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**THE DETECTION AND CHARACTERIZATION OF RAT PROTEIN
RECOGNIZING DNA DAMAGED BY N-ACETOXY-
ACETYLAMINOFLUORENE**

MONIKA PIETROWSKA¹, JOANNA LANUSZEWSKA¹, ZOFIA WALTER²,
JOANNA RZESZOWSKA-WOLNY¹ and PIOTR WIDLAK^{1*}

¹Department of Experimental and Clinical Radiobiology, Center of Oncology,
44-100 Gliwice, Poland; ²Department of Molecular Genetics, University of
Lodz, 90-237 Lodz, Poland.

Abstract: Proteins recognizing DNA damaged by the chemical carcinogen N-acetoxy-acetylaminofluorene (AAAF) were analyzed in nuclear extracts from rat tissues, using a 36 bp oligonucleotide as the substrate and an electrophoretic mobility shift assay. Two major proteins that form complexes with DNA damaged by AAAF were detected; one of them also bound DNA damaged by *cis*-diammine-dichloroplatinum. The complex specific for AAAF-damaged DNA contained protein loosely attached to nuclear components. It was extracted with 0.1 M NaCl. The amount of this protein was estimated at about 10⁵ copies per liver cell nucleus, and its probable size was about 42 kDa as detected by the Southwestern blotting assay. Its affinity for DNA damaged by AAAF was ~10-fold higher than that for undamaged DNA. Analogous AAAF-DDB (damaged-DNA-binding) proteins were also detected in extracts from rat brain, testis and kidney tissue. The levels of such proteins were not affected in rats treated with the carcinogen 2-acetylaminofluorene.

Key Words: Damage Recognition, Damaged-DNA Binding Proteins, N-acetoxy-acetylaminofluorene

* Corresponding author: phone: (48 32) 278 9672. fax: (48 32) 231 3512.
e-mail: widlak@onkol.instonko.gliwice.pl

INTRODUCTION

All organisms are constantly exposed to genotoxic agents that damage the DNA structure, and have evolved several mechanisms to repair DNA damage. Damage recognition is the first step in any repair pathway. In some repair systems (direct repair and base excision repair) damage is recognized by proteins with enzymatic activity (e.g. DNA-photolyases or DNA-glycosylases). In other systems damage is recognized by specific proteins that recruit other components of the repair machinery. XPA/RPA, XPC/HR23B and UV-DDB (XPE) are among such proteins in eukaryotic cells, involved in nucleotide excision repair [1, 2]. Many other proteins that preferentially bind damaged DNA have been detected in different organisms, but their relevance to DNA repair has not been established. Among these damaged-DNA-binding (DDB) proteins are abundant chromatin proteins HMG-1/2 (and other HMG-box containing proteins) and histone H1, which show preferential binding to DNA damaged by *cis*-diamminedichloroplatinum (*cis*-DDP) [3]. It has been proposed that such proteins may compete with *bona fide* DNA repair proteins, making repair less efficient and facilitating apoptosis [4].

The aromatic amine 2-aminofluorene (2-AF) and its acetylated derivative 2-acetylaminofluorene (2-AAF) are known hepatocarcinogens. After their metabolic activation, 2-AF derivatives form a mixture of non-acetylated and acetylated adducts, mostly at guanine residues. The formation of covalent DNA adducts seems to be an essential step in carcinogenesis as induced by these compounds [5]. DNA adducts induced by 2-AAF and its derivatives are repaired by nucleotide excision repair. However, some aspects of their repair differ from that of other bulky DNA adducts, e.g. those induced by benzo(a)pyrene or UV-radiation [6]. Proteins which specifically recognize DNA damage induced by 2-AAF derivatives are more poorly characterized than proteins recognizing UV-irradiated or *cis*-DDP-damaged DNA. Two such proteins were partially purified from human cells; one of them also binds to DNA damaged by methylating agents [7], while the other also binds to UV-irradiated DNA [8]. However, neither were purified to homogeneity and identified, nor was their relevance to DNA repair established.

Using an electrophoretic mobility shift assay two different proteins which form complexes with DNA damaged by N-acetoxy-acetylaminofluorene (AAAF) have been previously detected, present in protein extracts from rat tissues [9]. One of the detected complexes contains a protein of about 24 kDa and shows increased affinity for DNA damaged by *cis*-DDP. Here we characterize the other protein that specifically binds DNA damaged by AAAF.

