Poly-ADP-ribosylated histones: potent DNA suppressors

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Received 12 August 1985

When rat liver nuclear chromatin was sonicated in buffer containing 0.35 M $(NH_4)_2SO_4$ to release the engaged RNA polymerases, a potent inhibitor was also released. This inhibitor elicited dramatic inhibition of RNA synthesis regardless of whether the free or engaged RNA polymerase was used. On further analysis, it became apparent that the site of inhibition was on the DNA template, not on the enzyme. This inhibitor could be extracted into 0.25 N HCl by the standard procedure for the isolation of histones. This acid-soluble inhibitor, showing typical histone band on gel, was RNase A and DNase I resistant, but was sensitive to both pronase and snake venom phosphodiesterase digestion, as well as to 0.1 N KOH hydrolysis. Furthermore, when [^{14}C]adenine labeled poly-ADP-ribosylated histones were digested by snake venom phosphodiesterase, the release of radioactivity was in parallel to the loss of inhibitor activity. We conclude that the inhibitor substances are poly-ADP-ribosylated histones and propose that the poly-ADP-ribosylated histones rather than the histones are the natural suppressors of the gene.

Poly(ADP-ribose) Histone RNA polymerase RNA synthesis Snake venom phosphodiesterase Gene regulation

1. INTRODUCTION

Mammalian RNA polymerases are known to exist in the cell nucleus in 2 functional states, referred to as free and engaged RNA polymerases [1]. The procedure to isolate the free RNA polymerases requires the isolation of the nuclei in hypertonic sucrose, followed by extraction of the free enzyme with isotonic medium [2]. The release of the engaged RNA polymerases, on the other hand, is achieved by sonication of nuclear chromatin in buffer containing 0.35 M $(NH_4)_2SO_4$ [3]. A few years ago, we noticed that a potent inhibitor of the RNA polymerases was released from the chromatin together with the solubilized engaged enzyme under high salt sonication [4]. This inhibitor could be separated from the engaged enzyme after reduction of the $(NH_4)_2SO_4$ concentration to 50 mM followed by high-speed centrifugation [4]. The characterization of this inhibitor has been elusive. We now present experimental evidence showing that the principal inhibitor substances are poly-ADP-ribosylated histones. The fundamental property of this inhibitor is that it requires both the histone and the poly(ADP-ribose) components with the chemical linkages between them intact in order to function. This finding strongly suggests that the poly-ADP-ribosylated histones rather than the histones per se are the natural suppressors of the gene.

2. MATERIALS AND METHODS

2.1. *Materials*

Bovine pancreas RNase A (Boehringer Mannheim) was dissolved in H_2O to 1 mg/ml before use. Bovine pancreas DNase I (Cappel/Worthington) was dissolved in 0.15 M NaCl to 1 mg/ml for use. Pronase (protease from *Streptomyces griseus*) (Sigma) was dissolved in TGMEM buffer containing 50 mM $(NH_4)_2SO_4$ to 1 mg/ml and was self-digested for 2 h at 37°C. Snake venom phosphodiesterase (*Crotalus adamanteus* venom) (Pharmacia) was dissolved in

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00145793/85/$3.30

0.1 M Tris-HCl, pH 8.0, 50 mM $MgCl_2$ to 100 units/ml for use.

2.2. *Isolation of the RNA polymerase inhibitor from rat liver nuclear chromatin*

