glycohydrolase is present in mitotic chromosomes.

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In this work we examined whether metaphase-arrested HeLa S3 cell chromosomes contain poly(ADP-ribose) glycohydrolase. Results showed that they do contain this enzyme in an active form.

Materials and Methods

Materials —— [Adenosine-2,8-^{3}H]NAD (3.4 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. Hydroxyurea, 3',5'-cyclic GMP and ADP-ribose was purchased from Sigma Chemical Co. St Louis, Mo. 3',5'-Cyclic AMP was obtained from Kohjin Co., Ltd. Japan. 5'-AMP, ADP, ATP, NAD, calf intestine alkaline phosphatase and snake venom phosphodiesterase were purchased from Boehringer Mannheim GmbH, Mannheim, W. Germany. Cellulose thin-layer sheets were from Merck, W. Germany.

Cell Culture and Metaphase Synchronization —— HeLa S3 cells were maintained in monolayer culture in Eagle's minimal essential medium supplemented with 10% calf serum (growth medium). The cells were synchronized at the G1/S boundary with growth medium containing 1 mM hydroxyurea. The cells were then incubated in Ca^{2+},Mg^{2+}-free growth medium for 8 hr, and then for another 2 hr in the same medium with 0.03 μg/ml of Colcemid. Mitotic cells were collected by selective detachment from monolayers. The mitotic index of the cells collected was more than 95% (12).

Preparation of Metaphase Chromosomes —— HeLa S3 metaphase cells were suspended in buffer A (10 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 1 mM Na_2EDTA, 2 mM 2-mercaptoethanol and 0.1 mM phenylmethylsulfonyl-fluoride) containing 0.03% NP-40 at a concentration of 2 x 10^7/ml and allowed to swell for 7 min at 0 - 4°C. The cell suspension was homogenized with 15 strokes in a Potter-Elvehjem type Teflon-glass homogenizer. The homogenate was centrifuged at 800 x g for 5 min to precipitate metaphase chromosomes, and these were washed twice with buffer A with centrifugation as before (12).

Assay of poly(ADP-ribosyl)ation —— The standard reaction mixture consisted of 10 mM $MgCl_2$, 3 mM 2-mercaptoethanol, 82.5 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.5 mM [^{3}H]NAD (0.5 μCi/μmol) and metaphase chromosomes. Incubation was carried out at 25°C, and the reaction was stopped by adding cold trichloroacetic acid (TCA) to a final concentration of 10%. The amount of radioactivity incorporated into acid-insoluble material was measured in a liquid scintillation spectrometer (12, 13).

Assay of poly(ADP-ribose) Glycohydrolase —— The standard reaction mixture consisted of 50 mM potassium phosphate (pH 7.5), 10 mM KF, 10 mM 2-mercaptoethanol, 100 μg/ml of bovine serum albumin, and ^{3}H-labeled poly(ADP-ribosyl)ated metaphase chromosomes equivalent to 1 x 10^6 nuclei. Incubation was carried out at 37°C. The reaction was stopped by adding cold TCA to a final concentration of 5% for determination of acid-soluble radioactivity or by boiling the mixture for 3 min for identification of the hydrolytic product. Acid-soluble material was separated by centrifugation at 3,000 r.p.m. for 10 min for radioassay.

Thin-Layer Chromatography —— The hydrolytic product was examined by cellulose thin-layer chromatography. The reaction mixture with added markers, ADP-ribose and AMP, was spotted on a cellulose thin-layer plate and developed with solvent 2, consisting of 0.1 M sodium phosphate buffer (pH 6.8), ammonium sulfate and *n*-propanol (100:60:2, v/w/v) at room temperature (15). The thin-layer plate was cut into strips and radioactivity was counted in a liquid scintillation spectrometer.

