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Oligo(3′-deoxyADP-ribosyl)ation of the nuclear matrix lamins from rat liver utilizing 3′-deoxyNAD as a substrate

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It has previously been shown that the levels of poly(ADP-ribose)polymerase and polymers of ADP-ribose that co-purify with the nuclear matrix in regenerating liver fluctuate with the levels of in vivo DNA replication [(1988) FEBS Lett. 236, 362–366]. We have now electrophoretically identified lamins A and C, and poly(ADP-ribose)polymerase as the main protein targets for poly(ADP-ribosyl)ation in isolated nuclear matrices from adult rat liver. The identification of these protein acceptors was facilitated by the utilization of ^{32}P-radiolabeled 3′-deoxyNAD as a substrate for nuclear matrix extracts in the presence of exogenously added DNA-dependent poly(ADP-ribose)polymerase from calf thymus. The extent of protein modification was time- and substrate concentration-dependent. These results are consistent with the hypothesis that the poly(ADP-ribose) modification of the lamins A and C and poly(ADP-ribose)polymerase are important to modulate chromatin-nuclear matrix interactions in rat liver.

3′-DeoxyNAD; Lamin; Nuclear matrix; LiDS-PAGE; Poly(ADP-ribose)polymerase; Rat liver

1. INTRODUCTION

The nuclear matrix is composed of a proteinaceous structure that gives shape to the nucleus [1–3]. This structural framework may be isolated in an intact form by the sequential treatment of purified nuclei with either endogenous or exogenous nucleases and high salt extraction [1–3]. The nuclear scaffold obtained is totally free of histones and only 1% or less of the total DNA remains tightly associated with it. A number of physiological functions other than structural roles have recently been proposed for the nuclear matrix [1–4]. Thus, it has been suggested that both DNA replication [5,6] and DNA-excision repair [7,8] take place in this subnuclear compartment. The molecular role of the nuclear matrix in DNA replication has recently been substantiated by convincing enzymological data [9–11]. For example, activities corresponding to DNA primase [9], DNA polymerase α [10], and other enzymes from the macromolecular replicational complex or replisome [11], have recently been identified in nuclear matrix preparations. It is important to note, however, that the main protein components of this subnuclear fraction are the lamins A, B, and C [1–4]. These proteins of 60–75 kDa in molecular mass form the membrane laminar structure of the nucleus. They appear to provide this organelle with a dynamic structural framework to perform its physiological functions more efficiently.

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Nuclear matrix proteins have been shown to be covalently modified with both phosphate groups [12] and ADP-ribose polymers [13–16]. This latter reaction is catalyzed by poly(ADP-ribose)polymerase, a DNA-dependent enzyme that utilized NAD as a substrate. In fact, we have recently shown that the levels of nuclear matrix-associated poly(ADP-ribose)polymerase are initially decreased and later recover to normal values concomitantly with in vivo DNA replication.

Most of the proteins that have been identified as acceptors for poly(ADP-ribosyl)ation to date [17] are characterized for having DNA-binding affinity. Due to the fact that the nuclear matrix lamins have significant DNA-binding affinity [18], we decided to test whether these proteins could also function as in vitro targets for poly(ADP-ribosyl)ation utilizing [^{32}P]3′-dexoyNAD as a substrate in the presence or absence of exogenously added poly(ADP-ribose)polymerase purified from calf thymus.

2. MATERIALS AND METHODS

Male Sprague-Dawley rats (200–250 g) were obtained from Charles Ribers Laboratories. Rat livers were utilized as the source for the nuclear matrices used in the experiments described below.

2.1. *Isolation of nuclear matrices*

The nuclear matrix fraction from adult rat liver was isolated as previously described [16]. Briefly, nuclei were first isolated by centrifugation through a 2.2 M sucrose cushion. Isolated nuclei were subsequently incubated for 45 min at 37°C to endogenously degrade DNA. The incubation mixture was centrifuged at 15 000 × *g* and the pellet obtained was sequentially extracted with a low salt-containing buffer, a high salt-containing buffer and 0.1% Triton X-100. These

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nuclear matrices typically contained less than 2% of total DNA and approximately 12–15% of the total nuclear protein. The levels of endogenous poly(ADP-ribose)polymerase activity observed in the nuclear matrix fraction were 15–30% of the total nuclear enzyme activity [16].