Sprague-Dawley rat liver nuclei were isolated by the hypertonic sucrose method as in [2]. A nuclear suspension of 12 ml in 0.34 M sucrose (12 g original liver) was homogenized at 600 rpm for 3 up and down strokes in a glass homogenizer and centrifuged at 3000 × *g* for 10 min to extract the free RNA polymerase [2]. The nuclear pellet was suspended in 12 ml of 0.01 M Tris-HCl buffer, pH 7.9, containing 1 M sucrose, 5 mM $MgCl_2$ and 20 mM 2-mercaptoethanol. After mixing with 1/10 vol. of 3.5 M $(NH_4)_2SO_4$ (pH 7.9), the nuclear suspension was then sonicated to solubilize the engaged RNA polymerase according to Roeder and Rutter [3]. The sonicated nuclear suspension was diluted with 2 vols of TGMEM buffer (0.05 M Tris-HCl, pH 7.9, 25% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 20 mM mercaptoethanol), mixed and centrifuged at 105000 × *g* for 1 h at 4°C. The supernatant was adjusted by adding solid $(NH_4)_2SO_4$ to a final concentration of 0.42 g/ml, stirred in the cold room for 30 min, and then centrifuged again at 105000 × *g* for 1 h at 4°C. The precipitate was dissolved in 2 ml TGMEM buffer and dialyzed against TGMEM buffer containing 50 mM $(NH_4)_2SO_4$ at 4°C overnight (17 h) with several changes of the buffer. A fine precipitate was formed during the dialysis and it was collected by centrifugation at 105000 × *g* for 1 h. The supernatant was the solubilized engaged RNA polymerase. The precipitate, which contained the inhibitor, was resuspended by homogenization in 2.0 ml TGMEM buffer containing 50 mM $(NH_4)_2SO_4$. The yield was about 3.5–4.5 mg protein per ml.

2.3. *Assay of RNA polymerase inhibitor activity*

The inhibitor activity was measured under conditions where RNA polymerase activity is normally assayed [5]. As indicated in fig.1, various amounts of inhibitor (1 mg/ml) in volumes of 5–50 μl with compensatory volumes of TGMEM buffer were added to 0.2 ml RNA polymerase assay medium [20 μmol Tris-HCl (pH 7.9 at 23°C), 0.4 μmol $MnCl_2$, 5.6 μmol 2-mercaptoethanol, 10 μmol $(NH_4)_2SO_4$, 0.04 μmol each of ATP, GTP, UTP and CTP, 1.0 μCi [8-^{3}H]GTP and 2.5 μg poly(dI-dC)]. Then, the engaged enzyme or the free enzyme in volumes of 50 or 100 μl, respectively, was added to start the reaction. Tubes were incubated in a water bath at 37°C for 20 min with shaking. The reaction was stopped by adding 4.0 ml of 10% trichloroacetic acid containing 1% sodium pyrophosphate. After sitting in ice for 20 min, the acid-insoluble material was collected on Whatman GF/C filters and washed 7-times with 5 ml of 5% trichloroacetic acid containing 1% sodium pyrophosphate and twice with 5 ml cold 60% ethanol. After drying, the filters were counted in 5 ml of Brays' solution [5].

For the studies presented in tables 1–3, the basic assay procedures were the same as described in fig.1 with two exceptions. In one case (table 1) an assay medium containing either 2.5 or 25 μg poly(dI-dC) was used. In another case (tables 2 and 3), 10 μg in 50 μl buffer of the acid-soluble inhibitor or the commercial calf thymus histone sample was first treated with various agents as indicated, followed by boiling for 10 min at 100°C before assaying for RNA polymerase activity.

2.4. *Preparation of acid soluble inhibitor by 0.25 N HCl extraction*

The standard procedure for the extraction of histones was used [6,7]. The extraction was carried out in 0.25 N HCl with mechanical stirring at 4°C for 17 h. It was then centrifuged at 12000 × *g* for 10 min. The precipitate was re-extracted with 0.25 N HCl for 10 min and centrifuged as before. The combined supernatant constituted the acid-soluble inhibitor fraction. The inhibitor was dialyzed against TGMEM buffer containing 50 mM $(NH_4)_2SO_4$ overnight before use. However, for snake venom phosphodiesterase digestion and KOH hydrolysis, the inhibitor was first precipitated with 2 vols alcohol and then resuspended in buffer B (0.05 M Tris-HCl, pH 7.9, 5 mM $MgCl_2$) before use.

2.5. *Polyacrylamide gel electrophoresis of acid-soluble inhibitor*

Gel electrophoresis of both the acid-soluble inhibitor and the standard calf-thymus nuclear histones was carried out according to Alfageme et al. [8]. The gel contained 12% acrylamide, 0.08% methylenebisacrylamide, 5% glacial acetic acid,

0.5% *N,N,N',N'*-tetramethylethylenediamide, 0.06% ammonium persulfate, 6 M urea and 0.38% Triton DF-16. The gel was prerun at a constant voltage of 200 V until no further decrease in current (80 to 7.8 mA in 3 h). Then, 100 μl of 1 M cysteamine in 5% acetic acid was added to each of the troughs and run at 140 V for 30 min. At the end, the remaining cysteamine was removed. 50 μl (50 μg) each of the acid-soluble inhibitor and the histone standard in 5% acetic acid containing 0.02% pyronine y, 4 M urea and 4% β-mercaptoethanol were applied onto the gel and it was run at 200 V for 5 h. The electrophoresis buffer was 5% acetic acid containing 0.1% Triton DF-16. The gel was stained with 0.4% amido black in acetic acid/methanol/H_2O (1:5:16) for 17 h and destained with 7.5% acetic acid in 5% alcohol.

