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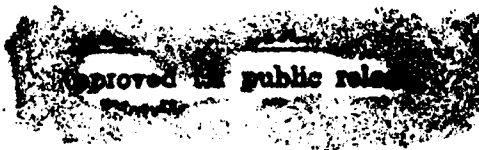
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BY:

Ernest Kun
Project Director

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

1a REPORT SECURITY CLASSIFICATION			1b RESTRICTIVE MARKINGS			
2a SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION/AVAILABILITY OF REPORT			
2b DECLASSIFICATION/DOWNGRADING SCHEDULE			Approved for public release; distribution unlimited.			
4 PERFORMING ORGANIZATION REPORT NUMBER(S)			5. MONITORING ORGANIZATION REPORT NUMBER(S)			
			AFOSR-TR- 94 0287.			
6a NAME OF PERFORMING ORGANIZATION		6b OFFICE SYMBOL (if applicable)		7a NAME OF MONITORING ORGANIZATION		
SFSU, Romberg Tiburon Ctr.				AFOSR/NL		
6c ADDRESS (City, State, and ZIP Code)				7b ADDRESS (City, State, and ZIP Code)		
3150 Paradise Drive Tiburon, CA 94920				110 Duncan Ave Suite B115 Bolling AFB DC 20332-0001		
8a NAME OF FUNDING/SPONSORING ORGANIZATION		8b OFFICE SYMBOL (if applicable)		9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER		
AFOSR		NL		F49620-92-J-0232		
8c ADDRESS (City, State, and ZIP Code)				10. SOURCE OF FUNDING NUMBERS		
110 Duncan Ave Suite B115 Bolling AFB DC 20332-0001				PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.
				61102F	2312	AS
11. TITLE (Include Security Classification)						
Molecular Toxicology of Chromatin Progress Report (2nd year, Grant number AFOSR F49620-92-J-0232-DEF)						
12. PERSONAL AUTHOR(S)						
Dr. Ernest Kun						
13a. TYPE OF REPORT		13b. TIME COVERED		14 DATE OF REPORT (Year, Month, Day)		15. PAGE COUNT
Annual Technical		FROM 4/1/93 TO 3/31/94		April 8, 1994		74
16 SUPPLEMENTARY NOTATION						
17 COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)			
FIELD	GROUP	SUB-GROUP	Phosphorylation of poly(ADP-ribose) polymerase protei and, pecific binding domains for histones and for the self associating enzyme protein			
19 ABSTRACT (Continue on reverse if necessary and identify by block number)						
<p>Intracellular phosphorylation of poly (ADP-ribose) polymerase was assayed in streptolysin-O-permeabilized human lymphocytes. Whereas ^{32}P incorporation from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into immuno-precipitated enzyme protein was undetectable in resting cells, significant phosphorylation of this enzyme was observed in lymphocytes treated with phytohemagglutinin. The phosphorylation of poly (ADP-ribose) polymerase in permeabilized cells was not stimulated by phorbol ester, but phosphorylation of other proteins and of a specific oligopeptide substrate of protein kinase C was increased by phorbol ester. The specific inhibitory pseudosubstrate peptide of protein kinase C blocked the phosphorylation of poly (ADP-ribose) polymerase induced by phytohemagglutinin. A potential role of a member of the protein kinase C family in the intracellular regulation of poly (ADP-ribose) polymerase by phosphorylation appears probable.</p> <p>The structure of poly (ADP-ribose) polymerase has been augmented by the identification of polypeptide sequences which define histone and self association. Both protein binding domains are components of 64-67 kDa basic moiety of poly ADP-ribose polymerase, obtained by degradation by chymotrypsin or plasmin. Two discrete histone binding domains are interspersed and contiguous with "self binding" domains and are located at 186-290 and 446-525 residues. Self binding is confined to the 29 kDa N-terminal moiety of poly ADP-ribose polymerase and to two smaller polypeptide sequences 291-395 and 526-606 residues. Bound zinc is not required for self binding. No selectivity of histone binding could be ascertained in the protein-polypeptide two component binding system used here, and higher order structures and NAD may be required to demonstrate specificity towards various histone species. Both self and histone binding augment poly (ADP-ribose) synthesis activity of the enzyme and in a soluble system 95% of polymers are bound to the enzyme protein. Histone poly ADP-ribosylation is greatly augmented by nucleosome-like structure.</p>						
20 DISTRIBUTION/AVAILABILITY OF ABSTRACT				21 ABSTRACT SECURITY CLASSIFICATION		
<input checked="" type="checkbox"/> UNCLASSIFIED UNLIMITED <input type="checkbox"/> SAME AS RPT <input type="checkbox"/> DTIC USERS						
22a NAME OF RESPONSIBLE INDIVIDUAL				22b TELEPHONE (include Area Code)		22c OFFICE SYMBOL
Dr Walter Kozumbo				(202) 767-5021		NL

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**PHOSPHORYLATION OF POLY (ADP-RIBOSE)
POLYMERASE PROTEIN IN HUMAN PERIPHERAL
LYMPHOCYTES STIMULATED WITH
PHYTOHEMAGGLUTININ**

Intracellular phosphorylation of poly (ADP-ribose) polymerase was assayed in streptolysin-O-permeabilized human lymphocytes. Whereas ^{32}P incorporation from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into immuno-precipitated enzyme protein was undetectable in resting cells, significant phosphorylation of this enzyme was observed in lymphocytes treated with phytohemagglutinin. The phosphorylation of poly (ADP-ribose) polymerase in permeabilized cells was not stimulated by phorbol ester, but phosphorylation of other proteins and of a specific oligopeptide substrate of protein kinase C was increased by phorbol ester. The specific inhibitory pseudosubstrate peptide of protein kinase C blocked the phosphorylation of poly (ADP-ribose) polymerase induced by phytohemagglutinin. A potential role of a member of the protein kinase C family in the intracellular regulation of poly (ADP-ribose) polymerase by phosphorylation appears probable.

In vitro experiments with purified enzymes have revealed that poly (ADP-ribose) polymerase (EC 2.4.2.30) is inactivated by phosphorylation catalyzed by protein kinase C (1). We have found that simultaneously with the phosphorylation of the polypeptide chain at two different sites, poly (ADP-ribose) polymerase also loses its propensity to bind DNA and added DNA inhibits the phosphorylation of poly (ADP-ribose) polymerase by protein kinase C (2). Thus protein kinase C may control both catalytic and colligative activities of this abundant nuclear protein which in turn influences chromatin structure and gene function (3). However, *in vitro* enzymatic models are insufficient proofs of existing cellular mechanisms. In the present work resting and mitogen-stimulated human lymphocytes served as a model system for the testing of the hypothesis that proposes a connection between protein phosphorylation and intracellular poly (ADP-ribose) polymerase activity.

The idea of application of phytohemagglutinin, a nonphysiological stimulant of lymphocyte proliferation, for the study of the cellular role of poly (ADP-ribose) polymerase has an unusual background. It is known that the propagation of immunodeficiency virus (HIV) in human lymphocytes *in vitro* requires stimulation by phytohemagglutinin and the inactivation of both retroviral *gag* protein zinc fingers and those of poly (ADP-ribose) polymerase by specific drugs abrogates HIV proliferation (4,5). Furthermore, phytohem-

agglutinin stimulates poly (ADP-ribose) polymerase mRNA production (6) and increases the *de novo* synthesis of poly (ADP-ribose) polymerase in S phase (7). We presumed that a transient and probably reversible inactivation of poly (ADP-ribose) polymerase, induced by phosphorylation, might have a regulatory role in the course of cell proliferation. Here we demonstrate that phytohemagglutinin stimulates also the phosphorylation of poly (ADP-ribose) polymerase in permeabilized whole cells.

Materials and Methods

Human blood lymphocytes were maintained in RPMI 1640, supplemented with 10% fetal calf serum, 2 mM L-glutamine (all from Gibco), 50 IU penicillin and 50 µg/ml streptomycin, at 37 °C, in humidified air containing 5% CO₂.

Peripheral blood was collected from healthy volunteers. Mononuclear cells separated on Ficoll-Iodamide (Pharmacia, Uppsala, Sweden and Bracco, Milano, Italy) gradient by the method of Boyum (8), washed twice and cultured at 10⁶ cells/ml density. Mitogen stimulation was achieved by purified phytohemagglutinin (Wellcome Diagnostics, Dartford, England) at 1 µg/ml concentration. Control samples were maintained at suboptimal stimulation by 0.03 µg/ml phytohemagglutinin. Cells were harvested after treatment for 16-60 hours, pelleted by low speed centrifugation (1500 x g, 10 minutes) to

eliminate cell debris, resuspended in RPMI 1640 and viable cells were separated on a Ficoll-Iodamide gradient. Viability was always higher than 94% determined by propidium iodide (Sigma, USA) dye exclusion and light scattering in a flow cytometer (Cytoson Absolute, Ortho Diagnostic System, Raritan, NJ, USA).

Cell count, blast cell content and cell-cycle stages were assayed in a flow cytometer. For cell-cycle measurement samples containing 100 μ l cell suspension from the original cultures were diluted with 900 μ l lysis solution (0.1% v/v Triton X-100 and 0.1% w/v Na-citrate in water) (9), and propidium iodide (20 μ g/ml final concentration) was then added and the suspension incubated at room temperature for 10 minutes before analysis. Percentages of cells in different cell-cycle phases were evaluated by the software of Cytoson Absolute.

Permeabilization of cells by streptolysin-O was carried out according to the method described by Alexander et al. (10) with minor modifications and the phosphorylation of intracellular proteins was performed in the medium used for permeabilization. The final concentration of components in this medium were 8 mM MgCl_2 , 120 mM KCl, 12 mM HEPES (pH 7.4), 10 mM EGTA, 0.5 μ M free Ca^{2+} , 0.1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (about 10^5 cpm/nmol) and 0.8 U/ml streptolysin-O freshly reconstituted from freeze-dried powder. Cells were incubated in the streptolysin-O containing medium (3×10^6 cells in 0.3 ml medium) for 12 minutes at 37 °C. At the end of the incubation

about 90% of the cells appeared to be permeabilized on the basis of staining with trypan blue. After 12 minutes incubation the cells were centrifuged (1500 x g, 2 minutes) and the pellet used for immunoprecipitation of poly (ADP-ribose) polymerase. In some other experiments the incubation was stopped by adding trichloroacetic acid (5% w/v final concentration) and after centrifugation the pellet was subjected to SDS-PAGE on 12% polyacrylamide gels and the phosphoprotein pattern was detected by autoradiography.

