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# State of Oncomarker Protein B23/Nucleophosmin in HeLa Cells

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**Abstract**—Western blot after SDS-PAGE for protein separation showed two immunoreactive bands corresponding to monomers (38-40 kDa) and oligomers (210-230 kDa) of nucleophosmin in HeLa cell lysates. Decreasing the buffer ionic strength during the incubation of cells and nuclei destabilized these oligomers. We also showed the existence of two B23/nucleophosmin pools in nuclei of HeLa cells with different sensitivity to hypotonic buffer treatment: one extractable from the nucleus and the other non-extractable and tightly bound to the nucleus. A detailed structural analysis of the extractable B23 pool was carried out: two closely related nucleophosmin isoforms (B23.1 and B23.2) were identified as a result of analysis of C-terminal amino acid sequences using carboxypeptidase hydrolysis; the N-termini of both isoforms are blocked by an acetyl group. As a result of sequencing of the deacetylated proteins, it has been established that the N-terminal amino acid sequence of nucleophosmin in these preparations is truncated by nine amino acid residues and the acetylated residue is Ser. The truncated monomer of nucleophosmin (represented only by the extractable part of the protein) on addition of magnesium ions to low ionic strength buffer or increase in buffer ionic strength was shown to form oligomers with molecular weights (210-230 kDa) similar to those revealed in the total cell lysate. It should be noted that the set of oligomers in this case differs from the one in total cell lysate. Our strategy of characterization of B23 forms for HeLa cells can be applied for other tumor cells.

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Nucleophosmin (protein B23, NPM1) belongs to the family of nuclear chaperones, nucleoplasmins. In interphase, most of the nucleophosmin is present in the nucleolus; hence, it is often called a nucleolar protein. However, it is also localized in the nucleoplasm and makes up >20% of total protein content of the nuclear matrix [1]. It has also been demonstrated that B23 is a "shuttle" that runs between the nucleus and the cytoplasm [1-3] and has an intricate localization during mitosis [4].

Nucleophosmin is a polyfunctional protein involved in a wide range of processes in cells. It has a chaperone activity, transports proteins and pre-ribosomal particles, is involved in biogenesis of ribosomes, controls cell response to stress, maintains genomic stability, and regulates transcription. The role of nucleophosmin in carcinogenesis is very intricate and diverse. Nucleophosmin is considered as an oncomarker and possible protooncogen participating in regulation of the activity and stability of tumor suppressors Arf and p53 [5-8].

Polyfunctionality characterizes both the protein structure with a polypeptide chain of complex domain organization [9-16] and existence of a family of its structural forms (isoforms B23.1 and B23.2 showing various posttranslational modifications and functioning as monomers and homo/heterooligomers) [16-18]. Both isoforms have an N-terminal domain significant for oligomerization, chaperone activity [9-11], and binding to Arf [12]. The nuclear export signal is localized in this domain. The central domain of the polypeptide chains of both isoforms includes two acidic regions and two signals of nuclear localization. This domain is critical for the binding of histones and ribosomal proteins [10, 13-15]. The C-terminal domain carrying a signal of nucleolar

*Abbreviations*: cp A, carboxypeptidase A; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FITC, fluorescein isothiocyanate; MA, monoclonal antibodies; MALDI-MS, matrix assisted laser desorption ionization mass spectrometry; 2-ME, 2-mercaptoethanol; PBS, phosphate buffered saline; PFA, paraformaldehyde; PMSF, phenylmethylsulfonyl fluoride; PVP-40, polyvinyl pyrolidone-40; RSB, reticulocyte standard buffer.

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localization (NoLS) is important for the binding of nucleic acids and for ribonuclease activity [16, 17]. It should be noted that this domain is present only in isoform B23.1. Therefore, it is localized mainly in the RNA-enriched nucleolus, while isoform B23.2 is localized in the nucleoplasm. However, isoform B23.2 is able to form heterooligomers with isoform B23.1 and can also be indirectly localized in the nucleolus and modulate the RNA-binding and endonuclease activities of isoform B23.1 [19].

Unfortunately, most of the properties of isoforms B23.1 and B23.2 have been determined by analyzing protein preparations obtained during the expression of plasmids carrying cDNA of these isoforms in *Escherichia coli* [9-13, 17-20]. Gel filtration, sedimentation analysis, and "native" gel electrophoresis showed that the preparations of both isoforms produced oligomers corresponding to proteins with molecular weight 210-255 kDa [9, 20]. In cells with content of monovalent cations of 140-160 mM and $Mg^{2+}$ ~ 0.5 mM, nucleophosmin was supposed to exist mainly as oligomers [9]. It was established that the oligomers of protein B23 participate in the binding to Arf [12] and RNA [17, 18] and show chaperone [10, 13] and RNase [19] activities, and only the monomers of isoform B23.1 are bound to DNA [21].

However, the properties of proteins obtained by expression often differ from the properties of endogenous proteins. The most complicated situation is typical of tumor cells, where the content of nucleophosmin (particularly isoform B23.1) abruptly increases and this protein undergoes various structural modifications both at the genetic level and at the level of the protein, resulting in emergence of new structural forms of nucleophosmin [5, 6, 22]. It was shown that the gene of nucleophosmin most frequently underwent modifications (mutations, deletions, translocations) during malignant blood disorders [23, 24]. Such mutations and translocations lower the content of the wild type protein in the nucleolus (due to formation of its modified forms and heterooligomers consisting of mutated/chimeric and normal protein forms), change in normal localization, and disturbance of interaction with oncosuppressors p53 and Arf [6, 25].