Results

Characterization of Poly(ADP-ribose) Hydrolytic Activity in Metaphase Chromosomes —— We developed the " chromatin system " described in the Materials and Methods to measure enzymatic activity for hydrolysis of poly(ADP-ribose) in metaphase chromosomes of HeLa S3 cells. With this chromatin system, hydrolytic enzymes present in metaphase chromosomes are detected by measuring acid-soluble radioactivity liberated from chromosome-bound prelabeled poly(ADP-ribose) (average chain length 14 Fig. 2A). The optimum conditions for the hydrolysis of poly(ADP-ribose) in metaphase chromosomes of HeLa S3 cells were similar to those for partially purified calf thymus poly(ADP-ribose) glycohydrolase (16), which was assayed with labeled poly(ADP-ribose) free from proteins by digestion with pronase. In our chromatin system, the hydrolytic activity was low in This-HCl buffer than in potassium phosphate buffer, but the optimum pH was about 7.5. The activity exhibited a requirement for sulfhydryl groups and was maximal with 10 mM 2-mercaptoethanol or dithiothreitol. Neither monovalent (Na^+, K^+) nor divalent (Ca^{2+}, Mg^{2+}) cations were required for the activity. KF, a potent inhibitor of phosphodiesterase , which hydrolyzes the pyrophosphate bonds of poly(ADP-ribose), had no inhibitory effect at a concentration of 10 mM.

Effect of Nucleotides —— As shown in table 1, ADP-ribose, which is a product of poly(ADP-ribose) glycohydrolase, inhibited the hydrolytic activity 30 and 79% at concentrations of 1 and 10 mM, respectively. Cyclic AMP at concentrations of 1 and 10 mM inhibited the activity 17 and 59%, respectively, but cyclic GMP had little effect at the same concentrations. NAD at concentrations of 1 and 10 mM caused 11 and 29% inhibition, respectively. ADP and ATP were slightly inhibitory at a concentration of 10 mM.

Analysis of the Hydrolytic Product —— The hydrolytic product of poly(ADP-ribose) in metaphase chromosomes was analyzed by chromatography on a cellulose thin-layer plate (Fig. 2). With solvent 2 as developing solvent, ADP-ribose is separated from phosphoribosyl-AMP, which is

Table I

Effect of Nucleotides on Hydrolysis of Poly(ADP-ribose) Bound to Metaphase Chromosomes

Compound	(mM)	% Inhibition
ADP-ribose	1	30.4
	10	78.5
3',5'-cyclic AMP	1	17.2
	10	59.2
3',5'-cyclic GMP	1	5.2
	10	8.6
ADP	1	3.1
	10	12.5
ATP	1	6.1
	10	17.2
NAD	1	10.7
	10	29.0

Hydrolysis of poly(ADP-ribose) bound to metaphase chromosomes was assayed as described under Materials and Methods. The indicated amounts of nucleotides were added to the reaction mixture. Incubation was carried out at 37°C for 20 min.

produced from poly(ADP-ribose) by the action of phosphodiesterase (15). As shown in Fig. 2B, the radioactivity appeared in the position of ADP-ribose and at the origin. In this solvent system, unhydrolyzed poly(ADP-ribose) and oligo(ADP-ribose) with a chain length of more than 5 ADP-ribose residues remained at the origin. The absence of oligo-(ADP-ribose) suggests that the digestion is exoglycosidic.

The hydrolytic product was examined further by treating it with alkaline phosphatase or snake venom phosphodiesterase. If the product is ADP-ribose, it should be digested to 5'-AMP and ribosyl-phosphate by phosphodiesterase but not by alkaline phosphatase. On the other hand, if the product is phosphoribosyl-AMP, it should be digested to ribosyl-adenosine and/or ribosyl-AMP by alkaline phosphatase but not by phosphodiesterase. The position on the chromatogram of the hydrolytic product was not altered by the treatment of alkaline phosphatase (Fig. 2C), but when the reaction product was then treated with phosphodiesterase, two radioactive peaks were found on the chromatogram which migrated to