Immunoprecipitation (11) was carried out immediately after phosphorylation. After centrifugation the supernatant of cells was carefully removed and the cells lysed in 100 μ l of lysis buffer composed of 50 mM Tris-HCl (pH 7.5), 0.1% SDS, 1% NP-40 (Nonidet P-40), 0.5 M NaCl, 1 μ g/ml aprotinin and 10 μ g/ml phenylmethylsulphonyl fluoride, for one h at 4 °C. Then 400 μ l of NET buffer (50 mM Tris-HCl, pH 7.5, 0.1% v/v NP-40, 0.4 M NaCl, 1 mM EDTA, 0.25% w/v gelatin) was admixed and the solution cleared by centrifugation (12000 x g, 5 minutes) at 4 °C. The samples were incubated with 3 μ l/assay (see above) of the antibody, specific for the poly (ADP-ribose) polymerase protein (12), for 3 h at 4 °C followed by an overnight incubation in the presence of protein A-Agarose (Oncogene Sci., 50 μ l slurry/tube). The unbound compounds were removed by subsequent washings (4 times with NET buffer, once with Tris-HCl buffer (pH 8.0), containing 1 mM

EDTA ; 0.4 ml each wash). Finally the resin was boiled with 50 μ l of Laemmli sample buffer for 10 minutes and 40- μ l aliquots from the supernatant of the centrifuged samples were loaded onto 10% SDS-PAGE gels. After electrophoresis gels were stained with coomassie brilliant blue, destained, dried and autoradiographed.

Intracellular protein kinase C activity was checked with the aid of the selective oligopeptide substrate of protein kinase C Ala-Ala-Ala-Ser-Phe-Lys-Ala-Lys-Lys-amide (13,14). Aliquots of cells were incubated for 10 minutes at 37 °C in the absence or presence of 40 ng/ml phorbol myristyl acetate (PMA, Sigma) and the cells were then pelleted and resuspended in the medium used for permeabilization. Samples (10^6 cells in 100 μ l medium) were permeabilized and incubated in the absence or presence of the oligopeptide (0.7 mg/ml) for 12 minutes and the incubation stopped with ice-cold glacial acetic acid (0.4 ml). After centrifugation (10000 x g, 2 minutes) aliquots were taken from the supernatant and the radioactivity of 32 P incorporated into the oligopeptide was measured as described previously (13,14). All assays were performed in duplicate. The activation caused by PMA was expressed as the difference between the amounts of phosphate incorporated into the oligopeptide in cell samples incubated in the presence and absence of PMA.

Poly (ADP-ribose) polymerase and protein kinase C (type II, β) were purified as described previously (15,16). The cyclin-dependent

kinase 1-cdc2 was obtained from UBI (USA) and the protein kinase C pseudosubstrate prototope inhibitory peptide was a generous gift of Dr. Doreen Cantrell (Imperial Cancer Research Fund, London). Phosphorylation of the purified poly (ADP-ribose) polymerase protein was performed as described previously (2).

Results

Intracellular phosphorylation of poly (ADP-ribose) polymerase was studied in human peripheral blood lymphocytes permeabilized by streptolysin-O. In our preliminary experiments the phosphorylation of poly (ADP-ribose) polymerase was not detectable in granulocytes or in resting lymphocytes, therefore we attempted to demonstrate the phosphorylation in artificially induced proliferating cells. Figure 1 shows that in lymphocytes stimulated by phytohemagglutinin the poly (ADP-ribose) polymerase protein is phosphorylated. As shown previously *in vitro* poly (ADP-ribose) polymerase is a good substrate for type II (β) and type III (α) isoenzymes of protein kinase C (2). Since these isoenzymes are present in lymphocytes (17), we presumed that the phosphorylation in permeabilized cells was catalyzed by protein kinase C. However, when protein kinase C was activated by treatment of resting cells with PMA no phosphorylation of poly (ADP-ribose) polymerase could be observed (Figure 1A). At

the same time the PMA-induced activation of protein kinase C was demonstrated by the phosphorylation of a number of other intracellular proteins, among them an about 116 kDa protein which on the basis of immunoprecipitation was not identical to poly (ADP-ribose) polymerase (Figure 2). The intensity of ^{32}P incorporation into the poly (ADP-ribose) polymerase protein was not increased when phytohemagglutinin stimulated cells were further treated with PMA (Figure 1A) although the effect of PMA on the phosphorylation of several other proteins, even in mitogen-stimulated cells was readily detectable (Figure 2). Activation of protein kinase C by PMA was also tested by the incorporation of ^{32}P into the specific oligopeptide substrate of protein kinase C (13,14) and PMA caused about 700% and 420% increase in the phosphotransferase activity in resting and mitogen-stimulated cells, respectively (Table I).

The possible role of enzymes besides protein kinase C in the intracellular phosphorylation of poly (ADP-ribose) polymerase was also considered. poly (ADP-ribose) polymerase has already been reported not to be a substrate for protein kinase A (1). On the other hand the amino acid sequence (18) in the DNA-binding domain of this nuclear enzyme contains a region (Thr-Pro-Lys) that could be a target for the cyclin-dependent protein kinase 1-cdc2. The cell population stimulated by phytohemagglutinin for 60 hours contained a significant number of cells in G2 phase, where cdc2 kinase is known to be active (19). For these reasons we tested *in vitro* the activity of cdc2 kinase

with poly (ADP-ribose) polymerase as a substrate. While the cdc2 kinase phosphorylated H1 histone intensively, it was unable to catalyze the phosphorylation of poly (ADP-ribose) polymerase (Figure 3A,B). Its role was also excluded when we found that poly (ADP-ribose) polymerase was phosphorylated in a cell population treated for only 16 hours with phytohemagglutinin (Figure 1B) which contained only a small percentage of cells in the G2 phase (Table II).

However, in the course of experiments with cdc2 kinase we observed that our purified poly (ADP-ribose) polymerase preparations contained traces of a protein kinase activity, apparently as a contaminant, thus under appropriate circumstances poly (ADP-ribose) polymerase phosphorylation was detectable without the addition of protein kinase C (Figure 3B,C,D). The final step in the purification of poly (ADP-ribose) polymerase was affinity chromatography on m-benzamide agarose (15) and the poly (ADP-ribose) polymerase protein accounted for about 95% of isolated proteins. At this stage of purity a trace of protein kinase C phosphorylating poly (ADP-ribose) polymerase may be adsorbed to the enzyme because of the high affinity of protein kinases for their substrates. Therefore it is conceivable that this kinase catalyzes the intracellular phosphorylation of poly (ADP-ribose) polymerase. Although the contaminating kinase activity was not increased by the physiological activators of protein kinase C, it was inhibited by staurosporin and H7 (Figure 3C) and by the pseudosubstrate inhibitory peptide which is a

specific inhibitor of protein kinase C (20), and by DNA which inhibits the phosphorylation of poly (ADP-ribose) polymerase by protein kinase C (2) (Figure 3D).

We also investigated the intracellular phosphorylation of poly (ADP-ribose) polymerase in the presence of inhibitors of protein kinase C. Under our experimental circumstances (i.e. the medium used for permeabilization contained both ³²P-labeled ATP and the inhibitor compound) the pseudosubstrate peptide of protein kinase C was the most effective inhibitor of phosphorylation of poly (ADP-ribose) polymerase in phytohemagglutinin-stimulated lymphocytes (Figure 4).

Discussion

Evidence presented supports the mechanism that connects protein kinase C activity with the regulation of poly (ADP-ribose) polymerase in a cellular system, albeit during cellular stimulation by a nonphysiological stimulant, phytohemagglutinin. This model may be eventually useful for the study of physiological cell stimulants (e.g. lymphokines). However the poly (ADP-ribose) polymerase inactivating effect of phytohemagglutinin-induced phosphorylation may explain the increased cellular toxicity of retroviral zinc finger oriented antiviral drugs in the presence of phytohemagglutinin (5)

since both drugs and phytohemagglutinin depress cellular poly (ADP-ribose) polymerase activity by differing molecular mechanisms.

The mode of protein kinase C-catalyzed phosphorylation of poly (ADP-ribose) polymerase appears complex and requires further comments. Since the effect of the pseudosubstrate peptide is accepted as a specific inhibition of protein kinase C activity in lymphocytes (10) we believe that in spite of the absence of stimulation by PMA the intracellular phosphorylation of poly (ADP-ribose) polymerase is probably catalyzed by a member of the protein kinase C family. Atypical subtypes of protein kinase C are not activated by PMA, therefore an atypical subtype may be responsible for the observed phosphorylation (21).

On the other hand, the possible role of a "classical" member (21) of the protein kinase C family is also conceivable. The endogenous protein kinase phosphorylating poly (ADP-ribose) polymerase that was found in the purified preparation of poly (ADP-ribose) polymerase protein may be identical with a proteolytically activated form of a classical protein kinase C isoenzyme (13,22). The failure of PMA to increase the intracellular phosphate incorporation into the poly (ADP-ribose) polymerase protein can be explained as well. One of the possible interpretations of this observation is that the poly (ADP-ribose) polymerase protein may not be available for protein kinase C in resting cells but becomes available in

phytohemagglutinin-stimulated cells even in the absence of PMA. Although protein kinase C may be associated with the nucleus (23) the phosphorylation of poly (ADP-ribose) polymerase could occur in the cytoplasm. In resting cells the major part of poly (ADP-ribose) polymerase is localized in the nucleus and we have shown that the binding of poly (ADP-ribose) polymerase to DNA prevents the phosphorylation of the enzyme by protein kinase C (2). Phytohemagglutinin has been reported to increase the level of mRNA for poly (ADP-ribose) polymerase (6), thus it promotes the *de novo* synthesis of the enzyme protein mainly in the S phase (7). In phytohemagglutinin-treated cells a fraction of protein kinase C may become activated and the newly synthesized poly (ADP-ribose) polymerase protein could be a target of protein kinase C activity. Phosphorylation by protein kinase C inhibits the binding of poly (ADP-ribose) polymerase to DNA (2), and thus, with the aid of a phosphatase (24), may regulate the binding of newly synthesized poly (ADP-ribose) polymerase to nascent DNA in the appropriate phase of the cell cycle.

