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Interaction of Poly(ADP-ribose) Polymerase 1 with Apurinic/Apyrimidinic Sites within Clustered DNA Damage

M. M. Kutuzov, E. S. Ilina, M. V. Sukhanova, I. A. Pyshnaya, D. V. Pyshnyi, O. I. Lavrik, and S. N. Khodyreva*

Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, pr. Akademika Lavrent'eva 8, 630090 Novosibirsk, Russia; fax: (383) 363-5153; E-mail: svetakh@niboch.nsc.ru

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Abstract—To study the interaction of poly(ADP-ribose) polymerase 1 (PARP1) with apurinic/apyrimidinic sites (AP sites) within clustered damages, DNA duplexes were created that contained an AP site in one strand and one of its analogs situated opposite the AP site in the complementary strand. Residues of 3-hydroxy-2-hydroxymethyltetrahydrofuran (THF), diethylene glycol (DEG), and decane-1,10-diol (DD) were used. It is shown for the first time that apurinic/apyrimidinic endonuclease 1 (APE1) cleaves the DNA strands at the positions of DEG and DD residues, and this suggests these groups as AP site analogs. Insertion of DEG and DD residues opposite an AP site decreased the rate of AP site hydrolysis by APE1 similarly to the effect of the THF residue, which is a well-known analog of the AP site, and this allowed us to use such AP DNAs to imitate DNA with particular types of clustered damages. PARP1, isolated and in cell extracts, efficiently interacted with AP DNA with analogs of AP sites producing a Schiff base. PARP1 competes with APE1 upon interaction with AP DNAs, decreasing the level of its cross-linking with AP DNA, and inhibits hydrolysis of AP sites within AP DNAs containing DEG and THF residues. Using glutaraldehyde as a linking agent, APE1 is shown to considerably decrease the amount of AP DNA-bound PARP1 dimer, which is the catalytically active form of this enzyme. Autopoly(ADP-ribosyl)ation of PARP1 decreased its inhibitory effect. The possible involvement of PARP1 and its automodification in the regulation of AP site processing within particular clustered damages is discussed.

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Key words: affinity modification, poly(ADP ribose) polymerase 1, Schiff base, apurinic/apyrimidinic sites and their analogs

The base excision repair (BER) system is responsible for repair of damage caused by ionizing radiation and exogenous oxidizing and alkylating agents that act similarly to endogenous factors [1]. The same system also repairs apurinic/apyrimidinic (AP) sites. AP sites are produced by spontaneous hydrolysis of the N-glycoside bond and also by elimination of an incorrect or damaged base by DNA glycosylases. Under physiological conditions, ~10,000 AP sites are produced in mammalian cells per day [2]. Clustered damage, or multiple lesions in which oxidized bases, AP sites, and strand breaks are combined within one or two turns of the DNA helix and can be located in both strands of DNA present a significant problem for the cell. Such damage is characteristic for ionizing radiation and therapeutic radiomimetics [3].

Depending on the type of ionizing radiation, 20-30% of damages are direct double-strand breaks in DNA, whereas other damages are located within clusters [4]. The repair of such damage requires careful control of the succession of repair of individual damages to prevent formation of double-strand breaks, which are most toxic for cells [3]. Studies *in vitro* with purified BER proteins and nuclear extracts have shown that repair of clustered damage can be associated with formation of double-strand breaks [5]. The presence of breaks within clusters and of AP sites in the opposite DNA strand increases the probability of formation of double-strand breaks compared to the case of multiple damages as different oxidized bases [6]. Despite the numerous studies on repair of multiple damages by BER proteins that have been performed using cell extracts and repair systems reconstructed from individual proteins, exact regulatory mechanisms and factors

Abbreviations: A, dAMP; APE1, human apurinic/apyrimidinic (AP) endonuclease 1; AP site, apurinic/apyrimidinic site; BER, base excision repair; DD, decane-1,10-diol residue; DEG, diethylene glycol residue; DTT, dithiothreitol; PARP1, human poly(ADP-ribose) polymerase 1; THF, 3-hydroxy-2-hydroxymethyltetrahydrofuran residue.

* To whom correspondence should be addressed.

responsible for the efficient repair of cluster damages are not established. Perhaps there are some proteins specifically interacting with clustered damages and regulating their repair. Among the known proteins of the BER system, this function may be fulfilled by poly(ADP-ribose) polymerase 1 (PARP1). PARP1 recognizes breaks in DNA produced not only under the influence of genotoxic agents (e.g. free radicals, ionizing radiation, monofunctional alkylating agents), but also as a result of functioning of BER enzymes [7]. On interacting with DNA breaks in the presence of NAD^+, the enzymatic activity of PARP1 is manifested resulting in synthesis of negatively charged polymer, poly(ADP-ribose), covalently bound to PARP1 itself and to acceptor proteins [8]. The automodification of PARP1 is thought to result in its dissociation from DNA, which promotes the regulation of repair. We have recently found that PARP1 can interact with AP sites via formation of Schiff base [9], and it was suggested that this protein should be involved in the regulation of processing of AP sites within clustered damages. In this connection it seems very interesting to study interactions of PARP1 with DNA containing AP sites within clustered damages and also mutual influences of PARP1 and APE1 (apurinic/apyrimidinic (AP) endonuclease 1) on interaction with such DNA structures. APE1 is the major enzyme processing AP sites in the cells of higher eukaryotes [10].

Formation of Schiff base is a reversible process, but the binding of protein with DNA can be stabilized by reduction with sodium borohydride [9]. Such covalent binding of proteins to nucleic acid is often used when protein–nucleic acid interactions are studied [9, 11-13].

In the present work the interaction of PARP1 with APE1 was studied using DNA duplexes with structure imitating DNAs with a definite type of clustered damages represented by two AP sites located opposite to each other. On using such model DNAs, it was desirable that one of the AP sites would be an analog maximally resistant to the action of APE1, and 3-hydroxy-2-hydroxymethyltetrahydrofuran (THF), diethylene glycol (DEG), and decane-1,10-diol residues (DD) were used as analogs of AP sites. The THF residue is most widely used as a mimetic of AP sites hydrolyzed by AP endonuclease-1 [14, 15]. The earlier used acyclic analogs of AP sites, ethylene glycol and propandiol residues, are cleaved by APE1 with approximately the same catalytic efficiency as a THF residue [15]. New analogs of AP sites, DEG and DD residues, were expected to be more resistant to the action of APE1.

