Monoclonal Antibodies to Poly(adenosine diphosphate ribose) Recognize Different Structures†

Hisae Kawamitsu, Hiro-o Hoshino, Hidechika Okada, Masanao Miwa,* Hironao Momoi, and Takashi Sugimura

ABSTRACT: Two hybridomas producing monoclonal antibodies to poly(adenosine diphosphate ribose) [poly(ADP-Rib)] were established. One antibody, 10H (IgG3,κ), bound to most of the poly(ADP-Rib) preparation, which consisted of molecules of various sizes of more than 20 ADP-Rib residues. The binding of this antibody was inhibited by not only poly(ADP-Rib) but also a monomer unit of poly(ADP-Rib), Ado(P)-Rib-P. The sites protected by antibody 10H were isolated and analyzed by hydrolysis with alkaline phosphomonoesterase and then snake venom phosphodiesterase. The sites contained the same amounts of monomer units and branched portions [Ado(P)-Rib(P)-Rib-P] as the original poly(ADP-Rib) molecules but a lower average number of branched portions per molecule than in the original molecules. The other antibody, 16B (IgM,λ), reacted with only 50% of the radioactive poly(ADP-Rib), and its binding was not inhibited by a monomer unit. This antibody protected 25% of all the poly(ADP-Rib) molecules from hydrolysis by snake venom phosphodiesterase. The protected sites contained twice as many branched portions per molecule as the original poly(ADP-Rib) molecules. These results show that the two monoclonal antibodies recognize different structures of poly(ADP-Rib); 10H antibody recognizes the linear structure with ribose–ribose linkages, and 16B antibody may recognize specific structures, including the branched portions of poly(ADP-Rib).

Poly(ADP-Rib)[1] is synthesized from NAD^+ by poly(ADP-Rib) polymerase in the nuclei of eukaryotes (Sugimura, 1973; Hilz & Stone, 1976; Hayaishi & Ueda, 1977; Purnell et al., 1980; Mandel et al., 1982). It has been suggested to have important biological functions, such as in repair of damaged DNA (Smulson, 1977; Juarez-Salinas et al., 1979; Durkacz et al., 1980; Creissen & Shall, 1982), cell differentiation (Tereda et al., 1979; Kanai et al., 1982b), or cell transformation (Miwa et al., 1977), but its exact functions are still unknown. This polymer has long been supposed to have a linear structure of ADP-Rib residues linked with α(1→2) ribose–ribose glycosidic bonds, but recently, we have shown the presence of a branching structure linked with α(1‴→2″) and α(1″→2′) ribose–ribose glycosidic bonds (Miwa et al., 1979, 1981; see also Figure 6). This structure has been demonstrated to exist in vivo (Kanai et al., 1982a; Juarez-salinas et al., 1982), but its function is also unknown.

Poly(ADP-Rib) is a potent immunogen and produces antibodies in mice and rabbits (Kanai et al., 1974, 1978). We also found natural antibodies to poly(ADP-Rib) in the sera of patients with systemic lupus erythematosus (SLE) (Kanai et al., 1977). However, they are a mixture of various antibodies with different specificities and cannot be used to select a special antigenic structure in poly(ADP-Rib) molecules. Monoclonal antibodies are of great value in studying structures and functions of biological substances since they recognize one specific antigenic structure. Thus, a monoclonal antibody would be helpful in further studies on the structures and functions of molecules of poly(ADP-Rib), such as branched portions or unknown structures, and could also be a useful reagent for analysis of natural antibodies in the sera of SLE patients. We immunized mice with poly(ADP-Rib) and obtained two hybridomas producing monoclonal antibodies that recognized different structures of poly(ADP-Rib).

Materials and Methods

Chemicals and Enzymes. [U-^{14}C]ATP was purchased from the Radiochemical Centre, Amersham, England. [^{14}C]NAD^+ labeled in NMN in the ribose moiety was kindly supplied by Dr. Fujiki of this institute. Proteinase K, snake venom phosphodiesterase (EC 3.1.4.1), and alkaline phosphomonoesterase (EC 3.1.3.1) from *Escherichia coli* were obtained from E. Merck, Darmstadt, FRG, Worthington Biochemical Corp., Freehold, NJ, and Sigma Chemical Co., St. Louis, MO, respectively. Snake venom phosphodiesterase was purified as previously reported by Oka et al. (1978), and 1 unit of the enzyme released 1 μmol of *p*-nitrophenol from (*p*-nitrophenyl)thymidine 5′-monophosphate per min at pH 8.8 at 25 °C. One unit of alkaline phosphomonoesterase hydrolyzes 1 μmol of *p*-nitrophenyl phosphate per min at pH 10.4 at 37 °C. Dulbecco's minimum essential medium was from Grand Island Biological Co., Grand Island, NY. CNBr-activated Sepharose 4B was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Pansorbin (*Staphylococcus aureus* cells wearing a coat of protein A) was from Calbiochem-Behring Corp., La Jolla, CA.

Preparation of Poly(ADP-Rib), Ado(P)-Rib-P, and Ado(P)-Rib(P)-Rib-P. Poly(ADP-Rib) was prepared from [U-^{14}C]ATP and NMN or from [*ribosyl*-^{14}C]NAD^+ with rat liver nuclei and from NAD^+ with calf thymus nuclei, as described previously (Sugimura et al., 1971). Purified poly(ADP-Rib) was separated into three fractions by hydroxylapatite column chromatography. Fraction I contained poly(ADP-Rib)

† From the Virology Division, National Cancer Center Research Institute, 1-1 Tsukiji, 5-chome Chuo-ku, Tokyo 104, Japan (H.K., H.H., H.O., M.M., and T.S.), and The First Department of Internal Medicine, Tokyo Medical and Dental University, 4-45 Yushima, 1-chome Bunkyo-ku, Tokyo 113, Japan (H.M.). *Received November 29, 1983.* This work was partly supported by Grants-in-Aid from the Ministry of Education, Science and Culture and from the Health and Welfare of Japan and by a grant from the Princess Takamatsu Cancer Research Fund.

