Poly(ADP-ribose) Has a Branched Structure in Vivo*

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We have searched for the presence of branching in the chromosomal polymer poly(ADP-ribose) as it occurs in vivo. Treatment of the polymer with phosphodiesterase and phosphomonoesterase results in the conversion of internal residues to the nucleoside ribosyladenosine and the conversion of points on branching to diribosyladenosine. We have detected diribosyladenosine in digests of the polymer derived from carcinogen-treated SV40 virus-forming 3T3 cells and in normal rat liver, kidney, and spleen. The frequency of residues involved in branching varied from 0.8 to 1.6 mole % over a 50-fold range of total levels of poly(ADP-ribose). Thus, branching seems to be a general feature of poly(ADP-ribose) as it occurs in vivo.

Poly(ADP-ribose) is a protein-bound chromosomal polymer whose cellular function is not well established (1, 2). Several lines of evidence suggest that it may play a role in DNA repair (3-7), cell growth regulation (8-12), and cell differentiation (13). Poly(ADP-ribose) has structural characteristics distinct from those of other polynucleotides including pyrophosphate linkages and $\alpha(1' \rightarrow 2')$ ribose-ribose glycosidic linkages. Recently, it was concluded that the polymer synthesized in vitro utilizing rat liver nucleus preparations has a branched structure (14, 15). This conclusion was based on the identification in phosphodiesterase digests of the polymer of the nucleotide $O-\alpha-D$-ribofuranosyl(1' $\rightarrow 2')$-$O-\alpha-D$-ribofuranosyl(1' $\rightarrow 2'$)-adenosine-5'-5'-$\beta$-triphosphate or Ado(P)-Rib(P)-Rib-P (see Fig. 1). We now provide evidence for the existence of a branched structure in vivo. Furthermore, we provide evidence that a branched structure is a general feature of poly(ADP-ribose) as it occurs in intact cells.

**EXPERIMENTAL PROCEDURES**

Preparation of $\epsilon$-Diribosyladenosine—Authentic Ado(P)-Rib(P)-Rib-P was kindly provided by Dr. T. Sugimura, National Cancer Center Research Institute, Tokyo, Japan. Diribosyladenosine was obtained by incubating 250 pmol of Ado(P)-Rib(P)-Rib-P with 6 units of bacterial alkaline phosphatase type IIIS (Sigma) in 30 mm Tris-HCl, pH 7.4 (300 $\mu$l, final incubation volume) for 3 h at 37 °C. At the end of this period, protein was precipitated by adjusting the reaction mixture to 20% (v/v) trichloroacetic acid. After centrifugation at 800 $\times$ g for 10 min, the supernatant was extracted 5 times with an equal volume of diethyl ether. The resulting solution containing diribosyladenosine was then adjusted to 200 mm sodium citrate buffer, pH 4.5, 20 mm chloroacetaldehyde. The fluorescent derivative 1-N-ethyldiribosyladenosine (epsilon-diribosyladenosine) was obtained by incubating this mixture at 30 °C for 8 h. Excess chloroacetaldehyde was removed by extraction 5 times with an equal volume of diethyl ether. This preparation was used as the epsilon-diribosyladenosine standard.

