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TIGHTLY-BOUND TO DNA PROTEINS IN RAT EXPERIMENTAL HEPATOMAS AND NORMAL LIVER CELLS

D. Labeikytė[1,], V. Borutinskaitė[2], N. Legzdiņš[3], N. Sjakste[3,4]*
[1]*Department of Biochemistry and Biophysics, Vilnius University, Vilnius LT-03101, Lithuania*
[2]*Department of Developmental Biology, Institute of Biochemistry, Vilnius university, Vilnius LT-08662, Lithuania*
[3]*Latvian Institute of Organic Synthesis, Riga LV1006, Latvia*
[4]*Faculty of Medicine, University of Latvia, Riga LV1001, Latvia*

Proteins tightly bound to DNA (TBP) comprise a group of proteins that remain bound to DNA even after harsh deproteinization procedures. The amount of these proteins is 20–100 µg for mg of DNA depending on eukaryotic source. This experimental paper examines the possibility to use some TBP for clinical biomarker discovery, e.g. for identification of prognostic and diagnostic cancer markers. *The main aim* of this study was to designate differences between tightly DNA binding protein patterns extracted from rat liver and rat experimental hepatomas (Zajdela ascites hepatoma and hepatoma G-27) and to evaluate possibility that some of these proteins may be used as biomarkers for cell cancer transformation. *Methods:* We used proteomics aproach as a tool for comparison of pattern of TBP from rat experimental hepatomas and normal liver cells. Combination of 2DE fractionation with mass spectrometry (MALDI TOF-MS) suitable for parallel profiling of complex TBP mixtures. *Results:* Intriguingly 2DE protein maps of TBP from rat liver and rat experimental hepatomas (Zajdela acites hepatoma and hepatoma G-27) were quite different. We identified 9 proteins, some of them shared in all TBP patterns. Among identified tightly bound to DNA proteins there were three proteins considered as nuclear matrix proteins (lamin B1, scaffold attachment factor B1, heterogeneous nuclear ribonucleoprotein). Also we identified DNA repair protein RAD50, coiled-coil domain-containing protein 41, structural maintenance of chromosomes protein1A and some ATP –dependent RNA helicases indicating that TBP are of interest with respect to their potential involvement in the topological organization and/or function of genomic DNA. *Conclusions:* We suppose that proteomic approach for TBP identification may be promising in development of biomarkers, also obtained results may be valuable for further understanding TBP functions in genome.

***Key Words*: tightly to DNA bound proteins, nuclear matrix proteins, biological markers, proteomic analysis.**

INTRODUCTION

Search for novel tumour markers is an important research branch in modern oncology. Several tumour markers have been found among the nuclear matrix proteins [1]. The nuclear matrix, operationally defined as nuclear structure resistant to high salt and detergent extraction, contains proteins that contribute to the preservation of nuclear shape and its organization. Nuclear matrix enables spatial organization of DNA replication, transcription and repair processes; it harbours numerous enzymes and transcription factors [2–4]. One of the bladder cancer-specific nuclear matrix proteins (NMP-22) was proposed for use in diagnostics commercial kit for its detection in urine is available from Matritech, Cambridge, Massachusetts. Utility of the marker and restrictions for its application are discussed in more than hundred publications [5]. Development of other nuclear-matrix-derived tumour markers is on line [6]. Nuclear matrix proteins are tightly bound to DNA as DNA-protein bonds in this structure are resistant to high salt and mild detergent treatments. Some researchers doubt in existence of nuclear matrix in living cells. Isolation procedure is prone to many artefacts and isolated nuclear matrix structures are supposed to form due to "molecular crowding" [7]. Another group of proteins forming tight complexes with DNA cannot be suspected in artefactual nature due to above considerations. These are the so-called tightly-bound proteins (TBP). These proteins remain attached to DNA with covalent or non-covalent bonds after harsh deproteinization procedures like treatment with phenol, chloroform, ionic detergents, proteases, etc. Enrichment of the TBP in specific DNA sequences was a special interest with respect to speculation on the potential function of such sequences in higher order structures of the genome of different organisms including human, mouse, and chicken [8, 9 and references therein]. Unlike nuclear matrix isolation procedure that is prone to many artefacts [7], TBP are easily isolated, their spectrum is well-reproducible. To our opinion the TBP are prospective for search of novel tumour markers. Finding of a tissue-specific spectrum of TBP in plants [9, 10] and animals [11] has encouraged us to compare spectrum of TBP in rat normal tissue (liver) and experimental hepatomas with a goal to find prospective proteins for tumor marker development.

