Antibody to poly(adenosine diphosphate-ribose) polymerase and its use in chromatin analysis

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ABSTRACT

To facilitate investigations on the organization of poly (ADP-Rib) polymerase in chromatin, and to elucidate its biological function, polymerase purified from HeLa nuclei was used to elicit antibodies in mice. The anti-polymerase sera was found to be specific by multiple criteria.

The association of polymerase with oligonucleosomes of differing chain size was determined by the specific binding of polymerase antibody (and as control, anti-histone H3) to nitrocellulose transfers of native electrophoretic gels of these particles. By this technique, polymerase appears to be associated with a select subclass of nucleosomes. Further resolution of the polymerase binding sites within nucleosome classes was achieved with the antibody by two-dimensional native polyacrylamide gel electrophoresis and nitrocellulose transfer.

INTRODUCTION

An understanding of the interaction of NHP with specialized domains of chromatin is of primary importance for the assignment of role for these macromolecules in regulation of the eukaryotic genes. Limited progress has been accomplished in this field because of the lack of availability of purified and well characterized NHP for structure/function studies. In contrast to the histones which are abundant and essentially homogeneous, the NHP proteins are diverse and heterogeneous and differ from tissue to tissue. These facts have prevented extensive purification of many singular NHP (with the exception of the HMG proteins) for use in elucidation of the interaction of these proteins with domains of chromatin. However, more recently, Elgin et al. (1) have provided considerable data on the association with chromatin and chromosomes of specialized classes of NHP related to the heat shock system of *Drosophila*; This investigation was made possible by the availability of antisera to certain of these NHP proteins. Current models for chromatin structure rarely take into consideration the interaction of NHP with DNA; however,

before a final physical or topographic determination of higher order folding of the genome is possible, more information must be obtained on the binding of these proteins with the polynucleosomal structural domains of chromatin.

Our laboratory has recently provided considerable information on the interaction with chromatin of one specific NHP, the enzyme poly (ADP-Rib) polymerase. These studies have been facilitated by the fact that this protein is bound to oligonucleosomes and has a defined enzymatic activity, which can be conveniently and sensitively assayed with radioactive substrate (NAD). The enzyme catalyzes a successive covalent transfer of ADP-Rib moieties from NAD to various nuclear protein acceptor molecules including core histones and histone H1 (2-4). In addition, a major NHP protein acceptor (112 K) for the enzymatic reaction was shown by Jump and Smulson (5) to copurify with the polymerase itself.

Previous in vitro data indicated that the specific activity of poly (ADP-Rib) polymerase increases with the size of nucleosomal chains. Utilizing the forementioned incorporation of ADP-Rib to the 112 K protein as a marker, it was tentatively concluded that the maximal enzymatic "activity" on oligonucleosomes (8-10 N) represents preferred binding of this enzyme to specialized domains of chromatin at a periodicity of approximately 8-10 nucleosomes. It is of interest that one turn of the 300 Å chromatin fiber is reported to consist of approximately 7-8 nucleosomes. Accordingly, information on the interaction of poly (ADP-Rib) polymerase with specialized regions of polynucleosomes could provide a useful and sensitive probe for elucidation of general relationships between non-histone proteins and chromatin structural domains.

Here, we report the preparation and characterization of an antibody specifically directed against poly (ADP-Rib) polymerase. The use of this antibody seems particularly suitable to study various aspects of the structure and function of poly ADP-ribosylation reactions with respect to polynucleosomal domains of chromatin.

EXPERIMENTAL PROCEDURES

Materials: [³²P] NAD was purchased from New England Nuclear, and [¹²⁵I] Protein A from Amersham. In all experiments asynchronously growing HeLa S₃ cells at mid log density (8-10 x 10⁵ cells/ml) were maintained in suspension culture at 37° C.

Preparation of HeLa Chromatin: HeLa cell nuclei were treated with micrococcal nuclease (30 units/ml) for 3 min at 37°C. by the method of Butt et al. (6). Under above digestion condition, 9-10% of the chromatin DNA was rendered acid soluble. Digested chromatin was separated on 10-30% linear sucrose gradients by centrifugation at 38K for 5 1/2 hr. in an SW40 rotor.

