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SIMILARITIES IN THE MOLECULAR WEIGHT OF POLY(ADPR) POLYMERASE FROM DIFFERENT TISSUES AND SPECIES

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SUMMARY

Using the protein blotting technique, we determine the molecular weight of polyADPR polymerase from different tissues of beef, rat, chicken and pig directly in nuclear homogenates. We report here that the molecular weight of the enzyme is 130,000 in all samples tested.

INTRODUCTION

PolyADPR polymerase is a nuclear enzyme bound to chromatin. It catalyzes the successive addition of the ADPR moiety of the substrate nicotinamide adenine dinucleotide (NAD) to various nuclear proteins. After the discovery of the enzymatic activity in chicken liver nuclei [1] several authors demonstrated the presence of the enzyme and its product in eukaryotic cell nuclei isolated from a number of tissues, from several different animal species. It has been proposed that polyADP-ribosylation is involved in the regulation of DNA repair, DNA replication, the maintenance of chromatin architecture and cell differentiation (see for review,[2, 3]).

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ABBREVIATIONS

ADPR : adenosine diphosphate ribose

SDS : sodium dodecyl sulfate

EDTA : ethylene diamine tetraacetate

EGTA : ethylene glycol bis (β-aminoethyl ether) N,N'-tetraacetate

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Several laboratories have purified polyADPR polymerase from various tissues or cells in culture. Molecular weight determinations of these enzyme preparations, using SDS-polyacrylamide gel electrophoresis, have given different values. Poly ADPR polymerase from calf thymus [4, 5], bovine thymus [6], Ehrlich ascites tumor cells [7] and HeLa cells [8] are reported to have a molecular weight of 120,000 ± 10,000, while the enzymes from pig thymus [9] and rat liver [10] are reported to have a molecular weight of 63,500 and 50,000 respectively.

To establish whether these differences in molecular weight could be the result of tissue or species specific forms of polyADPR polymerase we have determined the molecular weight of polyADPR polymerase using the protein blotting technique, a recently developed serological method for the detection of small amounts of proteins [11]. Proteins present in nuclear homogenates were separated on SDS-polyacrylamide gels and transferred to nitrocellulose sheets and the polyADPR polymerase was identified using a specific antiserum [12]. The sensitivity of this technique allowed us to determine the molecular weight of the enzyme present in several tissues derived from beef, rat, chicken or pig without the need of extensive purification.

MATERIALS AND METHODS

Purified poly ADPRpolymerase. PolyADPR polymerase was purified from calf thymus as described by Mandel *et al.* [4]. One µg carrier protein [bovine serum albumin (BSA) Sigma] was added to each sample of purified enzyme layered on the gel.

Thymus homogenates. Homogenates from calf thymus and pig thymus were prepared as described for the purification of polyADPR polymerase [4] with 0.3 M NaCl and 0.5 M NaCl respectively.

Nuclear homogenates. Nuclei were isolated from beef or rat liver, pancreas, brain and kidney and from chicken brain as described by Chauveau *et al.* [13]. All solutions contained 0.1 mM phenylmethyl sulfonyl fluoride (PMSF ; Sigma, U.S.A.). Neuronal and glial nuclei were isolated from beef brain as described by Ittel and Mandel [14].

The nuclear pellets were immediately homogenized (Potter-Elvehjem) in water with 0.1 mM PMSF and stirred for 30 min. After centrifugation at 4,400 xg for 15 min, the pellets were homogenized (1 g wet weight tissue/ml) with a Potter-Elvehjem homogenizer in 50 mM Tris HCl buffer, pH 7.5 containing 1 mM EDTA, 0.5 mM EGTA, 30 % glycerol, 0.1 mM PMSF, 2 mM β-mercaptoethanol, 0.2 % Triton X-100 and stirred for 30 min. All steps in the preparation of nuclear homogenates were carried out at 4°C. Samples were taken from the nuclear

homogenates for the determination of polyADPR polymerase activity and protein concentration and the rest of the homogenates were diluted (v/v : 2:1) with 3 times concentrated electrophoresis sample buffer containing 60 mM Tris HCl, pH 6.8, 3 % SDS (BDH, England), 6 % β-mercaptoethanol and 9 % glycerol, boiled for 10 min and stored at - 70°C.

Assay of polyADPR polymerase. PolyADPR polymerase activity was measured as described by Rochette-Egly *et al.* [15].

Antiserum. The specific antiserum against polyADPR polymerase was obtained and characterized as described by Okazaki *et al.* [12]. Antiserum dilutions were made in Tris buffered saline (TBS : 10 mM Tris-HCl buffer, pH 7.0, 0.9 % NaCl) containing 3 % normal sheep serum (Institut Pasteur, France).