MATERIALS AND METHODS

Animals. Animals were obtained from the Laboratory of Experimental Animals, Riga Stradins University, Riga, Latvia. All experimental procedures were carried out in accordance with guidelines of the Directive 86/609/EEC "European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes" (1986) and were approved by the

Received: June 14, 2011.
*Correspondence: Fax: 370-2398231
E-mail: danute.labeikyte@gf.vu.lt
Abbreviations used: TBP – the tightly bound to DNA proteins; 2DE – two dimensional gel electrophoresis.

Animal Ethics Committee of the Food and Veterinary Service (Riga, Latvia). Wistar male rats, each weighing 215.00±5.63 g at the beginning of the experiments, were used in all the work. The environment was maintained at a temperature of 22±0.5 °C with a 12-h light/dark cycle. The animals were fed a standard laboratory diet. Strains of Zajdela ascites hepatoma (ZAH) and G-27 hepatoma were obtained from the Cancer Research Centre (Moscow). 0.5 ml of ZAH ascetic liquid was inoculated to rats with five day interval. To passage the G-27 hepatoma 1 ml of tumor suspension was inoculated, tumor tissue was collected 14 days later [12].

DNA isolation. Chopped liver or hepatoma tissue was placed in a Dounce homogenizer, 10-fold extent of homogenization buffer (w/v) was added (0.25M sucrose, 0.05M Tris-HCl, pH 7.4; 0.002M $CaCl_2$), 10 frictions were produced. Homogenate was centrifuged at 1000 g for 10 min. The pellet was again homogenized by 15 frictions in a buffer containing 50 mM Tris-HCl pH 7.5; 0.25 M sucrose, 2 mM $CaCl_2$, 1% Triton X-100. Crude nuclei were pelleted at 1000 g for 10 min. Extraction was repeated. The nuclear pellet was mixed with appropriate amount of buffer (100 mM Tris HCl pH7.5; 500 mM NaCl, 50 mM Na_2EDTA, 1.25% SDS; 3.8 g/l of sodium bisulfite, 4ml/l of 2-mercaptoethanol). Two latter components were added to the buffer just before the extraction to obtain a slightly viscous suspension. The lysate was incubated for 45 minutes at 65 °C. DNA was extracted with the same volume of chloroform/isoamylic alcohol mixture (24:1), the suspension was centrifuged and the water phase was separated. RNA was separated by precipitation in 4M LiCl at 4 °C for at least one hour, 12M LiCl solution was added to the water phase to reach the necessary salt concentration. RNA was pelleted for 10 min at 10000 g. The chloroform extraction procedure of the supernatant was repeated again. DNA was precipitated from the water phase with two volumes of ethanol and washed in 70% ethanol.

Isolation of TBP complexes with DNA by means of exhaustive nuclease digestion.

The DNA was digested with DNase I (Fermentas) (0.01 U/μg, room temperature, overnight) in 10 mM Tris-HCl, pH 7.6; 5 mM $MgCl_2$. Digestion was performed in a dialysis sack with constant dialysis against digestion buffer. Completeness of digestion was monitored by gel electrophoresis. Protein concentration was determined with the BCA according [13], using crystalline bovine serum albumine as standard [13]. Proteins were precipitated with ice-cold 10% (v/v) TCA for 1 h on ice, and protein pellet was washed twice with ice-cold acetone. Resulting pellet were dissolved in rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, 0.5% IPG buffer, 40 mM DTT, 0.002% bromphenol).