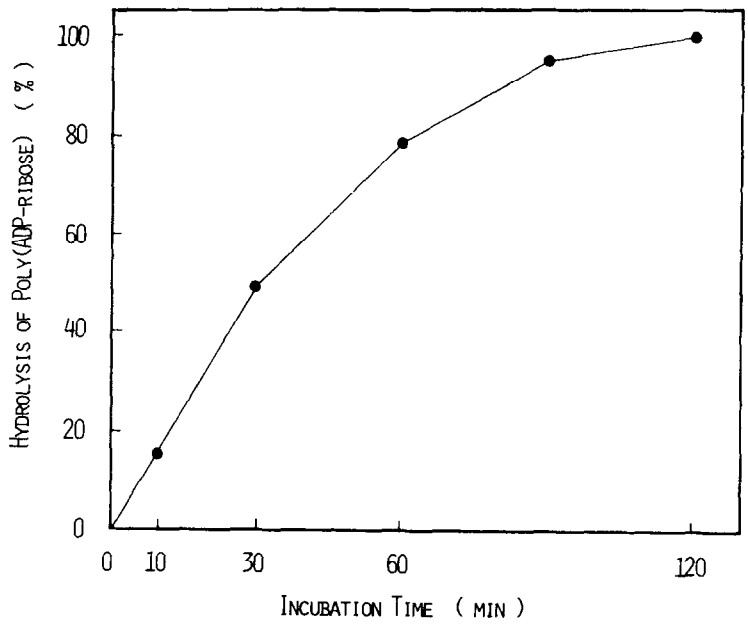

Figure 1. Time course of hydrolysis of poly(ADP-ribose) bound to metaphase chromosomes. Hydrolysis was determined by the measuring acid-soluble radioactivity as described under Materials and Methods.

the positions of 5'-AMP and phosphoribosyl-AMP (Fig. 2D). Judging from the amounts of radioactivity in these positions, these products formed by digestion of ADP-ribose and unhydrolyzed poly(ADP-ribose), and unhydrolyzed poly(ADP-ribose), respectively.

Discussion

The present results demonstrated that poly(ADP-ribose) glycohydrolase is present in an active form in metaphase chromosomes of HeLa S3 cells. The optimum conditions for hydrolysis of metaphase chromosome-bound poly(ADP-ribose) by the glycohydrolase were similar to those for partially purified calf thymus poly(ADP-ribose) glycohydrolase (16). Cyclic AMP inhibited the glycohydrolase activity and its inhibition seemed to be specific, since cyclic GMP and other related nucleotides had essentially no effect. Cyclic AMP may have some role in regulation of poly(ADP-ribose) glycohydrolase. ADP-ribose may cause product inhibition. These findings are consistent with the observations on cytoplasmic poly(ADP-ribose) glycohydrolase activity in rat liver (17,

