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Ku80 interaction with apurinic/apyrimidinic sites depends on the structure of DNA ends

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Aim. *The identification of a protein from human cell extract which specifically interacts with the apurinic/apyrimidinic (AP) site in the partial DNA duplex containing 5′- and 3′-dangling ends (DDE-AP DNA) and mimicking clustered DNA damage.* ***Methods***. *The Schiff base-dependent cross-linking of a protein to AP DNA (borohydride trapping), MALDI-TOF-MS, chromatography, and gel electrophoresis.* ***Results***. *A human cell extract protein which forms a major covalent adduct with the AP DNA duplex with dangling ends was identified as the Ku80 subunit of Ku antigen by peptide mass mapping based on MALDI-TOF-MS data. The Ku antigen purified from the HeLa cell extract was shown to form the covalent adducts with the same mobility as observed in cell extracts.* ***Conclusions***. *The Ku80 subunit of Ku antigen can specifically interact with AP DNA forming the Schiff base-mediated adducts which electrophoretic mobility depends on the structure of DNA ends. The difference in electrophoretic mobility can be caused by the cross-linking of AP DNA to distinct target amino acids that appears to reflect unequal positioning of AP DNAs in the complex with Ku antigen.*

Keywords: Ku antigen, apurinic/apyrimidinic site, protein-DNA cross-linking, clustered DNA damages.

Introduction. The abasic (AP) sites are among the most frequent DNA damages. Up to 50 000 AP sites arise in a mammalian cell per day [1] as a result of spontaneous hydrolysis of N-glycosidic bond or under the action of DNA glycosylases during the base excision repair [2]. If unrepaired, the AP sites are mutagenic and cytotoxic. The repair of AP sites in clustered DNA damages presented by combinations of the AP sites, oxidized bases and single strand breaks within 1–2 turns of DNA helix is of particular interest [3]. Such lesions arise in DNA under the action of ionizing radiation or radiomimetic drugs.

The cross-linking of proteins to a baseless deoxyribose in DNA via reduction of a Schiff-base intermediate is often called «borohydride trapping» (BHT). BHT in combination with mass spectrometry has been used in a proteomic approach to identify the human proteins reactive to AP sites [4–6]. The set of DNA duplexes containing the AP site in the middle of 32-mer oligonucleotide and different structural features has been used. With all AP DNA, except DNA with both 5'- and 3'-dangling ends of 8 nt (DDE-AP DNA), the predominant products of cross-linking were of the same electrophoretic mobility (an apparent molecular mass of 90 kDa). The protein cross-linked to the AP DNA duplex with blunt ends (BE-AP DNA) was identified as the Ku80 subunit of Ku antigen [4]. Ku antigen consisting of two subunits with molecular masses about 70 kDa (Ku70) and 83 kDa (Ku80) is a eukaryotic DNA-binding component of DNA-dependent protein kinase. The main function of Ku antigen is participation in the double-strand break repair by non-homologous end joining [7]. In the present work we identified the protein which specifically interacts with DDE-AP DNA forming the product with an apparent molecular mass of 100 kDa.

Materials and methods. *Chromatographic fractionation of cell extract proteins*. HEK 293 cell extract prepared as described elsewhere [8] was diluted 4 times with the buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 0.025 % NP-40 and was applied to a heparin-sepharose column. After washing with the same buffer, the proteins were eluted with linear gradient of NaCl (0.05–1 M) in the same buffer. The presence of a target protein in the fractions was determined by BHT. The fractions were concentrated and supplemented with glycerol (final concentration of 10 %).

Borohydride trapping and MALDI-TOF-MS analysis. All procedures were carried out as described previously [4] with a few exceptions. The concentration of AP DNA in the reaction mixtures was 0.2 M. The preparative cross-linking for MALDI-TOF-MS analysis was performed using a fractionated cell extract enriched in the target protein by chromatography on heparin-sepharose. The peptides derived from the cross-linked protein were additionally purified on Zip-tip C-18 prior to the MS analysis.

Purification of Ku antigen. Ku antigen was purified from the HeLa cell extract prepared as described in [8] by ammonium sulfate fractionation (45–65 % of saturation) followed by the successive chromatographies on DEAE-support («BioRad», USA), Q-sepharose («GE Healthcare») and DS-DNA-cellulose («ICN», USA).

Results and discussion. The BHT experiments using the DDE- and BE-AP DNAs and several human cell extracts, including the HeLa and HEK 293 cells, reveal nearly identical patterns of the cross-linked proteins. Data for HeLa cell extract are shown in Fig. 1.