In solid tumors (liver, breast, ovary, prostate, large bowel, and stomach cancer), nucleophosmin is overexpressed as well [26, 27] but its structural state is less studied. It is only known that patients with hepatocellular carcinoma have a truncated (by about 1 kDa) form of nucleophosmin making oligomers, which are cleaved by granzyme B and detected by SDS-PAGE [27]. Such oligomers, termed by the authors as SDS-resistant, have not been found in normal and cirrhotic liver. At the same time, oligomeric forms of B23 exist in normal and cirrhotic liver cells and are visible under "native" gel electrophoresis (without SDS). We and other authors [11, 28, 29] have found SDS-resistant nucleophosmin oligomers also in human uterine neck carcinoma (HeLa) cells.

We have developed a strategy for isolation and structural analysis of nucleophosmin from HeLa cells. Nucleophosmin forms functioning in human tumor cells have been characterized for the first time by the arsenal of protein chemistry methods. The site of protein truncation has been established and the ability of truncated nucleophosmin forms to form SDS-resistant oligomers has been shown for the first time.

## MATERIALS AND METHODS

**Materials.** The following materials were used in this work: 10% embryonal bovine serum, DMEM culture medium (HyClone, USA); penicillin, streptomycin (Gibco, Great Britain); versene solution (PanEco, Russia); aprotinin, calibration standard proteins (SDS-6H), trifluoroacetic acid, leupeptin, goat antibodies to mouse (IgG + IgM) conjugated with horseradish peroxidase, phenylmethylsulfonyl fluoride (PMSF), sheep antibodies to mouse IgG conjugated with fluorescein isothiocyanate (FITC), paraformaldehyde (PFA), 4′,6-diamidino-2-phenylindole (DAPI) dihydrochloride, PMSF-treated carboxypeptidase A (cp A) from bovine pancreas (50 U/mg protein), polyvinyl pyrolidone-40 (PVP-40) (Sigma, USA); reagents for electrophoresis and blotting (BioRad, USA); Immobilon-NC and -P membranes, pore diameter 0.45 μm (Millipore, USA); monoclonal antibodies (MA) to B23 (3C9) kindly provided by the Laboratory of Clinical Immunology, Hematological Research Center of the Russian Academy of Medical Sciences (described in [30, 31]); Moviol (Hoechst, USA). Other reagents were domestic products of "chemical purity" or "high purity" grade.

**Cell cultures.** HeLa cells were grown in culture flasks or on cover glasses on the Dulbecco's modified Eagle's medium (DMEM) containing 10% embryonal bovine serum, 10 μg/ml penicillin, and 10 μg/ml streptomycin at 37°C in the presence of 5% $CO_2$.

**Cell suspension preparation.** Cells were washed free of the medium with phosphate buffered saline (PBS) containing 2.7 mM KCl, 140 mM NaCl, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$ (pH 7.2), removed from culture flask surface with versene solution (0.2% EDTA in PBS) for 5 min at 37°C, and precipitated by centrifugation at 1672 rpm (500*g*) for 10 min at 4°C (5804R centrifuge; Eppendorf, Germany). Then the cells were washed by suspension in PBS and repeatedly centrifuged under the above conditions. In some experiments precipitated cells were suspended in other buffers: PBS diluted 2, 5, and 10 times; reticulocyte standard buffer (RSB) (10 mM Tris-HCl buffer, pH 7.4, containing 10 mM NaCl and 1.5 mM $MgCl_2$). In some cases, NaCl or $MgCl_2$ were added to the cell suspension to 140 and 7 mM, respectively. Protease inhibitors were added to the buffers immediately before use: PMSF to $10^{-4}$ M, leupeptin and aprotinin to 1 μg/ml.

**Isolation of nuclei.** Nuclei were isolated by the method described in [11] but in our modification. HeLa cells in the logarithmic growth phase (four culture flasks, 175 $cm^2$) were washed free of the medium with PBS, removed with versene solution, precipitated by centrifugation, and washed by suspension in PBS with repeated centrifugation as described. Then the cells were suspended in 25 ml of hypotonic RSB buffer and incubated for 30 min on ice. After complete swelling (according to light microscopy), the cells were not centrifuged as described [11], but NP-40 detergent was added to the suspension to final concentration of 0.5%. Then the cells were broken using a homogenizer (Wheaton, USA) (8-10 up-down movements). The homogenate was layered onto a three-step (not two-step as in [11]) gradient of sucrose concentrations (10 ml of 0.25 M sucrose, 10 mM $MgCl_2$; 15 ml of 0.88 M sucrose, 0.05 mM $MgCl_2$; 15 ml of 1.9 M sucrose, 0.05 mM $MgCl_2$ (additional step)) and centrifuged at 5752 rpm (1720*g*) for 20 min at 4°C. The following protease inhibitors were added to the sucrose solutions (immediately before use): PMSF to $10^{-4}$ M, leupeptin and aprotinin to 1 μg/ml. The fraction of nuclei localized on the boundary between the second and third gradient steps was taken, diluted 3 times with RSB, and centrifuged for 10 min under the same conditions. The pellet with purified nuclei was suspended in RSB or exposed to hypotonic treatment.