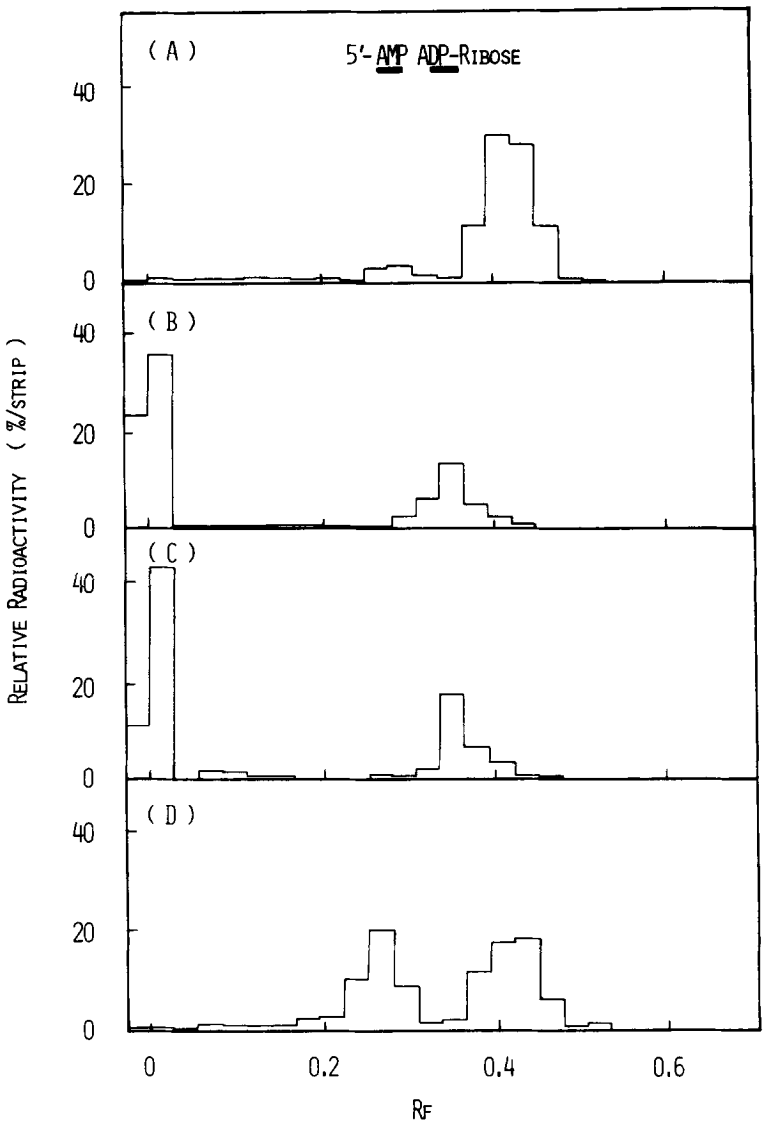

Figure 2. Thin-layer chromatograms of hydrolytic products. ^{3}H-Labeled poly(ADP-ribose) bound to metaphase chromosomes, which had been incubated in boiling water for 3 min, were digested with snake venom phosphodiesterase (25 µg/ml) for 1 hr at 37°C (A). The standard reaction mixture described under Materials and Methods was incubated for 2 hr at 37°C. The reaction was stopped by immersing the tubes in boiling water for 3 min (B). Parallel reaction mixtures were treated in the same manner and, after stopping the reaction, the mixtures were further treated with alkaline phosphatase (25 µg/ml) (C) and venom phosphodiesterase (25 µg/ml) (D) for 1 hr at 37°C. An aliquot of each reaction mixture was applied to a cellulose thin-layer plate and developed with solvent 2.

18), LS cells (19) calf thymus (16) and rat testis (20), and they imply that metaphase chromosomes contain poly(ADP-ribose) glycohydrolase activity. Analysis of the hydrolytic product by treating

it with phosphodiesterase or alkaline phosphatase showed that it was entirely ADP-ribose, again indicating the presence of poly(ADP-ribose) glycohydrolase in metaphase chromosomes. These results also show that hydrolysis of poly(ADP-ribose) by glycohydrolase is exoglycosidic. This enzyme seems to be an integral part of metaphase chromosomes; it is not merely a contaminant derived from the cytoplasm.

We have shown (12) that the level of poly(ADP-ribosyl)ation increases in the G2 - M phase of the cell cycle of HeLa S3 cells and decreases in the G1 phase. On immunofluorescent staining of poly(ADP-ribose) *in situ* in synchronously growing HeLa S3 cells, the nuclei of G2 - M phase cells stained most strongly for poly(ADP-ribose) (21, unpublished results). These observations suggest that poly(ADP-ribosyl)ation of chromosomal proteins is involved in structural changes during chromosomal condensation before cell division . The exact role of poly(ADP-ribose) glycohydrolase in metaphase chromosomes is unknown. It may be involved in extension of condensed chromosomes. The interrelation between this enzymes and poly(ADP-ribose) polymerase, and their physiological roles in the structure and function of chromatin remain to be elucidated. The present studies should serve as a basis for more detailed investigations on the biological significance of poly(ADP-ribosyl)ation of chromosomal proteins.

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