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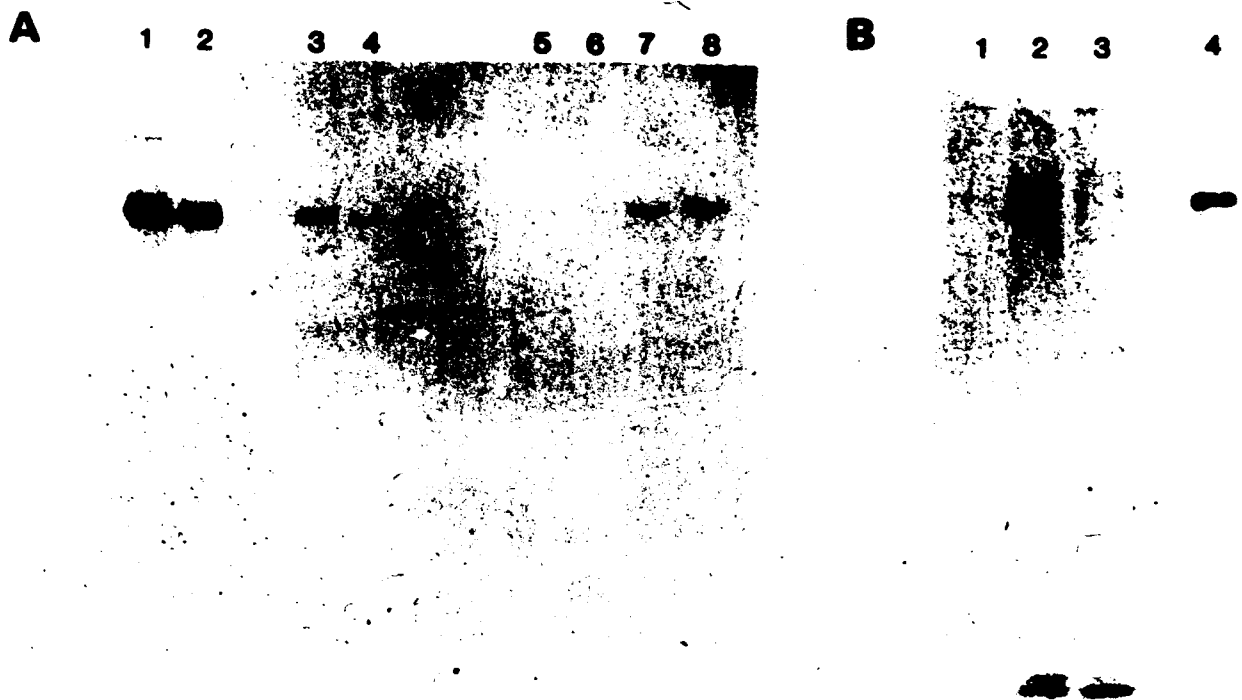


Figure 1

Figure 1. Phosphorylation of poly (ADP-ribose) polymerase in phytohemagglutinin-treated blood lymphocytes. Poly (ADP-ribose) polymerase protein was separated from lymphocytes by immunoprecipitation followed by SDS-PAGE and the phosphorylation was detected by the autoradiography of the [³²P]-labeled band of poly (ADP-ribose) polymerase. Lanes 1-4 of Figure A show the results of control experiments in which samples of purified poly (ADP-ribose) polymerase were phosphorylated *in vitro* by protein kinase C type II (β) isoenzyme. Lanes 1 and 2 of Figure A show the band of *in vitro* phosphorylated poly (ADP-ribose) polymerase without immunoprecipitation, in Lane 2 [³²P]-labeled sample was mixed with 3 μ g unlabeled poly (ADP-ribose) polymerase. Lane 3 shows a [³²P]-labeled sample added to permeabilized control (resting) lymphocytes, Lane 4 shows a [³²P]-labeled sample mixed with 3 μ g unlabeled poly (ADP-ribose) polymerase and added to permeabilized control lymphocytes. Lanes 5-8 show the phosphorylation of poly (ADP-ribose) polymerase in permeabilized cells. Control cells (Lanes 5,6) and cells stimulated by phytohemagglutinin for 60 hours (Lanes 7,8) were permeabilized in the absence (Lanes 5,7) or presence (Lanes 6,8) of 40 ng/ml PMA. Lane 2 in Figure B shows the phosphorylation of poly (ADP-ribose) polymerase in cells stimulated by phytohemagglutinin for 16 hours, Lanes 1 and 3 are identical samples from control cells and Lane 4 shows the band of poly (ADP-ribose) polymerase phosphorylated *in vitro*.

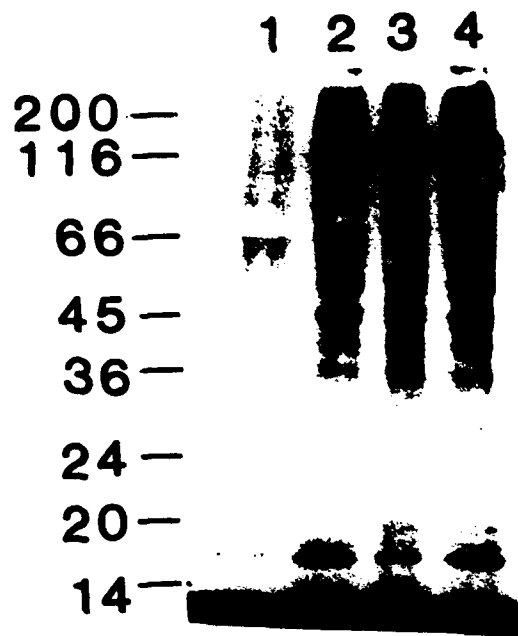


Figure 2

Figure 2. Phosphorylation of proteins in permeabilized lymphocytes and the effect of PMA. The phosphoprotein patterns are demonstrated by autoradiography of the [³²P]-labeled protein bands obtained by SDS-PAGE. Lane 1: control cells, Lane 2: control cells treated with PMA (40 ng/ml) in the course of permeabilization, Lane 3: cells stimulated by phytohemagglutinin for 60 hours, Lane 4: cells stimulated by phytohemagglutinin for 60 hours and treated with PMA (40 ng/ml) in the course of permeabilization.

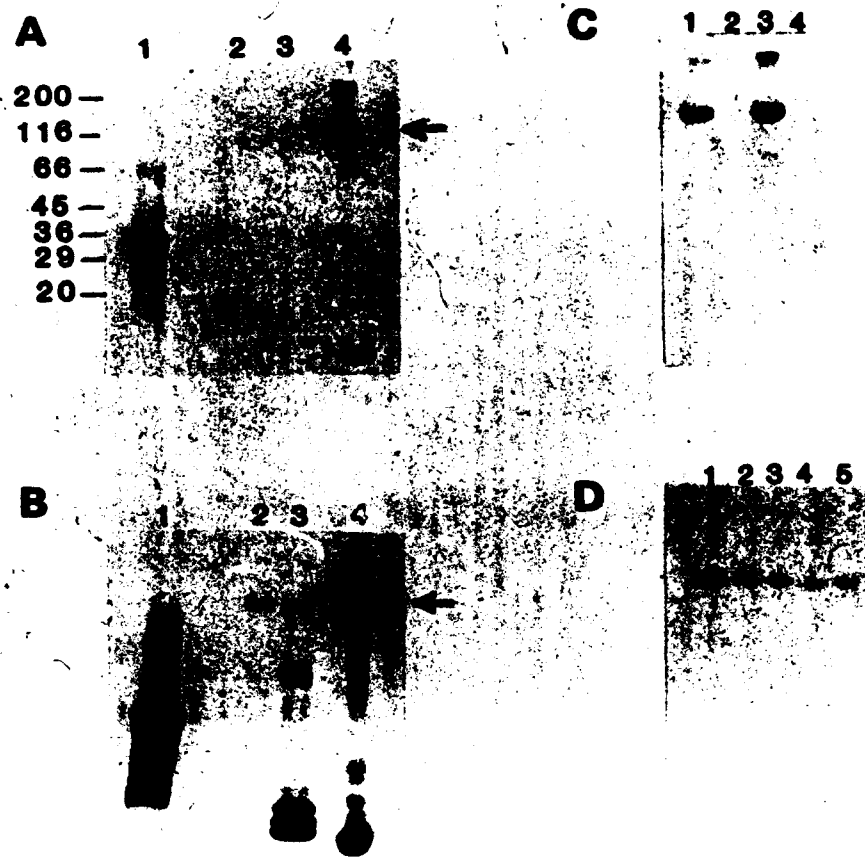


Figure 3

Figure 3. Phosphorylation of purified poly (ADP-ribose) polymerase protein by a contaminating protein kinase and the ineffectivity of protein kinase 1-cdc2. Figures A and B show the results of attempts to phosphorylate poly (ADP-ribose) polymerase by the cyclin dependent kinase 1-cdc2: sample of H1 histone phosphorylated by cdc2 kinase (Lane 1); a sample of purified poly (ADP-ribose) polymerase protein incubated in the absence of exogenous kinase (Lane 2); sample of poly (ADP-ribose) polymerase protein incubated in the presence of cdc2 kinase (Lane 3); poly (ADP-ribose) polymerase protein phosphorylated by protein kinase C (Lane 4). The amount of cdc2 kinase preparation was 50 ng and the radioactivity of [³²P]ATP was 3 x 10⁵ cpm per reaction mixture in experiments of Figure A and 200 ng cdc2 kinase preparation and 12 x 10⁵ cpm per reaction mixture were used for experiments of Figure B. Arrows indicate the position of the protein band of poly (ADP-ribose) polymerase. Figures C and D show the phosphorylation of poly (ADP-ribose) polymerase by the contaminating kinase in the absence of activators or inhibitors (Figure C Lanes 1,3 and Figure D Lane 1); in the presence of 0.1 mM Ca²⁺, 25 µg/ml phosphatidylserine and 250 ng/ml diacylglycerol (Figure C Lane 1); in the presence of 1µM staurosporin (Figure C Lane 2); in the presence of 200 µM H7 (Figure C Lane 4 and Figure D Lane 2); in the presence of the pseudosubstrate inhibitory peptide of protein kinase C (200 uM and 1 mM in Figure D Lanes 3 and 4, respectively); in the presence of DNA (12.5 µg per reaction mixture, Lane 5).