For identification of the target protein we used the fraction of HEK 293 cell extract proteins eluted from heparin-sepharose at 450–500 mM NaCl and enriched in the target protein. Use of the enriched extract ensures the yield of target protein cross-linking with AP DNA and reduces the likelihood of sample contamination with the proteins nonspecifically bound to DNA. After the electrophoretic separation of the protein covalent adducts with biotinylated DNA bound to streptavidin-coated beads, the gel was stained by Coomassie Brilliant Blue R-250 (Fig. 2, *A*) and autoradiography was carried out (Fig. 2, *B*). Five well-defined protein bands with apparent molecular masses from 100 to 70 kDa (Fig. 2, *C*) were excised from the gel and subjected to in-gel trypsin digestion.

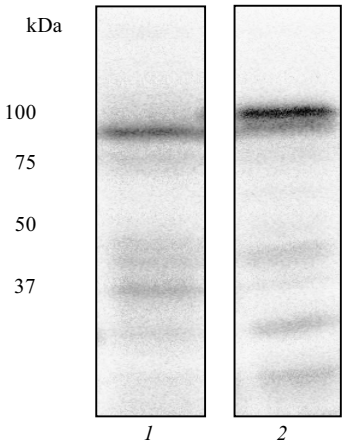

Fig. 1. Borohydride trapping of HeLa cell extract proteins with ^{32}P-labeled AP DNA: *1* – BE-AP DNA; *2* – DDE-AP DNA

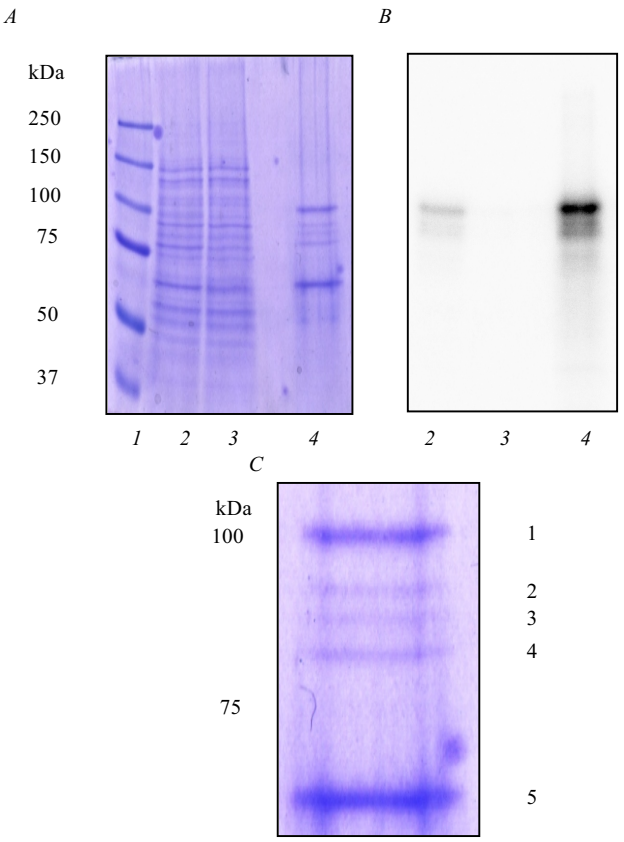

Fig. 2. Purification of cell extract protein(s) cross-linked with AP DNA: *A* – Coomassie Brilliant Blue R-250 stained gel (*1* – molecular weight markers; *2* – an aliquot (25 l) of reaction mixture before incubation with streptavidin-coated beads; *3* – an aliquot (25 l) after incubation (unbound proteins); *4* – covalent adducts and proteins bound to beads); *B* – autoradiograph of the gel from Fig. 2, *A*; *C* – zoomed fragment of Fig. 2, *A*, lane *4*

The bands N 1–4 have the matching radioactive bands, thus corresponding to the protein-DNA covalent adducts.

Table 1
Parameters of the first rank candidates for the samples N 1–5

Sample number	First rank candidate	Molecular mass, Da	MOWSE score	Mass values matched	Protein sequence coverage, %
1	Ku80	82652	176	19	27
2	Ku80	82652	149	33	38
3	Ku80	82652	67	13	17
4	N-terminal proteolytic fragment of Ku80	64064	85	19	22
5	Ku70	69799	71	16	35

Table 2
Observed masses in MALDI-TOF mass spectrum of the sample N 1 which correspond to theoretical peptides of Ku80

