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NON-COVALENT INTERACTION BETWEEN POLY(ADP-RIBOSE) AND CELLULAR PROTEINS: AN APPLICATION OF A POLY(ADP-RIBOSE)-WESTERN BLOTTING METHOD TO DETECT POLY(ADP-RIBOSE) BINDING ON PROTEIN-BLOTTED FILTER

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We describe a sensitive method for the detection of interactions between poly(ADP-ribose) and proteins. Proteins were blotted onto nitrocellulose filters and incubated with ^{32}P-labeled poly(ADP-ribose). Purified core histones and poly(ADP-ribose) polymerase were found to bind poly(ADP-ribose) polymer. Blots of HeLa cell protein extracts revealed a 48 kDa protein and several others of smaller than 35 kDa likewise bound the polymers even at high salt concentrations. Those proteins, along with a 69 kDa protein, also showed resistance to competitor DNA. Polymer binding of aforesaid HeLa extract proteins was restricted to polymers above 20 residues in length. Thus poly(ADP-ribose)-protein affinities were polymer-length dependent.

DNA damaging agents, which introduce DNA strand breaks, induce rapid poly(ADP-ribose) formation which is catalyzed by a chromatin-associated enzyme, poly(ADP-ribose) polymerase [EC2.4.2.30] using the substrate NAD (1). Under conditions of extensive DNA breakage, large amounts of polymers are rapidly synthesized and enzymatically degraded, resulting in a half-life of <1 min (2). Covalent modification of nuclear proteins with poly(ADP-ribose) has long been suggested to alter function of proteins which are involved in DNA repair (1).

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Since poly(ADP-ribose) polymerase recognizes DNA strand breaks and this activates its poly(ADP-ribosyl)ation activity, we considered that poly(ADP-ribose) polymerase may act as a DNA breakage signal generator. The signal may be poly(ADP-ribosyl)ation of nuclear proteins, poly(ADP-ribose) polymer or poly(ADP-ribose) polymerase itself. If poly(ADP-ribose) is the signal, it may interact with the proteins in the signal transduction pathway. Althaus *et al.* applied poly(ADP-ribose) affinity matrix for studying poly(ADP-ribose)-protein interactions and showed that histone H1 had strong affinity for poly(ADP-ribose) (3). They also demonstrated non-covalent interaction of poly(ADP-ribose) with histones and protamine by phenol partitioning assay (4). Although poly(ADP-ribose) glycohydrolase is also reported to have affinity for poly(ADP-ribose)-matrix (5), interaction of poly(ADP-ribose) with other proteins has not been observed by these methods. Thus, a more sensitive method is required to detect interaction of poly(ADP-ribose) and proteins. In this report, we applied poly(ADP-ribose) binding assays using filter onto which proteins were blotted to study non-covalent interactions of poly(ADP-ribose) with proteins.

MATERIALS AND METHODS

Preperation of poly(ADP-ribose). Poly(ADP-ribose) was synthesized in a reaction mixture containing 50 mM Tris-Cl (pH 8.0), 1 mM dithiothreitol (Sigma), 5 mM $MgCl_2$, 100 μM [^{32}P]NAD (0.6 Ci/mmol, New England Nuclear), 45 μg/ml activated DNA (Sigma), 45 μg/ml calf thymus core histones (typeIIA, Sigma), 5 μg/ml purified *Sarcophaga* poly(ADP-ribose) polymerase (6). This mixture was incubated at 25 °C for 1 hr, then treated with 1N NaOH at 37°C for 1 hr, digested with 100 μg/ml of proteinase K in the presence of 0.1% SDS at 37 °C for 30 min., extracted with phenol-chloroform and precipitated with ethanol. After treatment with 20 μg/ml of DNase I, poly(ADP-ribose) polymer was extracted with phenol-chloroform and precipitated with ethanol. Chain-length distribution of poly(ADP-ribose) was determined by electrophoresis on 20% polyacrylamide gel (7). Mean chain-length was determined by phosphodiesterase digestion (8).
Preperation of HeLa cell extracts. Crude cell extracts from HeLa cell were prepared with Laemmli buffer [10% glycerol, 2% SDS, 50 mM Tris-Cl (pH 8.0), 10% β-mercaptoethanol, 5 μg/ml leupeptin]. After sonication, the extracts were centrifuged at 10,000 x *g* at 2 °C for 30 min., and the supernatant was stored. Protein concentration was quantified with Protein Assay Kit (Bio Rad).
Poly(ADP-ribose)-western blot. Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gel, then renatured in a reduction buffer [0.7 M β-mercaptoethanol, 190 mM glycine, 25 mM Tris-Cl (pH 8.0), 0.1% SDS] at 37°C for 1 hr (9), and transferred to nitrocellulose filter (BA85, Schleicher & Schnell) electrophoretically. The filter was incubated with ^{32}P-labeled poly(ADP-ribose) in binding buffer [50 mM Tris-Cl (pH 8.0), 0.1 M NaCl, 1 mM dithiothreitol, 0.3% Tween 20] at 25°C for 3 hrs. The incubated filter was washed in binding buffer at 25°C for 1 hr with several changes, then analyzed with Image Analyzer (BAS 2000, Fuji Film). ^{32}P-oligomer DNA was prepared by labeling three oligodeoxynucleotides, 5'-GCTGGGTAGTCCCCACCTTT-3', 5'-CTTATGAGTATTTCTTCCAGGGTA-3' and 5'-GTTTTCCCAGTCACGAC-3'