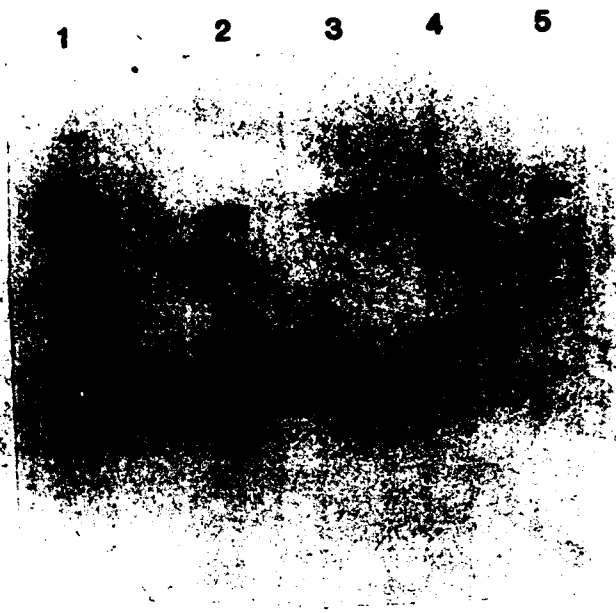


Figure 4

Figure 4. The effect of the pseudosubstrate inhibitory peptide of protein kinase C on the phosphorylation of poly (ADP-ribose) polymerase protein in phytohemagglutinin-stimulated lymphocytes. Phosphorylated poly (ADP-ribose) polymerase protein was immunoprecipitated, subjected to SDS-PAGE and the [³²P]-labeling was demonstrated by autoradiography of the protein bands. Purified poly (ADP-ribose) polymerase protein phosphorylated by protein kinase C in vitro (Lane 1), poly (ADP-ribose) polymerase [³²P]-labeled in permeabilized lymphocytes (Lanes 2-5): without inhibitor (Lane 2), in the presence of 200 μ M H7 (Lane 3), in the presence of 1 μ M staurosporin (Lane 4) and in the presence of 1 mM protein kinase C pseudosubstrate inhibitory peptide (Lane 5). Cells were treated with phytohemagglutinin for 40 hours.

Table I

Activation of protein kinase C by PMA in streptolysin-o-permeabilized cells with the specific oligopeptide as phosphate acceptor

Cells	Treatment with PMA	Phosphotransferase activity (cpm)
Resting	-	26,000
Resting	+	183,100
Stimulated	-	33,130
Stimulated	+	140,860

Cells were stimulated by phytohemagglutinin for 60 hours and resting cells were maintained at suboptimal stimulation. Samples of stimulated and resting cells were incubated in the absence and presence of 40 $\mu\text{g/ml}$ PMA for 10 minutes at 37 °C, then were pelleted and resuspended in the medium used for permeabilization (10^6 cells in 100 μl medium). The activity of protein kinase C was measured in the course of 12 minutes permeabilization. The phosphotransferase activity is expressed as the radioactivity of [^{32}P] phosphate incorporated into the selective oligopeptide substrate of protein kinase C. The specific radioactivity of [$\gamma\text{-}^{32}\text{P}$]ATP was 280000 cpm/nmol.

Table II

Cell cycle distribution of lymphocytes and phosphorylation of poly (ADP-ribose) polymerase

Cells	G1 (%)	S (%)	G2 (%)	Phosphorylation of poly (ADP-ribose) polymerase
I. Resting	93	3	4	undetectable
II. Resting	95	2	3	undetectable
I. Stimulated for 60 h	65	23.5	11.5	detectable*
II. Stimulated for 16 h	87	8	5	detectable*

*The specific radioactivity of these samples cannot be determined because of the unknown phosphorylation state by unlabeled ATP in cells.

Cell cycle distribution and phosphorylation of poly (ADP-ribose) polymerase was determined as described under Materials and Methods.

B

SPECIFIC BINDING DOMAINS FOR HISTONES AND FOR THE SELF-ASSOCIATING ENZYME PROTEIN OF POLY (ADP-RIBOSE) POLYMERASE

The structure of poly (ADP-ribose) polymerase has been augmented by the identification of polypeptide sequences which define histone and self association. Both protein binding domains are components of 64-67 kDa basic moiety of poly ADP-ribose polymerase, obtained by degradation by chymotrypsin or plasmin. Two discrete histone binding domains are interspersed and contiguous with "self binding" domains and are located at 186-290 and 446-525 residues. Self binding is confined to the 29 kDa N-terminal moiety of poly ADP-ribose polymerase and to two smaller polypeptide sequences 291-395 and 526-606 residues. Bound zinc is not required for self binding. No selectivity of histone binding could be ascertained in the protein-polypeptide two component binding system used here, and higher order structures and NAD may be required to demonstrate specificity towards various histone species. Both self and histone binding augment poly (ADP-ribose) synthesis activity of the enzyme and in a soluble system 95% of polymers are bound to the enzyme protein. Histone poly ADP-ribosylation is greatly augmented by nucleosome-like structure.

Poly (ADP-ribose) polymerase (ADP-ribose transferase, polymerizing, EC 2.4.2.30) is a highly abundant nuclear protein of higher eukaryotes (1,2) that exhibits at least two catalytic functions: ADPR polymerizing and NAD glycohydrolase activities which are also regulated by the state of differentiation of particular cell types (3). Assuming the same molecular activity *in vitro* and *in vivo*, poly (ADP-ribose) polymerase in maximally stimulated cells appears to account for only one percent of the molecular activity of this enzyme (1,2), approximating the magnitude detectable in the isolated homogeneous protein in the absence of coenzymic DNA (4), an activity present even in the 56 kDa polypeptide fragment of poly (ADP-ribose) polymerase (5) that contains no DNA recognition sites (6). Immunochemical estimation of native and automodified poly (ADP-ribose) polymerase in intact cells in culture. (AA-2 and MT-2 cells) indicated that only 4% (for AA-2) or 20% (for MT-2) of poly (ADP-ribose) polymerase was auto-ADP-ribosylated, thus a significant portion of this protein is available for macromolecular associations (7). Indeed recent results (8) show that *in vitro* poly (ADP-ribose) polymerase binds to and significantly stimulates DNA polymerase α but not β . Microinjection of the 46 kDa DNA-binding polypeptide fragment of poly (ADP-ribose) polymerase into human fibroblasts inhibits MNNG-induced unscheduled DNA synthesis (9), further supporting the contention that protein-protein or protein-DNA interactions involving poly (ADP-ribose) polymerase may have biological consequences. A potentially important cellular function of

poly (ADP-ribose) polymerase in NAD-dependent repair of damaged DNA has been also traced to the release of the binding of poly (ADP-ribose) polymerase from DNA sites by auto-poly-ADP-ribosylation of the enzyme protein (10,11). Self association (dimerization) of poly (ADP-ribose) polymerase has been shown to be essential for auto-poly-ADP-ribosylation of the enzyme protein (12,13). Furthermore binding to and trans-ADP-ribosylation (14) to an assortment of highly significant enzymes that act on DNA, such as the calcium/magnesium-dependent endonuclease (15), DNA polymerase α and β , terminal deoxynucleotidyl transferase, DNA ligase II (16,17), topoisomerase I (18,19), topoisomerase II (20) are also of critical cell biochemical significance, because trans-ADP-ribosylation to these enzymes produces a down regulation of the catalytic activities.

By far the most abundant nuclear proteins that are trans-ADP-ribosylated by poly (ADP-ribose) polymerase are histones (cf. 21) and we have demonstrated the *in vivo* occurrence of poly-ADP-ribosylated histones by specific antibodies directed against the polymer (22). Besides serving as ADPR acceptors (cf. 23) under certain conditions histones also activate rates of poly-ADP-ribosylation (24).

The examples cited clearly indicate the cell biological significance of macromolecular associations of poly (ADP-ribose) polymerase with

specific nuclear macromolecules. With the exception of the binding of DNA termini to zinc fingers of poly (ADP-ribose) polymerase (25) molecular details of protein-protein associations of poly (ADP-ribose) polymerase are unknown. We report here the identification of binding domains on poly (ADP-ribose) polymerase for histones and for the poly (ADP-ribose) polymerase enzyme protein itself and provide evidence for the catalytic importance of these sites.

Materials and Methods

Electrophoretically 98% homogeneous poly (ADP-ribose) polymerase and coenzymic DNA were isolated from calf thymus as reported (26,27). Unavoidable traces of lower mass bands were peptides of poly (ADP-ribose) polymerase produced by proteases as identified by immunoblots (26). The synthetic octadeoxyribonucleotide duplex 5'GCATGCAT3' (octamer C) was synthesized as described (28). The commercial sources of reagents were as follows: CNBr-activated Sepharose 4B and the Mono S column from Pharmacia (Piscataway, NJ), Affi-Gel 10 from Bio-Rad (Richmond, CA), [³²P]-NAD⁺ and the [¹²⁵I]-Bolton-Hunter reagent from ICN (Irvine, CA), [⁶⁵Zn]-ZnCl₂ from NEN-Du Pont (Wilmington, DE) and Centricon concentrators from Amicon (Danvers, MA). All other chemicals used were of the highest purity available.

Isolation of polypeptides of poly (ADP-ribose) polymerase obtained by proteases or CNBr cleavage

Partial digestion of poly (ADP-ribose) polymerase with chymotrypsin (29) was done as follows. poly (ADP-ribose) polymerase (2 mg/ml in 50 mM Tris-HCl, 200 mM NaCl, 20 mM 2-mercaptoethanol, pH 8.0) was digested with 3.3 µg/ml chymotrypsin for 30 minutes. at 25°C, then the reaction stopped with 1 mM PMSF and the polypeptides isolated by ion exchange chromatography on Mono S column as reported (6). The basic N-terminal 64 kDa polypeptide eluted at 0.45 M NaCl from Mono S cation exchanger column. The C-terminal 56 kDa polypeptide, which did not bind to the cation exchanger, was isolated on a benzamide-Affigel 10 affinity column as described earlier (6).

Cyanogen bromide fragments of poly (ADP-ribose) polymerase were prepared by the following technique. Homogeneous poly (ADP-ribose) polymerase (200 µg) was precipitated with 20% TCA, and pelleted by centrifugation, washed with 70% ethanol and dried. The dried protein was dissolved in 100 µl of 88% formic acid, and 300 µl of 0.1 M HCl and 20 µl of CNBr (100 mg/ml in ethanol) were added and the mixture allowed to stand for 48 hours at room temperature. Then the solvent was evaporated by freeze-drying and the residue dissolved in 400 µl of "renaturation buffer"(34).

Digestion with plasmin was carried out as described earlier (6).

Labeling of poly (ADP-ribose) polymerase or its fragments by [¹²⁵I]

The polypeptides to be labeled with [¹²⁵I] were dissolved in 25-50 μ l of 150 mM phosphate (pH 8.2) and pipetted into Eppendorf centrifuge tubes, which contained 2 μ l [¹²⁵I]-Bolton-Hunter reagent (1888 Ci/mmol, 33 μ Ci in benzene-dimethylformamide) previously evaporated to dryness by a stream of N₂. After mixing by aspiration into a micropipette several times, the iodination reaction was allowed to proceed at 6°C for 1 hour. The unreacted Bolton-Hunter reagent was quenched with 5 μ l of 1 M Tris-HCl (pH 9.0) and the reaction mixture gel-filtered through an 1.5 ml Sephadex G25 (fine) column equilibrated with 0.1 M sodium phosphate buffer (pH 7.5) containing 0.1% gelatin. The exclusion volume contained the iodinated polypeptides. The incorporation of [¹²⁵I] was in the range of 3 to 5%. The [¹²⁵I] labeled protein was used on the same day of its preparation for binding experiments.