MATERIALS AND METHODS

Reagents used were as follows: dNTP, EDTA, Tris, TEMED, imidazole, SDS, ammonium persulfate, dithiothreitol (DTT), and Coomassie G-250 (Sigma, USA); $MgCl_2$, formamide, and NP-40 (Fluka, Switzerland); acrylamide and glycerol (ICN, USA); β-mercaptoethanol (Serva, Germany); N,N′-methylene bisacrylamide (BioRad, USA); T4 phage polynucleotide kinase and uracil-DNA glycosylase from *Escherichia coli* (Biosan, Russia); [γ-^{32}P]ATP (>110 TBq/mmol) (Laboratory of Biotechnology, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences (ICBFM SB RAS)). Other reagents were of domestic production.

Expressing vectors containing cDNA of human APE1 and PARP1 were kindly provided by S. Wilson (National Institutes of Health, USA) and M. S. Sato (University of the Laval city, Canada), respectively. Recombinant proteins were isolated as described in works [13, 14]. Cell cultures of HeLa, MCF-7, human lung fibroblasts, K-562, and BL-2 were kindly provided by coworkers of ICBFM SB RAS M. A. Zenkova, V. A. Matveeva, and P. P. Laktionov. Whole-cell and nuclear extracts were prepared as described in work [11]. Protein concentrations were determined by the Bradford method [15] with BSA as a standard.

Oligodeoxyribonucleotides without modifications in the strands were synthesized in the Laboratory of Medical Chemistry (ICBFM SB RAS). Modified oligonucleotides were prepared by incorporation into them of inserts based on DEG, DD, and THF using corresponding phosphoramidite synthons synthesized by the method described in [16]. The 5′-end of oligonucleotides was radiolabeled using phage T4 polynucleotide kinase [17]; the products were purified by electrophoresis in denaturing polyacrylamide gel with 7 M urea [17].

Oligonucleotides of the following sequences were used: 5′-GGAAGACCCTGACGTT**DEG**CCCAACTTAATCGCC-3′; 5′-GGAAGACCCTGACGTT**DD**CCCAACTTAATCGCC-3′; 5′-GGAAGACCCTGACGTT**THF**CCCAACTTAATCGCC-3′; 5′-GGAAGACCCTGACGTT**A**CCCAACTTAATCGCC-3′; 5′-GGCGATTAAGTTGGG**U**AACGTCAGGGTCTTCC-3′; 5′-GGCGATTAAGTTGGGCAACGTCAGGGTCTTCC-3′; where U is dUMP.

Preparation of DNA duplexes containing AP sites using uracil-DNA glycosylase from *E. coli*. Reaction mixtures (5-10 μl volume) contained ^{32}P-labeled uracil-containing DNA (1 μM), uracil-DNA glycosylase (4 U/pmol DNA), and also the following standard components: 50 mM Tris-HCl (pH 8.0), 50 mM NaCl. The reaction was performed during 15 min at 37°C. AP sites in DNA were generated immediately before the experiment.

Determination of APE1 activity. Reaction mixtures (10 μl) contained ^{32}P-labeled AP DNA (0.1 μM), 25 nM APE1, 5 mM $MgCl_2$ ($CaCl_2$), and also standard components: 50 mM Tris-HCl (pH 7.8), 40 mM NaCl, 1 mM DTT, 0.1 mg/ml BSA. The reaction was performed for 15 min at 37°C and stopped by addition of sodium borohydride and EDTA to the concentration of 20 mM. The

reaction mixtures were incubated for 30 min at 0°C. The products were analyzed by electrophoresis in 20% polyacrylamide gel in the presence of 7 M urea [17] with a subsequent radioautography. On determination of substrate properties of AP sites within AP DNA containing analogs of AP sites the APE1 concentration was 3 nM, and the time was varied from 0.25 to 5 min. On determination of the substrate properties of AP site analogs the APE1 concentration was 30 nM, and the time of incubation is indicated in the caption to Fig. 1. Radioactivity of the products was measured using a Molecular Imager device and Quantity One software (BioRad).

Modification of PARP1 by DNA duplexes containing AP sites. Reaction mixtures (10 μl) contained ^{32}P-labeled AP DNA (0.1 μl), 5 mM EDTA or 5 mM $MgCl_2/CaCl_2$, 50 mM Tris-HCl (pH 7.8), 40 mM NaCl, 0.1 mg/ml BSA, 0.24 μM PARP1, 25 nM APE1. The reaction was carried out at 37°C for 15 min, then sodium borohydride was added to the final concentration of 20 mM, and the incubation was continued on ice for 30 min. The reaction products were analyzed by electrophoresis in 10% polyacrylamide gel in the presence of SDS [18] with subsequent radioautography.

Modification of extract proteins by DNA duplexes containing AP sites. AP DNA extract proteins were modified as described for recombinant PARP1 (without addition of BSA) at the extract protein concentrations 1.4 and 0.16 mg/ml for the whole-cell and nuclear extracts, respectively. The further procedures were similar to those described above.

Detection of protein–protein interactions by cross-linking with glutaraldehyde. Reaction mixtures (10 μl) contained ^{32}P-labeled AP DNA (0.1 μM), 0.24 μM PARP1, 25 nM APE1, 5 mM $CaCl_2$, and also standard components: 50 mM Tris-HCl (pH 7.8), 40 mM NaCl, 1 mM DTT. The reaction mixtures were incubated at 37°C for 15 min, then glutaraldehyde was added to concentration of 0.1%, and incubation was continued at the same temperature for 10 min. Then to stabilize Schiff bases the reaction mixtures were supplemented with sodium borohydride to the concentration of 20 mM and incubated for 30 min at 0°C. The reaction products were analyzed by electrophoresis in 10% polyacrylamide gel in the presence of SDS [18] with subsequent radioautography.