2D electrophoresis (IEF/SDS-PAGE). An 11 cm Immobiline DryStrips with linear pH gradient 5.3–6.5, and Excel Gel SDS, gradient 8–18% (GE Healthcare, Piscataway, NJ,USA) were used for 2DE. The TBP corresponding 1 mg of DNA dissolved in 200 μl of rehydration buffer and were loaded onto IPG gel strip; then were reswollen overnight. The isoelectrofocusing was carried out up to a total of 70 kVh. Prior to second dimension gel stripes were reduced and acylated according to the manufacturer's recommendations. IEF/ SDS PAGE was performed with Multiphor II device (GE Healthcare, Piscataway, NJ, USA). Gels were silver stained according to Shevchenko A et al. [14].

Preparation of samples for MALDI-TOF. After staining, the spots of interest were excised from gel, crushed to 1 mm^2 sized slices and dehydrated with 50% acetonitrile. The gel slices were then dried under vacuum and rehydrated with 30 μl 25 mM NH_4HCO_3 (pH 8.3). Proteins in the gel slices were digested overnight with 100 ng of modified trypsin (Promega, Madison, WI, USA) at 37 °C. The peptides were then washed twice from the gel with 50 μl 5% TFA in 50% acetonitrile. The wash-outs were collected and dried under vacuum. For MALDI-TOF, peptides were diluted in 3 μl of 0.01% trifluoracetic acid (TFA) in 30% acetonitrile. 0.8 μl of each sample with matrix (α-Cyano-4-hydroxycinnamic acid) were loaded on MALDI plate.

MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight) analysis. Samples were analyzed with a MALDI–TOF MS using a Voyager-DE™ Pro (Applied Biosystems, Framingham, MA). Positive ionization, acceleration voltage 20 kV, grid voltage 75%, guide wire 0.02 and the extraction delay time 200 ns were used to collect spectra in the mass range of 700–4000 Da. Reflector mass spectra were acquired and calibrated either externally or internally, using trypsin autolysis peptides (*m/z* 842.5200, 1045.5642, 2211.1046).

Data processing of the spectra was performed with Data Explorer™ Version 4.0 (Applied Biosystems). Protein identification was performed by searching in protein sequence database (SwisPro) using PeptIdent and MS-Fit programs available on the ExPaSY server (http://us.expasy.org/). The following parameters were used for databases searches: monoisotopic mass accuracy 50–100 ppm, missed cleavages 0–1 and complete carbomethylation of cysteines.

RESULTS

2D electrophoresis. We screened three separate sets of TBP for changes of the proteome. The separation by *pI* was limited to the range of 5.3–6.5 for a better resolution of TBP proteins**.** Proteins separated by 2D electrophoresis were visualized by staining with $AgNO_3$. As seen in Figure, the resulting 2DE maps of tightly bound protein patterns from rat liver and rat experimental hepatomas (Zajdela ascites hepatoma and G-27 hepatoma) appeared quite different. 2DE TBP patterns of Zajdela ascites hepatoma (Figure, b) and hepatoma G-27 (Figure, *c*) appeared different also. It was expected that these proteomas might share similar spot patterns. We suggest that protein spot pattern alterations hardly occur due to different protein abundance in TBP patterns. For all 2-DE fractionations we subjected TBP amount which

corresponded to 1 mg of DNA. As seen in Figure, the least amount of TBP is characteristic for hepatoma G-27 cells. TBP is represented by numerous peptides in all TBP samples. The images of 2DE gels could not be readily overlapped to determine differences. Protein spot profiles were analyzed using image analysis software — Melanie 7 (Swiss Institute of Bioinformatics). Due to the different spot pattern of the three sets of TBP, software matching did not generate useful identification of protein differences. Using the same spot detection parameters different number of spots was detected in all preparations. For example, comparative analysis of corresponding gel sections as denoted in Figure by frames (pH range of 5.8–6.4 and molecular mass within the range of 20–80 kDa) revealed 35 spots in Zajdela acites hepatoma TBP pattern, 24 spots in liver TBP patern and 18 in hepatoma G-27 TBP pattern. Only 7 spots were shared among the all TBP sets. In general, the TBP pattern appeared to be different in normal and malignant tissues. Zajdela ascites hepatoma TBP pattern appear to be more numerous and heterogenous. Fraction of proteins with lower pI is more pronounced in G-27 TBP pattern.

