Antigenic determinant and interspecies cross-reactivity of a monoclonal antibody to poly(ADP-ribose) synthetase

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A monoclonal antibody (1F4) was prepared against calf thymus poly(ADP-ribose) synthetase. It was classified as IgG_1/κ and its antigenic determinant was localized on the 46 kDa portion of the enzyme molecule which contains the site for the binding of DNA. When calf thymus DNA-binding proteins were subjected to immunostaining after electrophoresis and transblotting to a nitrocellulose filter, the native enzyme (120 kDa) and its endogenous degradation products (80, 64 and 32 kDa) were detected. When the interspecies cross-reactivity was examined using DNA-binding proteins from 6 different sources, 1F4 reacted with the 120- and 32-kDa protein bands in HeLa cells, mouse testis and chicken liver as in the case of calf thymus. These results indicate that the antigenic structures of poly(ADP-ribose) synthetase and its degradation products are highly conserved in various animal cells.

Monoclonal antibody *Poly(ADP-ribose) synthetase* *Immunoblotting* *Species specificity* *DNA-binding protein*

1. INTRODUCTION

Poly(ADP-ribose) synthetase is a chromatin-bound enzyme which synthesizes a protein-bound homopolymer of ADP-ribose utilizing NAD as a substrate [1–4]. The enzyme has been purified to homogeneity from various sources [5–12], but the physiological function and molecular structure of the enzyme are not yet fully understood. Using a technique of limited proteolysis, we recently demonstrated that poly(ADP-ribose) synthetase from calf thymus (120 kDa) consists of 3 separable domains, the first one of 54 kDa possessing the site for the substrate binding, the second of 46 kDa retaining the site for DNA binding, and the third of 22 kDa containing the sites for accepting poly(ADP-ribose) [13,14].

To analyze the molecular characteristics of poly(ADP-ribose) synthetase common to various species, we prepared a monoclonal antibody against the calf thymus enzyme, which recognizes the DNA binding domain of the enzyme. This paper provides evidence that the antigenic structures of poly(ADP-ribose) synthetase and its endogenous degradation products are all common to various animal cells.

2. EXPERIMENTAL

2.1. *Monoclonal antibody*

Antisera to the intact poly(ADP-ribose) synthetase and those to its proteolytic fragments were elicited by injecting BALB/c mice at multiple intradermal sites with 50–100 μg proteins emulsified with Freund's complete adjuvant. Intraperitoneal booster injections were administered 3 times at 10-day intervals with 15–20 μg proteins in phosphate-buffered saline (PBS). Three days after the final immunization, one of the mice having the highest antibody titer was killed, and spleen cells were fused with P3U1 myeloma cells by standard techniques [15]. Hybridoma cultures producing antibodies to poly(ADP-ribose) synthetase were identified both by enzyme-linked immunoadsorbent assay [16] and by dot immunobinding assay [17]. Clones selected were recloned to ensure

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monoclonality until the cloning efficiency reached 100%. Unique monoclonal hybridomas were expanded as ascites tumors in syngeneic mice. Antibodies were purified from these ascites with a column of Protein A-Sepharose (Pharmacia) equilibrated with PBS and eluted with 0.1 M sodium citrate buffer (pH 3.0) containing 0.15 M NaCl.

2.2. *DNA-binding proteins*

Various organs, tissues or cells were suspended in 5 vols of extraction buffer (50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 50 mM $NaHSO_3$, 10 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride) and homogenized by Polytron (Kinematica) at 4°C for 20 s. *Saccharomyces cerevisiae* was ruptured by grinding with almina and sea sand. The homogenate was fractionated by 40–80% ammonium sulfate precipitation and then applied onto a column of DNA-cellulose column which had previously been equilibrated with Buffer A (50 mM Tris–HCl (pH 8.0), 10% glycerol, 10 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride) containing 0.2 M NaCl, washed with Buffer A containing 0.4 M NaCl, and eluted with Buffer A containing 1 M NaCl. The protein fractions eluted with 1 M NaCl were designated as DNA-binding proteins.