RESULTS AND DISCUSSION

Non-nucleotide insertions as analogs of AP sites. DNA duplexes were created using 32-meric synthetic oligonucleotides containing in the middle of the strand non-nucleotide insertions of THF, DD, and DEG residues [19, 20]. Analysis of the DNA strand cleavage by positions of the AP site analogs within the DNA duplexes DEG-DNA, THF-DNA, and DD-DNA (Fig. 1) revealed that APE1 hydrolyzed DNA with acyclic analogs of AP sites (DD and DEG residues) significantly less efficiently than THF-containing DNA. On one hand, DD and DEG residues can be considered as analogs of AP sites because APE1 cleaves the DNA strand by their positions. On the other hand, when DD and DEG residues are used for imitating AP sites within clustered damages, the DNA cleavage by their positions is insignificant as compared to the cleavage in an AP site. Note that as opposed to natural AP sites these analogs are unable to produce Schiff bases, and this can be useful in some studies.

According to the available data, the rate of cleavage by APE1 of intact or borohydride-reduced AP sites is 50-80% lower in the presence of oppositely located AP sites (reduced AP sites) [21-23]. To determine the efficiency of imitating AP sites within clustered damages by the studied analogs, we compared the rates of cleavage of AP sites within DNA containing opposite the AP site the following residues: dAMP (AP DNA-A), THF (AP DNA-THF), DD (AP DNA-DD), and DEG (AP DNA-DEG). The results of this comparison presented in Fig. 2 show that the AP site is not as well imitated by a DD residue as by residues of THF and DEG, because the efficiency of hydrolysis of AP sites within AP DNA-DD is higher than within AP DNA with the two other insertions. As expected, the cleavage rate was maximal for AP DNA-A containing a single AP site. Thus, considering the decreased efficiency of hydrolysis of AP sites within AP DNAs containing non-nucleotide insertions and the ability of APE1 to cleave DNA strands at the positions of these groups, such DNAs can be considered as structures imitating a particular type of clustered damage. Note that residue DEG was more resistant to the action of APE1 than

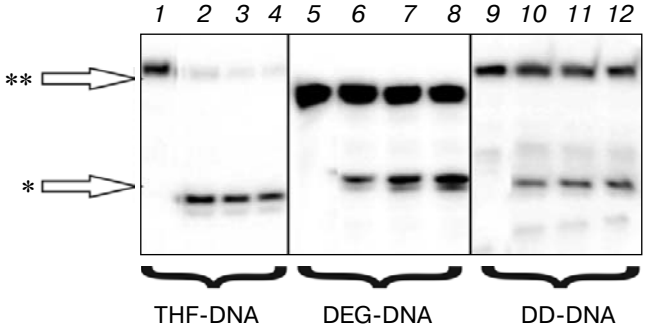

Fig. 1. Substrate features of non-nucleotide insertions as analogs of AP sites in the reaction catalyzed by APE1. Reaction mixtures (10 μl) containing standard components, 0.1 μM DNA with non-nucleotide insertions, and 30 nM APE1 were incubated at 37°C. The incubation time for DEG-DNA (*6-8*) was 5, 10, and 15 min and for THF-DNA (*2-4*) and DD-DNA (*10-12*) it was 0.5, 1.0, and 1.5 min, respectively. *1*, *5*, *9*) Control (incubation without APE1). The products were separated by electrophoresis in 20% polyacrylamide gel in the presence of 7 M urea. Arrows with one and two asterisks indicate cleaved and uncleaved oligonucleotides, respectively.

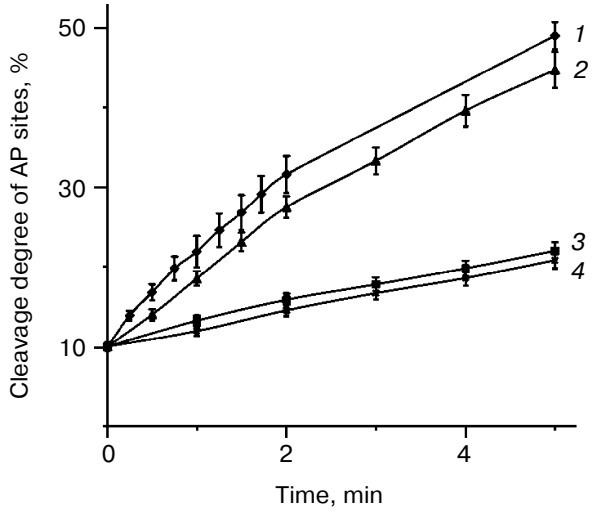

Fig. 2. Kinetic curves of cleavage of AP sites within AP DNA by AP endonuclease 1. Reaction mixtures (10 μl) containing standard components, 0.1 μM AP DNA with non-nucleotide insertions, and 3 nM APE1 were incubated at 37°C. At the indicated time intervals, the reaction was stopped, AP sites were reduced with borohydride (20 mM) at 0°C for 30 min, and the products were separated by electrophoresis in 20% polyacrylamide gel in the presence of 7 M urea. *1*) AP DNA-A; *2*) AP DNA-DD; *3*) AP DNA-THF; *4*) AP DNA-DEG. Mean values and standard errors are presented, $n = 3$.

residues DD and THF (Fig. 1), whereas hydrolysis of an AP site within AP DNA-DEG by AP endonuclease 1 was also the least efficient.

Interaction of PARP1 and APE1 with AP DNA. We have earlier shown that PARP1 interacts with AP sites producing Schiff bases [9] that can be stabilized by reduction with borohydride. In addition to photoaffinity modification, this covalent binding of proteins to nucleic acid can be used in studies of protein–nucleic interactions [9, 11, 12]. To assess the efficiency of the interaction of PARP1 with AP DNA having different structure and the influence of APE1 on the interaction of PARP1 with these DNAs, the levels of AP DNA binding to PARP1 were determined in the presence and in the absence of APE1. The binding of DNA with PARP1 and APE1 does not require the presence of bivalent metal ions, but the ions are necessary for catalytic activity of both proteins [10, 15, 24-26]. PARP1 can catalyze poly(ADP-ribosyl)ation in the presence of Mg^{2+} and Ca^{2+} [24], whereas APE1 does not use Ca^{2+} as a cofactor in hydrolysis of AP sites [25]. Therefore, interactions of PARP1 with AP DNA and influence of APE1 were studied in both the absence and presence of cofactor ions.