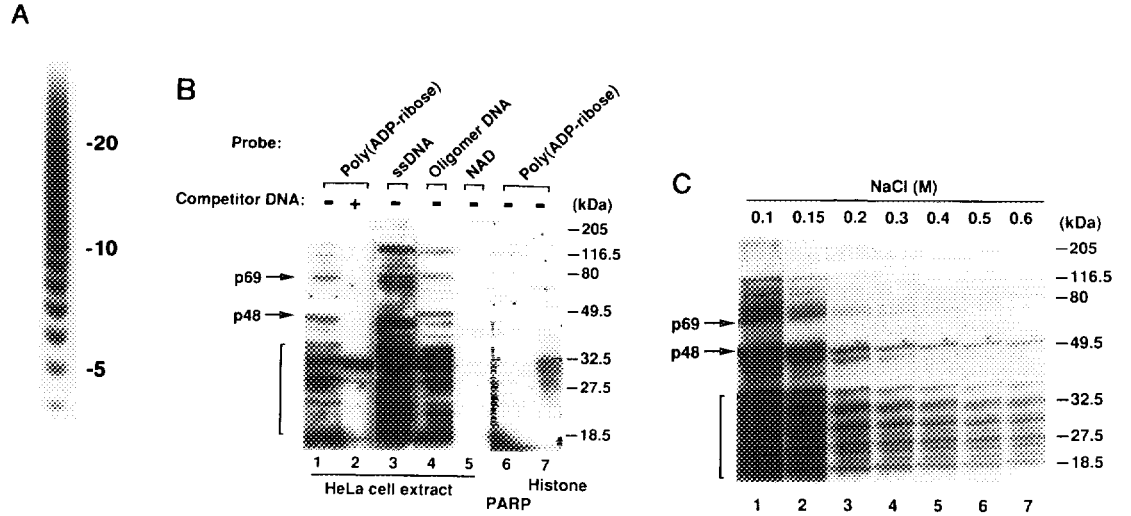

Fig. 1. Binding of poly(ADP-ribose) to proteins. (A) Size distribution of poly(ADP-ribose). 10,000 cpm of poly(ADP-ribose) was analyzed on a 20% polyacrylamide gel (7). (B) Comparison of various probes. Lanes 1 to 5: HeLa cell extract 100 μg protein/lane, Lane 6: 2 μg of purified poly(ADP-ribose) polymerase (PARP) from human placenta, Lane 7: 10 μg of calf thymus core histones (type IIA, Sigma). Binding and washing were done in buffer containing 0.1 M NaCl. Lanes 1, 2, 6, & 7: ^{32}P-poly(ADP-ribose) probe (2x10^5 cpm/180 ng/ml); lane 3: ^{32}P-single stranded salmon sperm DNA (2x10^5 cpm/180 ng/ml); lane 4: ^{32}P-oligomer DNA (2x10^5 cpm/180 ng/ml); lane 5: ^{32}P-NAD probe (2x10^5 cpm/ml). (C) Salt concentration dependency on binding. Blots of HeLa cell crude extracts (100 μg protein/lane) were incubated with ^{32}P-poly(ADP-ribose) (same specific activity as in (B)) in buffer containing 0.1 M NaCl and washed at 0.1 to 0. 6 M concentration of NaCl.

with T4 polynucleotide kinase as described (10). Single stranded salmon sperm DNA (Sigma) was labeled using ^{32}P-dCTP by a Multiprime DNA Labeling System (Amersham).