**Hypotonic treatment of nuclei.** An aliquot of precipitated nuclei was suspended in 10 mM Tris-HCl buffer, pH 7.4, and kept on ice for 15 min. Then, the nuclei were precipitated by centrifugation at 5752 rpm (1720*g*) for 10 min at 4°C and the supernatant was separated. The hypotonic treatment was repeated three times. The supernatants were combined, and the nuclei were resuspended in 10 mM Tris-HCl buffer, pH 7.4. Then protein concentration in the preparations was measured by a modified Lowry method [32] followed by electrophoretic and immunochemical analyses.

**Immunocytochemical staining.** Nuclear suspension was applied onto a cover glass, kept for 20 min at room temperature, and then fixed with 2% PFA prepared in the corresponding buffer (RSB, 10 mM Tris-HCl, pH 7.4) for 20 min at room temperature. The specimens for microscopic analysis were immunostained and handled as described [31]. Monoclonal antibodies 3C9 to B23 were used as primary antibodies, and sheep antibodies to mouse IgG conjugated with FITC were used as secondary antibodies. The preparations were analyzed in an Axiovert 200 microscope. Records were made with a $CoolSnap_{cf}$ 12-bit monochromatic CCD camera (Roper Scientific, USA). The images were processed in Adobe Photoshop 8.0.

**Sample treatment for electrophoresis.** Before addition of the lysing solutions to cell samples suspended in PBS (in some experiments, 2-, 5-, or 10-fold diluted PBS or 10-fold diluted PBS with 1, 3, 5, 7 mM $MgCl_2$ were used as suspension buffers), PBS (or respective suspension buffer) and glycerol were added so that the protein and glycerol concentrations in a sample would be 1-1.5 mg/ml and 30%, respectively. Then the samples were usually treated according to the procedure described in [28]: lysing solution containing SDS and 2-mercaptoethanol (2-ME) was added up to the concentrations of 5 and 2.5%, respectively. The mixture was thermostatted at 100°C for 1 min, followed by addition of 0.1 volume of staining solution (0.1% bromphenol blue in 50% glycerol). In one of the experiments, NP-40 and dithiothreitol (DTT) were added to the cell suspension in PBS up to 0.1% and 2 mM, respectively, instead of SDS-containing lysing solution. The mixture was incubated at 37°C for 20 min followed by addition of the staining solution.

The samples for "native" electrophoresis were processed under the conditions described in [11]: the cells were suspended in the lysing 10 mM Tris-HCl buffer, pH 7.4, containing 150 mM KCl, 1 mM $MgCl_2$, and 1 mM DTT. The mixture was incubated at 37°C for 20 min followed by addition of 0.1 volume of the staining solution (0.1% bromphenol blue in 50% glycerol).

During the treatment of samples for electrophoresis by the Laemmli method [33], the cells were suspended in the lysing 62.5 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 5% 2-ME, 10% glycerol, and 0.002% bromphenol blue and incubated at 100°C for 5 min.

**SDS-PAGE and electroblotting.** Electrophoresis was performed by the Laemmli method [33]; "native" electrophoresis was performed by the method described in [11]; electroblotting and immunostaining were performed by the methods described in [34, 35]. Proteins were electrotransferred to Immobilon-NC (for immune staining) or Immobilon-P (for analysis of N- and C-terminal amino acid sequences) membranes at 12°C, constant current 400 mA, in 0.025 M sodium bicarbonate buffer (pH 9.0) containing 20% $CH_3OH$ and 0.1% SDS. The primary antibodies for immunostaining were monoclonal antibodies to B23 (3C9), and the secondary antibodies were peroxidase conjugated goat antibodies to mouse IgG + IgM.

**Elimination of acetyl protection and analysis of N-terminal amino acid sequence.** The proteins were deacetylated out directly on the Immobilon-P membrane by the method described in [36]. Membrane strips with the protein B23 (according to the data of immunostaining) were moistened in methanol and kept in the TFA/MeOH vapor (non-aqueous), 1 : 1, for 72 h at 47°C in a sealed ampoule in an argon atmosphere. The ampoule was opened after hydrolysis was completed. The reaction mixture was removed by washing the membrane three times with 100% MeOH (30 μl portions). Then the membrane was used for protein structure analysis. Protein B23 was sequenced in a Procise 491 gas phase sequencer (Protein Sequencing System), and the phenylthiohydantoic derivatives of amino acids were identified in a 785A PTH analyzer (Applied Biosystems, USA).

**Carboxypeptidase A hydrolysis and C-terminal amino acid sequence analysis.** The proteins were hydrolyzed directly in the Immobilon-P membrane. For prevention of nonspecific adsorption of carboxypeptidases, the membrane was treated with PVP-40 as described in [35]. The proteins were hydrolyzed by cp A in N-ethylmorpholine acetate buffer (pH 8.3) containing 1 μg cp A at 37°C for 2 h. After the reaction was completed, the supernatant was separated and the membrane was successively washed with buffer, Milli-Q water, and methanol (20-μl portions). Washing solutions were combined with the supernatant. The cleaved amino acids were analyzed as dansyl derivatives as described in [34].

**MALDI-mass spectrometric (MS) analysis.** The proteins were trypsinolyzed in polyacrylamide gel and the samples prepared as described in [37]. Mass spectra were obtained in a MALDI-MS Reflex III instrument (Bruker, Germany) equipped with a UV laser (337 nm) in the positive-ion mode using a reflectron; the accuracy of masses measured after final calibration by the peaks of trypsin autolysis was 0.015%. Proteins were identified using Mascot (www.matrixscience.com) and NCBI database with the above accuracy, with allowance for the possibility of oxidation of methionine residues by atmospheric oxygen and modification of cysteine residues by acrylamide.