Formation of Schiff bases with AP sites is characteristic for base excision repair enzymes, such as bifunctional DNA glycosylases, where this intermediate is involved in the catalysis: cleavage of the sugar-phosphate skeleton of DNA at the AP site through the β-elimination mechanism [2, 12]. But in some cases the formation by proteins of Schiff bases with AP DNA is not accompanied by cleavage of the DNA strand. Thus, MutY, unlike other monofunctional DNA glycosylases, forms such an intermediate with AP DNA but is unable to cleave the DNA [12]. Incubation of PARP1 with AP DNA under conditions used in the subsequent experiments did not result in considerable cleavage of AP DNA (data not presented), which allowed us to use PARP1 cross-linking to AP DNA for studies on the interaction of the protein with AP DNA.

The first series of experiments was performed in the absence of Mg^{2+} to prevent a possible hydrolysis of AP sites by APE1. Figure 3 shows radioautographs of gels upon separation of products of AP DNA binding to PARP1. Note that in the case of AP DNA-DEG a rather intensive product of AP DNA cross-linking is observed, which can be assigned to APE1 by the apparent molecular weight (*7* and *9* in Fig. 3b). Upon prolonged exposure of the gel with the recording screen, similar but less intensive products are observed for AP DNA-THF and AP DNA-DD, but not for AP DNA-A. It seems that distortions in the structure of AP DNA duplexes caused by insertions of analogs of AP site can draw together a lysine of the enzyme to deoxyribose of AP site. Note that the formation of Schiff base is not involved in catalysis of cleavage of AP sites by APE1 [10, 22, 23].

The efficiency of interaction of PARP1 with DNA was quantitatively characterized by the level of PARP1 modification, which was determined as the fraction of DNA bound to PARP1 (Table 1). Under certain conditions the level of protein modification makes it possible to judge its affinity for the DNA duplex. Thus, a positive correlation between the level of modification and protein affinity for DNA intermediates of BER with different structure was demonstrated for DNA polymerase β and APE1 using photoactivated DNAs and the method of gel retardation [27]. Considering this, the lowest affinity for PARP1 should be specific for AP DNA-THF. However, it may be that the yield of PARP1 cross-linking to AP DNAs with different structure can depend on conformation of the PARP1–AP DNA complex. The mutual orientation of acceptor amino acid and deoxyribose of the AP site can be different in complexes with different AP DNAs due to distortions in the structure caused by the analog of the AP site.

The addition of APE1 decreases the level of PARP1 modification for all DNA duplexes. This effect is the most pronounced in the cases of AP DNA-A and AP DNA-DD. In the presence of two proteins in the reaction mixture the binding efficiency of each with DNA depends on the ratio of affinities of these proteins for DNA. In the presence of APE1, the level of PARP1 modification decreases differently for all AP DNAs. APE1 competes more efficiently with PARP1 for binding with AP DNA-A and AP DNA-DD, whereas AP DNA-THF and AP