MATERIALS AND METHODS

DNA substrates

A synthetic double-stranded 36bp-long oligonucleotide (5'-AATTCGTAGG CCTAAGAGCA ATCGCACCTG TGCGCG-3', with blunt ends) was used as a molecular DNA probe. The oligonucleotide was damaged by AAAF, BPDE, cis-DDP or UV-C radiation as described elsewhere [9, 10]. Such treatments introduced single lesions into about 15% of the DNA molecules (on average) [9]. The oligonucleotide was end-labelled by transfer of ^{32}P from $[\gamma^{32}\text{P}]\text{ATP}$ using T4 polynucleotide kinase and purified by polyacrylamide gel electrophoresis [11].

Preparation of nuclear extracts

Nuclei were purified from homogenized tissues of adult male WAG rats. To obtain extracts of nuclear proteins, the nuclei were incubated for 30 minutes at 4°C with a buffer consisting of 10 mM Hepes-NaOH pH 7.9, 1.5 mM MgCl_2 , 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, a protease inhibitor mixture (Complete™, Boehringer), and NaCl at different molarities ranging from 0.1 to 0.8 M. The nuclei extracted with 0.6 and 0.8 M salt were briefly (2-3 sec.) sonicated. Insoluble remnants of the nuclei were pelleted by centrifugation for 30 minutes at 16,000 rpm at 4°C. The protein concentration was assayed with the Protein Assay Kit (BioRad). Proteins were resolved on 13% polyacrylamide/SDS gels and stained with Coomassie Brilliant Blue [11].

Electrophoretic mobility shift assay (EMSA)

A radioactive oligonucleotide (20 ng) was incubated with proteins of different extracts (5µg) for 30 minutes at 4°C. The binding buffer consisted of 20 mM Tris-HCl pH 7.6, 5 mM MgCl_2 , 0.5 mM EDTA, 1 mM DTT, 5% glycerol and 150 mM NaCl. Complexes were formed in a final volume of 20 µl in the presence of a 100-fold excess of non-radioactive alternative copolymer poly dI-dC or sonicated *E. coli* DNA used as non-homologous competitors. Protein-oligonucleotide complexes were resolved by electrophoresis on a 6% polyacrylamide gel in a 0.5 x TBE running buffer (45 mM Tris-borate, 1 mM EDTA). The gels were dried and autoradiographed. Gel fragments containing radioactive complexes were excised and quantitated by scintillation counting.

Southwestern blot analysis

50 µg of nuclear proteins were fractionated on 13 % polyacrylamide/SDS gels [11] and electrotransferred onto PVDF membranes (Hybond-P; Amersham) in 25 mM Tris, 190 mM glycine and 20% methanol. Filter-bound proteins were renatured by incubation in a hybridisation oven for 5 hours at 25°C with 25 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 mM MgCl_2 and

2.5% BSA. After washing with a binding buffer (the same composition as above but with 0.25% BSA), the filters were incubated for 5 hours at 25°C in the binding buffer supplemented with 250 ng of ^{32}P -end-labelled oligonucleotide and an 80-fold excess of non-radioactive competitors in a final volume of 8 ml. The filters were then washed with the binding buffer and autoradiographed.

Assay of DNA adducts

Rats were injected intraperitoneally with 2-AAF (50 mg/kg of body weight, dissolved in DMSO) and tissue samples were collected at different times after the injection. The DNA isolated from the tissues, as well as the oligonucleotide used for EMSA, were assayed for the presence of adducts by the ^{32}P -postlabelling method, as described elsewhere [9].