Labeling of DNA

Sonicated calf thymus DNA, average length 250 bp, was used for labeling. The DNA (50 μ g) was dissolved in 100 μ l of Klenow buffer containing 50 μ M dNTP-s and 10 mCi of [³²P]TTP and enzymatically end-labeled with Klenow fragment of PolI as described (38). The labeled DNA was purified by phenol extraction and ethanol precipitation.

Electrophoretic techniques

poly (ADP-ribose) polymerase and its chymotryptic fragments were separated by 10% SDS-PAGE (31), and histones or CNBr-fragments of poly (ADP-ribose) polymerase on a 17.5% acrylamide-SDS system as described (32). [³²P]-Labeled poly (ADP-ribose) polymerase and its crosslinked products were separated on 10% acid-urea gels as described earlier(30).

Electroblotting was carried out in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS)/NaOH (pH 11.0) buffer containing 15% methanol for 90 minutes using nitrocellulose membranes with 0.45 mm pore size. When amino acid sequencing was performed, PVDF membranes (BroBlott membrane, Applied Biosystems, Foster City, CA) were used and the excised pieces containing the transblots of peptides were sequenced using the Model 470-A gas-phase sequencer and an on-line 120A PHT-analyzer (Applied Biosystems, Foster City, CA) according to a published method (33).

Preparation of Histone-Sepharose affinity matrix

Histones-Sepharose affinity matrix was prepared from CNBr-activated Sepharose 4B and histones (Type II-AS, Sigma, St. Louis, MO) according to the manufacturer's prescription (3 mg histones per ml of packed gel). The amount of histones bound to the matrix was determined in 0.1 ml aliquots of the settled resin by a published

method (35). On the average 0.9-1.2 mg of histones were covalently bound per 1 ml packed bed of gel matrix.

Crosslinking of poly (ADP-ribose) polymerase and Histone H2B

The association of the histone H2B with poly (ADP-ribose) polymerase was shown by crosslinking with dimethyl-3,3'-dithiobispropionimidate as reported (12).

For the identification of poly (ADP-ribose) polymerase on acid urea gels (30), the enzyme was labeled with [³²P] by mono-ADP-ribosylation with 100 nM [³²P]-NAD⁺ (14). The isolated mono-ADP-ribosylated poly (ADP-ribose) polymerase protein was then mixed with 20 fold excess unlabeled enzyme and this, diluted with respect to radiolabel poly (ADP-ribose) polymerase was used for crosslinking and was applied to gels (Figure 1). Control experiments indicated that the low amount of ADP-ribosylation did not influence the electrophoretic mobility of poly (ADP-ribose) polymerase when compared to the unmodified enzyme protein (not illustrated).

Binding Assays

Binding of Radioiodinated poly (ADP-ribose) polymerase or its polypeptides to transblotted peptides

Membranes containing electroblotted peptides were soaked in "renaturation buffer", 50 mM Tris-HCl, 100 mM NaCl, 1 mM DTT and 0.3% Tween 20, pH 8.0, (34) for 1 hour then blocked by incubation with 2% defatted powdered milk (dissolved in PBS containing 0.1 mM PMSF) for 2-6 hours at 25°C (34). The membranes were then washed with a "low salt buffer" (50 mM Tris-HCl, 10 mM 2-mercaptoethanol, 0.1 mM PMSF, 0.05% BSA and 0.05% Tween 20, pH 7.5). The incubation with labeled poly (ADP-ribose) polymerase ($2-5 \times 10^5$ cpm, 2-5 mg) was done in the same buffer (15-25 ml) as the one used for washing, for 30 minutes. at 25°C. Then the membranes were washed once with the low salt buffer, then 2 times with the low salt buffer containing 50 mM NaCl. Finally they were dried and exposed overnight to X-ray film.

Binding of Labeled DNA to Transblotted Peptides

Membranes renatured (34) and blocked with defatted milk, were washed once with 0.1 M potassium phosphate buffer, pH 7.6 containing 1 mM EDTA, then incubated in the same buffer (15 ml) with [32 P]-DNA ($5-10 \times 10^4$ cpm, 2-4 mg) for 20 minutes. at 25°C. After four washings with the same buffer the membranes were dried and exposed to X ray films overnight.

Incorporation of [⁶⁵Zn] into transblotted zinc finger containing peptides

Transblotted peptides were renatured (34) in the presence of 5 mCi [⁶⁵Zn]-ZnCl₂ in 10 ml renaturation buffer for one hour at 25°C, then the membranes were washed with renaturation buffer until no radioactivity over background was found in the washings. The membranes were exposed to X-ray films to locate [⁶⁵Zn]-containing bands.

Binding of the polypeptide fragments of poly (ADP-ribose) polymerase to Histones-Sepharose affinity column

Histones-Sepharose (1 ml bed volume) was loaded with 50 mg of unseparated CNBr fragments dissolved in 0.4 ml of 50 mM Tris-HCl, 10 mM 2-mercaptoethanol (pH 8.0) and was allowed to bind for 30 minutes at 25°C. Then the column was stepwise eluted with 2 ml aliquots of buffer containing increments of NaCl: 50, 100, 200, 400 and 1000 mM. The eluted fractions were concentrated on Centricon 3, one third of the total amount of the concentrates applied onto 17.5% SDS-PAGE. After separation, the gel was stained with Coomassie blue, or transblotted onto PVDF membranes for sequencing.

Poly(ADP-ribose) polymerase assay

The assays were carried out by published techniques (6) with modifications as given in the legends.

Nucleosome reconstruction (39)

Coenzymic DNA , 600 mg and an equal amount of core histones (Type II-AS, Sigma) were dissolved in 1.5 ml of TE buffer (10 mM Tris-HCl, 0.2 mM EDTA, pH 7.4) containing 0.8 M NaCl. This solution was dialyzed (at 4°C) stepwise against TE buffer that contained progressively lower concentrations of NaCl (0.8, 0.6, 0.4 and 0.2 M NaCl). Each dialysis step lasted 90 minutes except the final dialysis against TE buffer containing no added NaCl was extended overnight. The DNA content was adjusted to 200 mg/ml by dilution.

Results

The binding of histone H2B (molecular mass 13.8 kDa) to poly (ADP-ribose) polymerase was first determined by chemical crosslinking (12) as illustrated in Figure 1. In Lane 1 the monomeric poly (ADP-ribose) polymerase (116 kDa) and its dimer (200 kDa) were separated. When histone H2B was added, a new band appeared that was located between the mono and dimeric form of poly (ADP-ribose) polymerase (Lane 2).

Chemical reduction of the cross linker (12), as would be predicted, yielded the monomer of poly (ADP-ribose) polymerase only as a single [³²P]-labeled protein band (Lane 3). The unlabeled H2B, commensurate to its low molecular mass, migrated below the poly (ADP-ribose) polymerase bands and it is not visible in this gel. The H2B histone species could be replaced by any other histones indicating a nonselective binding of histones to poly (ADP-ribose) polymerase. We demonstrate the behavior of H2B only because this species was available to us in relatively homogenous form. Polylysine or polyarginine did not replace or displace histones as polycationic ligands to poly (ADP-ribose) polymerase (not shown).

The binding of polypeptides derived from poly (ADP-ribose) polymerase by chymotryptic digestion to radioiodinated core histones was assayed on nitrocellulose membranes. The separation of chymotryptic polypeptides of poly (ADP-ribose) polymerase, mainly representing a molecular masses of 64 kDa, 56 kDa and 42 kDa are shown (Coomassie stain) in Lane 1 of Figure 2, coinciding with published results (29). When the chymotryptic polypeptides of poly (ADP-ribose) polymerase were transblotted onto nitrocellulose membranes and incubated with radioiodinated core histones, only the basic polypeptide of poly (ADP-ribose) polymerase (64 kDa) indicated histone binding (Figure 2, Lane 2).

The correctness of this conclusion was tested also by "opposite" labeling, i.e. radioiodination of polypeptides of poly (ADP-ribose) polymerase. The same results as given in Figure 2 were obtained, i.e. only the basic half of poly (ADP-ribose) polymerase bound to histones, regardless which protein species was labeled with ^{125}I . In Figure 3, Lane 1 shows the Coomassie blue stained histones (mixed histones; 8 $\mu\text{g}/\text{Lane}$). Lane 2 illustrates that the 56 kDa polypeptide (labeled with ^{125}I) of poly (ADP-ribose) polymerase (the catalytic domain) did not bind to transblotted histones at all. The binding of the labeled basic 64 kDa polypeptide to transblotted histones is apparent from Lane 3, and in Lane 4 as a positive control, the binding of labeled intact poly (ADP-ribose) polymerase protein to histones is shown. The intact protein bound to transblotted histones in the same manner as the 64 kDa basic polypeptide (compare Lanes 3 and 4).

A more detailed localization of histone binding sites on poly (ADP-ribose) polymerase was achieved by percolating CNBr-generated peptide fragments of poly (ADP-ribose) polymerase through a histone-Sepharose affinity matrix and identifying the bound fragments. From the known amino acid sequence of bovine poly (ADP-ribose) polymerase (36) it is predictable that CNBr fragments of poly (ADP-ribose) polymerase in the region between zinc finger II and the catalytic domain, yield peptides of 8-18 kDa size, large enough to bind to poly (ADP-ribose) polymerase. In contrast, CNBr fragmentation of the zinc finger region produces some very short

peptides which would not signal binding to poly (ADP-ribose) polymerase because of their small size. Adsorption of CNBr fragments to histone matrix, followed by elution with a salt solution of stepwise increasing ionic strength permits an estimation of the relative binding strength of polypeptides to the histone matrix. This experiment is illustrated in Figure 4.

Electrophoretic separation of CNBr fragments of poly (ADP-ribose) polymerase is shown in Figure 4 Lane 1. In Lane 2, the non-adsorbed (flow-through) fragments, eluted by 50 mM Tris. HCl (pH 8.0) containing 10 mM 2-mercaptoethanol, are demonstrated. Lanes 3 to 7 show fragments emerging at 50 (Lane 3), 100 (Lane 4), 200 (Lane 5), 400 (Lane 6) and 1000 mM NaCl (Lane 7) as components of the elution buffer. The polypeptide with an apparent mass of 14 kDa eluted between 50 and 200 mM NaCl accompanied (Lanes 3 and 4) by a broader immunopositive band which most probably consists of proteolytic breakdown products of this fragment.