[1] Abbreviations: poly(ADP-Rib), poly(adenosine diphosphate ribose); ADP-Rib, adenosine diphosphate ribose; Ado(P)-Rib-P, 2′-(5″-phosphoribosyl)adenosine 5′-monophosphate or 2′-(1″-ribosyl)adenosine 5′,5″-bis(phosphate), which is a monomer unit of poly(ADP-Rib); Ado(P)-Rib(P)-Rib-P, 2′-[2″-(5‴-phosphoribosyl)-5″-phosphoribosyl]-adenosine 5′-monophosphate or 2′-[2″-(1‴-ribosyl)-1″-ribosyl]adenosine 5′,5″,5‴-tris(phosphate), which is a branched portion of poly(ADP-Rib); Ado-Rib-P, 2′-(5″-phosphoribosyl)adenosine or 2′-(1″-ribosyl)adenosine 5″-monophosphate; Ado(P)-Rib, 2′-ribosyladenosine 5′-monophosphate or 2′-(1″-ribosyl)adenosine 5′-monophosphate (see Figure 6); Ado-Rib(P)-Rib-P, 2′-[2″-(1‴-ribosyl)-1″-ribosyl]adenosine 5″,5‴-bis(phosphate); Ado(P)-Rib(P)-Rib, 2′-[2″-(1‴-ribosyl)-1″-ribosyl]adenosine 5′,5″-bis(phosphate); Tris, tris(hydroxymethyl)aminomethane.

0006-2960/84/0423-3771$01.50/0

molecules of less than 10 ADP-Rib residues, fraction II those of 10–30 residues, and fraction III those of more than 20 residues including those of as much as 60 residues or more (Tanaka et al., 1978). Fraction III was used as the immunogen to produce monoclonal antibodies. It was also used as the antigen in assays for detection and determination of antibody activities. Ado(P)-Rib-P and Ado(P)-Rib(P)-Rib-P were purified by DEAE-Sephadex A-25 column chromatography of hydrolytic products of poly(ADP-Rib) by the method of Miwa et al. (1979).

Cell Fusion and Selection of Clones Producing Antibodies. Female BALB/c mice of 6–8 weeks old were immunized by three intraperitoneal injections of 50 μg of poly(ADP-Rib) mixed with 50 μg of methylated bovine serum albumin and emulsified with an equal volume of Freund's complete adjuvant. Three or four days after the last injection, 10^8 spleen cells were removed and fused with 10^7 NS-1, mouse myeloma cells in 50% poly(ethylene glycol) by the method of Köhler & Milstein (1976). The fused cells were cultured in HAT medium (Dulbecco's minimum essential medium supplemented with 100 μM hypoxanthine, 0.4 μM aminopterine, and 16 μM thymidine). Hybridomas were screened for production of antibodies to poly(ADP-Rib) by passive hemagglutination assay. Briefly, in this assay, mouse erythrocytes were coated with poly(ADP-Rib) by a similar procedure to that used for coating erythrocytes with DNA (Lamlis, 1958) and were incubated with culture supernatants. Antibody activities in culture supernatants were determined by hemagglutination of the poly(ADP-Rib)-coated erythrocytes. Antibody-producing hybridomas were then subcloned 3 or 4 times by limiting dilution. Clones were mass cultured after subcloning.

Purification of Monoclonal Antibodies. Culture supernatants were adjusted to 50% saturation of ammonium sulfate, and the precipitates obtained by centrifugation were suspended in phosphate-buffered saline (PBS), pH 7.4, and dialyzed against the same buffer. It was then used as γ-globulin fraction. The ammonium sulfate fraction was incubated with poly(ADP-Rib)–Sepharose 4B, which was prepared by coupling poly(ADP-Rib) with Sepharose 4B in 0.1 M sodium phosphate buffer, pH 8.0, and subsequent blocking with 1 M ethanolamine, pH 8.0. The antibody bound to poly(ADP-Rib) was eluted with 3 M NaSCN and immediately dialyzed against PBS. The isotypes of monoclonal antibodies were determined by immunodiffusion and immunoelectrophoresis on 0.9% agar in PBS, pH 7.4, and in 0.05 M veronal buffer, pH 8.6, respectively.

Measurement of Activities of Monoclonal Antibodies. The binding activities of monoclonal antibodies to poly(ADP-Rib) were determined by radioimmunoassay, as described previously (Kanai et al., 1974, 1977). Poly([^{14}C]ADP-Rib) (1500 cpm, 60 ng) was incubated with γ-globulin fraction for 30 min at 37 °C in 1 mL of PBS, and then the mixture was allowed to stand for 30 min at 4 °C before filtration through a Millipore filter membrane (Millipore Corp., pore size 0.22 μm, GS type). The radioactivity in the materials trapped on the filter was measured with a Packard liquid scintillation counter. The activity of the antibody is expressed as the percentage binding, that is, the radioactivity trapped on the filter as a percentage of the total radioactivity of the antigen added.

Competitive Binding Assay. Various amounts of competitors were mixed with poly([^{14}C]ADP-Rib), and the mixtures were incubated with monoclonal antibodies. The amount of antibodies corresponding to 50% of maximal binding to poly([^{14}C]ADP-Rib) was chosen. The binding of the antibodies to the radioactive poly(ADP-Rib) in the presence of competitors was determined as described above. The binding in the absence of competitors was taken as 100%.

Polyacrylamide Gel Electrophoresis of Immunoprecipitates. Poly([^{14}C]ADP-Rib) (80000 cpm, 60 ng) was incubated with 1 mg of γ-globulin fraction from a culture fluid of hybridomas or with 10 μL of anti-poly(ADP-Rib) rabbit serum prepared as described previously (Kanai et al., 1974). The antigen–antibody complexes were then precipitated with Pansorbin after incubation with 100 μL of anti-mouse IgM serum in the case where IgM monoclonal antibody 16B was used. The precipitates were digested with 1 mg of proteinase K and treated with water-saturated phenol. The poly([^{14}C]ADP-Rib) recovered in the aqueous layer was precipitated with 95% ethanol and subjected to electrophoresis on 20% polyacrylamide gel in 50 mM Tris–boronate buffer, pH 8.3 (Tanaka et al., 1978). The gel was dried and exposed to Kodak X-ray film.