Position	$(M + H)_{obs}^{+}$	$(M+H)_{clc}^{+}$	Ku80 peptides
37–44	993.5544	992.5477	K.VITMFVQR.Q
37–44	1009.5442	1008.5426	K.VITMFVQR.Q + Oxidation (M)
82–97	1914.9439	1913.9444	R.HLMLPDFDLLEDIESK.I
82–97	1930.9707	1929.9394	R.HLMLPDFDLLEDIESK.I + Oxidation (M)
131–141	1317.6786	1316.6725	R.HIEIFTDLSSR.F
145–155	1266.7332	1265.7343	K.SQLDIIIHSLK.K
185–195	1109.6030	1108.6029	R.LGGHGPSFPLK.G
243–250	1035.4896	1034.4868	R.HSIHWPCR.L
251–260	1073.6282	1072.6240	R.LTIGSNLSIR.I
266–271	745.4070	744.4130	K.SILQER.V
308–315	977.5023	976.4978	K.EDIIQGFR.Y
355–363	1083.5626	1082.5583	R.FFMGNQVLK.V
355–363	1099.5442	1098.5532	R.FFMGNQVLK.V + Oxidation (M)
401–413	1377.7583	1376.7565	R.ANPQVGVAFPHIK.H
444–465	2321.1723	2320.1620	K.YAPTEAQLNAVDALIDSMSLAK.K
470–481	1380.7484	1379.6820	K.TDTLEDLFPTTK.I
546–565	2243.0201	2242.0138	K.DQVTAQEIFQDNHEDGPTAK.K
569–599	3121.5137	3120.4749	K.TEQGGAHFSVSSLAEGSVTSVGSVNPAENFR.V
649–654	795.3550	794.3559	K.FSEEQR.F

Furthermore, the 100 kDa-band has the highest intensity with the both methods of visualization. Peptides were analyzed by MALDI-TOF-MS and data were searched against a database.

The first rank candidates for all the samples with their scores, the number of the matched peptide mass values and the protein sequence coverage are shown in Table 1. Obviously, Ku80 forms several covalent adducts with DDE-AP DNA. Ku70 is retained on the beads due to the strong interaction with Ku80 [4, 9].

The data for the matching peptides in the sample N 1 are shown in Table 2. Interestingly, the set of Ku80 peptides identified in this work is almost overlapped with those obtained in the other works [4, 10]. This is

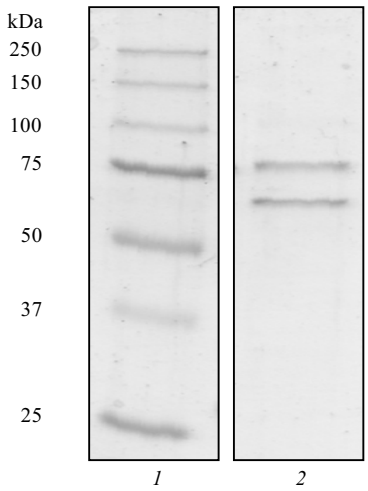

Fig. 3. Purified Ku antigen (Coomassie Brilliant Blue R-250 stained gel): *1* – molecular weight markers; *2* – purified Ku antigen (0.6 g)

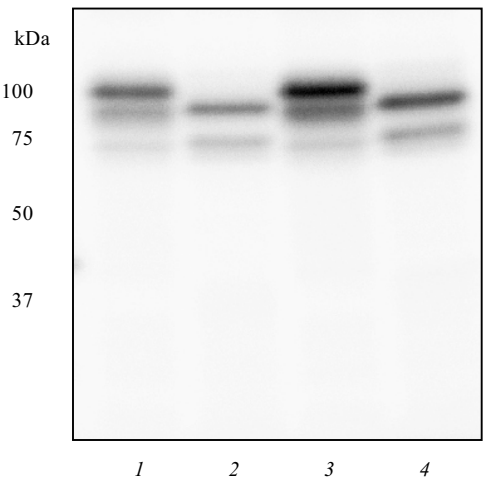

Fig. 4. Borohydride trapping of purified Ku antigen (*1*, *2* – 0.6 g and *3*, *4* – 1.2 g of Ku antigen): *1*, *3* – DDE-AP DNA; *2*, *4* – BE-AP DNA

probably due to an easy ionization of these peptides under the analysis conditions.