RESULTS

Poly(ADP-ribose)-western blotting system. Prepared poly(ADP-ribose) length distributed from 3 residues to 30 residues, as shown in Fig. 1 (A). HeLa cell crude extract was blotted on a filter and incubated with ^{32}P-labeled poly(ADP-ribose) in binding buffer containing 0.1 M NaCl. Several proteins in HeLa cell extract showed binding to poly(ADP-ribose). Proteins of 69 and 48 kDa and several proteins smaller than 35 kDa gave relatively strong signals (Fig. 1 (B) lane 1). However, most of those bands disappeared when an excess amount of single stranded salmon sperm DNA 50 times that of poly(ADP-ribose) was present during incubation and washing, except for a few bands smaller than 35 kDa (Fig. 1 (B) lane 2). Those bands may include the core histones, as purified core histones showed strong affinity for poly(ADP-ribose) (Fig. 1 (B) lane 7). Purified poly(ADP-

ribose) polymerase also showed binding to poly(ADP-ribose) (Fig. 1 (B) lane 6). When poly(ADP-ribose) probe was compared with single stranded DNA and oligomer DNA probe, most bands smaller than 35 kDa were commonly observed with these three probes, however, larger size bands differed in binding pattern. Several proteins including 69 and 48 kDa protein were specific to poly(ADP-ribose). When NAD was used as a probe, no significant band was observed. When salt concentration in washing buffer was changed, most bands gradually disappeared with the increase of salt concentration. However, 48 kDa and several proteins smaller than 35 kDa (marked with arrows and a bracket in Fig. 1 (C)) were resistant to salt, suggesting a high affinity for poly(ADP-ribose).

Competition of binding with DNA and RNA. The increase in concentration of competitor resulted in the decrease in intensity of poly(ADP-ribose) bound signals (Fig. 2). When only 2.5 times single stranded salmon sperm DNA was included in incubation and washing buffer, 71, 44 and 40 kDa protein bands were competed out. In contrast, 69 and 48 kDa and several proteins smaller than 35 kDa were more resistant to competitor DNA. But with the 50 times DNA (10 μg/ml), most bands were competed out; however, when 10 μg/ml of yeast total RNA was added instead of DNA, the proteins, 69, 48 kDa and several proteins smaller than 35 kDa were still observed. Thus RNA was comparatively ineffective competitor vis-a-vis DNA.

Dependency of protein binding on poly(ADP-ribose) length. Various lengths of polymer were prepared by gel filtration on Sephacryl S-300 and Sephadex G-100 column and used as probes. For this purpose another batch of poly(ADP-ribose) was prepared because the previous batch only contained relatively short polymer (Fig. 1 (A)). When the synthesis reaction was prolonged to two hours, we were able to obtain longer poly(ADP-ribose), as seen in Fig. 3 (A) leftmost lane (mean length: 47 residues). With longer polymer (>40 residues) several additional bands were observed (Fig. 3 (B), lanes 1 & 2, marked with arrows). With probes longer than 20 residues, poly(ADP-ribose) binding could be observed (Fig. 3 (B), lanes 3 &4); however, with probes shorter than 20 residues, only weak binding of low molecular weight proteins were observed (Fig. 3 (B), lanes 5-7).

DISCUSSION

To elucidate non-covalent interaction between poly(ADP-ribose) and cellular proteins, we applied filter binding technique: poly(ADP-ribose)-western blotting method, which was similar to south-western blotting method (11). To determine the specifity of binding, we examined the effects of salt concentration and DNA or RNA competition. Because poly(ADP-ribose) is a large molecule with a highly negative charge, the ionic interaction and competition with DNA and RNA are readily expected. Interestingly, a 48 kDa protein and several

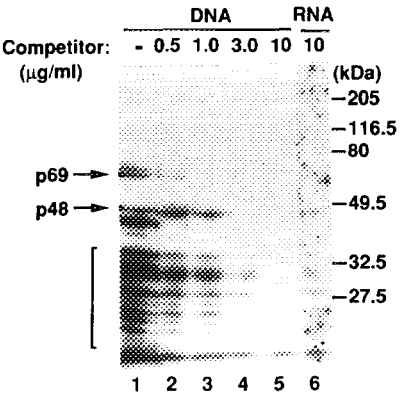

Fig. 2. Competition of DNA or RNA in poly(ADP-ribose)-protein binding. HeLa cell crude extract (100 μg protein/lane) was used in each lane. Indicated amount of competitor single stranded salmon spermDNA or yeast total RNA (Sigma) was present during incubation and washing. The filter was incubated with ^{32}P-poly(ADP-ribose) ($2x10^5$ cpm/180 ng poly(ADP-ribose)/ml) in binding buffer containing 0.1 M NaCl and washed at 0.1 M NaCl concentration.