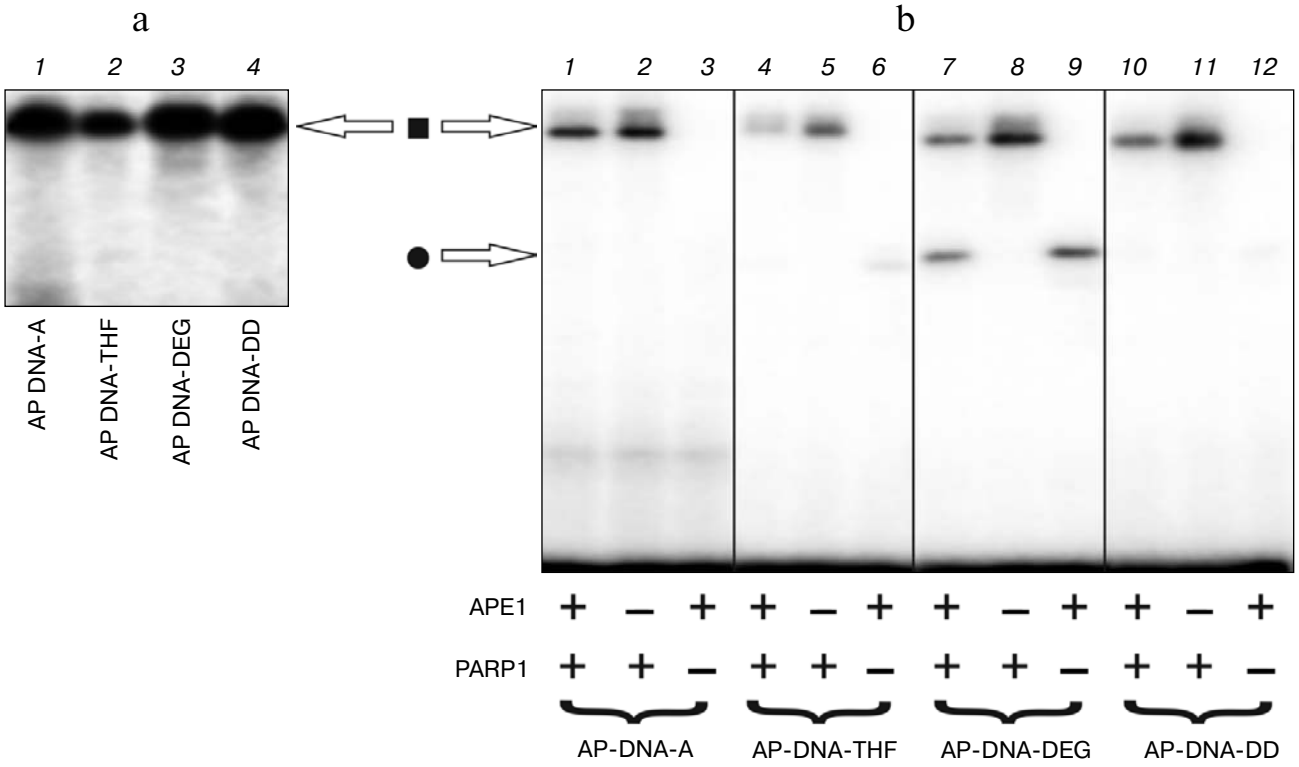

Fig. 3. Modification of PARP1 by AP DNA (a) and the influence of APE1 on modification of PARP1 (b). Reaction mixtures (10 μl) containing standard components, [^{32}P]AP-DNA (0.1 μM), 0.24 μM PARP1 (where indicated), and 25 nM APE1 (where indicated) were incubated at 37°C for 15 min. Then the Schiff bases were reduced with sodium borohydride (20 mM) at 0°C for 30 min. The reaction products were separated by electrophoresis in 10% polyacrylamide gel by the Laemmli method. The arrows with the square and with the dot indicate covalent adducts PARP1–AP DNA and APE1–AP DNA, respectively.

DNA-DEG are less efficiently displaced by APE1 from the complexes with PARP1.

On using DNAs with residues of deoxyribitol (an analog of an AP site) opposite to dAMP or two deoxyribitol residues opposite to each other, it was shown for APE1 that insertion into DNA of another analog of the AP site resulted in a significant decrease in the affinity of APE1 for DNA: the value of K_{dis} increased 70-fold [23]. Overall, this is consistent with the less pronounced influence of APE1 on the levels of PARP1 modification by AP DNA-THF and AP DNA-DEG than by AP DNA-A and AP DNA-DD, based on the suggestion that within the clustered damage a DD residue does not imitate an AP site as well as residues of THF and DEG. Note that there are no data in the literature on the influence of single or multiple AP sites (or their analogs) on the affinity of DNA for PARP1.

Table 1. Influence of APE1 on level of PARP1 modification

AP DNA	Level of PARP1 modification, %					
	in absence of APE1			in presence of APE1		
	without Me^{2+}	Ca^{2+}	Mg^{2+}	without Me^{2+}	Ca^{2+}	Mg^{2+}
AP DNA-A	5.2 ± 0.7	6.0 ± 1.0	6.2 ± 0.8	2.6 ± 0.8	3.3 ± 0.6	4.6 ± 0.3
AP DNA-THF	4.0 ± 0.3	7.6 ± 0.4	6.5 ± 0.4	3.7 ± 0.8	4.0 ± 0.9	4.3 ± 0.5
AP DNA-DEG	4.7 ± 0.6	8.0 ± 1.0	6.1 ± 0.2	3.5 ± 0.6	4.1 ± 0.7	5.0 ± 1.0
AP DNA-DD	5.0 ± 1.0	6.5 ± 0.1	6.0 ± 1.0	2.6 ± 0.8	2.6 ± 0.1	3.6 ± 0.7

Note: Mean values and standard errors are presented, $n = 3$.

Data on the influence of Ca^{2+} on the level of PARP1 modification by AP DNA presented in Table 1 indicate an increase in the efficiency of the interaction of PARP1 with all AP DNAs, but for AP DNAs containing non-nucleotide insertions this effect is more pronounced. There is no literature data on the direct influence of Ca^{2+} on the affinity of PARP1 for DNA, but Ca^{2+} is a cofactor in the reaction of autopoly(ADP-ribosyl)ation [24]. The increase in the PARP1 modification in the presence of Ca^{2+} can be caused either by an increase in the protein affinity for DNA or by conformational changes in the enzyme complex with DNA leading to optimization of positions of deoxyribose of the AP site and of the primary amino acid of the protein. The presence of APE1 decreases the level of PARP1 modification for all AP DNAs, but the decrease is the most pronounced in the case of AP DNA-DD. Overall, in the presence of Ca^{2+} APE1 decreases the level of PARP1 modification a little more effectively. There is also no literature data on the influence of Ca^{2+} on the efficiency of DNA binding with APE1; therefore, it is difficult to take this factor into consideration. However, because Ca^{2+} is not a cofactor on hydrolysis of AP sites by APE1 [25], the decrease in the level of PARP1 modification in the presence of APE1 is not associated with cleavage of AP DNA.

The level of AP DNA cross-linking to PARP1 and the influence of APE1 on this process were also assessed in the presence of Mg^{2+}. Moreover, in this case the efficiency of hydrolysis of AP sites was also determined. Overall, the efficiency of PARP1 modification for all AP DNAs in the presence of Mg^{2+} is higher than in the absence of bivalent metal ions, but the difference between the levels of cross-linking of AP DNAs used is less pronounced. The influence of APE1 is similar: the level of modification of PARP1 in its presence decreases in the reaction with any of DNA duplexes used in this work. The strongest decrease in the level of modification was observed in the case of AP DNA-DD. When the reaction is performed in the presence of Mg^{2+} the conditions are most similar to physiological ones, but in this case the cleavage of AP DNA occurs concurrently with the modification, and this also can influence the level of modification of PARP1.

Table 2. Influence of PARP1 on level of AP DNA cleavage by APE1

AP DNA	Efficiency of AP site cleavage by APE1, %	
	in absence of PARP1	in presence of PARP1
AP DNA-A	94 ± 5	93 ± 5
AP DNA-THF	93 ± 8	62 ± 10
AP DNA-DEG	94 ± 10	58 ± 12
AP DNA-DD	93 ± 7	90 ± 8

Note: Efficiency of AP site cleavage by APE1 is determined as the fraction of radiolabeled product corresponding to cleaved oligonucleotide in the total contents of radiolabeled products in the sample. Mean values and standard errors are presented, $n = 3$.