2.3. *Immunoblotting*

Polyacrylamide gel electrophoresis was performed by the method of Laemmli [18] in the presence of SDS. The electrophoretic transfer of proteins from the SDS gel to a nitrocellulose sheet was performed at 4°C for 1 h in Tris-glycine buffer (25 mM Tris, 192 mM glycine (pH 8.3), 10% methanol and 0.05% Triton X-100) according to the method of Towbin et al. [19]. Immunostaining of proteins on a nitrocellulose sheet was performed according to [17]. The peroxidase reaction was developed by 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in 50 mM Tris-HCl (pH 7.6).

3. RESULTS AND DISCUSSION

Monoclonal antibodies to poly(ADP-ribose) synthetase were prepared using a homogeneous preparation of calf thymus poly(ADP-ribose) synthetase. Among antibodies from 5 different clones obtained, 1F4 has the highest titer as detected by enzyme-linked immunoadsorbent assay and by dot immunobinding assay. When examined by immunodiffusion analysis, 1F4 was classified as an IgG_1/κ antibody type.

The antigenic determinant of 1F4 on the enzyme was then determined using the native poly(ADP-ribose) synthetase or its 5 different proteolytic fragments, each purified to homogeneity and adsorbed onto nitrocellulose filters as antigens. As shown in fig.1, the monoclonal antibody 1F4 reacts with the native enzyme (120K), the 66- and 46-kDa fragments, but does not react with the 74-, 54- and 22-kDa fragments which lack the DNA binding domain. In control experiments, antisera against the native enzyme or other proteolytic fragments were simultaneously examined. As observed in the figure, antisera against the intact enzyme ($\bar{a}$-120K) react with all enzyme species or fragments, indicating that each enzyme fragment has its own antigenic determinant(s) on its molecule. Antisera to the 66-kDa fragment ($\bar{a}$-66K) react with all fragments other than the 54-kDa fragment, whereas those to the 54-kDa fragment ($\bar{a}$-54K) react only with the native enzyme and the 54- and 74-kDa fragments. Antisera to the 46-kDa fragment ($\bar{a}$-46K) give consistent results with those obtained by 1F4.

In the next experiments, papain- and chymotryptic-digests of poly(ADP-ribose) synthetase were electrophoresed on SDS gels and transferred to nitrocellulose filters. The filters were then stained either with the antisera ($\bar{a}$-120K) or the monoclonal antibody 1F4 as shown in fig.2. All the protein bands derived from the native enzyme are stained with the antisera $\bar{a}$-120K (fig.2A, lanes 1,2,3), whereas the 46- and 66-kDa fragments in addition to the native enzyme are specifically stained with 1F4 (fig.2B, lanes 1,2,3). These results agree well with the data in fig.1. It is concluded, therefore, that the antigenic determinant detected by 1F4 is localized on the 46-kDa fragment which contains the site for the binding of DNA.

To investigate whether there exist precursor proteins and/or endogenous degradation products of poly(ADP-ribose) synthetase, DNA-binding proteins were prepared from fresh calf thymus and immunochemically stained with polyclonal or monoclonal antibodies (fig.2A,B, lane 4). In addition to the native enzyme, 80- and 64-kDa proteins