proteins smaller than 35 kDa showed resistance to high salt under which ordinary ionic binding should be disturbed. The above proteins and a 69 kDa protein showed relative resistance to competition with DNA and RNA. These

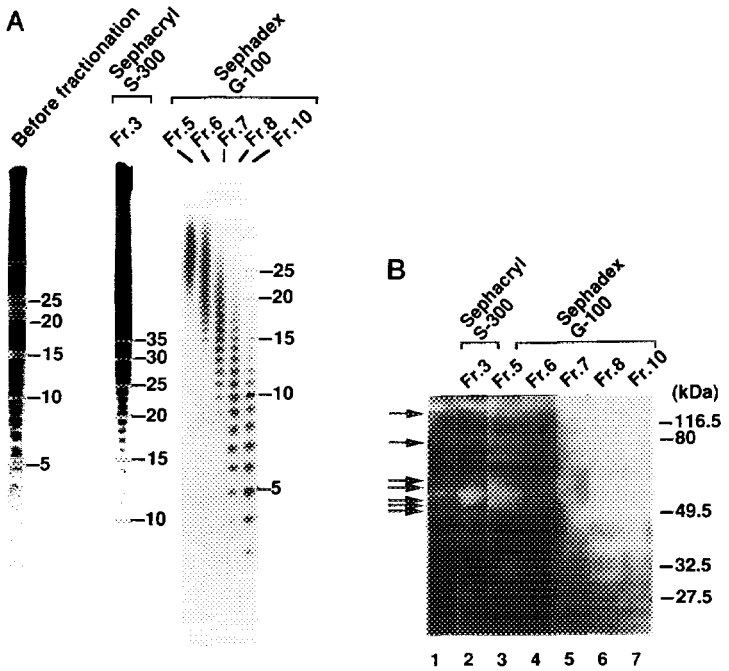

Fig. 3. Comparisons of different-length poly(ADP-ribose)s on binding. (A) Poly(ADP-ribose) was fractionated by gel filtration on Sephacryl S-300 and Sephadex G-100 (Pharmacia/ LKB). 2,000 cpm was loaded on each lane. (B) HeLa cell crude extract (100 μg protein/lane) was used in each lane and original or size fractionated poly(ADP-ribose) probe in (A) ($2x10^4$ cpm/0.5 ml) was used.

results indicated that the 69 kDa and the 48 kDa proteins and several proteins smaller than 35 kDa had significantly high specificity for poly(ADP-ribose). The 69 kDa and the 48 kDa proteins were observed in nuclear fractions (Nozaki *et al.*, unpublished data), and we are currently characterizing those proteins. Core histones are probably present in those proteins smaller than 35 kDa. The nature of those small proteins should soon be clarified. In the study with phenol-partitioning assay, the affinity of poly(ADP-ribose) for poly(ADP-ribose) polymerase itself was not detected (4); however, with this poly(ADP-ribose)-western blotting method, we could show that poly(ADP-ribose) polymerase itself had affinity for poly(ADP-ribose).

The length of poly(ADP-ribose) was a critical binding factor in this system. A length of 20 residues is required for efficient binding; longer polymer, greater than 40 residues, gave additional weak bands. Thus there are probably proteins which bind only long poly(ADP-ribose). Althaus *et al.* showed that histone H1 bound branched polymer preferentially in the phenol-partitioning assay (4). One of our preparation of poly(ADP-ribose) contained heavily branched polymers, which remained at the polyacrylamide gel origin (Fig. 3 (A) leftmost lane). If pure branched polymers can be made available for a probe, we should be able to identify the branched polymer recognition protein with this system.

Covalent binding of poly(ADP-ribose) polymer to nuclear proteins has long been studied (1). Poly(ADP-ribose) is a large molecule with highly negative charge even with branching structures. Recently, involvement of non-covalent binding of poly(ADP-ribose) to histones in regulation of the chromatin structure has been suggested (4). We consider that non-covalent binding of poly(ADP-ribose) to various proteins is important not only for chromatin structure regulation but also for signalling DNA strand breaks and for other cellular functions. Thus this poly(ADP-ribose)-western blotting method may facilitate identification and isolation of poly(ADP-ribose) binding proteins and also the characterizations of those proteins. By such studies, we may elucidate unknown physiological functions of poly(ADP-ribose).

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