Protein blotting. Protein blotting was performed as previously described by Towbin *et al.* [11]. After the separation of proteins by SDS-poly acrylamide (Bio Rad Laboratories, USA) gel electrophoresis [16] on 7 % gels (for 4.5 h, at 120 V) the proteins were electrophoretically transferred to nitrocellulose sheets (Millipore, France) for 1 h at 60 V, 1.5 A. Additional protein binding sites on the sheets were saturated by incubation with 3 % BSA in TBS for 2 to 3 h at 37°C. The sheets were then incubated overnight at room temperature in a humidified chamber with appropriate dilutions of specific antiserum or normal rabbit serum. The sheets were washed in two changes of TBS for 30 min, and incubated for 2 to 3 h at room temperature with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G antibodies (Nordic, Holland) diluted 1:1000 in TBS containing 3 % normal sheep serum, followed by washing and the colour reaction with o-dianisidine (Fluka, Switzerland) and hydrogen peroxide (Merck, Germany).

RESULTS

The value of the protein blotting method depends critically on the specificity of the serological reaction used to identify proteins after transfer to nitrocellulose sheets. Due to the non-specific antigen-antibody reactions which may occur at high protein concentration or at low antiserum dilution we have analyzed each cellular or nuclear homogenate by titration of both the amount of protein layered onto the gel and the antiserum dilution used for identification. We predicted that the band on the nitrocellulose sheet corresponding to the reaction of polyADPR polymerase with its specific antibodies would persist at lower antigen antibody concentrations than those bands corresponding to non-specific cross reactions. To test this prediction we determined the molecular weight of purified calf thymus polyADPR polymerase and compared it with the value obtained by analysis of whole calf thymus extracts. Figure 1 shows the sheets onto which different amounts of purified calf thymus enzyme were transferred (sheets a-c). Sheet b incubated with

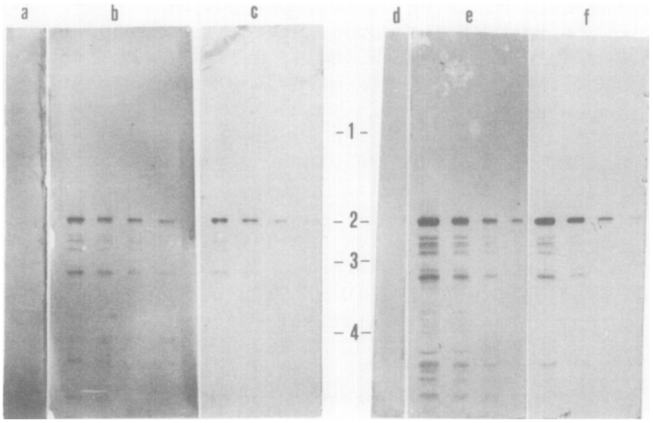

Fig. 1. Protein blotting analysis of calf thymus polyADPR polymerase. Blots were stained with antiserum against polyADPR polymerase (sheets b,c and e,f) or with non-immune rabbit serum (sheets a and d). Different amounts of purified polyADPR polymerase were transferred to sheets b and c (25 ng, 12 ng, 6 ng and 3 ng, from left to right). Sheet a received 25 ng purified enzyme. To sheets e and f calf thymus homogenates containing 16 µg, 8 µg, 4 µg and 2 µg proteins (from left to right) were transferred. Sheet d received 16 µg homogenate proteins. The middle column indicates the position of marker proteins which were transferred to nitrocellulose sheets and stained with amido black [11]. 1, myosin : 200,000 daltons, 2, β-galactosidase : 130,000 daltons 3, phosphorylase b : 94,000 daltons 4, BSA : 68,000 daltons.

antiserum diluted 1:100 shows a strong band with a molecular weight similar to that of β-galactosidase (130,000 dalton) as well as several additional bands of lower molecular weight which are weaker in intensity. These weaker bands disappear when less than 3 ng of enzyme is layered onto the gel. On sheet c, where the antiserum is diluted 1:2,500 and less than 6 ng of enzyme is used, these bands disappear while the band at 130,000 dalton remains visible for all samples. Sheet a was incubated with non-immune rabbit serum diluted 1:100. When 25 ng of enzyme was transferred to this sheet no bands were observed. From these results, we conclude that the molecular weight of the purified poly ADPR polymerase is 130,000 dalton, in agreement with the previously reported molecular weight [17]. The weak bands of lower molecular weight appearing together with the main enzyme band, could be the degradation products of the enzyme, possibly occuring during the boiling of the enzyme in the electrophoresis buffer before introducing the sample onto the gel. This was confirmed by the

fact that when the enzyme in electrophoresis sample buffer was boiled several times, these bands gained more intensity (data not shown). Sheets d to f show the results when calf thymus homogenates were layered onto a gel, care being taken to use the same amounts of enzyme and antiserum dilutions (the amount of enzyme in the homogenates was estimated by measuring the polyADPR activity as described by Mandel *et al.* [4], e.g. 1 unit of enzymatic activity corresponds to 1 µg of enzymatic protein). We observed a band at 130,000 dalton which is visible for 3 ng of enzyme at an antiserum dilution of 1:100 (sheet e) and with 6 ng enzyme at an antiserum dilution of 1:2,500 (sheet f). These results are similar to those obtained when using purified calf thymus enzyme. No bands are observed on sheet d which was incubated with non immune rabbit serum at a dilution of 1:100.