Analysis of Structures of Poly(ADP-Rib) Protected by Monoclonal Antibodies. Samples of 60 ng of poly([^{14}C]-ADP-Rib) were incubated with large excesses of the purified monoclonal antibodies in PBS and then hydrolyzed with 0.05 unit of snake venom phosphodiesterase for 1–3 h at 37 °C in the presence of 1 mM $MgCl_2$. Hydrolysis by the enzyme was monitored by following production of AMP and Ado(P)-Rib-P, a monomer unit of poly(ADP-Rib), by thin-layer chromatography on cellulose with a mixture of isobutyric acid/concentrated ammonium hydroxide/water (66:1:33 v/v) (Shima et al., 1969). The incubation mixture was digested with 1 mg of proteinase K and extracted with water-saturated phenol. The aqueous layer was subjected to gel filtration on a Bio-Gel P-10 or A-50m column that had been equilibrated with 10 mM triethylammonium bicarbonate buffer, pH 7.5. The fractions in the void volume from the Bio-Gel P-10 column were collected and digested with 0.6 unit of alkaline phosphomonoesterase at pH 8.0 for 1 h at 37 °C and then with 0.05 unit of snake venom phosphodiesterase after removal of alkaline phosphomonoesterase by water-saturated phenol. The hydrolytic products were then analyzed by descending paper chromatography on Toyo No. 51A filter paper with a mixture of 0.1 M sodium phosphate buffer (pH 6.8), ammonium sulfate, and *n*-propyl alcohol (100:60:2 v/w/v) (Shima et al., 1969).

Results

Isolation of Monoclonal Antibodies. Two kinds of monoclonal antibodies were obtained in separate cell fusion experiments. In experiment I, hybridomas grew in 108 of 192 wells, and production of antibodies to poly(ADP-Rib) was found in 16 wells, but only one clone, 10H, could be established as a clonal cell line after three subclonings. In experiment II, production of antibodies to poly(ADP-Rib) by hybridomas that were initially cultured in 192 wells was found in 6 of 183 wells, and clone 16B was obtained after three subclonings. Clone 10H produces IgG3,κ antibody, and clone 16B produces IgM,λ antibody.

Binding Activities of Monoclonal Antibodies. The binding activities of monoclonal antibodies were determined by radioimmunoassay as described under Materials and Methods (Figure 1). Antibody 10H bound most of the radioactivity of poly(ADP-Rib), but antibody 16B bound only about half of it, even with a large excess of antibody. The culture supernatant of NS-1 showed essentially no binding activity to poly(ADP-Rib).

Polyacrylamide Gel Electrophoresis of Poly(ADP-Rib) Immunoprecipitated with Monoclonal Antibodies. The amounts of poly([^{14}C]ADP-Rib) immunoprecipitated by

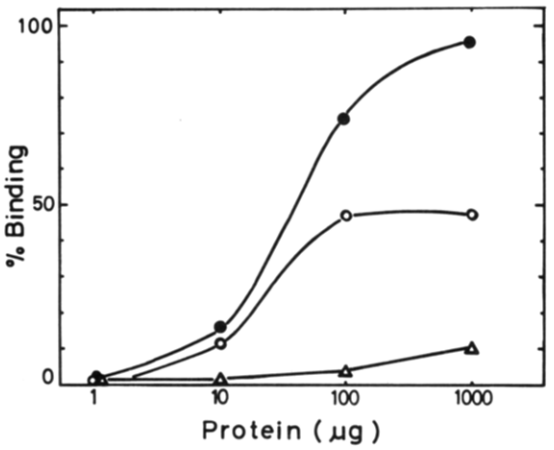

FIGURE 1: Binding of monoclonal antibodies to poly([^{14}C]ADP-Rib): antibody 10H (●), antibody 16B (○), and NS-1 (△). Culture supernatants were fractionated with 50% saturation of ammonium sulfate and used in determination of binding.

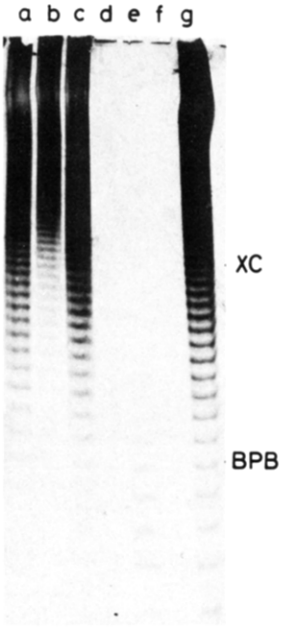

FIGURE 2: Polyacrylamide gel electrophoresis of poly(ADP-Rib) immunoprecipitated with monoclonal antibodies. The immunoprecipitates by antibodies 10H and 16B and anti-poly(ADP-Rib) rabbit serum were subjected to electrophoresis in lanes a–c, respectively. The supernatants after immunoprecipitation by each antibody were run in lanes d (10H), e (16B), and f (rabbit antiserum). Lane g shows the electrophoretic pattern of poly(ADP-Rib) molecules as a control.

monoclonal antibodies 10H and 16B and rabbit antiserum were 95, 60, and 100% of the starting radioactivity, respectively. A total of 60–70% of the poly([^{14}C]ADP-Rib) in the immunoprecipitates was recovered after phenol extraction and ethanol precipitation in all cases. The ethanol precipitates were subjected to electrophoresis (Figure 2). Antibody 10H or polyclonal anti-poly(ADP-Rib) rabbit serum bound almost all molecular sizes of poly(ADP-Rib), as seen from the electrophoretic pattern of control poly(ADP-Rib), whereas antibody 16B bound only larger molecules of poly(ADP-Rib), smaller molecules being recovered in the supernatant.