RESULTS AND DISCUSSION

Nuclear extracts from rat liver cells were analyzed for the presence of proteins binding to an AAAF-damaged DNA (AAAF-DDB proteins) using an electrophoretic mobility-shift assay. Figure 1A shows the results of an experiment in which nuclear proteins extracted with increasing concentrations of NaCl (from 0.1 to 0.8 M) were incubated with a radioactive oligonucleotide, either damaged by AAAF or undamaged, in the presence of a non-homologous competitor. When an excess of poly dI-dC was used to compete non-specific interactions, two complexes which contained AAAF-damaged DNA were detected. The complex that shows lower mobility (it co-migrates with 180 bp-long double stranded DNA, marked with an asterisk on Fig. 1A) contains proteins which were loosely attached to nuclear components and were extracted with a low salt buffer (0.1 M NaCl). The other complex has higher mobility (similar to 100 bp DNA, marked with a square on Fig. 1A) and contains proteins extracted from nuclei with 0.4 M and higher concentrations of NaCl. The proportion of both AAAF-DDB proteins to total extracted proteins markedly decreased in fractions extracted with higher salt concentrations. Different amounts of protein relative to the total nuclear content were extracted with increasing salt concentrations (about 10% and 80% in 0.1 and 0.8 M NaCl extracts, respectively). Thus, the observed decrease in the level of DDB proteins in the fractions extracted with higher salt concentrations was due to their "dilution" with other nuclear proteins (e.g. histones; Fig. 1B). When an excess of more heterogeneous DNA from *E. coli* was used as a non-radioactive competitor, the AAAF-DDB complex of lower mobility could not be detected. The formation of this complex was also inhibited when the reaction mixtures contained 0.2 M and higher NaCl, or 10 mM EDTA (not shown).

To examine whether 0.2 M NaCl nuclear extracts contain proteins which recognize other DNA lesions, the oligonucleotide damaged by different agents

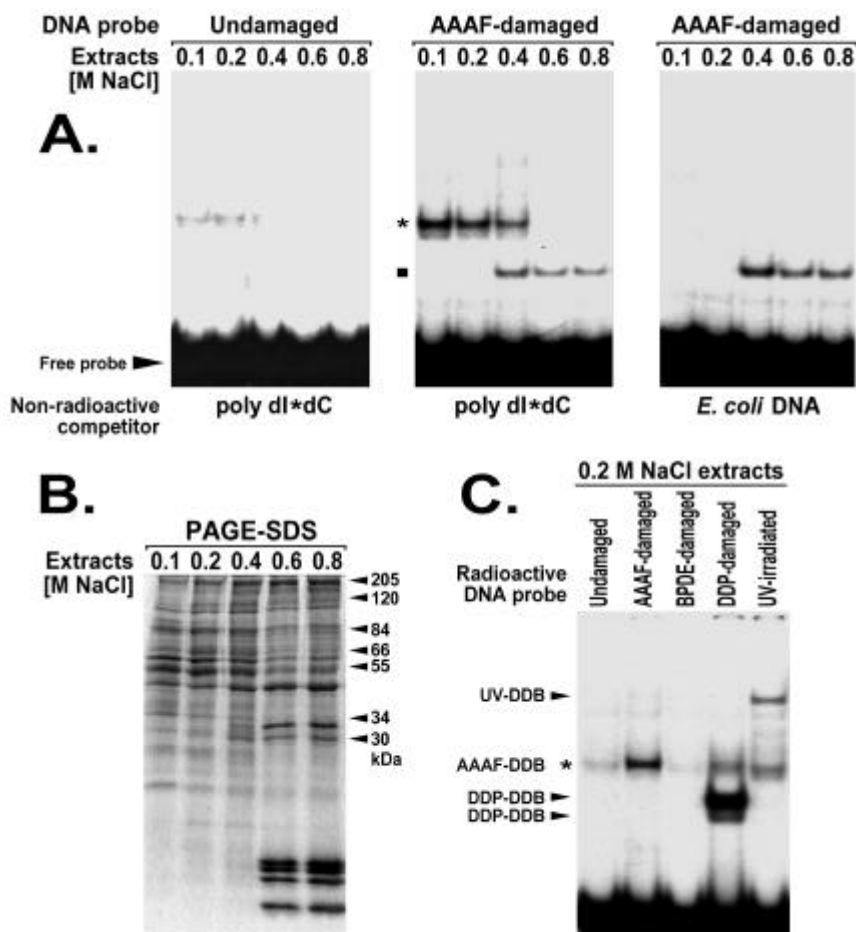


Fig. 1. Detection of rat liver proteins that recognize damaged DNA.