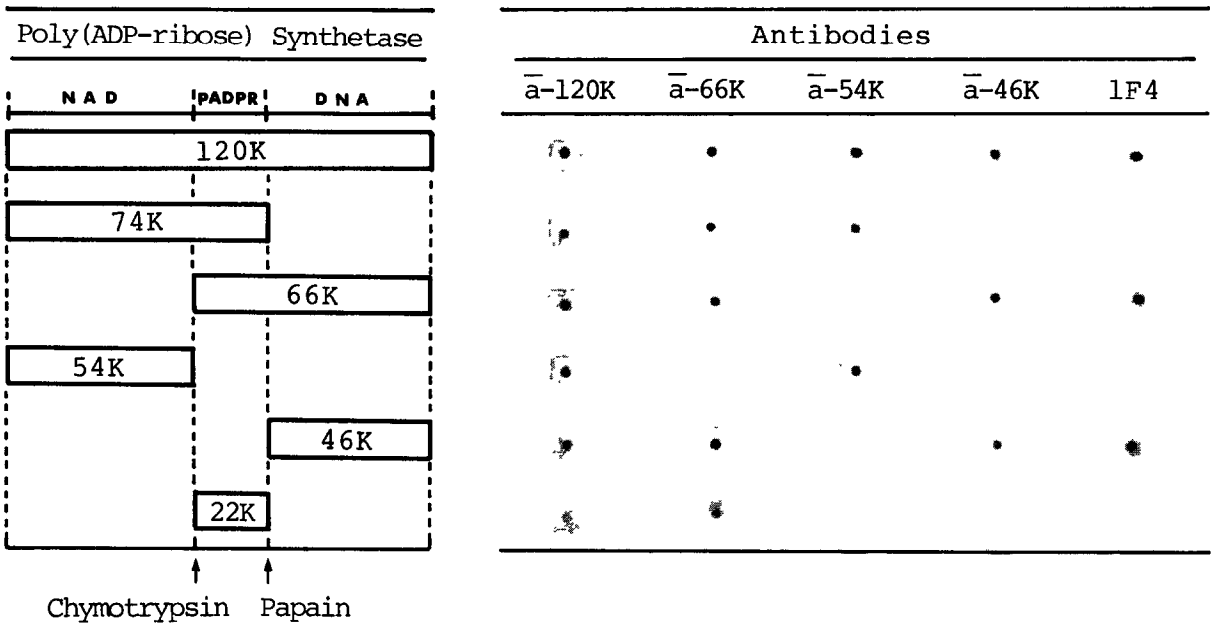

Fig.1. Determination of the antigenic site of the monoclonal antibody 1F4 on the enzyme molecule. The native poly(ADP-ribose) synthetase or its 5 different proteolytic fragments, each purified to homogeneity, were adsorbed onto nitrocellulose filters (4 × 4 mm) as small spots (0.17 pmol protein per spot), dried thoroughly and then immunostained with the antisera (× 500 dilution) against the native enzyme (ā-120K), the 66-kDa fragment (ā-66K), the 54-kDa fragment (ā-54K), and the 46-kDa fragment (ā-46K) or the monoclonal antibody 1F4 (× 10000 dilution).

were clearly detected by this procedure. When the samples were prepared from aged calf thymus kept at 4°C for 24 and 72 h, respectively, and analyzed by the same procedures, there appeared a major cross-reactive fragment (32 kDa) in addition to a number of smaller fragments with concomitant disappearance of the native enzyme and the 80-kDa protein (fig.2A,B, lanes 5,6). The 64-kDa protein appeared to be rather stable as compared with the native enzyme and the 80-kDa protein. These results indicate that the precursor protein(s) of poly(ADP-ribose) synthetase might not exist as judged by the present procedure employed, but the endogenous degradation products of the native enzyme are usually present, especially in aged calf thymus.

To examine the interspecies immunological cross-reactivity of poly(ADP-ribose) synthetase and its degradation products, DNA-binding proteins were prepared from 6 different sources and immunostained either with the polyclonal or the monoclonal antibodies as described above. As shown in fig.3, the protein corresponding to the native poly(ADP-ribose) synthetase was detected in HeLa cells, mouse testis, and chicken liver as in the case of calf thymus. Proteolytic digests of 32 kDa were also present in these animal cells. In contrast, no protein band corresponding to 120 kDa was stained in the cases of spinach leaf and yeast; however, cross-reactive materials of 44 and 40 kDa were stained in spinach and yeast samples, respectively (fig.3, lanes 5,6).