## RESULTS AND DISCUSSION

**Analysis of monomer/oligomer state of nucleophosmin in HeLa cells.** It should be noted that HeLa cells have been subjected to many studies on the functional properties of nucleophosmin, including its role in the apoptosis of tumor cells. Preparations were usually analyzed by Western blot; sample treatment and SDS-PAGE were performed by the Laemmli method under standard conditions. Under these conditions, only nucleophosmin monomers were detected. Analysis of the literature shows that inconsistency of the data on oligomer status of the protein might be due to the differences in sample treatment and electrophoresis and electroblotting conditions.

The blotting under standard transfer conditions (for 3-4 h) proved to be insufficient for the complete transfer of proteins with different molecular weights, in particular, nucleophosmin monomers (38-40 kDa) and oligomers (210-230 kDa). Hence, we used two-stage blotting: the bottom part of the gel containing proteins with molecular weights of <60 kDa was blotted for 3 h, while the top part with high molecular weight proteins was blotted for 15 h.

We also estimated the effects of different conditions of electrophoresis and sample treatment on the monomer/oligomer state of nucleophosmin. During electrophoresis by the conventional Laemmli method, where the gel and electrode buffer contained 0.1% SDS and the samples were treated with a lysing solution (62.5 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 5% 2-ME, 10% glycerol, and 0.002% bromphenol blue) and by boiling for 5 min, only a doublet band was detected within the region of nucleophosmin monomers on immunoblots (Fig. 1a, lane *1*). On shortening the period of thermal treatment, a weak band appeared in the region of nucleophosmin oligomers (data not presented). These results are in agreement with data of works where nucleophosmin was analyzed by the traditional Laemmli method. Under conditions of "native" electrophoresis, when SDS is excluded from the gel and electrode buffer and the samples are treated in 10 mM Tris-HCl buffer, pH 7.4, containing 150 mM KCl, 1 mM $MgCl_2$, and 1 mM DTT at 37°C for 20 min, we and the authors of [11] found bands in the region of nucleophosmin oligomers (210-230 kDa). These results suggest [11] that the oligomer state is a ground state of this protein in cells. However, under these conditions we found out that most of the proteins did not enter the gel and intensive immunostaining was observed in the zone of blotting (Fig. 1a, lane *2*). In this case probably some of the protein is present in a high molecular weight or aggregated state.

A series of analytical experiments showed that sample treatment conditions are important for preservation of nucleophosmin oligomers. In contrast to the method of Laemmli, in this case first PBS (buffer with composition and ionic strength close to physiological conditions) and glycerol were added to the samples (up to the protein concentration of 1-1.5 mg/ml and glycerol concentration of 30%). Immediately before the application to polyacrylamide gel, SDS- and 2-ME-containing lysing solutions were added to the samples up to the concentrations of 5 and 2.5%, respectively. The mixture was incubated at 100°C for 1 min (for preservation of nucleophosmin oligomers). Under these conditions, three major bands in the region of oligomers are detected on immunoblots (Fig. 1a, lane *3*) along with the monomeric form, and the oligomeric forms are prevalent. It should be noted that these oligomers are detected in the same range of molecular weights (210-230 kDa) as those revealed under the conditions of "native" electrophoresis [11]. Incubation under milder conditions (at 37°C for 20 min) gave analogous results (Fig. 1b, lane *1*).

The substitution of nonionic detergent NP-40 for the ionic detergent SDS during sample treatment resulted in lower intensity of monomer bands, higher intensity of three major bands corresponding to the oligomeric forms of nucleophosmin (210-230 kDa) detected in the presence of SDS, and appearance of additional bands in the region of 160-200 kDa (Fig. 1b, lane *2*). Thus, it has been shown for the first time that the HeLa tumor cells have two types of nucleophosmin oligomers: resistant and sensitive to SDS treatment. It is of particular interest to study the properties of nucleophosmin oligomers resistant to the treatment with SDS-containing lysing solution and visible under the conditions of SDS-PAGE (SDS-resist-