Specificities of Monoclonal Antibodies. The specificities of monoclonal antibodies were examined by competitive binding assay. Binding of antibody 10H to fraction III of poly([^{14}C]ADP-Rib) was completely inhibited by fraction III and fraction II of poly(ADP-Rib) and was inhibited 20% by Ado(P)-Rib-P, a monomer unit of poly(ADP-Rib), and

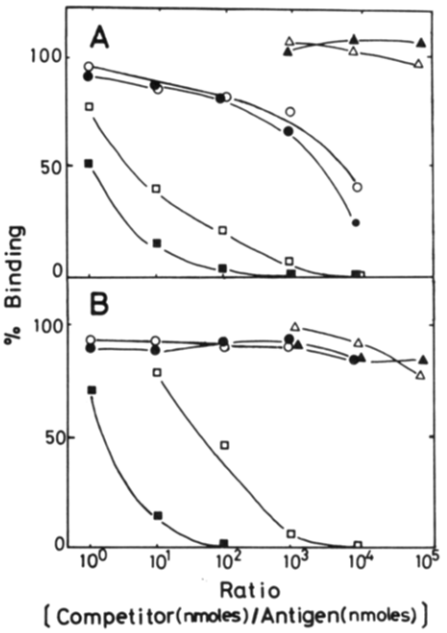

FIGURE 3: Specificity of monoclonal antibodies determined by competitive binding assay: (A) antibody 10H and (B) antibody 16B. The competitors are poly(ADP-Rib) fraction III (■), fraction II (□), Ado(P)-Rib-P (●), Ado(P)-Rib(P)-Rib-P (○), calf thymus DNA (▲), and denatured calf thymus DNA (△). Amounts of competitors are expressed as ratios of nanomoles of ADP-Rib residues or nucleotides to those of ADP-Rib residues of poly([^{14}C]ADP-Rib).

Ado(P)-Rib(P)-Rib-P, a branched portion, with a 100-fold excess of these competitors over the antigen (Figure 3A). Binding of antibody 16B was also strongly inhibited by fraction III, but less strongly by fraction II. No inhibition was observed with either Ado(P)-Rib-P or Ado(P)-Rib(P)-Rib-P (see Discussion) (Figure 3B). Other analogues of poly(ADP-Rib), such as ADP-Rib, 5′-AMP, and yeast RNA, did not inhibit the binding of either antibody (data not shown).

As shown in the above experiments on the binding of antibodies to poly([^{14}C]ADP-Rib) and the competitive inhibition of binding by poly(ADP-Rib) analogues, the two monoclonal antibodies recognize different antigenic sites on the polymer. Antibody 10H seems to recognize a structure that consists of several repeats of a monomer unit, because it bound most of the poly(ADP-Rib) molecules and the binding was inhibited by Ado(P)-Rib-P: antibody 16B may recognize a structure found in larger molecules rather than simple repeats of a monomer, as shown by electrophoresis and competitive binding assay. So, the detailed structures of the portions of poly(ADP-Rib) recognized by monoclonal antibodies were analyzed.

Time Course of Hydrolysis of Poly(ADP-Rib) Not Protected by Monoclonal Antibodies. After preincubation with normal mouse γ-globulin or without preincubation with an antibody, almost all the poly(ADP-Rib) molecules were rapidly hydrolyzed with snake venom phosphodiesterase (Figure 4). However, antibody 10H protected most portions of each poly(ADP-Rib) molecule from the hydrolytic action of snake venom phosphodiesterase. With antibody 16B, the hydrolysis by snake venom phosphodiesterase progressed gradually and reached a plateau after 3 h, when about 25% of the radioactivity of poly(ADP-Rib) remained resistant to hydrolysis.

Bio-Gel P-10 Column Chromatography of Snake Venom Phosphodiesterase Digestion Products of Poly(ADP-Rib) Preincubated with Monoclonal Antibodies. Poly([^{14}C]ADP-Rib) was treated with snake venom phosphodiesterase after preincubation with monoclonal antibodies, and the products

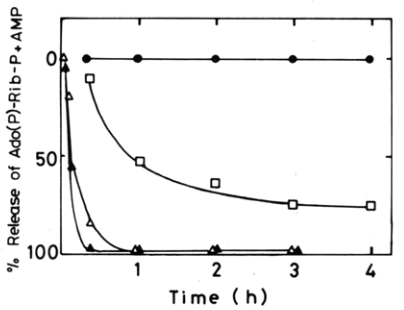

FIGURE 4: Time course of hydrolysis of poly(ADP-Rib). Poly(ADP-Rib) was hydrolyzed with snake venom phosphodiesterase after preincubation with antibody 10H (●), antibody 16B (□), or normal mouse γ-globulin (▲) or without an antibody (△). The progress of hydrolysis was shown as the production of Ado(P)-Rib-P plus AMP.

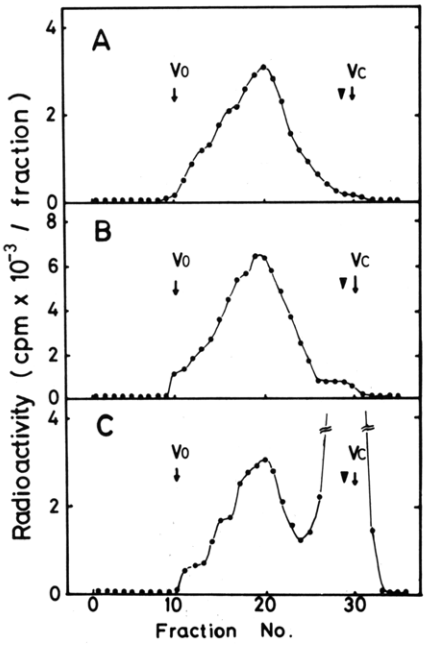

FIGURE 5: Molecular sizes of poly(ADP-Rib) protected by monoclonal antibodies. Poly(ADP-Rib) was incubated wth monoclonal antibody 10H (B) or 16B (C) and then hydrolyzed with snake venom phosphodiesterase. The hydrolytic products were isolated by phenol extraction and fractionated by Bio-Gel A-50m column chromatography: (A) unhydrolyzed poly(ADP-Rib); V_0, void volume; V_c, column volume; (▼) fraction where hydrolyzed product, Ado(P)-Rib-P, was eluted in the case of antibody 16B.

were extracted with phenol. About 85% of the total radioactivity was recovered in the aqueous layer, and the hydrolytic products were fractionated by Bio-Gel P-10 column chromatography. Without antibody, most of the hydrolytic products were eluted in the peak of low molecular weight with Ado(P)-Rib-P, which was added as a marker. Most of the poly(ADP-Rib) that had been incubated with antibody 10H before digestion with snake venom phosphodiesterase was eluted in the void volume. With antibody 16B, about 25% of hydrolytic products were eluted in the void volume, and 75% of them were eluted with Ado(P)-Rib-P. These results are compatible with the results on the time course of hydrolysis shown in Figure 4. The fractions in the void volume seem to be the sites of poly(ADP-Rib) protected from hydrolysis by antibody because they were clearly separated on the column from Ado(P)-Rib-P, a hydrolytic product with snake venom phosphodiesterase. So, these fractions were collected for more detailed study.