2.2. *Synthesis of protein-bound oligo(3'-deoxyADP-ribose) in nuclear matrix extracts*

Eighteen to 50 μg of nuclear matrix protein were incubated with different concentrations of [^{32}P] 3'-deoxyNAD at 37°C in the presence or absence of 0.2 μg/ml of pure DNA-dependent poly(ADP-ribose)polymerase. The times of incubation for each experiment are indicated for each individual experiment (vide infra). The incubation mixture also contained 100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM DTT, and 20 μg/ml of active calf thymus DNA. The reaction was typically stopped by adding trichloroacetic acid to 20% (w/v) final concentration. The total amount of acid-insoluble radioactivity was measured by liquid scintillation counting to determine the levels of enzyme activity. Alternatively, the reaction was stopped by a 1:1 dilution with electrophoresis loading buffer containing 2.0% LiDS (lithium dodecyl sulfate), 4 M urea, 20% glycerol, 20 mM β-mercaptoethanol, 60 mM Tris-phosphate buffer, pH 5.0, and 0.1% Bromophenol blue.

2.3. *Polyacrylamide gel electrophoresis*

Identification of (3'-deoxyADP-ribosyl)ated-proteins was achieved by autoradiography following polyacrylamide gel electrophoresis. Samples containing (3'-deoxyADP-ribosyl)ated-proteins were electrophoresed with 30 mM Tris-phosphate, pH 5.0, as the running buffer for 15 h at 15 mA. Slab gels contained 8% acrylamide, 60 mM Tris-phosphate buffer, pH 5.0, 4.5 M urea, and 0.1% LiDS. The gels were stained with Coomasie blue, dried, and exposed to Kodak X-AR2 X-ray film at room temperature.

2.4. *Miscellaneous*

DNA-dependent poly(ADP-ribose)polymerase was purfied from calf thymus as described by Zahradka and Ebisuzaki [19]. Protein and DNA were determined by the methods of Smith et al. [20] and Burton [21], respectively.

3. RESULTS AND DISCUSSION

3.1. *Utilization of 3'-deoxyNAD as a substrate by nuclear matrix-associated poly(ADP-ribose) polymerase*

It has previously been shown that 3'-deoxyNAD is a good substrate for highly purified DNA-dependent poly(ADP-ribose)polymerase from calf thymus [22]. This enzyme activity has also recently been found to copurify with the nuclear matrix [16]. In this report, we have performed experiments to determine whether nuclear matrix proteins, especially the lamins, function as covalent acceptors for (ADP-ribose) polymers utilizing 3'-deoxyNAD as a substrate for endogenous poly(ADP-ribose)polymerase activity associated with the nuclear matrix with or without exogenously added poly(ADP-ribose)polymerase purified from calf thymus. Table I, experiment 3, shows that the total poly(ADP-ribose)polymerase activity observed following incubation of a nuclear matrix ectract with NAD and pure polymerase (1491 pmol/min per mg of protein) was higher than the combined activity seen with either the nuclear matrix (44 pmol/min per mg of protein) or pure polymerase (567 pmol/min per mg of protein) alone. This synergistic effect strongly suggested that the nuclear matrix fraction contained efficient protein cceptors for poly(ADP-ribosyl)ation. Experiment 4 shows that addition of benzamide, a competitive inhibitor of poly(ADP-ribose) biosynthesis, completely blocks the incorporation of radiolabeled substrate into acid-insoluble material. An alternative explanation for the synergistic effect observed is that the average size of the polymers synthesized in experiment 3 was larger than the size of the polymers synthesized in experiments 1 and 2. This possibility is further substantiated by the known ability of the polymerase to synthesize polymers of more than 200 residues in size [22] when using NAD as a substrate. Therefore, instead of determining the average size of the polymers synthesized with NAD as a substrate, an approach that is usually complicted by the fact that the polymers formed with NAD are highly branched [22], we utilized 3'-deoxyNAD as a substrate to address this question. Utilization of 3'-deoxyNAD results in the synthesis of oligomers of no more than four residues in size [23]. Further, the electrophoretic mobility of oligo(3'-deoxyADP-ribosyl)ated-proteins appears not to be altered (unpublished observations).