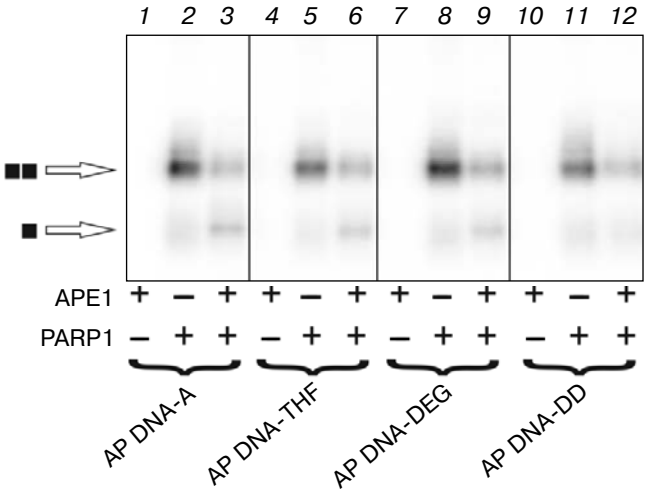

Fig. 4. Detection of protein–protein interactions of PARP1 and APE1 using cross-linking with glutaraldehyde. Reaction mixtures (10 μl) containing ^{32}P-labeled AP DNA (0.1 μM), 0.24 μM PARP1 (where indicated), 25 nM APE1 (where indicated), 5 mM $CaCl_2$, and standard components were incubated at 37°C for 15 min, then glutaraldehyde was added (to 0.1%) and incubation was continued for 10 min at the same temperature. Reduction with $NaBH_4$ and subsequent analysis were performed as described in the caption to Fig. 3. Arrows with one and two squares indicate PARP1–AP DNA and PARP1x2–AP DNA covalent adducts, respectively.

For different DNAs the degree of cleavage of AP sites by APE1 strongly depends on the presence of PARP1 (Table 2). Under the described conditions, no protective effect of PARP1 is observed for AP DNA-A and AP DNA-DD, whereas PARP1 significantly decreases the efficiency of cleavage of AP sites for AP DNA-THF and AP DNA-DEG. This is consistent with the hypothesis that the structure of the DD residue does not imitate an AP site as well as the structures of the two other non-nucleotide insertions, because it is known that the presence of AP sites in complementary DNA strands decreases the rate of AP site hydrolysis by APE1 [23]. The influence of APE1 varies depending on the conditions of the experiment and on the type of AP DNA, but it is reasonable to suppose that APE1 and PARP1 should compete for binding with AP DNA. However, it may be that formation of the PARP1–APE1–AP DNA triple complex can be associated with changes in the conformation of PARP1 leading to a decrease in the number of PARP1–AP DNA cross-links. Competitive interactions of PARP1 and APE1 in the presence of DNA duplexes

with an AP site analog cleaved by APE1 have been demonstrated in some works [27-30]. We have monitored the existence of PARP1–APE1–AP DNA triple complexes using two approaches: cross-linking with glutaraldehyde [31] and coprecipitation with antibodies against APE1 [29].

Data on the cross-linking of the components of the complex by glutaraldehyde are presented in Fig. 4. No product with molecular weight corresponding to the AP DNA–PARP1–APE1 complex was detected in the gel radioautograph. However, a product with higher molecular weight was recorded, the electrophoretic mobility of which corresponded to a protein with apparent molecular weight approximately equal to twice the molecular weight of PARP1. This product is likely to be AP DNA bound to PARP1 dimer with two protein molecules cross-linked by glutaraldehyde. It is known that on binding with damaged DNA PARP1 is dimerized, and then a mutual poly(ADP-ribosyl)ation of these molecules occurs [32, 33].

Addition of APE1 into the reaction mixture lowers the total amount of product of PARP1 cross-linking with AP DNA, and especially of the product assigned to the catalytically competent PARP1 dimer. Such an influence of APE1 on PARP1 labeling indicates that these proteins compete for binding to DNA. The coprecipitation with antibodies against APE1 also did not reveal PARP1–APE1–AP DNA complexes (data not presented).

Using photoactive DNA intermediates of BER, we showed earlier that PARP1 covalently bound to DNA can undergo poly(ADP-ribosyl)ation, which results in a decrease in the amount of product of the PARP1 cross-linking with DNA and in generation of products with the lower electrophoretic mobility corresponding to the covalent adduct poly(ADP-ribosyl)ated PARP1–DNA [34]. The generation of such products allows us to record the automodified form of PARP1 and in the case of cellular extract to identify PARP1-related products among other protein–DNA cross-links. This property of the protein was also confirmed for PARP1 covalently bound with AP DNA-A through Schiff base [9]. A similar property of PARP1 has also been shown for AP DNAs containing analogs of AP sites opposite the natural AP site. Figure 5 exemplifies data for AP DNA-THF, PARP1, and the whole-cell extract of MCF-7.

Automodification of PARP1 is considered to be a mechanism regulating the interaction of this protein with DNA due to decrease in the affinity of poly(ADP-ribosyl)ated PARP1 for damaged DNA. The weaker binding of PARP1 with DNA provides for the availability of a damaged site for enzymes of repair and this, in turn, can increase the efficiency of consequent stages of repair. Such regulation has been considered for stages of BER upon formation of a break in the DNA strand [8]. To elucidate a possible regulatory role of PARP1 in the APE1-catalyzed processing of AP sites (single and within DNA with analogs of AP sites), we compared the influences of