Distribution of Poly(ADP-Rib) Protected by Monoclonal Antibodies on Bio-Gel A-50m Column Chromatography. The size of poly(ADP-Rib) protected by monoclonal antibodies was

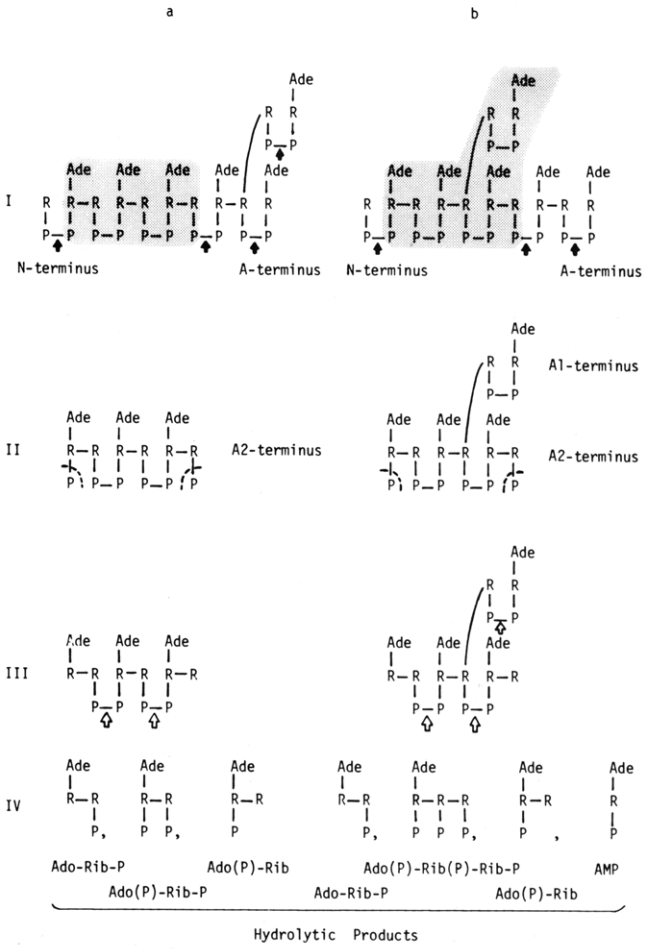

FIGURE 6: Strategy for analysis of poly(ADP-Rib) protected by monoclonal antibodies. The stippled areas show the sites of poly(ADP-Rib) protected by monoclonal antibodies. Alkaline phosphomonoesterase releases phosphates from the termini of poly(ADP-Rib) (dotted arcs), and snake venom phosphodiesterase hydrolyzes poly(ADP-Rib) at pyrophosphate bonds (solid and open arrows).

examined by gel filtration on a Bio-Gel A-50m column (Figure 5). Fraction III of poly(ADP-Rib) is heterogeneous in size, as shown by its wide distribution on the column (Figure 5A), and poly(ADP-Rib) protected by antibody 10H had the same distribution as fraction III on the column (Figure 5B). The molecules protected by antibody 16B also showed a very similar distribution on the column and were recovered in about 25% of the total fractions (Figure 5C).

The sizes of the sites of poly(ADP-Rib) protected by monoclonal antibodies were expected to be homogeneously small, but actually their sizes on the column were very heterogeneous. With antibody 10H, it seems that excess antibody may protect most of the poly(ADP-Rib) molecules because the antibody seems to bind to a structure of several repeats of Ado(P)-Rib-P. However, antibody 16B protected only 25% of the molecules of heterogeneous sizes even when added in large excess. This implies that 25% of the poly(ADP-Rib) molecules of each size have a specific structure that is firmly protected by antibody 16B.

Analysis of Hydrolytic Products of Poly(ADP-Rib) Protected by Monoclonal Antibodies. Figure 6 shows the method and the principle of analysis of poly(ADP-Rib) protected by monoclonal antibodies. The sites of poly(ADP-Rib) not protected by antibodies will be hydrolyzed by snake venom phosphodiesterase, as shown by solid arrows in Figure 6I. The protected sites, which are shown in the stippled areas in Figure 6I, were isolated as described under Materials and Methods

Table I: Hydrolytic Products of Poly(ADP-Rib) Protected by Monoclonal Antibodies

poly(ADP-Rib) protected by	alkaline phosphomonoesterase	hydrolytic products (%)[a]			
		Ado-Rib-P	Ado(P)-Rib(P)-Rib-P	Ado(P)-Rib-P	AMP + Ado(P)-Rib
nothing	−	0	2.2 (2.0–2.3)	95.0 (94.6–95.6)	2.8 (2.4–3.1)
	+	0.4 (0–0.7)	2.1 (2.0–2.1)	93.7 (93.1–94.6)	3.8 (2.9–4.1)
antibody 10H	−	0.2 (0–0.6)	2.1 (2.0–2.2)	95.3 (94.4–95.8)	2.4 (2.2–2.8)
	+	1.7 (1.0–2.2)	1.5 (1.0–2.2)	91.0 (90.3–92.5)	5.8 (5.1–6.2)
antibody 16B	−	0.2 (0–0.4)	3.7 (3.6–3.7)	94.0 (93.6–94.5)	2.1 (1.9–2.2)
	+	0.6 (0.4–0.7)	2.3 (1.8–2.7)	92.0 (91.9–92.3)	5.1 (4.7–5.5)

[a] Values are averages and ranges (parenthesized) for two to three independent experiments.