Analysis of SVT2 Cells—SVT2 cells were grown as described previously (16). Ten tissue culture dishes (55 mm) containing 3.5 $\times$ 10^6 cells/dish were treated with 50 ng/ml MNNG for 20 min. Medium was removed and cells were rapidly washed on the dish with 10 ml of cold phosphate-buffered physiological saline followed by the addition of 5 ml of ice-cold 20% trichloroacetic acid. Dishes were kept on ice for 15 min and the resulting precipitate was collected by centrifugation at 800 $\times$ g for 10 min and washed twice with 20% trichloroacetic acid and once with diethyl ether. The pellet was dissolved in 50 ml of 0.1 M potassium phosphate buffer, pH 5.0, containing 5 mm guanidine-HCl. This solution was divided into 5 aliquots of 10 ml each containing an amount of material equivalent to 7.0$\times$10^6 cells. All 5 aliquots were subjected to our procedure for poly(ADP-ribose) determination (17) with modifications indicated below. One aliquot was spiked with 50,000 cpm of [14C]poly(ADP-ribose) synthesized in permeabilized cells to determine recovery. Recovery yields were approximately 65%. Adenosine deaminase treatment was accomplished as follows. The dihydroxyboryl column eluate (2 ml) was concentrated in vacuo to 100 $\mu$l of 100 mm Tris-HCl, pH 7.4, and 130 units of adenosine deaminase from calf intestine (Type 1, Sigma). After incubation, protein was precipitated by adjusting to 20% trichloroacetic acid. After centrifugation, the supernatant was extracted 5 times with an equal volume of diethyl ether. The sample was adjusted to 1 ml and made 200 mm in sodium citrate buffer, pH 4.5, incubated with chloroacetaldehyde, and prepared for chromatography as before (16). High pressure liquid chromatography was performed with a Beckman 110A liquid chromatograph equipped with an Altex Ultrasphere-ODS reversed phase column (250 mm x 4.6 mm inner diameter x 4-inch outer diameter). Fluorescence was detected with a Varian Fluorochrom Filter Fluorometer equipped with a deuterium light source. Excitation was performed using a 220-2 Varian interference filter (220 nm band pass) and a Varian 3-75 filter (370 nm cutoff) was used for emission. The sensitivity of the fluorometer was set at 20 mV full scale at the time of injection and increased to 2 mV full scale approximately 5 min after elution of epsilon-diribosyladenosine (Fig. 2, B, C, D, and E). This sensitivity change allowed the detection of epsilon-diribosyladenosine. Sample injection volume was 2 ml in all cases in 200 mm sodium citrate buffer, pH 4.5. Elution was performed isocratically at room temperature with 7 mm ammonium formate buffer, pH 5.8/100% methanol, 97:3 (v/v). The flow rate was 1.4 ml/min.

Analysis of Rat Tissues—For determination of rat tissues, 3 adult male Sprague-Dawley rats were killed by decapitation; liver, kidneys, and spleen were quickly removed, and each tissue was blended in ice-cold 20% (w/v) trichloroacetic acid using a Waring Blender. The mixture was kept cold during blending by adapting an ice water bath to the blending chamber. The resulting suspension was centrifuged at 800 $\times$ g for 20 min and the precipitate was washed twice with 20% trichloroacetic acid and once with diethyl ether. The pellet was subsequently dissolved in 100 ml of 0.1 M potassium phosphate buffer, pH 8.6, containing 0.1 M guanidine-HCl. Each sample was then divided in two aliquots of 50 ml each. One of the aliquots in each group was treated with 50,000 cpm of [14C]poly(ADP-ribose) as described above. Recovery values varied between 60 and 70% and the values shown are corrected for recovery. The remainder of the procedure for epsilon-diribosyladenosine and epsilon-diribosyladenosine determination was as described above except that it was scaled up by a factor of 10.

**RESULTS AND DISCUSSION**

Authentic Ado(P)-Rib(P)-Rib-P was subjected to digestion with bacterial alkaline phosphatase to yield $O-\alpha$-D-riboura-1. The abbreviation used is: MNNG, N-methyl-N'-nitro-N-nitosoguanidine.

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nosyl(1'' → 2'')-O-α-D-ribofuranosyl(1'' → 2')adenosine which we will refer to here as diribosyladenosine. Diribosyladenosine was then incubated with chloroacetaldehyde to form the fluorescent etheno derivative ε-diribosyladenosine (17). Fig. 2A shows the chromatographic elution of ε-diribosyladenosine and 4 other ethenoadenine-containing compounds including ε-ribosyladenosine, ε-deoxyadenosine, ε-adenosine, and ε-adenine as determined by fluorescence detection. Fig. 2B shows the chromatographic profile obtained from 7.0 × 10⁷ SV40 virus-transformed 3T3 cells (SVT2) which had been treated with MNNG for 20 min and then subjected to our previously published method for poly(ADP-ribose) determination (17). This method involves the conversion of all internal residues of the linear portion of poly(ADP-ribose) to ε-ribosyladenosine and would result in the conversion of branch points to ε-diribosyladenosine. It is clear that the material synthesized after MNNG treatment in vivo did show peaks of fluorescent material at the elution position of both ε-ribosyladenosine (peak 4) and ε-diribosyladenosine (peak 5). Characterization of these fluorescent compounds as ε-ribosyladenosine and ε-diribosyladenosine was accomplished by a series of control experiments.

**Fig. 1.** Structure of the nucleotide postulated to occur at the position of branch points in poly(ADP-ribose). The numbers refer to the carbon atoms of the ribose moieties.