3. RESULTS

Fig.1 shows that the inhibitor was effective in inhibiting the activities of both free and engaged RNA polymerases, and that the inhibition was dose dependent. When 50 μg of the inhibitor was used, close to 90% inhibition was observed for both enzymes.

To determine whether this inhibition was a result of suppression of the DNA template function or of a direct inhibition of the RNA polymerase per se, the inhibitor activity was measured at both 2.5 and 25 μg poly(dI-dC) with a fixed amount of 50 μg inhibitor per assay. The results presented in table 1 clearly show that this inhibition could be overcome by the addition of an excessive amount of DNA template. Therefore, the inhibition was due to a suppression of the DNA template function.

The inhibitor could be extracted into 0.25 N HCl by the standard procedure for the isolation of histones [6,7]. Indeed, fig.2 demonstrates the inhibitor gave the typical histone bands on polyacrylamide gel [8]. Because of this, and the findings (table 2) that the inhibitor was RNase A and DNase I resistant but pronase sensitive, we initially believed the inhibitor to be histones. However, on further analysis, we found that the inhibitor was also sensitive to snake venom phosphodiesterase, a unique enzyme known to hydrolyze specifically poly(ADP-ribose) [9,10]. We therefore conclude that these inhibitor

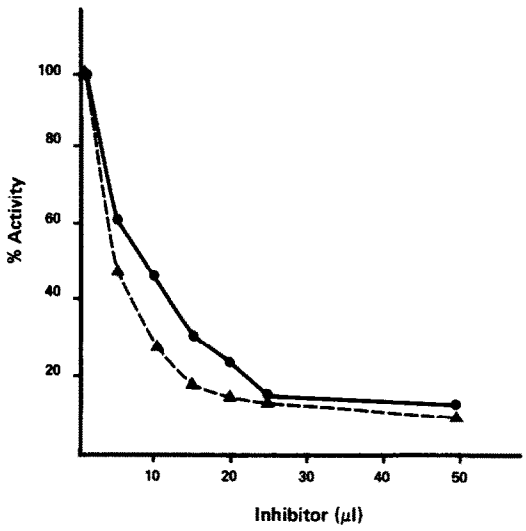

Fig.1. Dose-dependent inhibition of RNA polymerase activity by an inhibitor released from nuclear chromatin under high salt sonication. Rat liver nuclei were isolated by the hypertonic sucrose method [2]. After the free RNA polymerase was extracted [2], the nuclear pellet was resuspended in buffer containing 0.35 M $(NH_4)_2SO_4$ and sonicated according to Roeder and Rutter [3] to solubilize the engaged RNA polymerase. The RNA polymerase inhibitor was then isolated from the engaged RNA polymerase fraction after reduction of the $(NH_4)_2SO_4$ concentration to 50 mM followed by high-speed centrifugation [4]. Various amounts of inhibitor (1 mg/ml) in volumes of 5–50 μl were added to the 0.2 ml RNA assay medium [5]. The assay was carried out at 37°C for 20 min using either the free (▲) or engaged (●) RNA polymerase. For details, see section 2.