Table I

Synthesis of poly(3'-dADP-ribose) in isolated rat liver nuclear matrix either in the presence or absence of benzamide and purified DNA-dependent poly(ADP-ribose)polymerase from calf thymus

Exp. no.	Substrate (100 μM)	Nuclear matrix[a]	PADPRP[b]	Benzamide (1 mM)	Radioactivity (cpm)	Polymerase activity (pmol/min/mg)
1	NAD	+	–	–	156	44
2	NAD	–	+	–	2007	567
3	NAD	+	+	–	5279	1491
4	NAD	+	+	+	72	20
5	3'-dNAD	+	–	–	80	5
6	3'-dNAD	–	+	–	484	15
7	3'-dNAD	+	+	–	1275	39
8	3'-dNAD	+	+	+	ND	ND

The specific radioactivities of NAD and 3'-dNAD utilized were both equal to 3.29×10^7 cpm/μmol. ND = either non-detectable or below the levels of sensitivity

[a] The total amount of nuclear matrix protein utilized was 180 μg/ml and 500 μg/ml when using NAD and 3'-dNAD, respectively

[b] PADPRP; 0.2 μg of poly(ADP-ribose)polymerase/assay. Each assay was performed in triplicate

Therefore, we measured the polymerase activity of the nuclear matrix with 3'-deoxyNAD as a substrate in the absence or presence of exogenous polymerase. As shown in Table I, experiments 5–8, the enzyme activity measured in the presence of exogenous enzyme (39 pmol/min/mg of protein) was higher than the addition of the activities observed with either the nuclear matrix (5 pmol/min/mg of protein) or poly(ADP-ribose)polymerase (15 pmol/min/mg of protein) alone. Therefore, while differences in the average polymer size synthesized in these experiments may contribute to the synergism observed, our results argue that nuclear matrix proteins other than poly(ADP-ribose)polymerase, i.e. the lamins, function as covalent targets for poly(ADP-ribosyl)ation.

3.2. *Electrophoretic identification of lamins A and C as oligo(3'-dADP-ribose) acceptors in nuclear matrix extracts*

The main protein components of the nuclear matrix fraction correspond to three polypeptides of 60–75 kDa identified as lamins A, B, and C, respectively [1–3]. These proteins have recently been shown to possess DNA binding ability [18] and may therefore modulate nuclear functions via biochemical interactions of the nuclear matrix with chromatin. A common characteristic amongst poly(ADP-ribosyl)ated proteins is their ability to bind DNA [17]. Thus, we analyzed the products of experiment 7 (Table I) by polyacrylamide gel electrophoresis and autoradiography to determine whether polypeptides of 60–75 kDa were 3'-deoxyADP-ribosylated. Fig. 1, panel A shows the Coomasie blue-stained gel. Lane a corresponds to 0.2 μg of pure poly(ADP-ribose)polymerase which is not stainable with Coomasie blue at this concentration. Lanes c, and d show the electrophoretic patterns of nuclear matrix proteins following incubation with [^{32}P] 3'-deoxyNAD in the presence or absence of 0.2 μg of pure poly(ADP-ribose)polymerase, respectively. Lane b corresponds to nuclear matrix proteins incubated in the absence of 3'dNAD and poly(ADP-ribose)polymerase. Fig. 1, panel B, shows the autoradiogram of the same experiment. Lane a, shows the electrophoretic migration of automodified polymerase as a single band. This demonstrates that pure poly(ADP-ribose)polymerase is not contaminated with ADP-ribosylatable polypeptides that might electrophoretically co-migrate with the lamins. As expected, lane b, the minus 3'-dNAD control, shows no radiolabeled proteins. Lane c demonstrates that proteins co-migrating with lamins A and C are oligo(3'-deoxyADP-ribosyl)ated when incubated in the presence of exogenous poly(ADP-ribose)polymerase. Fig. 1, panel B also shows that the polymerase remains to be the main acceptor in the presence of nuclear matrix proteins. Lane d shows that there was no detectable protein-oligo(3'-deoxyADP-ribosyl)ation in the