Agemori et al. [12] previously demonstrated that mouse testis poly(ADP-ribose) synthetase has some characteristics common to those of calf thymus enzyme, particularly in regard to molecular mass, amino acid composition, and some other kinetic properties. Holtlund et al. [10] also reported that poly(ADP-ribose) synthetase from Ehrlich ascites tumor cells, pig thymus, and HeLa cells are closely related proteins as judged by similar criteria. Here, structural similarities of the enzyme have been demonstrated by interspecies cross-reactivity as analyzed by polyclonal and monoclonal antibodies. These results, taken together, indicate that the antigenic structures of

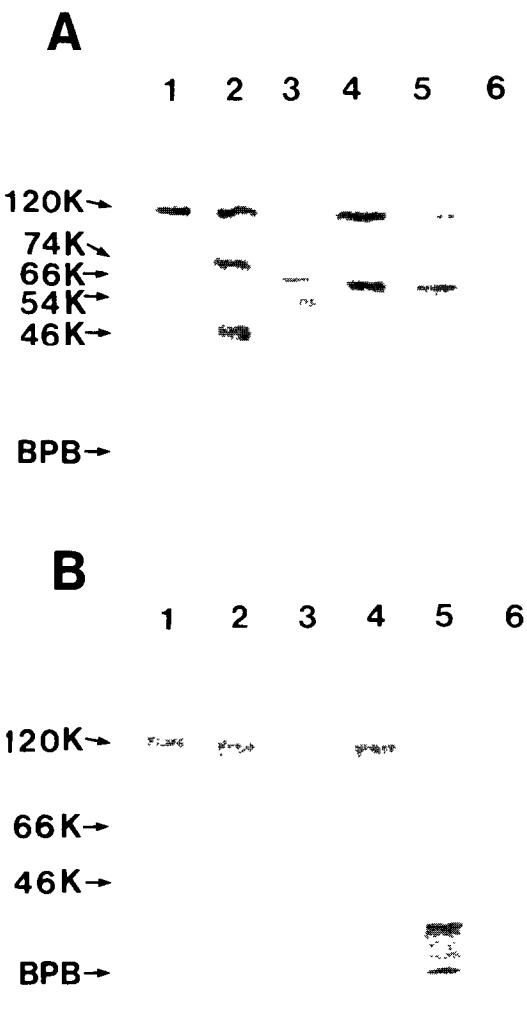

Fig.2. Detection of proteolytic degradation products of poly(ADP-ribose) synthetase by immunostaining. Proteins on a SDS polyacrylamide gel were transferred to a nitrocellulose filter and then immunostained with (A) the antisera against the native enzyme (a-120K) or (B) the monoclonal antibody 1F4. Lane 1, the native enzyme (0.12 μg); lane 2, papain-digests of the enzyme (0.12 μg); lane 3, chymotryptic-digests of the enzyme (0.12 μg); lane 4, DNA-binding proteins (2 μg) prepared from fresh calf thymus; lanes 5,6, DNA-binding proteins (2 μg) prepared from calf thymus left at 4°C for 24 and 72 h, respectively.