Table 1

Effect of template excess on the inhibitor activity

Group	Poly[dI-dC] (μg)	RNA polymerase activity	%
Control	2.5	5850 ± 348	100
Inhibitor	2.5	1455 ± 10	25
Control	25	8304 ± 919	100
Inhibitor	25	8740 ± 1123	105

RNA polymerase activity was measured in pmol [8-^{3}H]GMP incorporated per g liver. Values given are mean ± SE of 2 separate experiments

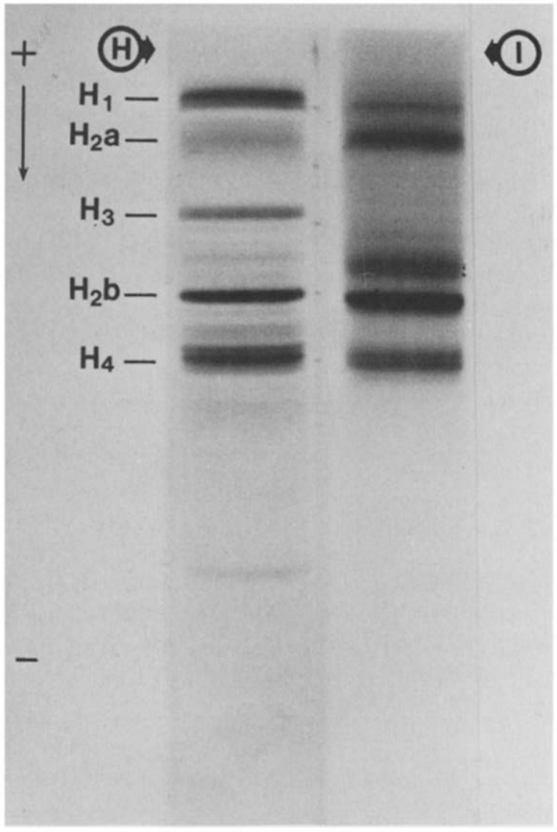

Fig.2. Polyacrylamide gel electrophoresis of the 0.25 N HCl soluble RNA polymerase inhibitor. Gel electrophoresis of the acid-soluble inhibitor along with the standard calf thymus nuclear histones was carried out according to Alfageme et al. [8]. The gel contained 12% acrylamide, 0.08% methylenebisacrylamide, 5% glacial acetic acid, 0.5% *N,N,N′,N′*-tetramethylethylenediamide, 0.06% ammonium persulfate, 6 M urea and 0.38% Triton DF-16. 50 μg of each sample was used. The electrophoresis buffer was 5% acetic acid containing 0.1% of Triton DF-16. The run was at 200 V for 5 h. The gel was stained with 0.4% amido black in acetic acid/methanol/H_2O (1:5:16) and destained with 7.5% acetic acid in 5% alcohol. (I) Acid soluble inhibitor, (H) standard histones.

substances are not histones, but rather poly-ADP-ribosylated histones.

Furthermore, since this inhibitor activity could be largely (70%) abolished by either pronase or snake venom phosphodiesterase treatment (table 2), these results strongly suggest that intact poly-ADP-ribosylated histone molecules are required

Table 2

Studies on the nature of the 0.25 N HCl soluble RNA polymerase inhibitor

Conditions	RNA polymerase activity	%
Control	6582 ± 268	100
0.25 N HCl soluble inhibition (10 μg)	56 ± 14	0.9
+ RNase A (10 μg), 37°C, 30 min	33 ± 8	0.5
+ DNase I (10 μg), 37°C, 30 min	941 ± 94	14
+ pronase (10 μg), 37°C, 2 h	4758 ± 130	72
+ snake venom phosphodiesterase (2.0 units), 37°C, 2 h	4549 ± 75	69
+ KOH (0.1 N), 100°C, 10 min	6767 ± 421	103

RNA polymerase activity was measured in pmol [8-^{3}H]GMP incorporated per g liver. Values given are mean ± SE of 2–4 separate experiments

for this inhibition. This interpretation is further supported by the finding that the inhibitor activity was totally abolished after it was boiled in 0.1 N KOH for 10 min. It is known that while both histones and poly(ADP-ribose) are alkaline stable, the chemical bonds between them are alkaline-labile ester linkages [9,10].

In view of the fact that histones are normally prepared by dilute acid extraction, this study suggests the possibility that the early reports investigating the suppressor roles of histones might have measured the effects not of histones but of poly-ADP-ribosylated histones. The data presented in table 3 clearly show that the commercial histone sample was as potent an inhibitor for RNA synthesis as our 0.25 N HCl soluble inhibitor preparation (table 2). Since it was also sensitive to snake venom phosphodiesterase digestion and to 0.1 N KOH hydrolysis, we believe that these 'histones' are in fact poly-ADP-ribosylated histones. Furthermore, these results (table 3) should also erase any doubt that the 0.25 N HCl soluble inhibitor might be the unknown band (between H3 and H2B) on the polyacrylamide gel (fig.2).