The 14 kDa polypeptide was identified by sequencing and corresponds to residues 186-290 on the sequence of poly (ADP-ribose) polymerase (105 residues, $M_r = 11,826$), its N-terminal sequences being GFSVL... . This polypeptide is located between the 29 and 36 kDa domains of poly (ADP-ribose) polymerase obtained by digestion with plasmin (6). It is only 22 residues downstream of the second zinc finger as apparent from the domain diagram (Figure 5).

We also sequenced the fragments which elute at 0.4 mM NaCl (apparent M_r 10 kDa and 18 kDa, shown in Figure 4, Lane 6). The N terminus of the 18 kDa peptide was XXLTLGXLSQ... . This sequence places the peptide on the bovine enzyme at 396-525 (130 residues, $M_r = 14,002$). Sequencing of the 10 kDa fragment yielded an N-terminal sequence of XEVKEANIRV... . The position of this peptide is at 446-525 (80 residues, $M_r = 8,603$). These two polypeptides thus partly overlap, the larger including the smaller one which is adjacent to the 56 kDa catalytic domain (Figure 5) as part of the automodifiable region of the enzyme (37).

Our results are consistent with the existence of two histone binding regions, one between residues 186 and 290, which is close to the second zinc finger, and a second histone binding sequence that is between residues 446 and 525 that is part of the automodifiable portion of poly (ADP-ribose) polymerase (Figure 5).

Since both direct chemical (12) and kinetic (13) evidence emphasizes the significance of self-association of poly (ADP-ribose) polymerase molecules, we attempted to identify polypeptide domains of poly (ADP-ribose) polymerase that are participatory in the self binding reaction. Chemical cross linking experiments (12) identified the 36 and 29 kDa polypeptides to bind to each other implying self association. When the poly (ADP-ribose) polymerase protein was digested with plasmin (6) a degradation pattern of polypeptides

occurs as visualized after electrophoresis on 10% SDS-PAGE and transblot to nitrocellulose membranes followed by Western blot with anti-poly (ADP-ribose) polymerase antiserum (26), as shown in Figure 6 (Lanes 1,2). The native enzyme protein (Lane 1, Figure 6) contains traces of polypeptide degradation products of poly (ADP-ribose) polymerase of a mass of 98, 80, 67 and 56 kDa (6). As shown in Lane 2 of Figure 6, digestion of poly (ADP-ribose) polymerase with plasmin (see legend to Figure 6) yielded 67, 56, 36 and 29 kDa polypeptides (6) and some minor peptides. When these transblotted polypeptides were probed with [¹²⁵I]-labeled poly (ADP-ribose) polymerase for self-binding, the intact poly (ADP-ribose) polymerase, the 67 kDa, the 36 kDa and the 29 kDa polypeptides exhibited strongest binding (Figure 6, Lanes 3 and 4). The same polypeptides also bound DNA (Figure 6, Lanes 5 and 6), and incorporated [⁶⁵Zn] (Lane 7, Figure 6). Since it is known that zinc-finger polypeptides of the 29 kDa fragment are required for the binding of DNA termini (25) the role of Zn²⁺ in self binding of poly (ADP-ribose) polymerase had to be clarified. As illustrated in Figure 6, (Lanes 8 and 9) zinc depleted (Lane 8) or replenished (Lane 9) polypeptides of 67 kDa, 36 kDa and 29 kDa mass equally bound radio-iodinated poly (ADP-ribose) polymerase, indicating that the basic (67 kDa) polypeptide fragment of poly (ADP-ribose) polymerase participates in self binding and Zn²⁺ is not critical in this self association reaction. Densitometric tracings of Lanes 8 and 9 confirm the conclusions deduced from visual inspection of the bands

that self association of poly (ADP-ribose) polymerase at 67 and 29 kDa peptide domains is independent of the presence of zinc ion. As shown in Figure 5, two discrete polypeptide domains specific for histone binding are also part of the 67 kDa proteolytic enzyme fragment. In further experiments, CNBr-generated peptides were separated by electrophoresis followed by transblot to PVDF membranes and incubation with radioiodinated poly (ADP-ribose) polymerase to detect self -association. This technique identified two discrete poly (ADP-ribose) polymerase-binding polypeptide fragments (separation not shown) and their N-terminal amino acid sequence was: VKTQT... for the 13 kDa fragment (291-395, 105 residues, M_r 13,056 and KKTLK... for the 9 kDa fragment (526-606, 81 residues, M_r 9,070).

The same 13 kDa polypeptide which was generated by CNBr coeluted with the poly (ADP-ribose) polymerase molecule in a Sephadex G75 column as identified by SDS-PAGE (not shown). Positioning of the 13 and 9 kDa CNBr fragments together with the histone binding polypeptides (Figure 5) indicates the existence of contiguous and intermittent histone and self binding regions in the poly (ADP-ribose) polymerase molecule. The 29 kDa N-terminal polypeptide which was not further fragmented comprises the third domain involved in self association. As stated above CNBr fragmentation of the 29 kDa polypeptides yielded too small fragments

for binding assays in our test system. These polypeptides had a tendency to self associate.

Both the binding of histones to poly (ADP-ribose) polymerase (24) and self association of poly (ADP-ribose) polymerase protein (12, 13) exert significant stimulation of poly ADP-ribosylation. We therefore devised experiments which can demonstrate a correlation between protein-protein binding, as described above, and the enzymatic activity of poly (ADP-ribose) polymerase. As illustrated in Figure 7A, a protein concentration-dependent association of poly (ADP-ribose) polymerase molecules produces an increase of enzymatic rates between 0 and 30 nM poly (ADP-ribose) polymerase and at higher enzyme concentrations activity decreases, probably due to inhibitory higher order self association of the monomer. Almost complete inhibition occurs at 1 μ M poly (ADP-ribose) polymerase concentration which coincides with the estimated nuclear concentration of this enzyme. In the presence of histone H3, a dilute - by itself inactive - poly (ADP-ribose) polymerase solution exceeds its maximal polymerase activity assayed in the absence of histone H3; however above 30 nM poly (ADP-ribose) polymerase, the activity diminishes parallel with the increase of poly (ADP-ribose) polymerase concentration, similar to the behavior of the enzyme in the absence of histone.

The activation of poly (ADP-ribose) polymerase by histone H3 was dependent on the histone concentration (Figure 7B). The most obvious explanation to the activation of poly (ADP-ribose) polymerase by histones would suggest that histone H3 merely serves as an ADP-ribose acceptor. However, as tested by gel electrophoresis (not shown) only 1 to 5% of poly ADP-ribose was detectable on histone H3 and the rest of ADP-ribose oligomers were covalently bound to poly (ADP-ribose) polymerase protein itself, comprising "self-ADP-ribosylation".

The apparent contradiction between these enzymological results and the known poly ADP-ribosylation of histones in nuclei was resolved by demonstrating that artificially reconstructed nucleosomes (39) indeed provided favorable conditions for extensive poly ADP-ribosylation of histones, as illustrated in Figure 8. In Lane 1 of Figure 8, auto-ADP-ribosylation of poly (ADP-ribose) polymerase is shown in presence of DNA. Addition of mixed histones (Lane 2) increased auto ADPR-ribosylation of poly (ADP-ribose) polymerase without detectable histone ADP-ribosylation. However, in reconstructed nucleosomes extensive poly ADP-ribosylation of histones was apparent (Lane 3, Figure 8).

Discussion

The previously defined domain structure of poly (ADP-ribose) polymerase (29) has been augmented by the identification of two protein binding regions, one involving histones, the second the self-association of poly (ADP-ribose) polymerase molecules, as shown in Figure 5. Both histones and self-association have significant influence on the polymerase activity of this enzyme (Figure 7) and it appears meaningful that both protein binding domains reside within the 67 kDa basic part of the poly (ADP-ribose) polymerase molecule that also contains DNA binding domains (29), the 29 kDa with its zinc fingers binding DNA termini (25) and the 36 kDa (6) binding to internal regions of certain double-stranded DNAs (40,41). Our technique of detection of protein-protein binding is limited by polypeptide mass of 8-13 kDa minimally required for a binding reaction to be identifiable. Interpretation of the two discrete histone binding sites in macromolecular terms would require detailed knowledge of the tertiary structure of poly (ADP-ribose) polymerase which is not available at this time. The apparent nonspecificity of histones as poly (ADP-ribose) polymerase binding proteins is in contrast to the well known specific biological function of histones in the nucleus. That basic polyamino acids cannot replace or displace histones as poly (ADP-ribose) polymerase binders appears to rule out the basic domains of histones as common denominators as poly (ADP-ribose) polymerase-binding histone moieties. It has been

shown (42) that in isolated polynucleosomes the concentration of NAD determines selectivity of poly ADP-ribosylation of various histone species, indicating that the substrate NAD and nucleosomal structure or both are required to exhibit histone specificity. Since our binding system consist of only protein and polypeptide components, it is not unexpected that physiologically important selectivities (e.g. histone specificity) cannot be demonstrated and our reductionist model illustrates only elementary association mechanisms. Similar mechanistic arguments may apply to the negligible poly ADP-ribosylation of histones with purified poly (ADP-ribose) polymerase *in vitro* (Figures 7,8) which is maximized when the soluble system is converted to one containing reconstructed nucleosomes (Figure 8). The lack of histone poly ADP-ribosylation by purified poly (ADP-ribose) polymerase has been reported (43) but the changes in the polymer-accepting function of histones as a consequence of supramolecular structural modifications has so far not been considered. Our results, identifying the 29 kDa and two smaller domains (9, 13 kDa) of poly (ADP-ribose) polymerase to be participatory in self association may be correlated with predictions made from the cDNA structure of poly (ADP-ribose) polymerase isolated from *Drosophila* (44). The leucine zipper motif has been identified in the automodification domain of the *Drosophila* enzyme (44). It is noteworthy that the amphipathic helix at 395-417 residues of the bovine poly (ADP-ribose) polymerase includes the "bZip" domain identified in *Drosophila* (44) and the conclusions regarding

the importance of this domain in self association of poly (ADP-ribose) polymerase (44) support our deductions.