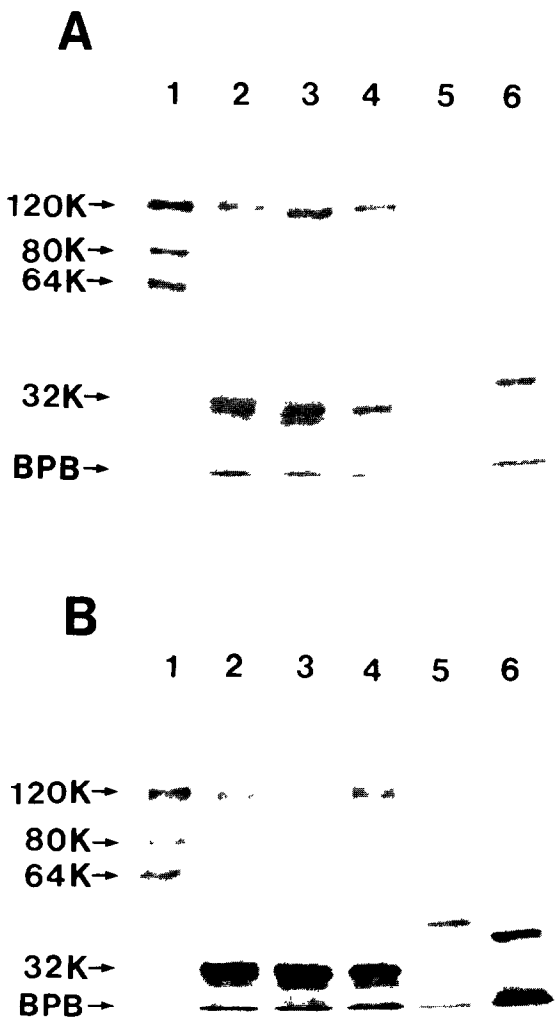

Fig.3. Interspecies cross-reactivity of the polyclonal and monoclonal antibodies to poly(ADP-ribose) synthetase. DNA-binding proteins prepared from the following 6 different sources were immunostained with (A) the antisera against the native enzyme (a-120K) or (B) the monoclonal antibody 1F4 after electrophoresis and transblotting. Lane 1, calf thymus (1 μg protein); lane 2, HeLa cells (5 μg); lane 3, mouse testis (5 μg); lane 4, chicken liver (5 μg); lane 5, spinach leaves (10 μg); and lane 6, the yeast (10 μg).

poly(ADP-ribose) synthetase are highly conserved in animal cells.

It is noted that the 120-kDa protein was not immunostained and yet cross-reactive 44- and 40-kDa proteins were clearly detected in spinach and yeast samples, respectively. Whether or not the cross-reactive proteins in these species are merely endogenous degradation products of the native enzyme is currently under investigation.

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REFERENCES

[1] Chambon, P., Weill, J.D., Doly, J., Strosser, M.T. and Mandel, P. (1966) Biochem. Biophys. Res. Commun. 25, 638–643.
[2] Nishizuka, Y., Ueda, K., Nakazawa, K. and Hayaishi, O. (1967) J. Biol. Chem. 242, 3164–3171.
[3] Sugimura, T., Fujimura, S., Hasegawa, S. and Kawamura, Y. (1967) Biochim. Biophys. Acta 138, 438–441.
[4] Hayaishi, O. and Ueda, K. (1977) Annu. Rev. Biochem. 46, 95–116.
[5] Mandel, P., Okazaki, H. and Niedergang, C. (1977) FEBS Lett. 84, 331–336.
[6] Okayama, H., Edson, C.M., Fukushima, M., Ueda, K. and Hayaishi, O. (1977) J. Biol. Chem. 252, 7000–7005.
[7] Yoshihara, K., Hashida, T., Tanaka, Y., Ohgushi, H., Yoshihara, H. and Kamiya, T. (1978) J. Biol. Chem. 253, 6459–6466.
[8] Tsopanakis, C., Leeson, E., Tsopanakis, A. and Shall, S. (1978) Eur. J. Biochem. 90, 337–345.
[9] Ito, S., Shizuta, Y. and Hayaishi, O. (1979) J. Biol. Chem. 254, 3647–3651.
[10] Holtlund, J., Kristensen, T., Østvold, A.-C. and Laland, S.G. (1981) Eur. J. Biochem. 119, 23–29.
[11] Petzold, S.J., Booth, B.A., Leimbach, G.A. and Berger, N.A. (1981) Biochemistry 20, 7075–7081.
[12] Agemori, M., Kagamiyama, H., Nishikimi, M. and Shizuta, Y. (1982) Arch. Biochem. Biophys. 215, 621–627.
[13] Nishikimi, M., Ogasawara, K., Kameshita, I., Taniguchi, T. and Shizuta, Y. (1982) J. Biol. Chem. 257, 6102–6105.
[14] Kameshita, I., Matsuda, Z., Taniguchi, T. and Shizuta, Y. (1984) J. Biol. Chem. 259, 4770–4776.
[15] Kennett, R.H. (1979) Methods Enzymol. 58, 345–359.
[16] Engvall, E. and Perlmann, P. (1972) J. Immunol. 109, 129–135.
[17] Hawkes, R., Niday, E. and Gordon, J. (1982) Anal. Biochem. 119, 142–147.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Towbin, H., Staehelin, T. and Gordon, J. (1978) Proc. Natl. Acad. Sci. USA 76, 4350–4354.