and digested with alkaline phosphomonoesterase to remove phosphates from the termini of protected sites (Figure 6II). Alkaline phosphomonoesterase was removed by phenol extraction, and the protected sites were hydrolyzed with snake venom phosphodiesterase, as shown by open arrows in Figure 6III. Ado-Rib-P, Ado(P)-Rib(P)-Rib-P, Ado(P)-Rib-P, AMP, and Ado(P)-Rib should be produced from the N-terminus, branched portions, linear portions, A1-terminus, and A2-terminus, respectively (Figure 6IV). When poly(ADP-Rib) was hydrolyzed in the absence of antibody, the amount of Ado(P)-Rib(P)-Rib-P, the branched portion, was 2.2% and the amount of Ado(P)-Rib-P was 95.0% (Table I). The amounts of all products except AMP and Ado(P)-Rib were almost the same in the presence or absence of alkaline phosphomonoesterase. The amount of A-terminus is reflected by the productions of AMP and Ado(P)-Rib in the presence of alkaline phosphomonoesterase, because Ado(P)-Rib will be produced by digestion with alkaline phosphomonoesterase and then phosphodiesterase (Figure 6). The amount of N-terminus is measured as the sum of Rib-P, derived from the complete form of N-terminus, and Ado-Rib-P, derived from the incomplete form of N-terminus that was partially hydrolyzed at the pyrophosphate bond. But, the Rib-P was not labeled with ^{14}C in poly([^{14}C]ADP-Rib), which we used. So, the amount of N-terminus was deduced by subtracting the amount of branched portions from the amount of A-terminus, since the number of A-terminus is theoretically always one more than that of the branched portions in a poly(ADP-Rib) molecule. Taking this into consideration, calculations from the data in Table I showed that the amount of N-terminus was 1.6% and the average number of branched portions per molecule was 1.4, which was a ratio of the amount of Ado-(P)-Rib(P)-Rib-P to the determined amounts of Rib-P and Ado-Rib-P. An additional experiment was performed in which a different part of the molecule was labeled. In this experiment, hydrolysis of poly(ADP-[^{14}C]Rib) in which [^{14}C]Rib-P and Ado-[^{14}C]Rib-P were released from the N-terminus showed that the amount of N-terminus was 2.0%. This value compared well with the 1.6% calculated amount of N-terminus determined from the experiment with poly([^{14}C]ADP-Rib).

In the presence of antibody 10H or antibody 16B, the amount of Ado(P)-Rib(P)-Rib-P decreased when the protected sites were digested with alkaline phosphomonoesterase. If a branched portion is located at the terminus in a protected site, it will release one phosphate on treatment with alkaline phosphomonoesterase, and partially dephosphorylated branched portions, Ado-Rib(P)-Rib-P or Ado(P)-Rib(P)-Rib, should be produced. These dephosphorylated branched portions might show very similar R_f values to Ado(P)-Rib-P on paper chromatography and would be difficult to be quantitated. So, the precise amount of Ado(P)-Rib(P)-Rib-P can be measured without treatment with alkaline phosphomonoesterase. The species and amounts of hydrolytic products of the sites protected by antibody 10H are almost the same as those of hydrolytic products of poly(ADP-Rib) without antibody except that the amounts of AMP and Ado(P)-Rib are larger than that without an antibody (Table I). The amount of N-terminus deduced was 3.7%, and the number of branched portions per molecule was 0.6. The amount of Ado(P)-Rib-(P)-Rib-P in sites protected by antibody 16B (3.7%) is about twice that in sites protected by antibody 10H (2.1%) (Table I), and the number of branched portions per molecule was calculated as 2.6.

Discussion

In this work, two monoclonal antibodies that recognized different structures of poly(ADP-Rib) were isolated. Antibody 10H seemed to recognize the structure of linear portions, which are repeats of Ado(P)-Rib-P, judging from experiments on the binding of the antibody to poly([^{14}C]ADP-Rib) (Figures 1 and 2) and the competitive inhibition of the binding (Figure 3). The binding was slightly inhibited by Ado(P)-Rib(P)-Rib-P as well as Ado(P)-Rib-P. The inhibition by Ado(P)-Rib-(P)-Rib-P may be explained by the presence of the Ado-(P)-Rib-P residue of Ado(P)-Rib(P)-Rib-P. The sites of poly(ADP-Rib) protected by antibody 10H showed heterogeneous sizes on gel filtration (Figure 5), so several molecules of antibody 10H seem to bind and protect whole poly(ADP-Rib) molecules of different sizes. Contrary to this, antibody 16B may recognize not simple repeats of Ado(P)-Rib-P but a structure containing branched portions, because the branched portions were most concentrated in sites of poly(ADP-Rib) protected by this antibody. About 25% of the poly(ADP-Rib) molecules of each size seem to have such structures to which the IgM antibody 16B binds firmly enough to protect the polymer from hydrolysis. The branched portion, Ado(P)-Rib(P)-Rib-P, did not inhibit the binding of antibody 16B to poly(ADP-Rib). But if the antibody binds to a structure consisting of the branched portion and several monomer units around it, the binding of the antibody to poly(ADP-Rib) molecules would not be inhibited by a small molecule such as Ado(P)-Rib(P)-Rib-P. As mentioned before, the calculated numbers of branched portions per molecule were 1.4, 0.6, and 2.6 in the cases without antibody, with antibody 10H, and with antibody 16B, respectively. Of the three cases, poly(ADP-Rib) protected by antibody 16B contains the most branched portions per molecule, and poly(ADP-Rib) protected by antibody 10H has less branched portions per molecule than control poly-(ADP-Rib). This implies that among the sites protected by antibody 10H there are several sites that are attacked by snake venom phosphodiesterase and produce N- and A-termini. This might cause the observed decrease in the average number of branched portions in the molecule. On the basis of these results, the model of the sites recognized by monoclonal antibodies shown in Figure 7 is proposed. There seem to be various kinds of molecules with respect to the number of branched portions among poly(ADP-Rib) molecules of similar size, and so, four types of poly(ADP-Rib) molecules with about 75 residues are shown. Here, four molecules contain 300

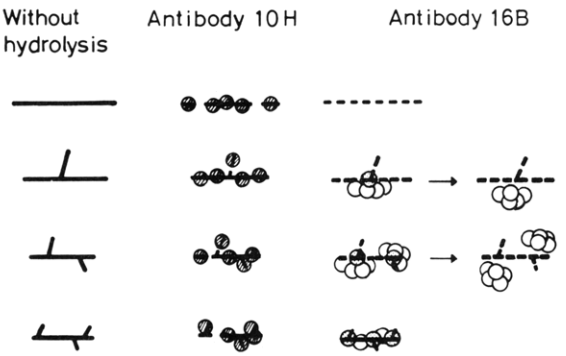

FIGURE 7: A model of the sites of poly(ADP-Rib) protected by monoclonal antibodies. One circle indicates one monomer of an immunoglobulin molecule. Crossed circles and stippled circles show immunoglobulin molecules bound to poly(ADP-Rib). Interrupted lines are sites that are hydrolyzed by snake venom phosphodiesterase.