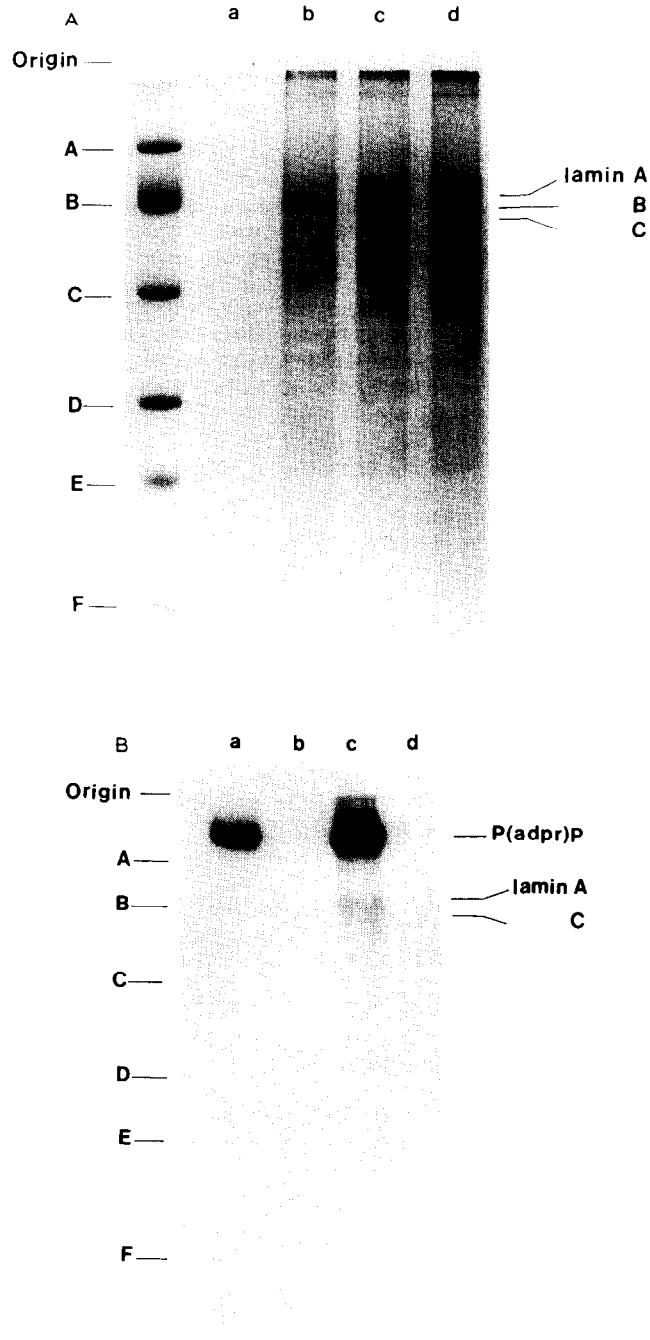

Fig. 1. Identification of oligo(3'-deoxyADP-ribosyl)ated proteins in nuclear matrix extracts following incubation with [^{32}P]3'-deoxyNAD for 40 min at 37°C in the absence or presence of pure poly(ADP-ribose)polymerase. Panel A shows the Coomasie blue-stained gel with relative migration of the molecular mass markers A, B, C, D, E and F which correspond to phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa), respectively. Lane (a) corresponds to 200 ng of pure polymerase. Lanes (b–d) show nuclear matrices following incubation either without (b) and with (c and d) [^{32}P]3'-deoxyNAD or with (c) and without (d) exogenously added enzyme. Panel B shows the autoradiograph of the same gel. The relative migration of the molecular mass markers is also indicated on the left side of the figure.

absence of pure enzyme indicating that the endogenous levels of poly(ADP-ribose)polymerase in the nuclear matrix were low. Therefore, as shown on lane c, we had to add pure DNA-dependent poly(ADP-ribose)polymerase in order to conclusively demonstrate that nuclear matrix proteins of identical electrophoretic mobility to the lamins are oligo(3'-deoxyADP-ribosyl)atable. Our results are consistent with previous findings by others [13,24] who also observed that nuclear matrix polypeptides of identical electrophoretic properties to the lamins were minor covalent acceptors for polymers of ADP-ribose when using [^{32}P]NAD as a substrate for isolated nuclei from HeLa cells.