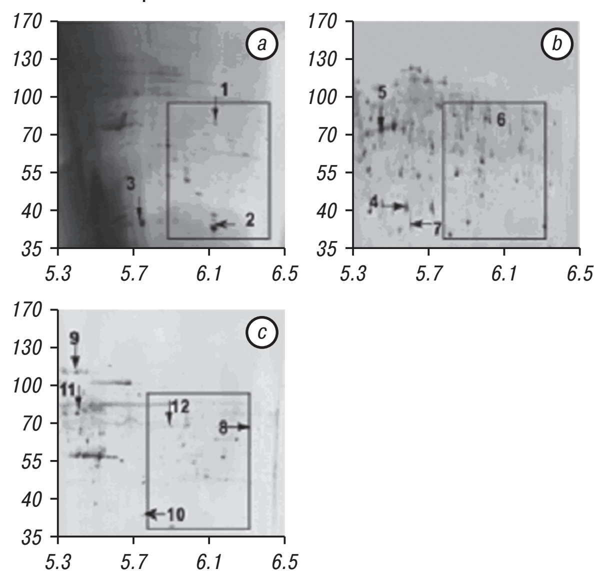

Figure. 2D electrophoresis patterns of TBP isolated from rat liver cells (*a*), experimental Zajdela ascites hepatoma (*b*) and experimental G-27 hepatoma (*c*). First dimension — Immobiline Dry Strips 5.3–6.5 pH; second dimension — Excel Gel SDS, gradient 8–18%. Gels stained with $AgNO_3$. Positions of molecular weight markers are indicated on the left, pH — below. Numbers denote spots which were identified by MALDI-TOF. Frame denoted the gel section comparatively analyzed with Melanie 7

Mass spectrometric analysis. Some of protein spots were subjected to mass spectrometry. Our criteria for accepting spots for mass spectrometry analysis were as follows: i) due to the limited sensitivity of the mass spectrometer only protein spots containing no less than 1 pmol of protein were subjected to analysis; we selected similar spots (*pI,* Mw) shared among all TBP sets, and some spots which were present only in separate TBP pattern; iii) spots for analysis were chosen following methodological approaches for proteomic analysis of nuclear matrix proteins [15, 16];

Protein spots were cut out from the gels and subjected to in gel tryptic digestion. Mass fingerprints of the peptide mixtures were obtained by MALDI TOF mass spectrometry. Protein identification was performed by searching in protein sequence database (SwisPro) using PeptIdent and MS-Fit programs. The following parameters were used for databases searches: monoisotopic mass accuracy 50–100 ppm, missed cleavages 0–1 and complete carbomethylation of cysteines. The list of proteins identified by PeptIdent program is presented in Table. The Table presents only proteins with a MASCOT total protein score > 50 so were considered as real and as high confidence proteins and covered by peptides at least 15% of the entire protein sequence.

Surprisingly, we found that the resulting list of identified proteins from different TBP patterns appeared quite similar. We consider that such result to some extent is determined by our criteria of accepting spots for mass spectrometry and the limited sensitivity of the mass spectrometer.

Spots (e.g., 3, 4, 7, 8, 10) were detected as fragments of identified proteins. Intriguingly similar spots (the same *pI*, Mw) from different gels contained the same TBP as identified by mass spectrometry. Peptides from spots 3 (liver), 7 (Zajdela acites hepatoma) and 10 (hepatoma G-27) are identified as DNA repair protein RAD50. Spots 5 (Zajdela acites hepatoma) and 11 (hepatoma G27) are homologous to lamin B1. Spots 1 (liver) and 12 (hepatoma G27) contain peptides homologous to coiled-coil domain-containing protein 41. In all above cases the spots occupy similar positions in all the three gels. However spots 2 (liver; Mw≈35 kDa; *pI* 6.2) and 8 (hepatoma-G27; Mw ≈60kDa; *pI* 6.4) are corresponding to peptides of different size. It was revealed that these peptides are homologous to “Structural maintenance of chromosomes protein 1A” in both cases. Spot 4 solely contained peptides homologous to ATP-dependent RNA helicase. Spot 9 was the only to be homologous to scaffold attachment factor B1.