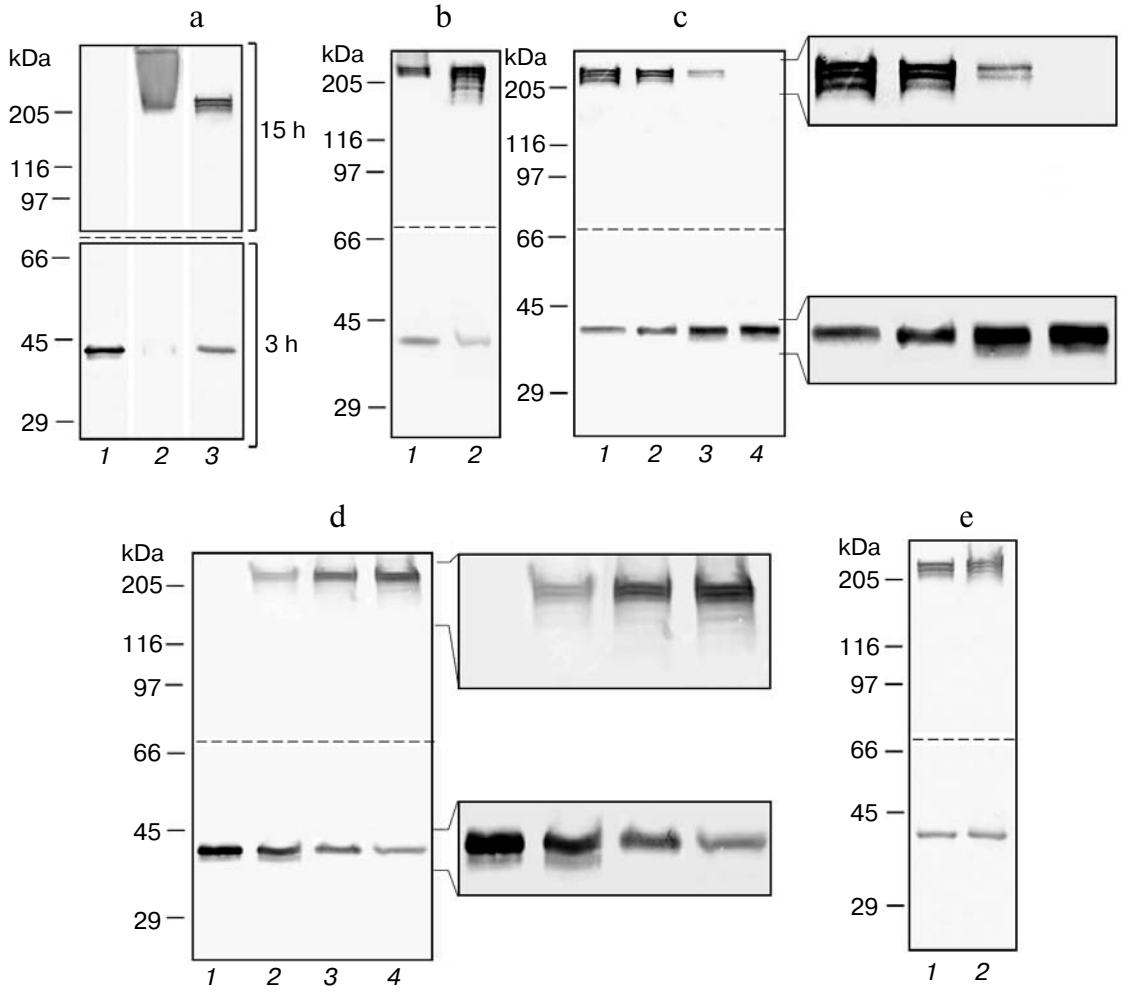


**Fig. 1.** Effects of different conditions of electrophoresis (a) and sample treatment (b-d) on the monomer/oligomer state of nucleophosmin in HeLa cells and comparative analysis of the monomer/oligomer state of nucleophosmin in HeLa and HepG2 cells (e). a) Under conditions of conventional Laemmli method (*1*), "native" electrophoresis (*2*), and modified Laemmli method (*3*). b) Sample treatment in PBS in the presence of SDS-containing (*1*) and NP-40-containing (*2*) lysing solutions. c) Sample treatment in PBS (*1*) and PBS diluted 2 (*2*), 5 (*3*), and 10 (*4*) times by the lysing solution with SDS. d) Sample treatment in tenfold-diluted PBS containing 1 (*1*), 3 (*2*), 5 (*3*), and 7 (*4*) mM $MgCl_2$ with the lysing solution with SDS. e) Treatment of HeLa (*1*) and HepG2 (*2*) cells in PBS with the lysing solution with SDS. Samples were treated at 100°C for 1 min (a, *3*; c-e) and 5 min (a, *1*) and at 37°C for 20 min (a, *2*; b). SDS-PAGE was performed by the Laemmli method in 7.5% polyacrylamide gel. The proteins were electrotransferred in two stages. Here and in Figs. 2 and 3 strokes show the lines of membrane section; MA to protein B23 (3C9) were used for immunostaining; positions of marker proteins are shown on the left.

ant oligomers), because, in accordance with literature data, they are detected in human tumor cells [27].

**Properties of SDS-resistant nucleophosmin oligomers in HeLa cells.** Practically all properties of nucleophosmin oligomers were studied in homooligomers from preparations of B23.1 and B23.2 isoforms obtained during the expression of plasmids carrying cDNA of these isoforms in *E. coli* [9, 17, 20]. However, the composition of actually functioning (particularly in tumor cells) oligomers might be different, because nucleophosmin *in vivo* forms not only homooligomers but also heterooligomers, including those of abnormal shape. Their properties can be different as well. It has been shown, for example, that nucleophosmin oligomers produced by its expressed forms, in contrast to oligomers of tumor cells, have no resistance to SDS treatment (like endogenous forms of nucleophosmin in normal cells) [27]. Gradient centrifugation and gel filtration showed that the content of uni- or bivalent cations is essential for preservation of oligomers formed by expressed protein isoforms. The Western-blot method was used to assess the influence of changing ionic strength of the medium on stability of SDS-resistant oligomers of nucleophosmin by incubation of HeLa cell suspension in PBS (buffer containing only the salts of univalent metals, ~160 mM) and 2-, 5-, and 10-fold diluted PBS (Table 1). According to data of [38], the storage of HeLa cells in fivefold diluted PBS resulted in translocation of nucleophosmin from the nucleolus

**Table 1.** Concentration of univalent cations in diluted PBS

| PBS dilution rate | Concentration, mM | |
|---|---|---|
| | $\Sigma Na^+$ | $\Sigma K^+$ |
| 0 | 156.20 | 4.20 |
| 2 | 78.10 | 2.10 |
| 5 | 31.24 | 0.84 |
| 10 | 15.62 | 0.42 |

into the nucleoplasm, which was reversible in the case of substitution of undiluted PBS. Immunoblot analysis of cell lysates incubated in buffers of different ionic strength shows that its decrease results in gradual decomposition of SDS-resistant nucleophosmin oligomers to monomers (Fig. 1c) and, in the case of tenfold-diluted PBS, the protein is detected only in the monomeric form as a doublet band. Decomposition of oligomers is accompanied by intensification of the major band and appearance of a faster migrating band in the zone of monomers.