Preparation of antigen and antisera: Poly (ADP-Rib) polymerase was purified from HeLa cell according to the method of Jump and Smulson (5). Anti-serum to polymerase was elicited by injecting BALB/c mice at multiple intradermal sites with 200 ug polymerase in 50% Freund's complete adjuvant. Booster injections were administered on two subsequent weeks. The mice were sacrificed and the total body blood was collected by cardiac puncture.

Electrophoretic transfer from polyacrylamide gels to nitrocellulose sheets: Electrophoretic blotting from SDS polyacrylamide gels: The nuclear protein samples resolved on SDS-polyacrylamide gels were electrophoretically blotted onto nitrocellulose sheets by the method of Towbin et al., (7).

Electrophoretic blotting of chromatin gels to nitrocellulose sheets: Chromatin fragments resolved on polyacrylamide gels (8) were electrophoretically transferred to nitrocellulose sheets. The method of transfer was identical to that used for SDS-gels. However, the composition of transfer buffer was 89mM Tris Borate buffer pH 8.0 containing 2mM EDTA.

Immunological Detection of Proteins on Nitrocellulose: The electrophoretic blots were soaked in 3% bovine serum albumin in phosphate buffer saline for 1 hr. at 37°C to saturate additional protein binding sites. The nitrocellulose sheets were rinsed in PBS at least 4 times and incubated with antisera appropriately diluted in 1% BSA containing 0.05% NP-40 (detergent) overnight at 4° C. The sheets were then rinsed with PBS containing 0.05% NP-40 at least 6 times. Subsequently, the sheets were treated with 3uCi [¹²⁵I] protein A, 10 ug cold protein A, 0.25% BSA, 0.05% NP-40 containing PBS for 4 hr at room temperature. Finally the sheets were washed about 4 times with PBS over 1 hr period and then twice with solution containing 0.25% BSA, 0.4% Sarkosyl and 1 M NaCl in PBS. The Sarkosyl wash eliminates the non-specifically bound [¹²⁵I] protein A. The sheets were finally washed with PBS, dried and autoradiographed using Cronex Film.

Immunoprecipitation of polymerase enzymatic activity by antisera: Small aliquot of chromatin or the free polymerase was incubated with appropriately diluted pre-immune or immune sera. The mixture was agitated at 4°C overnight. An aliquot of protein A Sepharose was added to the tubes and mixed for 4 hr. at 4°C. The mixtures were centrifuged at 8K for 5 min. The supernatant was used to assay polymerase activity using method previously described (5).

Solid Phase Radioimmunoassay: This procedure was performed according to the method of Romani et al. (9).

RESULTS

Poly (ADP-Rib) polymerase was purified from HeLa cell nuclei by the methods previously described (5). A coomassie blue stain of the purified polymerase preparation electrophoresed on a SDS-polyacrylamide gel indicated that the preparation contained essentially pure poly (ADP-Rib) polymerase (not shown). The purified polymerase was injected into mice to elicit antibodies as described in Methods. The sera of the mice were collected and checked for specificity.

We have previously described a sensitive method for microtiter analysis of nuclear protein - antibody reaction utilizing [¹²⁵I] protein A (9). A binding curve of [¹²⁵I] protein A to microtiter plates coated with 0.3M KCl nuclear extract containing approximately 0.06 units of polymerase activity (0.5 ugm protein) reacted with various dilutions of antisera is shown in Fig. 1A. The data indicate that the amount of protein-A bound is dependent on the concentration of antisera used, i.e. maximal amount of [¹²⁵I] counts incorporated were at lowest sera dilution.

The binding of antipolymerase sera to wells coated with partially purified polymerase (0.06 units) could be inhibited by incubation of antisera with a suspension of purified polymerase. A typical inhibition curve is presented in Fig. 1B. Under these conditions, 50% inhibition was obtained with approximately 5 ug (0.6 units) of purified polymerase.