ADP-Rib residues and six branched portions, occupying 2% of the total ADP-Rib residues.

In the model of antibody 10H, several antibody molecules cover a poly(ADP-Rib) molecule uniformly except for the sites shown as interrupted lines that are hydrolyzed by snake venom phosphodiesterase. Antibody 16B is an IgM that is a pentamer, and it is shown by five circles in Figure 7. If branched portions are close enough for two or more monomeric IgM to bind to each branched portion (shown as stippled circles in Figure 7), the IgM molecule will bind to the poly(ADP-Rib) molecule firmly and be resistant to the action of snake venom phosphodiesterase. However, if branched portions are too far apart from each other, only one binding site of monomeric IgM will bind to one branched portion and the binding of the antibody, which is in equilibrium between association and dissociation, may be weak. This could result in the gradual hydrolysis of poly(ADP-Rib) by snake venom phosphodiesterase, as shown by the arrows in Figure 7. Papalian et al. (1980) suggested that one anti-DNA antibody molecule in sera of patients with systemic lupus erythematosus might cover about 30 base pairs in the helix of double-stranded DNA. If we assume that the length of an ADP-Rib monomer is about twice the length of one base pair of DNA (Hayashi et al., 1983), one molecule of 10H IgG antibody and one monomer of 16B IgM molecule would cover about 15 ADP-Rib residues in the linear structure and 20–25 ADP-Rib residues in the structure including branched portions of poly(ADP-Rib), respectively. As IgM antibody has five monomers, it seems likely that one molecule of antibody 16B could easily cover a poly(ADP-Rib) molecule with 75 ADP-Rib residues in the model. The structure of poly(ADP-Rib), and especially the three-dimensional structure, is still unknown. Monoclonal antibodies like antibody 16B should be helpful in finding and studying unknown structures in vivo.

The antibodies described in this paper could also be useful in other ways. For example, preliminary data have indicated that there are similar antibodies to poly(ADP-Rib) in the sera of patients with SLE. Some of the sera of the patients inhibited the binding of antibody 10H, and several other sera inhibited that of antibody 16B. Therefore, it will be interesting to see if there is any relationship between the clinical conditions and the types of antibodies in the sera of SLE patients by using these monoclonal antibodies.

Another possible use of these monoclonal antibodies is purifying poly(ADP-ribosylated) oligonucleosomes in chromatin that have been isolated by polyclonal anti-poly(ADP-Rib) antibody (Wong et al., 1983). The monoclonal antibodies will be able to separate and characterize the oligonucleosomes modified by linear and branching poly(ADP-Rib) and analyze the biological functions of poly(ADP-ribosylation) involved in DNA repair, cell differentiation, and cell transformation.

Registry No. Poly(ADP-Rib), 26656-46-2; Ado(P)-Rib-P, 65281-70-1; Ado(P)-Rib(P)-Rib-P, 70028-80-7.

References

Creissen, D., & Shall, S. (1982) *Nature (London) 296*, 271–272.

Durkacz, B. W., Omidiji, O., Gray, D. A., & Shall, S. (1980) *Nature (London) 283*, 593–596.

Hayaishi, O., & Ueda, K. (1977) *Annu. Rev. Biochem. 46*, 95–116.

Hayashi, K., Tanaka, M., Shimada, T., Miwa, M., & Sugimura, T. (1983) *Biochem. Biophys. Res. Commun. 112*, 102–107.

Hilz, H., & Stone, P. R. (1976) *Rev. Physiol., Biochem. Pharmacol. 76*, 1–59.

Juarez-Salinas, H., Sims, J. L., & Jacobson, M. K. (1979) *Nature (London) 282*, 740–741.

Juarez-Salinas, H., Levi, V., Jacobson, E. L., & Jacobson, M. K. (1982) *J. Biol. Chem. 257*, 607–609.

Kanai, M., Miwa, M., Kuchino, Y., & Sugimura, T. (1982a) *J. Biol. Chem. 257*, 6217–6223.

Kanai, M., Miwa, M., Kondo, T., Tanaka, Y., Nakayasu, M., & Sugimura, T. (1982b) *Biochem. Biophys. Res. Commun. 105*, 404–411.

Kanai, Y., Miwa, M., Matsushima, T., & Sugimura, T. (1974) *Biochem. Biophys. Res. Commun. 59*, 300–306.

Kanai, Y., Kawaminami, Y., Miwa, M., Matsushima, T., Sugimura, T., Moroi, Y., & Yokohari, R. (1977) *Nature (London) 265*, 175–177.

Kanai, Y., Miwa, M., Matsushima, T., & Sugimura, T. (1978) *Immunology 34*, 501–508.

Köhler, G., & Milstein, C. (1976) *Eur. J. Immunol. 6*, 511–519.

Lamlis, J. F. (1958) *Proc. Soc. Exp. Biol. Med. 98*, 300–303.

Mandel, P., Okazaki, H., & Niedergang, C. (1982) *Prog. Nucleic Acid Res. Mol. Biol. 27*, 1–44.

Miwa, M., Oda, K., Segawa, K., Tanaka, M., Irie, S., Yamaguchi, N., Kuchino, T., Shiroki, K., Shimojo, H., Sakura, H., Matsushima, T., & Sugimura, T. (1977) *Arch. Biochem. Biophys. 181*, 313–321.

Miwa, M., Saikawa, N., Yamaizumi, Z., Nishimura, S., & Sugimura, T. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 595–599.

Miwa, M., Ishihara, M., Takishima, S., Takasuka, N., Maeda, M., Yamaizumi, Z., & Sugimura, T. (1981) *J. Biol. Chem. 256*, 2916–2921.

Oka, J., Ueda, K., & Hayaishi, O. (1978) *Biochem. Biophys. Res. Commun. 80*, 841–848.

Papalian, M., Lafer, E., Wong, R., & Stollar, D. (1980) *J. Clin. Invest. 65*, 469–477.

Purnell, M. R., Stone, P. R., & Whish, W. J. D. (1980) *Biochem. Soc. Trans. 8*, 215–227.