Introduction of Mg ions into tenfold-diluted PBS up to the concentration of 1 mM (Fig. 1d) does not change the monomer/oligomer state, and protein B23 is detected only as a monomer. However, on addition of Mg ions up to the concentration of 3 mM, the monomer/oligomer state visibly shifts towards oligomer stabilization. The number and intensity of protein bands corresponding to oligomeric nucleophosmin forms increases on introduction of Mg ions up to 7 mM, and their set becomes comparable with the set of oligomers in cell lysates in undiluted PBS (Fig. 1c, lane *1*). Thus, it has been shown that reduction of ionic strength results in deoligomerization of nucleophosmin, like in the case of expressed protein forms, while introduction of Mg ions results in oligomer stabilization. However, for stabilization of oligomers obtained from expressed isoforms it is enough to enhance Mg concentration (0.1-1 mM), while for stabilization of oligomers functioning in HeLa cells it is necessary to introduce 5-7 mM Mg ions, significantly exceeding physiological Mg concentration in a cell (0.5 mM). It can be supposed that the changes in the ionic composition and strength of the cell medium influence the localization and monomer/oligomer state of nucleophosmin or its individual forms.

The monomer/oligomer state of nucleophosmin in HeLa (uterine neck carcinoma) and HepG2 (hepatocellular carcinoma) tumor cells was analyzed under the developed conditions: the electrode buffer and the gel contained 0.1% SDS and the samples were treated in PBS containing 30% glycerol, 5% SDS, and 2.5% 2-ME at 100°C for 1 min. As one can see from Fig. 1e, the two types of tumor cells had similar sets of nucleophosmin oligomeric forms. The presence of such forms in human hepatocarcinoma cells [27] is associated with appearance in these cells of a protein form truncated by approximately 1 kDa, which is characterized by an extremely high tendency for oligomerization. Though the precise site of truncation was not determined, it was shown using nucleophosmin deletion mutants that the protein truncated by seven N-terminal amino acid residues was highly subject to the formation of oligomers visible under conditions of SDS-PAGE. Hence, it is extremely important to carry out the structural analysis of nucleophosmin in tumor cells.

**Extraction of nucleophosmin-containing protein fraction from nuclei.** Protein analysis by one- and two-dimensional electrophoresis during proteomic analysis of the nucleoli of HeLa cells revealed quite a number of nucleophosmin forms different in isoelectric points and electrophoretic mobility [39-41]. MALDI-MS analysis of tryptic protein hydrolysates identified only the B23.1 isoform beginning from Met1 [39, 40]. Taking into account the nuclear–nucleolus localization and probable existence of several nucleophosmin forms, we decided to assess its state in the nuclei of HeLa cells. For obtaining a nucleophosmin-enriched preparation, we elaborated the conditions of protein extraction from HeLa nuclei. The nuclei were isolated by the conventional method described in [11] using hypotonic RSB buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$). The same method was used to obtain HeLa nuclei for subsequent isolation and proteomic analysis of nucleoli [39, 40]. We obtained a fraction of intact nuclei not containing cytoplasmic material (Fig. 2a). When the nuclei are held in hypotonic RSB buffer, protein B23 (as shown by immunocytochemical staining (Fig. 2a)) is localized not only in the nucleoli but also in the nucleoplasm. The electrophoregram of cell lysates in PBS and RSB and of isolated nuclei in RSB is presented in Fig. 2b, and their immunoblot is presented in Fig. 2c. Immunoblot analysis of the monomer/oligomer state of nucleophosmin showed that decomposition of oligomers when cells were kept in RSB buffer (Fig. 2c, lane *2*) is analogous to decomposition of oligomers in tenfold-diluted PBS (Fig.