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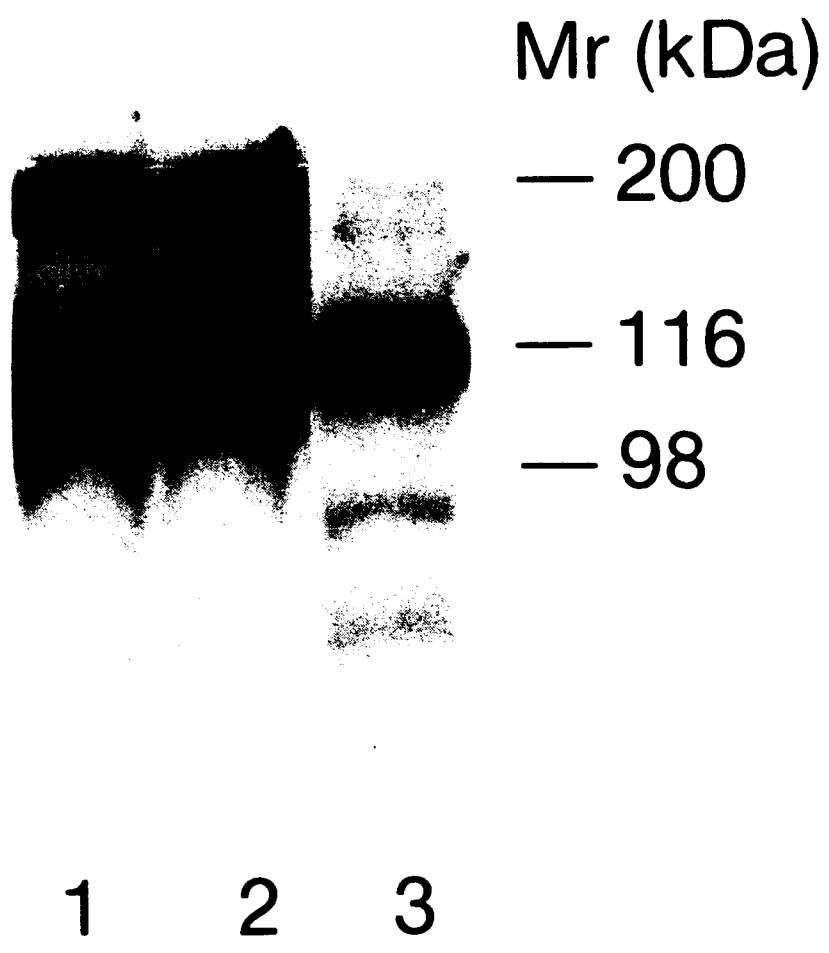


Figure 1

Figure 1. Crosslinking of histone H2B to poly (ADP-ribose) polymerase with dimethyl-3,3'-dithiobispropionimidate. Lane 1, cross linked poly (ADP-ribose) polymerase only; Lane 2, cross linked poly (ADP-ribose) polymerase + histone H2B; Lane 3 same as Lane 2, but reduced by boiling for 2 min. in the presence of 1% 2-mercaptoethanol. Techniques are given in Materials and Methods.

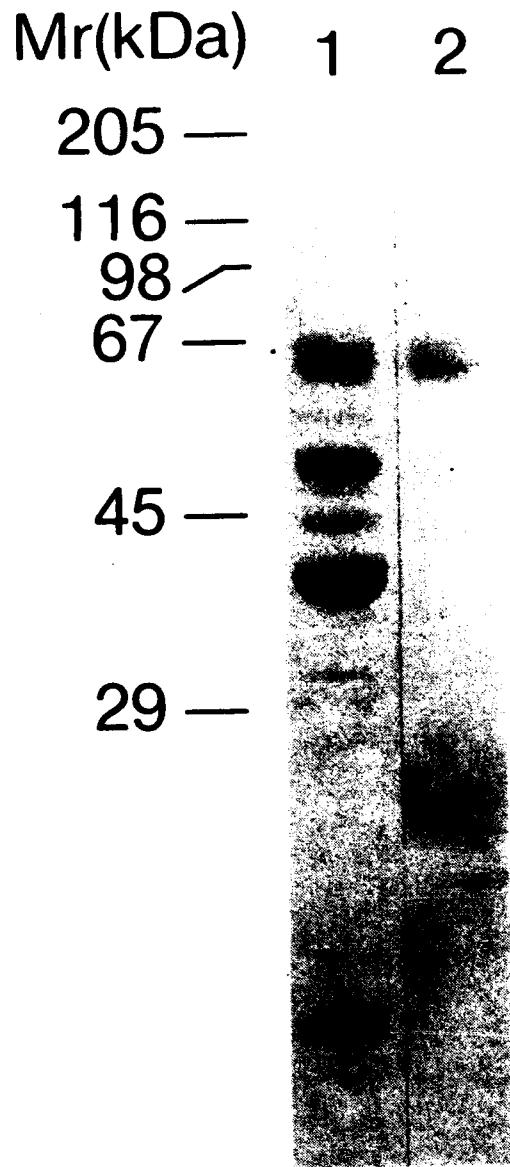


Figure 2

Figure 2. Binding of labeled histones to transblotted chymotryptic fragments of poly (ADP-ribose) polymerase. The digestion of poly (ADP-ribose) polymerase, separation of polypeptides, electroblotting onto nitrocellulose membrane and the procedure for binding [¹²⁵I]-histones are described in Materials and Methods. Lane 1: Coomassie stained peptides; Lane 2: autoradiography of bound [¹²⁵I]-histones to transblotted peptides of poly (ADP-ribose) polymerase.

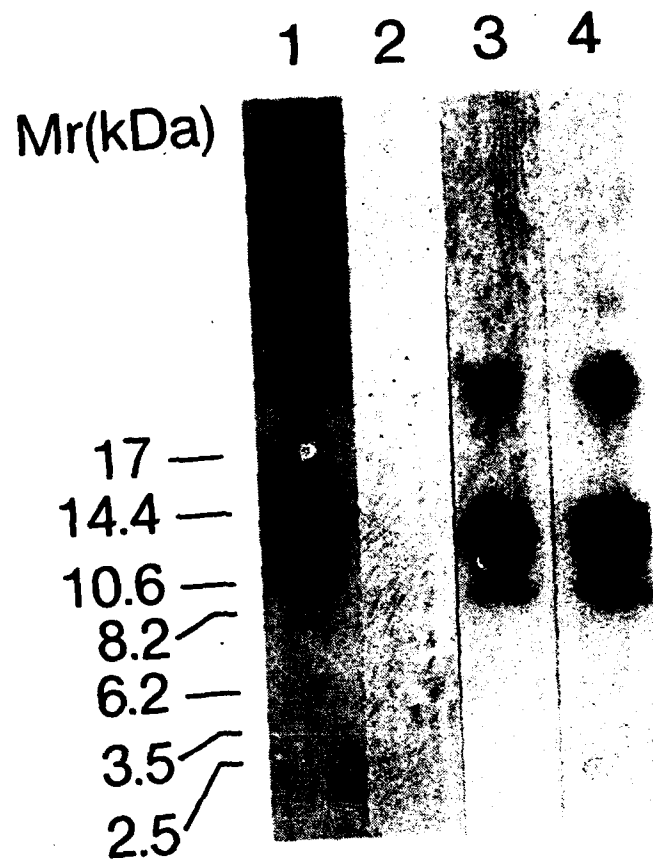


Figure 3

Figure 3. Binding of poly (ADP-ribose) polymerase and its N-terminal or C-terminal moieties to transblotted histones. Lane 1 shows Coomassie stained histones, all the other Lanes are autoradiograms. The absence of binding of radiolabeled 56 kDa polypeptide of poly (ADP-ribose) polymerase to histones is shown in Lane 2. Lanes 3 and 4 illustrate the histone binding of the labeled 64 kDa polypeptide and of the entire poly (ADP-ribose) polymerase protein as a control.

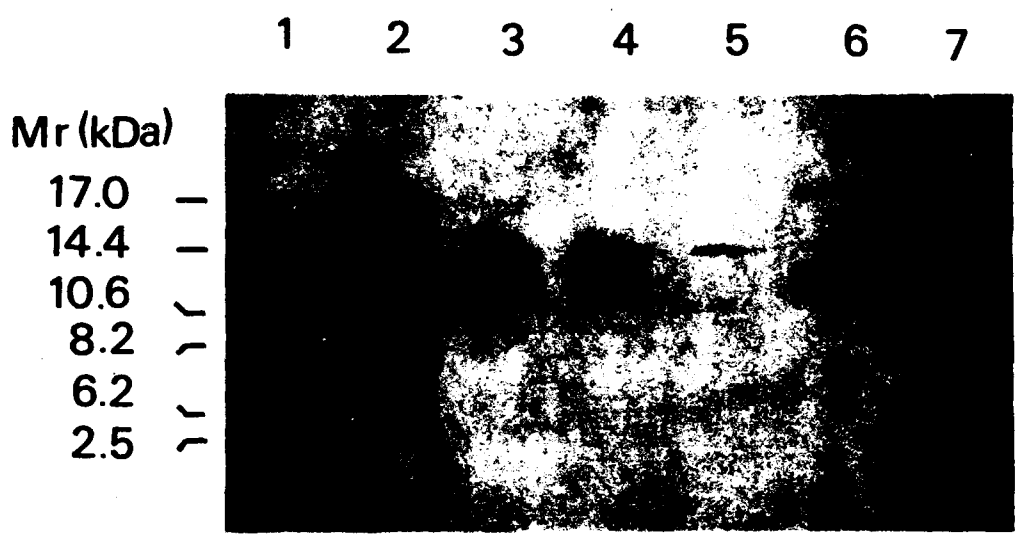
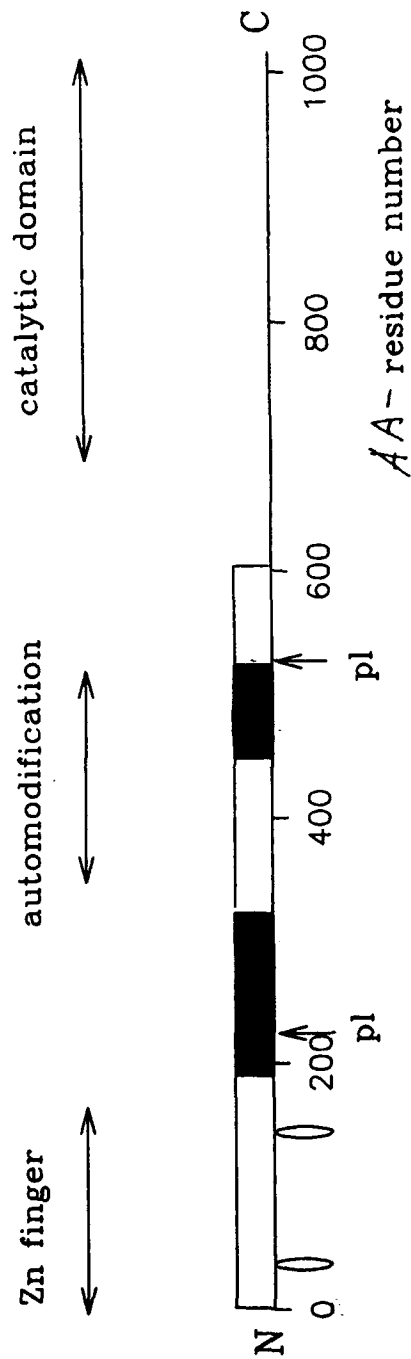


Figure 4

Figure 4. Electrophoretic separation of CNBr fragments of poly (ADP-ribose) polymerase eluted from a histone-Sepharose affinity column. Lane 1: CNBr- generated peptides prior to passing through the affinity matrix; Lane 2: flow-through CNBr fragments; Lanes 3-7 eluates with 50, 100, 200, 400, 1000 mM NaCl respectively. For technical details, see Materials and Methods.