From these results we conclude that we can use the protein blotting technique to determine the molecular weight of the enzyme in nuclear homogenates when both the antigen and antibody concentrations are carefully titrated.

Okazaki *et al.* [12] using a micro complement fixation test, reported that the antiserum which was used in the experiments shown in Fig. 1 cross reacts with polyADPR polymerase from different tissues and species. It therefore seemed possible that by using this antiserum we could extend the molecular weight determination to nuclear homogenates derived from tissues other than calf thymus. Fig. 2 shows the results obtained for different tissues from beef (sheets a-d) and rat (sheets e-g) origin, from chicken brain (sheet h) and from pig thymus (sheet i). In all tissues the enzyme exhibits a molecular weight of 130,000. All sheets were incubated with antiserum diluted 1:500. When the sheets were incubated with a similarly diluted non-immune rabbit serum, no bands were observed (data not shown).

DISCUSSION

Using the protein blotting technique, we have identified polyADPR polymerase in different organs from beef, rat, chicken and pig as having a molecular

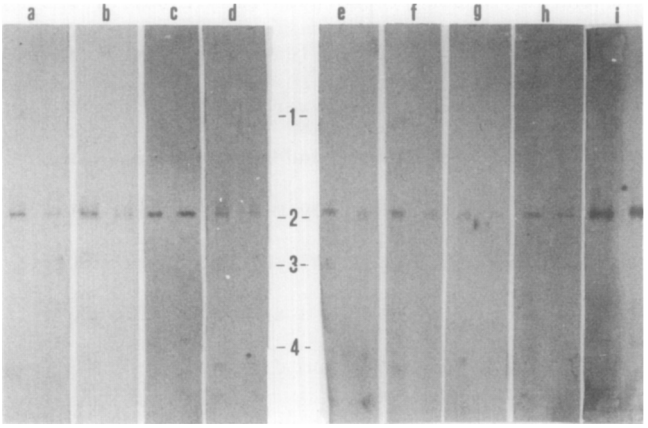

Fig. 2. Protein blotting analysis of polyADPR polymerase from different tissues and species : the amounts of protein layered on gels are indicated in parenthesis. a : beef liver (4 and 2 μg), b, beef pancreas (12 and 6 μg), c, beef brain (glial cells : 1 μg ; neurons : 2.5 μg), d, beef kidney (2 and 1 μg), e, rat liver (6 and 3 μg), f, rat pancreas (2.4 μg and 1.2 μg), g, rat brain (1.3 and 0.6 μg), h, chicken brain (4 and 2 μg), i, pig thymus (5 and 3 μg). All the sheets were incubated with specific antiserum as a dilution 1:500. The middle column indicates the marker proteins as mentioned in Fig. 1.

weight of 130,000. We were unable to find evidence which would indicate that species or tissue specific differences in the molecular weight of polyADPR polymerase exist. Our molecular weight determinations were made using appropriate antiserum dilution conditions such that only one protein was detected on nitrocellulose sheets. At higher concentrations of antiserum or with increasing amounts of antigen, additional proteins may react with the antiserum. However, the antigenicity and/or the quantity of these proteins differs from that of the enzyme. We consider these additional proteins to represent polypeptides derived from the degradation of the enzyme molecule rather than unrelated nuclear proteins cross-reacting with the antiserum because of the following observations. When nuclear homogenates are prepared without PMSF and are stored without the electrophoresis sample buffer, the additional bands on nitrocellulose sheets increased in intensity (data not shown). Furthermore all of the additional bands are of a lower molecular weight than 130,000 and seem to be similar in all sample preparations including that of the purified enzyme.

Also, we have noticed that repetitive freezing and thawing or boiling of the same enzyme sample resulted in a big increase in the intensity of these bands.

Our results indicating the molecular weight of polyADPR polymerase as 130,000 would agree with the reported values for the enzymes purified from calf thymus [4, 5] and beef liver as mentioned by OHGUSHI *et al.* [18]. Our results differ however from the values reported for the purified enzymes from rat liver [10] and pig thymus [9] which were 50,000 and 63,000 respectively. The molecular weight of the purified rat liver enzyme was recently revised to 110,000 [19] a value which is closer to our results.

We found the pig thymus enzyme to be very unstable. For example when we prolonged the time of preparation of pig thymus homogenate by first isolating nuclei in order to analyse nuclear homogenates, an additional protein with a molecular weight of 57,000 reacted specifically with the antiserum (data not shown). This protein is most likely generated by the degradation of the 130,000 dalton enzyme molecule. We do not know if this protein corresponds to the 63,500 molecular weight enzyme purified from pig thymus [9].

Our finding that polyADPR polymerase has an identical molecular weight in different tissues and species suggest that the enzyme's structure-function relationships have been well preserved during evolution. In this respect poly ADPR polymerase is comparable to other nuclear enzymes such as RNA polymerases [20], DNA polymerase α and β [21] and to histones [22] the major structural proteins of chromatin.

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