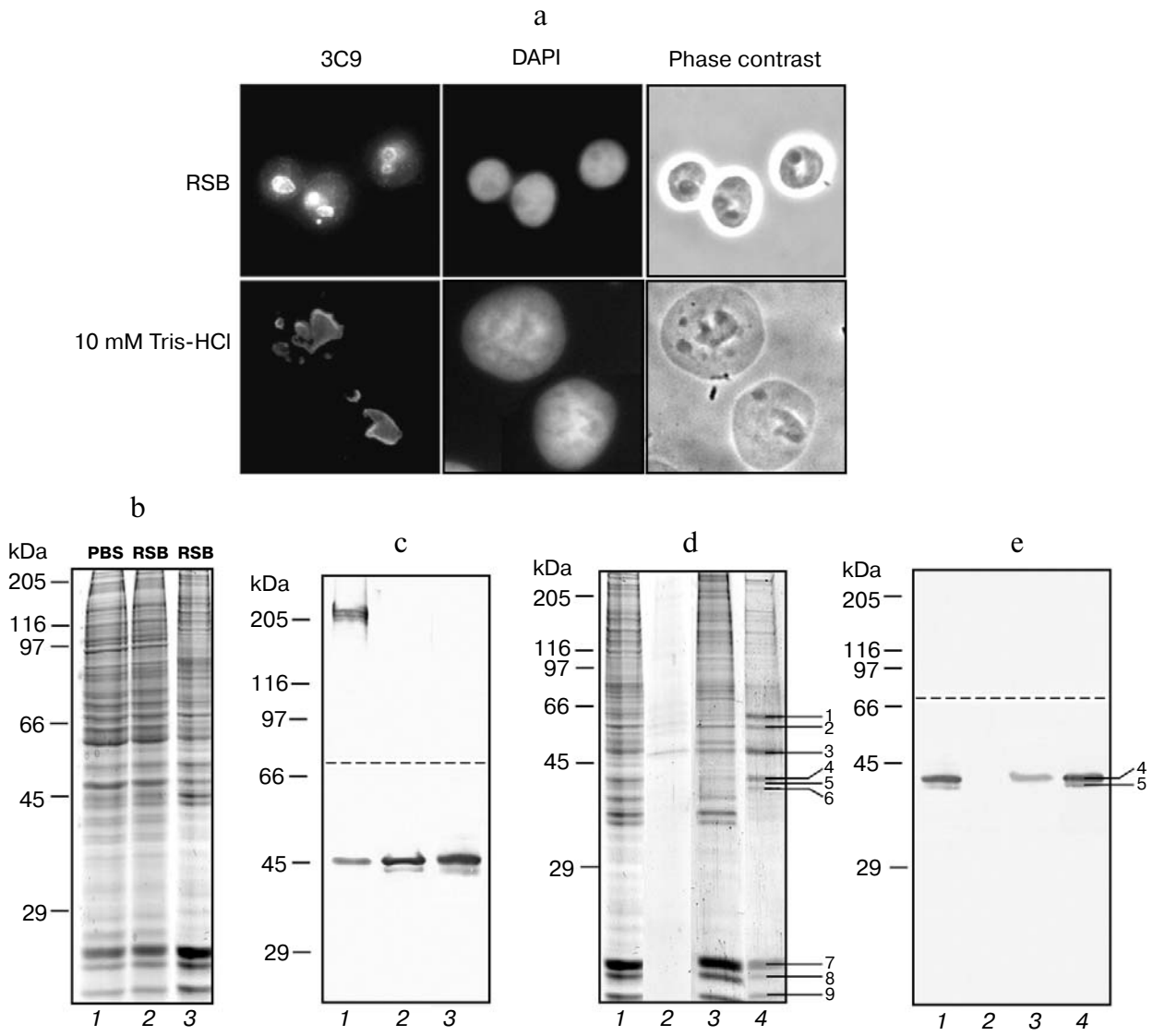


**Fig. 2.** Immunocytochemical (a), electrophoretic (b, d), and immunochemical (c, e) analyses of the influence of hypotonic conditions during treatment of nuclei on the state of nucleophosmin. a) Nuclei suspended in RSB buffer (top row) and in 10 mM Tris-HCl buffer (bottom row). b, c) Cells in PBS (*1*), RSB (*2*), and nuclei in RSB (*3*). d, e) Fraction of nuclei (*1*) and supernatant (*2*) after extraction in RSB buffer; fraction of nuclei (*3*) and the supernatant (*4*) after extraction with 10 mM Tris-HCl. Cells were treated in PBS or RSB buffers and nuclei were treated in respective suspension buffers with SDS-containing lysing solution (100°C, 1 min). SDS-PAGE was performed by the Laemmli method in 7.5% (b, c) and 12.5% polyacrylamide gel (d, e). The proteins were electrotransferred in two stages. Positions of the nine supernatant protein bands more intensively stained with Coomassie G-250 (d) are shown on the right; the two of them (4 and 5) contained nucleophosmin (e).

1c, lane *4*) accompanied by appearance of a low molecular weight band in the zone of monomers. The analogous band doublet was detected in the fraction of isolated nuclei incubated in RSB (Fig. 2c, lane *3*).

It is known that treatment of nuclei with hypotonic buffers results in extraction of a number of nuclear proteins [42, 43]. The set of these proteins and their quantitative content are largely determined by the buffer composition. Immunocytochemical analysis shows that the threefold treatment of nuclei with RSB buffer does not lead to significant changes in nucleophosmin localization (Fig. 2a) or to protein washout and appearance of nucleophosmin in the supernatant fraction (Fig. 2, d and e, lanes *2*), as shown by electrophoretic (Fig. 2d) and immunochemical analysis (Fig. 2e). However, the absence of Na and Mg ions in RSB buffer composition when keeping the nuclei in 10 mM Tris-HCl, pH 7.4, resulted in substantial swelling of the nuclei (Fig. 2a) accompanied by protein washout. Electrophoretic analysis showed the supernatant fraction (Fig. 2d, lane *4*) to have nine most intensely stained protein bands, two of which (4 and 5) contained nucleophosmin as demon-

strated by immunostaining (Fig. 2e, lane *4*). At the same time, the quantity of B23 in the pellet (nuclear fraction) considerably decreased and the immunoblot showed only one high molecular weight band (Fig. 2e, lane *3*). A certain quantity of B23 (usually high molecular weight form), remaining tightly bound to the nucleolar structures, was detected by other authors during different treatments of HeLa cells [17]. The following nine proteins were identified by MALDI-MS analysis of tryptic peptides in the supernatant: vimentin, keratin-8, β/γ-actin, nucleophosmin, GAPDH, hnRNP A2/B1, and histones H2A, H2B, and H4 [31]. The nucleophosmin fraction extracted from the nuclei during the treatment with a buffer of low ionic strength (10 mM Tris-HCl, pH 7.4) was used for structural analysis.