3.3. *Oligo(3'-deoxyADP-ribosyl)ation of the nuclear matrix lamins is substrate concentration-dependent*

In order to further determine optimum conditions for the poly(ADP-ribosyl)ation of the lamins, we also incubated nuclear matrix extracts with 0.2 μg of pure enzyme/assay at increasing concentrations of [^{32}P]3'-deoxyNAD. Samples were then electrophoresed on an 8% polyacrylamide gel and autoradiographed. Fig. 2 shows the autoradiograph of the gel. Lane a corresponds to the polymerase incubated in the absence of radiolabeled substrate. Lanes c, e, g, and i show the result of incubation of nuclear matrix extracts in the absence of exogenous enzyme. The substrate concentrations used were 1, 10, 20 and 40 μM [^{32}P] 3'-deoxyNAD, respectively. A barely detectable amount of automodified enzyme was observed in the reaction products of these incubations. The substrate concentrations utilized in this experiment reflect the apparent K_m of the polymerase for 3'-deoxyNAD of 20 μM as previously reported [23]. In marked contrast, when 0.2 μg of pure enzyme were added to identical incubation mixtures, the oligo(3'-deoxyADP-ribosyl)ation of nuclear matrix proteins co-migrating with lamins A and C was evident as observed in Fig. 2, lanes b, d, f, and h, respectively. The intensity of the radiolabeled proteins increased proportionately with increasing substrate concentrations. Interestingly, the polymerase remained to be the best acceptor protein for poly(ADP-ribosyl)ation at all substrate concentrations tested. Additional evidence that lamins A and C efficiently function as targets for covalent poly(ADP-ribosyl)ation was obtained with an experiment in which a time-dependent modification of these proteins was observed following incubation of isolated nuclear matrix with 3'-deoxyNAD in the presence of 0.2 μg of pure enzyme (data not shown). The reasons for the more efficient automodification of DNA-dependent poly(ADP-ribose)polymerase in the presence of nuclear matrix proteins remain to be established. In conclusion, our results are consistent with the notion that the ADP-ribose polymer modification of chromatin and nuclear matrix proteins is an important reaction in the modulation of nuclear functions such as DNA replication and DNA-repair. Finally, the covalent modification of DNA-binding

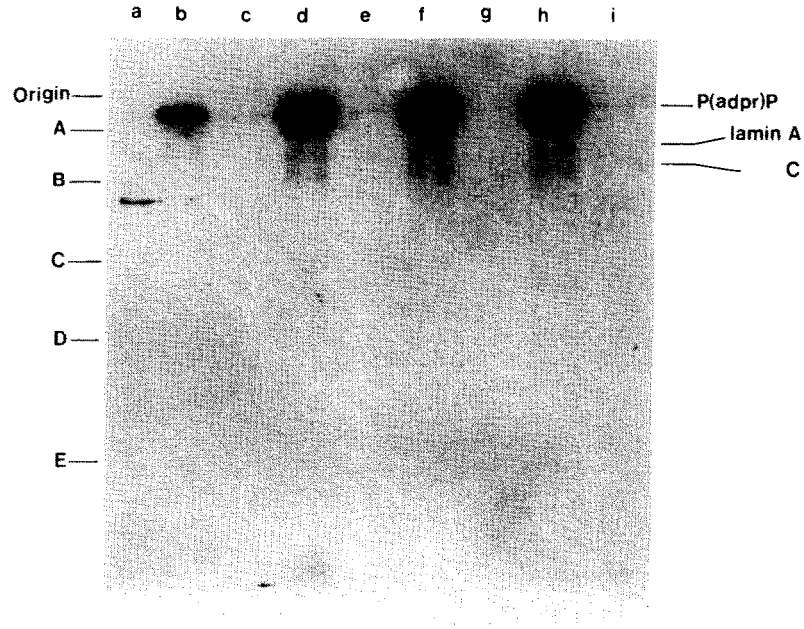

Fig. 2. Poly(3'-deoxyADP-ribosyl)ation of lamins A and C following incubation of nuclear matrices with and without pure poly(ADP-ribose)polymerase in the presence of 1 μM (lanes b and c), 10 μM (lanes d and e), 20 μM (lanes f and g) and 40 μM (lanes h and i) of [^{32}P]3'deoxyNAD as a substrate, respectively. Incubations were performed at 37°C for 30 min in the presence (lanes b, d, f, and h) or absence (c, e, g, and i) of exogenously added poly(ADP-ribose)polymerase [P(adpr)P]. The relative migration of the molecular weight markers is indicated to the left of the autoradiograph and they correspond to the same set of standards described in the legend to Fig. 1.

proteins with oligomers of 3'-deoxyADP-ribose should prove useful in characterizing the effects of this post-translational modification on the DNA-binding properties of chromatin and nuclear matrix acceptor proteins.

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