Spots 1, 2 and 6 were found to contain two proteins as identified by MALDI-TOF MS.

DISCUSSION

The identified proteins in all TBP samples are nuclear proteins (Table). This excludes possibly artefactual binding of the proteins *in vitro* observed in some cases [11, 17]. Moreover, ability to form very tight complexes with DNA was reported to several of these identified proteins.

As seen in Table some of identified proteins belong to nuclear matrix. Lamins, heterogeneous nuclear ribonucleoproteins and scaffold attachment factor B1 are considered as nuclear matrix proteins. The nuclear matrix is considered a proteinaceous structure spatially organizing the interphase nucleus. Nevertheles it was demonstrated that TBP-DNA and nuclear matrix-DNA complexes are different structures [9], most likely some of nuclear matrix proteins shared in TBP pattern.

Table. Description of TBP proteins identified after 2D electrophoresis and MALDI-TOF MS[1]. Proteins are listed with their respective SWISS-PROT primary accession numbers

Spot[2]	Identified Protein	pI	Swiss-Prot Access Nr	Mass, Da		Number of peptides[3]	% of sequence[4]
				Expected	Measured		
1(A)	Coiled-coil domain-containing protein 41	6,2	Q66H89	82	≈60	18	18
	Heterogeneous nuclear ribonucleoprotein M		Q62826	56		19	18
2(A)	Structural maintenance of chromosomes protein 1A	6,2	Q9Z1M9	56	35	54	30
	Nucleophosmin		P13084	33		18	46
3(A)	DNA repair protein RAD50	5,7	Q9JIL8	154	≈35	96	47
4(B)	Putative ATP-depen-dent RNA helicase DHX39	5,7	Q5BJS0	134	≈38	27	20
5(B)	Lamin-B1	5,4	P70615	66	66	43	48
6(B)	Heterogeneous nuclear ribonucleoprotein M	6,4	Q62826	74	≈70	71	40
	Structural maintenance of chromosomes pr.1A		Q9Z1M9	143		28	34
7(B)	DNA repair protein RAD50	5,7	Q9JIL8	154	≈35	65	33
8(C)	Structural maintenance of chromosomes pr.1A	6,4	Q9Z1M9	143	60	37	18
9(C)	Scaffold attachment factor B1	5,4	O88453	105	≈105	20	15
10(C)	DNA repair protein RAD50	5,7	Q9JIL8	154	≈35	84	42
	ATP-dependent RNA helicase DHX39		Q5U216	49	≈35	23	41
11(C)	Lamin-B1	5,4	P70615	66	≈70	18	22
12(C)	Coiled-coil domain-containing protein 41	6,0	Q66H89	69	≈70	27	25

Notes: [1]proteins characterized with a MASCOT total protein score > 50 were considered as real and as high confidence proteins; [2]Spots corresponds numbers in Fig. TBP isolated from rat liver cells (A); rat experimental Zajdela ascites hepatoma B); rat experimental G-27 hepatoma (C); [3]number of peptides – number of peptides of the protein identified by mass spectrometry as peptides belonging to the sequence of identified protein; [4]% of the the sequence – part of the sequence of identified protein covered by the above – mentioned peptides

Some TBP proteins are identified as RAD50 are components of a single protein complex, Mre11-Rad50-Nbs1 (MRN). The MRN complex consists of dimers of each subunit and this heterohexamer controls key sensing, signaling, regulation, and effector responses to DNA double-strand breaks including ATM activation, homologous recombinational repair, microhomology-mediated end joining and, in some organisms, non-homologous end joining. To organize the MRN complex, the Mre11 exonuclease directly binds Nbs1, DNA, and Rad50. Rad50, a structural maintenance of chromosome (SMC) related protein, employs its ATP-binding cassette (ABC) ATPase, Zn hook, and coiled coils to bridge DSBs and facilitate DNA end processing by Mre11 [18, 19]. Thus RAD50 keeps attached to DNA a huge multiprotein complex.