The solid phase radioimmunoassay satisfies the basic requirements for serological analysis of polymerase. The reaction is dependent on the concentration of the antigen and on the concentration of antisera and can be inhibited by the homologous antigen. To establish that the antibody in mouse sera was directed specifically against polymerase, the effects of preimmune and immune sera were determined on enzyme activity of both oligonucleosome bound polymerase and partially purified polymerase (Table I). The data indicates that preimmune sera had essentially no effect on

FIGURE 1: Dependence of the binding of [^{125}I] protein A on sera concentration at a fixed antigen concentration and inhibition of antibody binding by free antigen. Microtiter plates were coated with 0.5 μg of a 0.3 M KCl nuclear extract containing approximately .06 units of polymerase activity (5). A. The wells were incubated with various dilutions of antisera, followed by treatment with 0.01 μCi [^{125}I] protein A (30 mCi/mg) (9). B. Solutions containing differing concentration of purified polymerase (0.5 - 15 μg) were added to the wells followed by the addition of a 1:20 dilution of antisera. The microtiter plate-bound antigen-antibody complex was determined with [^{125}I] protein A as above.

the enzymatic activity. In contrast, immune sera inhibited activities of the oligonucleosomes and nuclear extracts by 81% and 75%, respectively.

For further determination of the specificity of the antibody reaction, a 0.3 M KCl extract of HeLa nuclei was electrophoresed on SDS polyacrylamide gels (Fig. 2). The electrophoresed material was transferred to nitrocellulose strips. Amido black staining of this transfer (Fig. 2A, lane 1) shows multiple bands, representing different nuclear proteins. To localize the position of polymerase, the automodification reaction of this enzyme was utilized (5). The 0.3 M KCl extract, was incubated with [^{32}P] NAD, histones and DNA under standard polymerase assay conditions (5). The electrophoretically separated proteins were transferred to nitrocellulose and autoradiographed (Fig. 2A, lane 2). Two major [^{32}P] ADP-ribosylated acceptors are evident, histone H1 and a 112 K protein, previously identified to be the automodified polymerase (5). The nitrocellulose blots were then incubated with pre-immune and immune mice sera and subsequently [^{125}I] protein A. No

FIGURE 2: Correlation between antipolymerase sera reactivity with protein band on nitrocellulose blot and electrophoretically separated [^{32}P] auto-modified polymerase. A. Lane 1 represents an amido black stain of typical 0.3 M KCl extract nitrocellulose transfers. Lane 2: The 0.3 M KCl extract was incubated with [^{32}P] NAD, DNA and histones under standard poly ADP-ribosylation conditions and electrophoresed (5). The lane in the gel containing this sample was transferred and autoradiography was performed. B. In a separate transfer as those shown in A, transferred proteins were incubated with 1:20 diluted polymerase antisera, followed by detection of antigen-antibody complex with [^{125}I] protein A described in Methods. An autoradiographic representation is shown. Lane 1: reaction with preimmune serum; lane 2: reaction with anti-polymerase sera.

reactivity was noted with any protein band when the strip was incubated with pre-immune serum (Fig. 2B, lane 1). The data (lane 2) show that only one protein band interacted with anti-polymerase sera from this preparation. Although performed with transfers from different electrophoretic runs, the relative migration rates of the automodified polymerase (Fig. 2A, lane 2) and the immunoreactive band (Fig. 2B, lane 2) were in agreement. In the next series of experiments, we tested whether

these gel transfer techniques could be exploited to study the detailed association of this NHP antigen with chromatin subfractions.