**Fig. 2.** Detection of diribosyladenosine in MNNG-treated cells. A shows the elution position of ε-adenine (peak 1), 15 min; ε-adenosine (peak 2), 35 min; ε-deoxyadenosine (peak 3), 47 min; ε-ribosyladenosine (peak 4), 50 min; and ε-diribosyladenosine (peak 5), 73 min. B shows the fluorescence profile obtained from 7.0 × 10⁷ MNNG-treated SVT2 cells which have been subjected to the entire procedure for poly(ADP-ribose) determination (16). Peak 4 represents fluorescence equivalent to 200 pmol of ε-ribosyladenosine and peak 5 represents fluorescence equivalent to 3.2 pmol of ε-diribosyladenosine. C, D, and E show the fluorescence profile obtained in each case from 7.0 × 10⁷ cells subjected to the same treatment as in B except that chloroacetaldehyde (C) or snake venom phosphodiesterase (D) treatment had been omitted or the sample had been treated with adenosine deaminase prior to treatment with chloroacetaldehyde (E).
Branching in Poly(ADP-ribose)

levels of poly(ADP-ribose) in SVT2 cells and rat tissues

Poly(ADP-ribose) was measured as described under “Experimental Procedures.” The values shown represent the mean of duplicate determinations that agreed within 10% of the mean.

| Total Residues | Ribosyladenosine plus diribosyladenosine | Diribosyladenosine |
|----------------|------------------------------------------|-------------------|
| MNNG-treated SVT2 cells | 423 pmol/mg DNA | 0.16 mole % |
| Rat liver | 14 pmol/mg DNA | 0.8 mole % |
| Rat kidney | 18 pmol/mg DNA | 1.1 mole % |
| Rat spleen | 8.0 pmol/mg DNA | 1.6 mole % |

The presence of branching in poly(ADP-ribose) raises a number of interesting questions related to the structure of this polymer. We do not know the percentage of chains that are branched or the location of branch points within a chain. Likewise, it will be of interest to determine whether the branch points are sites of branching of a single residue or are sites for the synthesis of long branches of polymer. The branching of poly(ADP-ribose) may play a role in the regulation of synthesis and/or degradation of the polymer. Alternatively, branching may be required to effect alterations in chromatin structure associated with one or more of the postulated functions of this polymer.

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REFERENCES

1. Hilz, H., and Stone, P. R. (1976) Rev. Physiol. Biochem. Pharmacol. 76, 1–59
2. Hayashi, O., and Ueda, K. (1977) Annu. Rev. Biochem. 46, 95–116
3. Juarez-Salinas, H., Sim, J. I., and Jacobson, M. K. (1979) Nature 282, 740–741
4. Durkacz, B. W., Omidiqi, O., Gray, D. S., and Shall, S. (1980) Nature 283, 593–596
5. Smulson, M. E., Schein, P., Mullins, D. W., Jr., and Sudhaker, S. (1977) Cancer Res. 37, 3006–3012
6. McCurry, L. S., and Jacobson, M. K. (1980) J. Biol. Chem. 255, 551–553
7. Berger, N. A., Sikorski, G. W., Petzold, S. J., and Kurohara, K. K. (1980) Biochemistry 19, 289–293
8. Miwa, M., Oda, K., Segawa, K., Tanaka, M., Irie, S., Yamaguchi, N., Kuchino, T., Shiromi, K., Shimoo, H., Sakurai, M., Matsushita, T., and Sugimura, T. (1977) Arch. Biochem. Biophys. 181, 313–321
9. Stone, P. R., and Shall, S. (1975) Exp. Cell Res. 91, 95–100
10. Berger, N. A., Weber, G., Kaichi, A. S., and Petzold, S. J. (1978) Biochim. Biophys. Acta 519, 105–117
11. Berger, N. A., Petzold, S. F., and Berger, S. J. (1979) Biochim. Biophys. Acta 564, 90–104
12. Smulson, M. E., and Rideau, C. (1972) Biochim. Biophys. Acta 272, 408–416
13. Caplan, A. I., and Rosenberg, M. J. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 1852–1857
14. Miwa, M., Saikawa, N., Yamaiumi, Z., Nishimura, S., and Sugimura, T. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 595–599
15. Miwa, M., Ishihara, M., Takishima, S., Takasuka, N., Maeda, M., Yamaiumi, Z., and Sugimura, T. (1981) J. Biol. Chem. 256, 2919–2921
16. Jacobson, E. L., and Jacobson, M. K. (1976) Arch. Biochem. Biophys. 175, 627–634
17. Sims, J. L., Juarez-Salinas, H., and Jacobson, M. K. (1980) Anal. Biochem. 106, 296–306

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3 M. K. Jacobson, H. Juarez-Salinas, and J. E. Moss, unpublished.
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