Panel A. Complexes were formed between labelled oligonucleotide (either AAAF-damaged or undamaged) and proteins extracted from rat liver nuclei with the indicated NaCl concentrations, in the presence of excess non-radioactive competitors (poly dI-dC co-polymer or *E. coli* DNA). The positions of the free oligonucleotide and of complexes containing AAAF-DDB proteins are denoted.

Panel B. SDS-PAGE analysis of proteins in the different nuclear extracts.

Panel C. Complexes were formed between labelled oligonucleotide (either undamaged or damaged with different agents) and 0.2 M NaCl nuclear extracts, in the presence of a poly dI-dC co-polymer as a non-radioactive competitor. Major complexes specific for damaged DNA are denoted.

was used as the substrate (Fig. 1C). DNA damaged by *cis*-DDP formed two major specific complexes (DDP-DDB), while UV-irradiated DNA formed a different complex (UV-DDB). However, a minority of DNA damaged by either *cis*-DDP or UV radiation forms complexes which migrate with mobility similar to that of the AAAF-DDB complex (analogous complexes with undamaged or BPDE-damaged DNA could be also detected after longer autoradiography). All such complexes are several-fold less abundant than the AAAF-DDB complex. To estimate the affinity of the AAAF-DDB protein for AAAF-damaged oligonucleotide, complexes were formed in the presence of increasing amounts of homologous competitor, either undamaged or AAAF-damaged (not shown). The affinity for AAAF-damaged oligonucleotide was 8-fold greater than that for undamaged DNA. To analyze whether the formation of such complexes was not specific for this oligonucleotide sequence, another 36bp-long duplex oligonucleotide (5'- ATCAGCGTGA TCTGAACGAT AACGCTCCAC GTGCGG -3') was used as a non-radioactive competitor. The affinity of this alternative oligonucleotide for the AAAF-DDB protein was similar to that of the originally studied oligonucleotide (not shown).

Another *in vitro* method that can be used as a supplementary analytical tool to study DDB-proteins is Southwestern blotting, in which a DNA probe binds to membrane-bound proteins after their electrophoretic resolution [12]. Figure 2A shows the results of an experiment in which electrophoretically resolved proteins from nuclear extracts (either 0.2 or 0.4 M NaCl) were probed with labelled oligo- nucleotide either undamaged or damaged by AAAF. When undamaged oligonucleotide was tested, the only complexes detected co-migrated with histone H1. When oligonucleotide damaged by AAAF was used as a probe, many faint bands ranging in size from about 80 to a few kDa were visible. To search for proteins which form the AAAF-DDB complex detected by EMSA, the pattern of protein bands was compared where *E. coli* DNA or poly dI-dC were used as competitors. The most promising protein for that role was one with an approximate size of 42 kDa (asterisk in Fig. 2A), which was detected almost exclusively in 0.2 M salt extracts where poly dI-dC was used as a competitor.

Some factors which damage DNA increase the level of damage recognition proteins, e.g. UV-DDB protein [13]. Here the aim was to examine whether treatment of animals with 2-acetylaminofluorene (2-AAF) affected the level of AAAF-DDB proteins. Rats were injected with the carcinogen and the levels of DNA adducts and of DDB complexes were compared in the liver at different times after the treatment (Fig. 2B). The level of AAAF-DDB proteins expressed in arbitrary units (defined as the amount in picograms of oligonucleotide bound by 1 µg of total protein in the extract in the conditions of the experiment) was not affected at any time after treatment with 2-AAF. Treatment of rats with another chemical carcinogen, benzo(a)pyrene, also did not affect the levels of AAAF-DDB proteins at 24 and 48 hrs after treatment (not shown). In contrast,

treatment with 2-AAF increased the level of the UV-DDB protein with a maximal effect observed 48 hrs after treatment (not shown).

The proteins detected in rat liver which recognize DNA damage and form AAAF-DDB complexes are abundant nuclear proteins. Assuming that each protein molecule binds a single molecule of damaged oligonucleotide and that they are completely extracted with 0.2 M NaCl, and based on the amount of DNA bound in the presence of a very low amount of a competitor (50 ng of poly dI-dC), it was calculated that a single liver nucleus contains at least 5×10^4 copies of this protein. To examine whether the AAAF-DDB proteins detected in liver tissue are present in other types of cells, their presence was studied in nuclear extracts from different rat tissues (Fig. 2C). AAAF-DDB proteins were found in different tissues, but at different levels; 580, 510, 180 and 320 units were found in brain, testis, kidney and liver tissue, respectively. It is difficult to evaluate whether these differences reflected differences in the amount or in the activity of this AAAF-DDB protein; furthermore different proteins could form complexes having similar electrophoretic mobility.