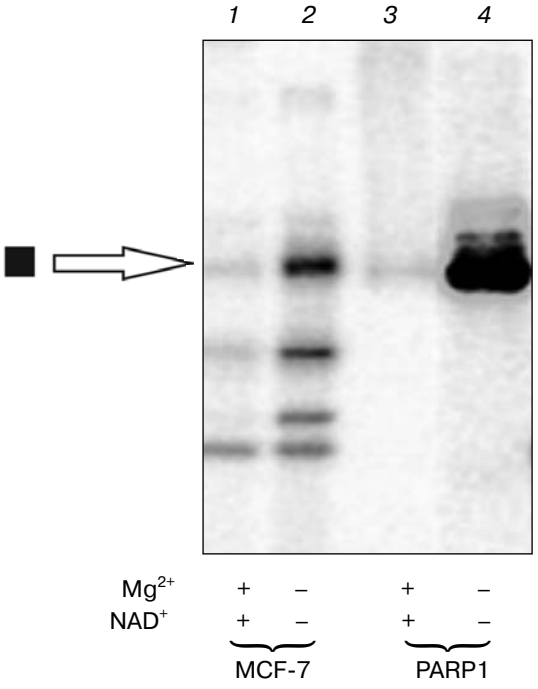

Fig. 5. Poly(ADP-ribosyl)ation of PARP1 covalently bound to AP DNA-THF. Reaction mixtures (10 μl) containing ^{32}P-labeled AP DNA-THF (0.1 μM), proteins of extract from MCF-7 (1.4 mg/ml), or 0.4 μM PARP1, and standard components were incubated at 37°C for 15 min, then 0.5 mM NAD^+ and 10 mM $MgCl_2$ were added and the incubation was continued for 5 min at the same temperature. Reduction by $NaBH_4$ and subsequent analysis were performed as described in the caption to Fig. 3. The arrow with square indicates a covalent PARP1–AP DNA adduct.

PARP1 and its poly(ADP-ribosyl)ated form on the activity of this enzyme. Results of this comparison are presented in Fig. 6. Under conditions of PARP1 automodification, the depth of cleavage of AP sites by APE1 increases. The effect is maximal for AP DNA-DEG (~25%), for AP DNA-THF and AP DNA-DD it is 10 and 3-5%, respectively. Thus, the efficiency of hydrolysis of AP sites within clustered damages can be regulated by functional interactions of APE1 with PARP1 and its poly(ADP-ribosyl)ated form. We showed earlier that PARP1 and its autopoly(ADP-ribosyl)ation are involved in the regulation of the latest stages of BER, and their influence on the long-patch pathway is more pronounced. Automodification of PARP1 weakens its inhibitory effect and seems to be a prerequisite for the Pol β-dependent variant of the long-patch pathway [17, 35, 36].

Interaction of AP DNA with proteins of cell extracts. The finding of interactions of recombinant PARP1 with AP sites within clustered damage has stimulated questions whether such interactions can occur in the presence of other cell proteins, e.g. in cell extracts. Data on AP DNA cross-linking with proteins of extracts from some cultured cell lines are presented in Fig. 7. These experiments were performed in the absence of Mg^{2+} to prevent

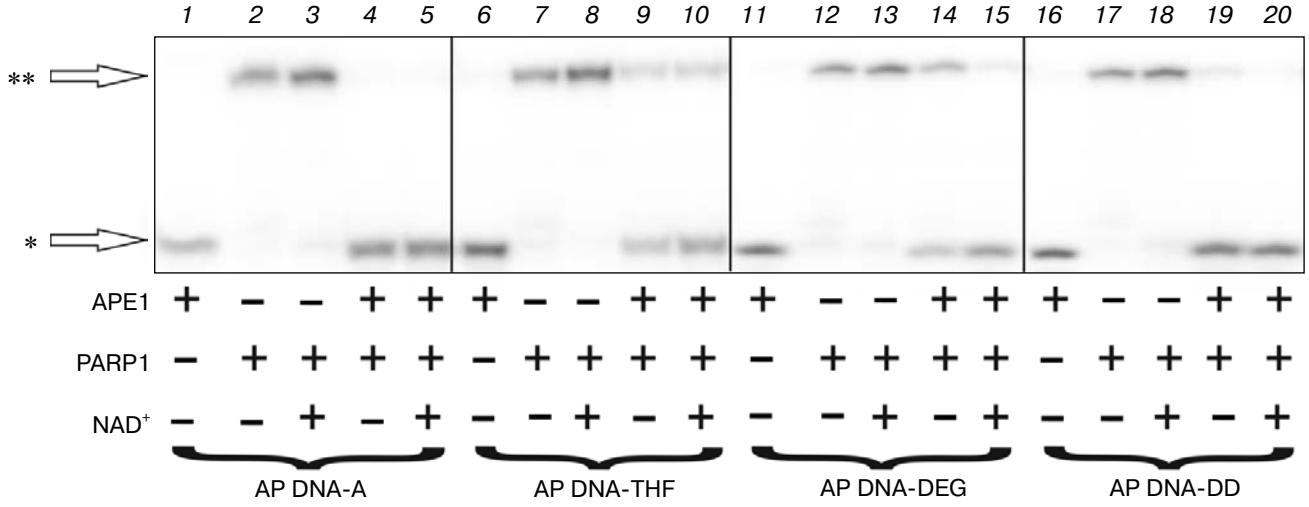

Fig. 6. Effect of PARP1 automodification on cleavage of AP sites within AP DNA. Reaction mixtures (10 μl) containing ^{32}P-labeled AP DNAs (0.1 μM), 25 APE1 (where indicated), 0.24 μM PARP1 (where indicated), 0.2 mM NAD^+ (where indicated), 5 mM $MgCl_2$, and standard components were incubated at 37°C for 15 min. Reduction by $NaBH_4$ and subsequent analysis were performed as described in the caption to Fig. 2. Arrows with one and two asterisks indicate cleaved and uncleaved oligonucleotides, respectively.