Shima, T., Hasegawa, S., Fujimura, S., Matsubara, H., & Sugimura, T. (1969) *J. Biol. Chem. 244*, 6623–6635.

Smulson, M. E., Schein, P., Mullins, D. W., Jr., & Sudhakar, S. (1977) *Cancer Res. 37*, 3006–3012.

Sugimura, T. (1973) *Prog. Nucleic Acid Res. Mol. Biol. 13*, 127–151.

Sugimura, T., Yoshimura, N., Miwa, M., Nagai, H., & Nagao, M. (1971) *Arch. Biochem. Biophys. 147*, 660–665.

Tanaka, M., Miwa, M., Hayashi, K., Kubota, K., Matsushima,

T., & Sugimura, T. (1977) *Biochemistry 16*, 1485-1489.
Tanaka, M., Hayashi, K., Sakura, H., Miwa, M., Matsushima, T., & Sugimura, T. (1978) *Nucleic Acids Res. 5*, 3183-3194.
Terada, M., Fujiki, H., Marks, P. A., & Sugimura, T. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6411-6414.
Wong, M., Kanai, Y., Miwa, M., Bustin, M., & Smulson, M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 205-209.

Investigation of Structure and Rate of Synthesis of Ornithine Decarboxylase Protein in Mouse Kidney†

Lo Persson,‡ James E. Seely,§ and Anthony E. Pegg*

ABSTRACT: An immunoblotting technique was used to study the forms of ornithine decarboxylase present in androgen-induced mouse kidney. Two forms were detected which differed slightly in isoelectric point but not in subunit molecular weight (~55 000). Both forms were enzymatically active and could be labeled by reaction with radioactive α-(difluoromethyl)-ornithine, an enzyme-activated irreversible inhibitor. On storage of crude kidney homogenates or partially purified preparations of ornithine decarboxylase, the enzyme protein was degraded to a smaller size (M_r ~53 000) without substantial loss of enzyme activity. The synthesis and degradation of ornithine decarboxylase protein were studied by labeling the protein by intraperitoneal injection of [^{35}S]methionine and immunoprecipitation using both monoclonal and polyclonal antibodies. The fraction of total protein synthesis represented by renal ornithine decarboxylase was increased at least 25-fold by testosterone treatment of female mice and was found to be about 1.1% in the fully induced androgen-treated female. Both forms of the enzyme were rapidly labeled in vivo, and the immunoprecipitable ornithine decarboxylase protein was almost completely lost after 4-h exposure to cycloheximide, confirming directly the very rapid turnover of this enzyme. Treatment with 1,3-diaminopropane which is known to cause a great reduction in ornithine decarboxylase activity did not greatly selectively inhibit the synthesis of the enzyme. However, 1,3-diaminopropane did produce an increase in the rate of degradation of ornithine decarboxylase and a general reduction in protein synthesis. These two factors, therefore, appear to be responsible for the loss of ornithine decarboxylase activity and protein in response to 1,3-diaminopropane.

There have been many studies of ornithine decarboxylase in mammalian cells because of the remarkable inducibility of the activity of this enzyme which catalyzes the first step in the polyamine biosynthetic pathway (Jänne et al., 1978; Russell, 1980; McCann, 1980; Pegg & McCann, 1982). Until recently, most of these investigations were limited to measurements of enzyme activity because of the small amount of ornithine decarboxylase protein present in mammalian cells even after maximal induction and the insensitivity or unavailability of methods for studying the protein itself. Therefore, although a number of groups have proposed that there are multiple forms of this enzyme or that it may be a substrate for regulatory posttranslational modifications (Richards et al., 1981; Kuehn & Atmar, 1982; Mitchell & Mitchell, 1982; Russell, 1983; Bullock et al., 1983), definitive experiments to test these possibilities have not been carried out. Also, there is solid evidence based on the use of immunoaffinity chromatography that yeast ornithine decarboxylase is present in the cell as a larger protein than that obtained after purification owing to a rapid proteolytic cleavage which occurs in crude cell extracts (Tyagi et al., 1982). In the present experiments, monospecific antibodies to mouse ornithine decarboxylase have been used to examine the size and possible existence of multiple forms of this protein in the kidney. Androgen treatment was used to induce a high level of the enzyme in this organ (Henningsson et al., 1978; Seely et al., 1982a,b; Isomaa et al., 1983).

A variety of approaches have been used to demonstrate that ornithine decarboxylase has a rapid rate of turnover in most mammalian cells (Russell, 1980; McCann, 1980; Seely et al., 1982c), but the identification of a rapidly labeled polypeptide corresponding to ornithine decarboxylase has so far been accomplished only with variant mouse cell lines (McConlogue & Coffino, 1983a,b). These cells were selected for resistance to an ornithine decarboxylase inhibitor and greatly overproduce the enzyme, but it is not known how similar the overproduced enzyme is to that found in normal cells. We have, therefore, studied the labeling of mouse kidney ornithine decarboxylase by administration of [^{35}S]methionine and determined the synthesis rate of this enzyme and demonstrated its rapid degradation.

It is well-known that ornithine decarboxylase activity is lost rapidly after treatment with exogenous diamines including 1,3-diaminopropane (Pösö et al., 1978; Pegg et al., 1978; Canellakis et al., 1979; McCann, 1980). The mechanism by which this decrease is brought about is not clear. A protein which binds to ornithine decarboxylase and inhibits its activity appears to be induced or released in response to 1,3-diaminopropane (Canellakis et al., 1979), but radioimmunoassay techniques which can detect the complex between this protein and the decarboxylase failed to reveal substantial quantities of such an inactive complex (Seely & Pegg, 1983a,b). In these experiments, there was a substantial loss of total immunoreactive ornithine decarboxylase protein in response to 1,3-diaminopropane, suggesting that the regulation of enzyme

† From the Department of Physiology and Cancer Research Center, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033. *Received January 18, 1984.* This investigation was supported by Grants CA-18138 and 1P30-CA18450 from the National Institutes of Health.
‡ Recipient of a grant from the Swedish Medical Research Council.
§ Recipient of a training fellowship (1T32 HL-0723) from the National Institutes of Health.

0006-2960/84/0423-3777$01.50/0