Chromatin fragments released by micrococcal nuclease digestion were separated on native chromatin gels by electrophoresis. An ethidium bromide stain of this separation is shown in Fig. 3A. We tested the feasibility of transferring the chromatin particles separated by this procedure to nitrocellulose. The fidelity of exchange of particles to nitrocellulose was monitored by restaining the gel with ethidium bromide after electrophoretic transfer. Chromatin was prepared from cells treated with [^3H] thymidine and [^3H] leucine to label the cellular DNA and protein. The chromatin fragments were run on polyacrylamide gel and transferred to nitrocellulose. The gel and nitrocellulose blots were cut and counted in a scintillation counter. The results indicate that 78% of

FIGURE 3: The reactivity of anti-histone H3 and anti-polymerase sera with transfers of nucleosome oligomers. A gradient sample containing oligonucleosomes (1-4N) was separated on native gels and was transferred by electrophoresis to nitrocellulose. The transfer was subsequently incubated with either anti-histone H3 sera (A) or antipolymerase sera (B), [^{125}I] protein A and exposed for autoradiography. Et Br is the stain before transfer; R is the autoradiograph

the transferred DNA and 71% of the transferred proteins are retained on the nitrocellulose.

Reactivity of the blot with anti-histone H3 sera and [^{125} I] protein A clearly shows that the proteins of oligonucleosomes were retained on nitrocellulose (Fig 3A.) The area of reactivity of the H3 antiserum within each oligomer repeat size corresponded closely to the ethidium bromide staining pattern. When anti-polymerase sera was used (Fig. 3B), the autoradiogram shows relatively sharp bands of immune complex compared to the broader bands noted on reaction with H3 antibody. The broadness of a given nucleosome repeat size is due to the heterogeneity of linker DNA length and nuclear protein composition of differing size particles within a single class. The data confirms the observation (Table I) that this preparation of anti-polymerase sera can react with the nucleosomal-bound polymerase, and furthermore that perhaps a limited population of nucleosomes within a subset contain this non-histone protein. To help resolve the polymerase-bound nucleosomes from the bulk, the nucleosomes were further separated by two dimensional electrophoresis.

Table I. The Effect of Anti-poly (ADP-Rib) Polymerase Sera on Nucleosomal and Nuclear Polymerase Activity.

Treatment Prior to Assay	Oligonucleosomal Activity	Nuclear Extract Activity
PBS	(100) ^a	(100) ^b
Preimmune Sera	100	100
Immune Sera	25	19

^a8.2 X 10⁴ CPM were incorporated. This activity was inhibited 99% by addition of the polymerase inhibitor, nicotinamide to the assay.

^b100% represents 7.6 X 10³ CPM.

20 ul of 0.3 M KCl HeLa nuclear extract (containing approximately 0.03 units of polymerase activity 0.25 ug protein) was added to 25 ul of either of the two sera or PBS and the volume was adjusted to 200 ul with PBS. Alternately, 0.05 A₂₆₀ of a sucrose gradient fraction of polynucleosomes containing predominately 6-8 N oligonucleosomes was added to 10 ul of either of the two sera or PBS and the volume adjusted to 100 ul with PBS. All reaction mixtures contained 0.1 M benzamidine. The reaction mixtures were incubated with agitation at 4°C for 14 hr, after which protein-A sepharose (30 ugm) was added and the mixtures left shaking for two hr. The reaction mixture was centrifuged at 10,000 RPM for 15 min and subsequently the supernatants assayed for poly (ADP-Rib) polymerase activity as previously described for either chromatin or DNA and histone-free enzyme (5).

FINER RESOLUTION OF POLYMERASE-BOUND NUCLEOSOMES

The resolution of mono- through tetra- nucleosomes in two dimensions is shown in the data of Fig. 4A. When one and two-dimensionally resolved nucleosomes were transferred onto nitrocellulose, incubated with anti-H3 sera, followed by detection of the immune complex using [^{125}I] protein A, all transferred sub-classes of nucleosomes were noted to react with H3 antibody (Fig. 4B). (The dotted lines represent the ethidium bromide staining area of the second dimension.) However, when an identical nitrocellulose strip was treated with anti-polymerase sera (Fig. 4C), not all the potentially available nucleosomes reacted with polymerase antibody.