Self and histone binding sites on the PARP primary sequence



- N: N-terminal;
- C: C-terminal;
- pl: plasmin cutting sites;
- ellipses: Zn fingers;
- closed rectangles: histone binding CNBr peptides;
- open rectangles: selfbinding peptides.

Figure 5

Figure 5. Self and histone binding sites of poly (ADP-ribose) polymerase. Shaded rectangles: histone binding CNBr peptides; open rectangles: self-binding peptides. Small ellipses mark the position of Zn-fingers. N: the N-terminal; C: the C-terminal; and pl: plasmin cutting sites. Polypeptide domains of poly (ADP-ribose) polymerase are also indicated with double ended arrows.

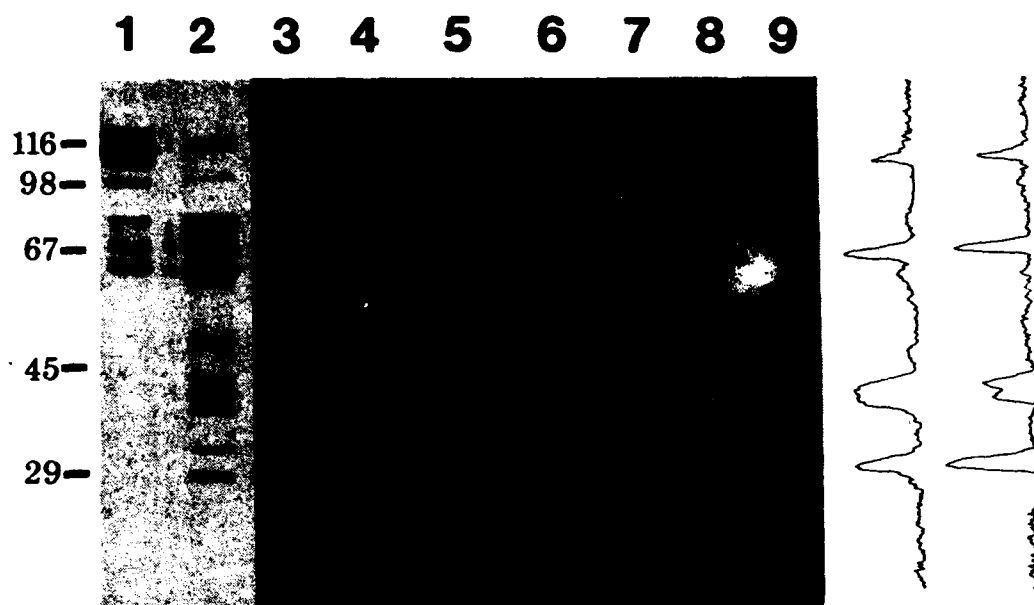


Figure 6

Figure 6. Identification of the basic polypeptide domain (64 kDa) as the self-binding portion of poly (ADP-ribose) polymerase

Lanes 1 and 2 are immunostained blots of poly (ADP-ribose) polymerase (Lane 1) and its plasmin digested (Lane 2) polypeptides, Lanes 3 and 4 are autoradiograms of transblotted poly (ADP-ribose) polymerase and its polypeptides both probed with [¹²⁵I]-poly (ADP-ribose) polymerase. Lanes 5 and 6 are autoradiograms of transblotted poly (ADP-ribose) polymerase and its peptides both probed with [³²P]-DNA. Lane 7 is polypeptides of poly (ADP-ribose) polymerase labeled with ⁶⁵Zn²⁺. Lanes 8 and 9 are [¹²⁵I]-poly (ADP-ribose) polymerase-binding polypeptides obtained by plasmin digestion of poly (ADP-ribose) polymerase and renatured in the nitrocellulose membrane either in the absence (Lane 8) or in the presence of (Lane 9) 1 mM EDTA, followed by probing with [¹²⁵I]-poly (ADP-ribose) polymerase. The two tracings on the far right are densitometric recordings of Lanes 8 and 9 in the same order.

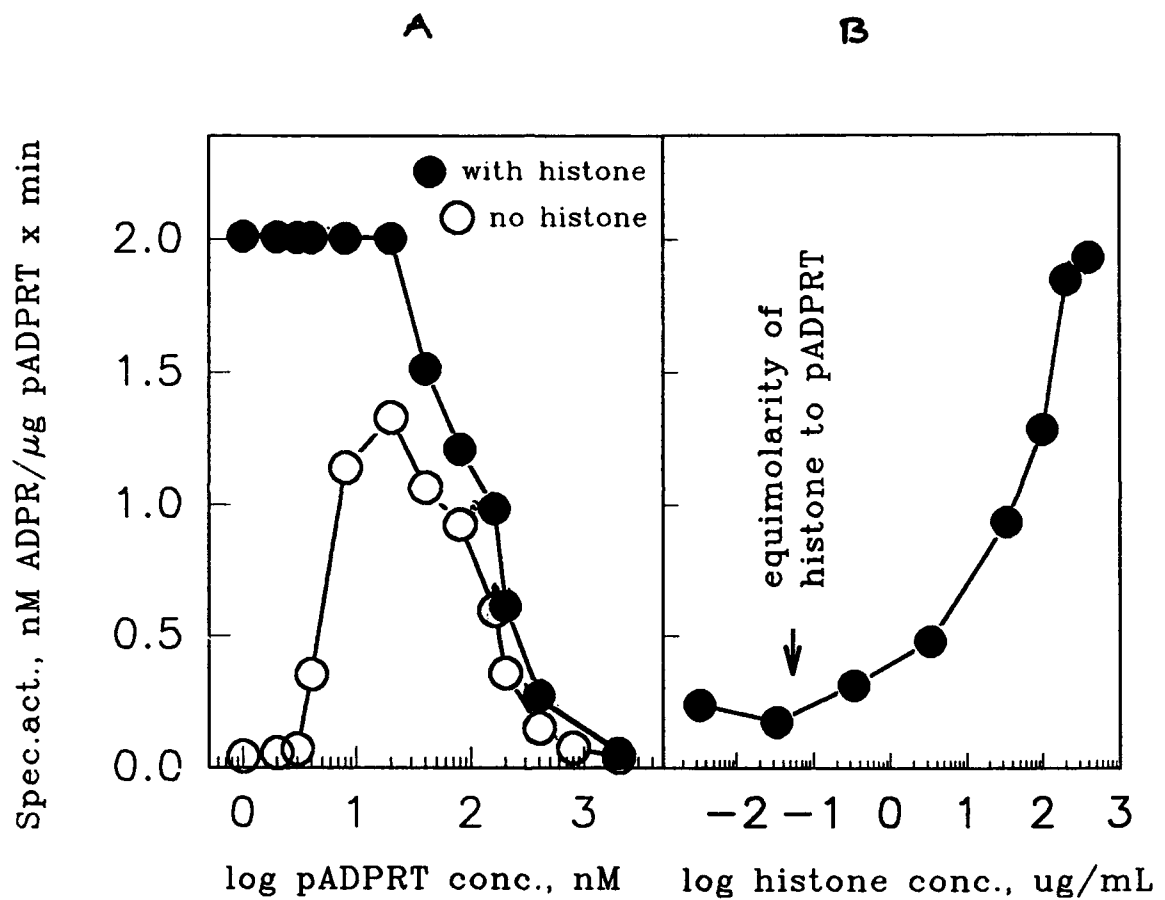


Figure 7

Figure 7A Effect of poly (ADP-ribose) polymerase concentration on the specific activity of the enzyme in the absence and presence of Histone H3. Poly (ADP-ribose) polymerase at varying concentrations was assayed (6) either in the absence (open circles) or in the presence (closed circles) of 200 mg/ml histone H3 for 1 minute at 23°C. The reactions were started by adding 1 ml of poly (ADP-ribose) polymerase to the mixture. The specific activity of the enzyme (ordinate) is plotted against the log of enzyme concentration (abscissa).

Figure 7B The effect of varying histone concentration on the specific activity of poly (ADP-ribose) polymerase. Poly (ADP-ribose) polymerase concentration was 5.5 nM and histone concentration varied between 0.03 to 200 mg per ml.

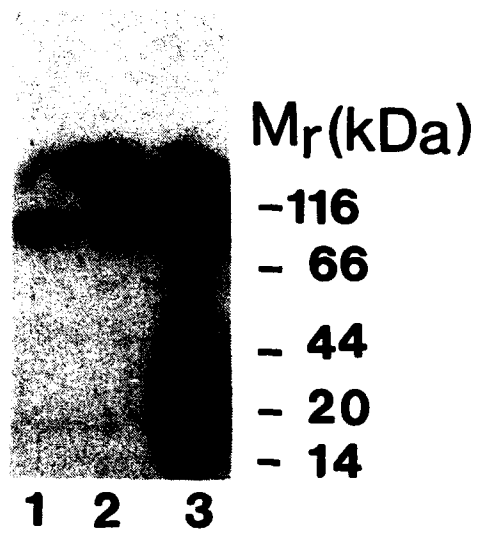


Figure 8

Figure 1. Poly-ADP-ribosylation of poly (ADP-ribose) polymerase and histones in reconstructed nucleosomes. Experimental conditions are detailed in Methods. Lane 1, poly (ADP-ribose) polymerase (1.2mg)+ coDNA(10mg); Lane 2, poly (ADP-ribose) polymerase (1.2mg)+ coDNA(10mg) + histone H3(10mg); Lane 3, poly (ADP-ribose) polymerase (1.2mg)+ reconstructed nucleosomes (equal to 10mg).

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