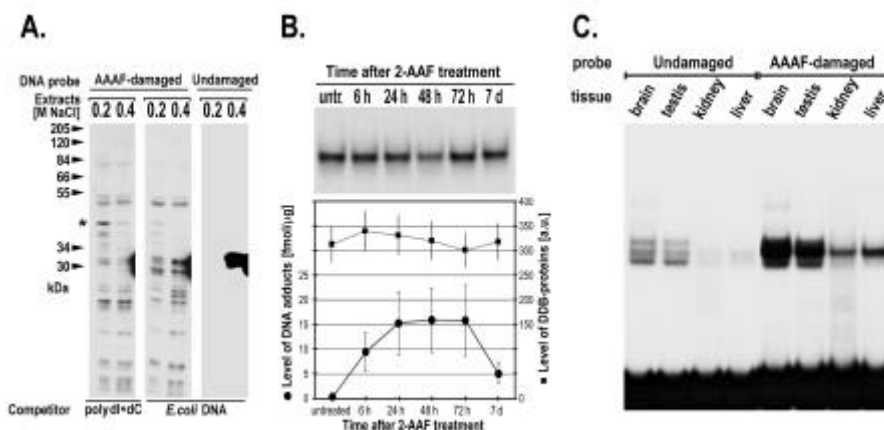


Fig. 2. Characterization of the AAAF-DDB complex.

Panel A. A Southwestern blot analysis of DNA damage recognition proteins. Electrophoretically separated proteins were probed with radioactive undamaged or AAAF-damaged oligonucleotide in the presence of poly dI-dC or *E. coli* DNA.

Panel B. An analysis of the level of AAAF-DDB complexes in animals treated with 2-AAF. The levels of hepatic DNA adducts and AAAF-DDB complexes are compared in 2-AAF-treated rats at different time periods after the drug injection. The values on the graph are means for 3 animals (\pm S.D.).

Panel C. Detection of AAAF-DDB complexes in different rat tissues. Complexes were formed between labelled oligonucleotide, either undamaged or AAAF-damaged, and nuclear proteins extracted with 0.2 M NaCl in the presence of poly dI-dC.

AAAF-DDB protein detected in rat tissues is an abundant protein loosely associated with nuclear components. The affinity of this protein for DNA damaged by AAAF was an order of magnitude higher than for undamaged DNA. The formation of such AAAF-DDB complexes when the competitor was an excess of poly dI-dC but not when it was the more heterogeneous *E. coli* DNA revealed possible specificity for a structure which is not present in the dI-dC co-polymer. Enhanced affinity for AAAF-damaged DNA suggested that the DNA adduct induced by this compound may promote the formation of such hypothetical structures. The major AAAF-induced form of DNA damage, dG-C8-AAF, induces local distortions in the DNA double helix leading to its unpairing, unwinding and the formation of a hinge point [14, 15]. DNA lesions induced by BPDE, *cis*-DDP or UV, which were not preferentially recognized by the AAAF-DDB protein, promote local DNA unwinding but induce rather direct bending than the formation of a hinge point [3, 15]. However, at this point we may not conclude which type of DNA helix distortion affects the binding of the AAAF-DDB protein.

DNA lesions induced by AAAF and BPDE are removed by nucleotide excision repair, yet the latter are substrates for preferential repair and may be repaired more rapidly in rat liver cells [6, 9]. Here, it can be seen that the binding of the AAAF-DDB protein to damage induced by BPDE was an order of magnitude weaker than that to AAAF-damaged DNA. The level of this protein was not affected in rats treated with the carcinogen 2-AAF. These data suggest that proteins present in the AAAF-DDB complex are DNA-binding proteins that are not involved in repair processes. We propose that these proteins may instead compete with repair proteins and shield DNA damage making its repair slower and less efficient.

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