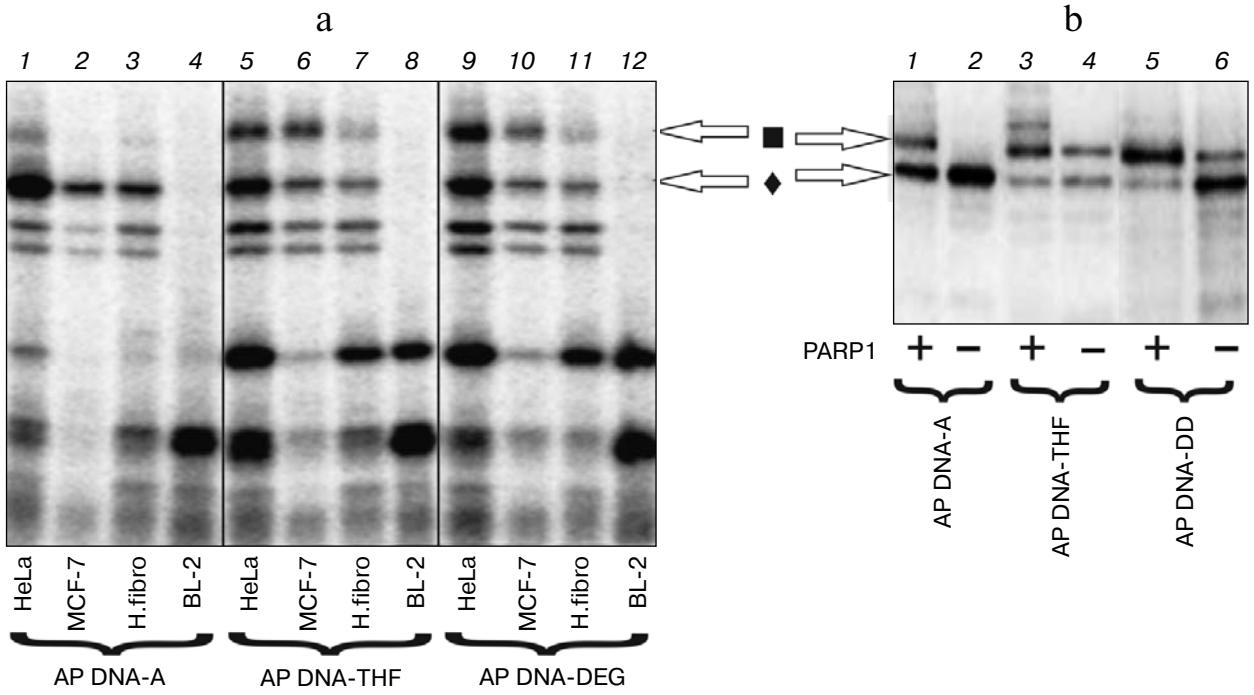

Fig. 7. Modification of extract proteins by DNA duplexes containing an AP site. Reaction mixtures (10 μl) contained ^{32}P-labeled AP DNAs (0.1 μM), extract proteins (1.4 mg/ml for whole-cell extracts and 0.16 mg/ml for nuclear extracts). Other conditions were similar to those described in the caption to Fig. 3. a) Whole-cell extracts, cell type is indicated under the radioautograph. b) Nuclear extract from the K-562 cells. Samples corresponding to *1*, *3*, and *5* additionally contained 0.1 μM recombinant PARP1. Arrows with square and diamond indicate PARP1–AP DNA and Ku80–AP DNA covalent adducts, respectively.

the cleavage of AP sites by endogenous AP nucleases of the extracts. The labeling of the extract proteins unambiguously indicates that PARP1 efficiently interacts with AP DNA in the presence of other proteins of the cells.

It should be noted that the amount of product of AP DNA cross-linking corresponding to PARP1 widely varies depending on the cell type. Addition of Mg^{2+} decreases the level of modification of all proteins (data

not presented). The identification of a designated product of AP DNA cross-linking with proteins is based on the apparent molecular weight of the product determined by its electrophoretic mobility and also on the partial disappearance of this product accompanied by concurrent appearance of products with higher molecular weight during the modification of proteins of AP DNA extracts in the presence of NAD^+ and Mg^{2+} (data not presented). As noted above, this approach can be used for discriminating products of PARP1 cross-linking with AP DNA from products of modification of other proteins. Note that in the extracts the levels of PARP1 modification for AP DNA-DEG and AP DNA-THF are higher than for AP DNA-A (compare *1-4* with *5-8* and *9-12* in Fig. 7a), as differentiated from specimens with recombinant PARP1 (Fig. 3 and Table 1). Such differences can be due to influences of cell proteins, which can either compete with PARP1 or promote its more efficient interaction with DNA. In similar experiments with addition of nuclear extract from K-562 cells, some of the reaction mixtures were supplemented with recombinant PARP1 (*1*, *3*, and *5* in Fig. 7b). The same tendency was observed without addition of PARP1: endogenous PARP1 binds to AP DNAs with analogs of AP sites more efficiently than to AP DNA-A (compare *2* with *4* and 6 in Fig. 7b). Upon addition of PARP1, the amount of the corresponding product increases (compare *1*, *3*, *5* with *2*, *4*, *6* in Fig. 7b) and the intensity of the product with the lower molecular weight decreases. This product was earlier identified by us as Ku80, a subunit of Ku-antigen, which is a multicopy nuclear protein possessing a high affinity for double-stranded termini of DNA [11]. Note that for AP DNA-A Ku80 is the major product of modification in extracts from all cells except BL-2 (*1-3* in Fig. 7a and *2* in Fig. 7b), as shown earlier for some extracts including those used in the present work [11]. The observed type of protein labeling suggests a competition of PARP1 with Ku-antigen for binding with DNA. However, it may be that a protein from the extract unable to form cross-linking with AP DNA influences the binding of PARP1 with AP DNA and promotes its more efficient interaction with AP DNAs containing analogs of AP sites in the complementary strand.

Thus, PARP1 can interact with both single AP sites and AP sites within clustered damages. PARP1 and its automodification seem to be involved in the regulation of hydrolysis of AP sites by APE1 within definite types of clustered damages. This regulation is especially important for minimizing formation of double-strand breaks, which are the most toxic for cells [3]. Double-strand breaks can arise under spontaneous or BER enzyme-catalyzed cleavage of AP sites within clustered damages. These results are consistent with the revealed ability of PARP1 to decrease formation of double-strand breaks in cellular DNA under conditions of intensive oxidative damage of DNA [37] when AP sites are often produced as intermediates. Moreover, PARP1 is also known as a protein involved in determination of cell sensitivity to ionizing radiation [38], whereas clustered damages are a specific type of DNA damage caused by ionizing radiation and some radiomimetics used as antitumor preparations [3].

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