Structural maintenance of chromosomes protein 1A (SMC1A) is structural component of cohesin. Cohesin regulates sister chromatid cohesion during the mitosis and meiosis. In addition, cohesin has been demonstrated to play a critical role in the regulation of gene expression. Furthermore, multiple proteins in the cohesin pathway are also involved in additional fundamental biological events such as double strand DNA break repair, chromatin remodeling and maintaining genomic stability. Composed of several essential subunits, cohesin forms a ring-like complex that is thought to embrace sister chromatids, thereby physically linking them until their timely segregation during cell division [20].

Several identified polypeptides were homologous to RNA helicases (Spots No. 4 and 10). These proteins are now of major interest because they are known to play important roles in virtually all aspects of RNA synthesis and function, including nuclear transcription, pre mRNA splicing, ribosome biogenesis, nucleocytoplasmic transport, translation, RNA decay and organellar gene expression, processes that involve multi-step association/ dissociation of large RNP complexes as well as the modulation of complex RNA structure [21]. Helicases were resently identified among barely TBP [10].

Lamin B1 is identified exclusively in preparations of tumour TBP (Spots No.5 and 11). Traditionally lamins were not considered to form very tight complexes with DNA. Actually besides being structural components of the nuclear envelope lamins turn out to be involved in multiple functions and complex interactions with other nuclear proteins and DNA. Lamin B1 is involved in sequestration of the transcription factors [22]. Lamin B1 maintains the functional plasticity of nucleoli interacting with nucleophosmin, intriguingly nucleophosmin also revealed in one of the spots [23]. In whole the nuclear envelope is considered to be a signalling node in development and disease [24, 25]. Some data indicate possible involvement of lamins and lamin receptors in tumour phenotype development [26]. Lamin B interacts with DNA via lamin B receptor [27]. The interaction occurs via linker DNA and is enhanced by DNA curvatures [28]. Certain genomic elements are attached to the nuclear lamina, this contributes to the spatial organization of chromosomes inside the nucleus. Sequences in the human genome that interact with the nuclear lamina *in vivo* have been already identified. A map of the interaction sites of the entire genome with the nuclear lamina shows that genome-lamina interactions occur through more than 1,300 sharply defined large domains 0.1–10 megabases in size. These lamina-associated domains (LADs) are typified by low gene-expression levels, indicating that LADs represent a repressive chromatin environment. The borders of LADs are demarcated by the insulator protein CTCF, by promoters that are oriented away from LADs, or by CpG islands, suggesting possible mechanisms of LAD confinement [29]. Interaction of silenced genes with the nuclear lamina is mediated by lamins [30]. Probably presence of Lamin B in the TBP preparations of rat hepatomas indicates tumour-progression associated modifications of the above DNA-lamin interactions.

The nucleus contains many potential cancer markers [1, 3]. The present study was designed to determine changes in TBP patterns from normal and malignant cells. We have analyzed TBP from rat liver and rat experimental hepatomas (Zajdela ascites

hepatoma and hepatoma G-27) to search for candidates of malignant transformation markers. Altogether we have identified some TBP by mass spectrometry. TBP pattern proteome alterations of normal and malignant cells evidenced by comparative 2DE-gel analysis proved that proteomic approach may be promising in development of biomarkers. At present the nature and function of many TBP have not been established, awaiting further investigation. Function of the tightly bound to DNA proteins *in vivo* remains an open question. Lamins were identified as prospective markers, however furher research is nessesery to test their utility for practical applications. Although the further elucidation of the TBP potential for biomarker trawling is necessary due to the limited number of identified TBP, the proteomic approach has proven to be promising.

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