Table 3

Properties of commercial 'histone' as inhibitor of DNA-dependent RNA synthesis

Conditions	RNA polymerase activity	%
Control	6494 ± 146	100
Commercial 'histone' (10 μg)	33 ± 15	0.5
+ RNase A (10 μg), 37°C, 30 min	24 ± 9	0.4
+ DNase I (10 μg), 37°C, 30 min	28 ± 5	0.4
+ pronase (10 μg), 37°C, 2 h	6352 ± 574	98
+ snake venom phosphodiesterase (2.0 units), 37°C, 2 h	3888 ± 190	60
+ KOH (0.1 N), 100°C, 10 min	6703 ± 284	103

RNA polymerase activity was measured in pmol [8-^{3}H]GMP incorporated per g liver. Values given are mean ± SE of 2 separate experiments

To prove directly that indeed poly-ADP-ribosylated histones rather than histones were responsible for the observed inhibition and that the release of inhibition was due to the digestion of poly(ADP-ribose) moiety by snake venom phosphodiesterase, radiolabeled poly-ADP-ribosylated histones were prepared using [U-^{14}C]adenine according to Nishizuka et al. [15]. Gel electrophoresis of this radiolabeled poly-ADP-ribosylated histones showed identical band patterns to the unlabeled 0.25 N HCl soluble inhibitor isolated from chromatin under high salt sonication (not shown). As shown in table 4, when 10 μg of this labeled poly-ADP-ribosylated histones was used, it produced 98% inhibition. This inhibition was stable after 2 h incubation in the absence of snake venom phosphodiesterase, and the inhibitor activity was almost totally lost when incubated for 2 h with snake venom phosphodiesterase. In parallel, the radioactive label was quite stable in the absence of snake venom phosphodiesterase and was 80% released after the enzyme treatment.

Table 4

Direct evidence for the requirement of poly-ADP-ribose moiety in the poly-ADP-ribosylated histone inhibition of DNA-dependent RNA synthesis

Conditions	RNA polymerase activity	%	Radioactivity	%
Control	3937 ± 461	100		
+ [^{14}C]adenine-labeled poly-ADP-ribosylated histones (10 μg), zero time preincubation	94 ± 22	2	11808 ± 195	100
+ [^{14}C]adenine-labeled poly-ADP-ribosylated histones (10 μg), 2 h preincubation	51 ± 9	1	10630 ± 416	90
+ [^{14}C]adenine-labeled poly-ADP-ribosylated histones (10 μg), 2 h incubation in the presence of snake venom phosphodiesterase (2.0 units)	3771 ± 141	96	2334 ± 163	20

RNA polymerase activity was measured in pmol [8-^{3}H]GMP incorporated per g liver. Radioactivity was expressed in pmol [^{14}C]adenine-labeled poly-ADP-ribosylated histones per mg protein. Values given are mean ± SE of 2 separate experiments

4. DISCUSSION

Poly(ADP-ribose) was discovered 20 years ago [11–13]. The phenomenon of ADP-ribosylation of various cellular proteins, including histones, is well known [9,10,14]. However, the physiological significance of the protein ADP-ribosylation is unclear. In view of their general occurrence in the cell, ADP-ribosylated proteins are thought to play an important role in the regulation of various cellular functions [10]. The present finding is important for at least 2 reasons. Firstly, nearly 20 years after their discovery [10,15–18], a possible biological role of the ADP-ribosylated histones is finally identified. Secondly, since the discovery of the nucleosome [19,20] and the finding that actively transcribed gene sequences are also found in the nucleosomal histone core [21,22], the classical concept that histones are gene suppressors [23,24] clearly requires modification. The finding that ADP-ribosylated histones rather than histones are the suppressors of the gene is therefore most timely, and it is conceivable that this new concept should make significant contributions toward the final understanding of gene regulation.

ACKNOWLEDGEMENTS

I would like to thank Irmanely Geronimo and Wanda Bender for their expert technical help, Dr T.G. Yap for the early part of the work, and Drs George R. Pack and B. Salafsky for critical reading of this manuscript. This investigation was supported by PHS grant number CA-30093 awarded by the National Cancer Institute, DHHS.

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