It was previously noted that polymerase specific activity increases with nucleosome chain length (6). Highest specific activity was noted in oligomers of approximately 7-10N. However, poly (ADP-Rib) polymerase activity in vitro measurements per se are complicated by a number of factors. There is a correlation between the extent of single strand breaks in DNA within a chromatin preparation and the level of (ADP-Rib) incorporation (10-12). The amount of endogenous poly (ADP-Rib) bound to acceptors would also be expected to influence in vitro activity values.

Oligonucleosomes of differing size ranges were assayed for polymerase enzymatic activity and the amount of antigen (polymerase) present in the nucleosomes using solid phase radio-immunoassay. The immunological results (not shown) indicated that the fraction with high polymerase activity contains higher levels of the polymerase antigen and the opposite was true for fractions with lower polymerase activity.

DISCUSSION

The work presented in this paper provides experimental evidence for the development of specific antibodies in mice against the NHP, poly (ADP-Rib) polymerase. Recently Ikai et al. (13) and Okazaki et al. (14) have also reported on the isolation of antibodies to calf thymus polymerase. The availability of specific antibodies to chromosomal proteins with established enzymatic activities should greatly accelerate the elucidation of their distributions and functions in chromatin.

Elgin et al. (1), using antisera to several *Drosophila* chromosomal NHP fractions, and fluorescent antibody techniques have studied the differential binding of these proteins to regions of the polytene chromosome. Peters et al. (15) using antibodies to HMG-T studied its

FIGURE 4: Reactivity of anti-H3 or anti-polymerase sera with nitrocellulose blots of oligonucleosomes resolved on polyacrylamide gels by two dimensional electrophoresis. Mono-tetra nucleosomes were separated on a polyacrylamide gel. The slab gel lane was cut and fused to 3% acrylamide, 0.5% agarose gel and electrophoresed in second dimension. The two-dimensionally resolved nucleosomes, as well as nucleosomes, resolved in one direction were transferred to nitrocellulose strips. A. Ethidium bromide staining pattern B. Autoradiogram of transfer treated with anti-H3. C. Autoradiogram of transfer after treatment with anti-polymerase sera.

distribution amongst oligonucleosomes derived from nuclease digested chromatin. Antibodies to histone H4 and to NHP proteins HMG-1 and HMG-17 were also used for this purpose (16).

Poly (ADP-Rib) polymerase catalyzes the poly ADP-ribosylation of core histones, and H1 histone. It also represents a major NHP acceptor for poly ADP-ribosylation via an automodification reactions (5). Although a definite biological function for the enzyme has not been established, various lines of investigation suggest that it may function in DNA replication/repair reactions (11, 12, 18, 19). The use of the antipolymerase sera characterized in this study hopefully will help to clarify a biological function for the enzyme.

The results presented suggest that the antisera developed in mice against the polymerase is highly specific. The immune reactions of anti-H3 and antipolymerase sera with transfers of nucleosomes of differing chain lengths appeared somewhat different in specificity (Fig. 4). The reaction of antipolymerase sera with nucleosomes separated in either one or two dimensions suggests that the polymerase may not be associated with all classes of nucleosomes, but more direct evidence is required to establish this fact. However, indirect evidence supporting this concept comes from the work of Jump et al. (2) who showed that amongst a single size nucleosome, those released preferentially by micrococcal nuclease have high polymerase specific activity as compared to those released later during digestion.

The antibody to polymerase may help in the future to clarify a number of as yet poorly understood characteristics of the poly ADP-ribosylation system. For example, the ability to detect small levels of polymerase, or to immunologically inhibit its activity, possibly in cells, might help to confirm its putative role in DNA repair (11, 18, 19). In addition, the enzyme may be associated with specific domains of chromatin which may be isolated by an immunological approach.

Butt and Smulson have recently demonstrated an NAD-dependent complex formation of ADP-ribosylated nucleosomes (8). It was subsequently determined that the chromatin aggregate is composed of poly ADP-ribosylated histone H1, core histones, and also automodified poly (ADP-Rib) polymerase (20). Accordingly, the antibody could be useful in the isolation of this polymerase-enriched class of nucleosomes, enabling a better understanding of the role of ADP-ribosylation in maintenance of the organization of chromatin.

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