The appearance of the Ku80 adduct with a lowered electrophoretic mobility characteristic for DDE-AP DNA can reflect the existence of two different modes of Ku80 binding with DNA (and, consequently, different points of interaction with the AP site) or the highly efficient cross-linking of DNA with another Ku80 isoform. Indeed, the lists of candidates for the samples N 1 and 2 include a Ku80 isoform with higher molecular mass (93464 Da). The biochemical properties of this isoform resemble those of the conventional Ku80, but it cannot fully replace Ku80 [9]. To discriminate these alternatives, we purified the Ku antigen from HeLa cells (see «Materials and methods») to near homogeneity (Fig. 3) and then tested the ability of the purified protein to interact with two aforementioned types of AP DNA (Fig. 4). The patterns of cross-linking of two AP DNAs to the purified Ku antigen and cell extract proteins have the analogous set of products: more abundant 100 kDa and less intensive 90 kDa adducts for DDE-AP DNA (lanes *1*, *3*) and 90 kDa adduct for BE-AP DNA (lanes *2*, *4*).

Conclusions. Thus, the Ku80 subunit of Ku antigen specifically interacts with the AP DNA structure with 5'- and 3'-dangling ends and forms a covalent adduct with an apparent molecular mass of 100 kDa after the treatment with $NaBH_4$. Further investigations are required to determine the role of these modes of the Ku antigen interaction with the AP sites. This type of damaged DNA structure can be used to monitor the binding activity of Ku antigen in the extracts of various human cells.

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Взаємодія Ku80 з апуриновими/апіримідиновими сайтами залежить від структури ДНК

А. А. Косова, С. Н. Ходирева, О. І. Лаврик

Резюме

Мета. *Ідентифікація білка з екстракту клітин людини, який специфічно взаємодіє з апуриновим/апіримідиновим (АР) сайтом у складі часткового дуплексу ДНК, що містить виступаючі 5'- і 3'-кінці та імітує кластерне пошкодження ДНК.* ***Методы***. *Зшивання білка з АР-ДНК, опосередковане утворенням основи Шиффа, MALDI-TOF мас-спектрометрія, хроматографія і гель-електрофорез.* ***Результаты***. *Білок клітинних екстрактів людиниа, який формує мажорний ковалентний адукт з АР-ДНК, що містить виступаючі кінці, ідентифіковано як субодиниця Ки80 Ки-антигену методом пептидного картування, заснованого на даних MALDI-TOF мас-спектрометрії. Показано, що Ки-антиген, виділений з клітин HeLa, формує ковалентні адукти, електрофоретична рухливість яких відповідає рухливості адуктів, утворених білками з клітинних екстрактів.* ***Висновки***. *Субодиниця Ки80 Ки-антигену може специфічно взаємодіяти з АР-ДНК, формуючи адукти, опосередковані утворенням основи Шиффа, електрофоретична рухливість яких залежить від структури кінців ДНК. Розбіжності в електрофоретичній рухливості можуть обумовлюватися зшиванням АР-ДНК з різними амінокислотними залишками білка, що в свою чергу може відображати різне розташування АР-ДНК у комплексі з Ки-антигеном.*

Ключові слова: Ku-антиген, апуриновий/апірімідиновий сайт, зшивання білок–ДНК, кластерні пошкодження ДНК.

Взаимодействие Ku80 с апуриновыми/апиримидиновыми сайтами зависит от структуры концов ДНК

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Резюме

***Цель**. Идентификация белка клеточных экстрактов человека, специфично взаимодействующего с апуриновым/апиримидиновым (АР) сайтом в составе частичного ДНК-дуплекса, содержащего выступающие 5′- и 3′-концы и имитирующего кластерное повреждение ДНК. **Методы**. Сшивка белка с АР-ДНК, опосредованная образованием основания Шиффа, MALDI-TOF масс-спектрометрия, хроматография и гель-электрофорез. **Результаты**. Белок клеточных экстрактов человека, формирующий мажорный ковалентный аддукт с АР-ДНК, содержащей выступающие концы, идентифицирован как субъединица Ku80 Ku-антигена методом пептидного картирования, основанного на данных MALDI-TOF масс-спектрометрии. Показано, что Ku-антиген, выделенный из клеток HeLa, формирует ковалентные аддукты, электрофоретическая подвижность которых соответствует подвижности аддуктов, формируемых белками клеточных экстрактов. **Выводы**. Субъединица Ku80 Ku-антигена может специфично взаимодействовать с АР-ДНК, формируя аддукты, опосредованные образованием основания Шиффа, электрофоретическая подвижность которых зависит от структуры концов ДНК. Различие в электрофоретической подвижности может быть обусловлено сшивкой АР-ДНК с разными аминокислотными остатками белка, что может отражать различное расположение АР-ДНК в комплексе с Ku-антигеном.*

Ключевые слова: Ku-антиген, апуриновый/апиримидиновый сайт, сшивка белок–ДНК, кластерные повреждения ДНК.

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