**Structural analysis of nucleophosmin.** Western-blot analysis showed protein bands 4 and 5 (Fig. 2, d and e) to contain nucleophosmin. As demonstrated by the MALDI-MS analysis of tryptic peptides, the five peptides identified in these bands correspond to nucleophosmin, and two of them (268-273, 278-291) belong to the C-terminal domain, which is present in isoform B23.1 only (Table 2). Unfortunately, these data were insufficient for concluding about the presence or absence of isoform B23.2, because its structure was completely identical to the structure of isoform B23.1 in the polypeptide chain region 1-257 and differed only in the presence of two additional amino acid residues (AH) in the C-terminal domain. It is also impossible to identify nucleophosmin isoforms based on their electrophoretic mobility only, because the appearance of band doublet might be due to posttranslational modifications, i.e. deletions. Antibodies that would specifically reveal isoforms are absent as well. Hence, it was obviously necessary to develop a strategy for identification of B23 structural forms by means of their direct structural analysis. Theoretically, it is possible to identify the isoforms by analyzing their C-terminal amino acid sequences as the isoforms have different C-terminal domains (Table 3): ...288WQWRKSL294 (B23.1) and ...253ASIEKAH259 (B23.2) [44, 45]. A

**Table 2.** Mass spectrometric analysis of nucleophosmin tryptic hydrolysate

| Identified peptides | Peptide molecular weight, Da | | Position in polypeptide chain [44] |
|---|---|---|---|
| | experiment | theory | |
| VDNDENEHQLSLR | 1568.76 | 1567.72 | 33-45 |
| DELHIVEAEAMNYEGSPIK | 2145.05 | 2144.01 | 55-73 |
| MSVQPTVSLGGFEITPPVVLR | 2227.21 | 2226.21 | 81-101 |
| MSVQPTVSLGGFEITPPVVLR | 2243.24 | 2242.20 | 81-101 |
| FINYVK | 783.33 | 782.43 | 268-273 |
| MTDQEAIQDLWQWR | 1819.88 | 1818.84 | 278-291 |
| MTDQEAIQDLWQWR | 1835.89 | 1834.83 | 278-291 |

**Table 3.** Structural analysis of nucleophosmin isoforms

| Isoform | N- and C-terminal amino acid sequences of nucleophosmin | Source |
|---|---|---|
| B23.1 | 1 12 285 294<br>M E D S M D M D M S P L.....Q D L W Q W R K S L | [44, 45] |
| B23.2 | 1 12 250 259<br>M E D S M D M D M S P L.....K M Q A S I E K A H | [45] |
| B23.1* | S P L R Q N Y L F G.....(Q D L W Q W)R K S L<br>→→→→→→→→→→ ←←←← | |
| B23.2** | S P L R Q N Y L F G.....(K M Q A S I E)KA H<br>→→→→→→→→→→ ←←← | |

* Bands 4 and 5 (Fig. 2, d and e).
** Band 5 (Fig. 2, d and e).

series of analytical experiments with variations in the set of carboxypeptidases (A, B, Y), pH of the buffers (5.6-8.3), and time of hydrolysis (1-4 h) proved it optimal to use cp A under conditions corresponding to the minimal cleavage of dicarboxylic acids (0.2 M N-ethylmorpholine acetate buffer, pH 8.3, 2 h). Under these conditions, the amino acids L > S, K were detected in band 4 (Fig. 2e, lane *4*) and the amino acids L, S, K > H, A were detected in band 5. Analysis of the set of cleaved amino acids and their comparison with the known structures of C-terminal domains of isoforms B23.1 and B23.2 in humans [44] and rats [45] made it possible to identify isoform B23.1 in band 4 and both isoforms in band 5 (Table 3).

It should be noted that the characteristics of the N-terminal domain of B23 in the normal and tumor cells are of particular interest. The authors of [27] believe that its truncation results in appearance in hepatocellular carcinoma patients of new structural protein forms strongly tending to form oligomers that can be seen under SDS-PAGE and absent in normal tissues. Our and other authors' attempts of protein sequencing (before and after deformylation) have failed. We suggest that the N-terminal amino group is probably blocked by acetyl protection. Interpretation of N-terminal amino acid sequences of proteins, according to the sequencing data, is often complicated by appearance of internal breakages in polypeptide chains during deacetylation. Besides nucleophosmin, we have used control proteins (GAPDH, hnRNP A2/B1) with known N-terminal amino acids sequences. For all proteins, the optimal deacetylation conditions (not resulting in internal breakages of polypeptide chains) were as described in [36]. Eleven cycles of degradation on deacetylated B23 (bands 4 and 5 in Fig. 2e, lane *4*) resulted in detection of a single amino acid sequence corresponding to a polypeptide chain region SPLRPQNYLFG (10-20) (Table 3). Truncation of the N-terminal domain by nine amino acid residues is typical of isoforms B23.1 and B23.2 present in these bands. Thus, for the first time direct structural analysis has shown the truncation of the N-terminal domain of nucleophosmin in HeLa cells, like in cells of hepatocellular carcinoma HepG2 [27]. For the first time it has been established that the acetylated residue is serine – the most frequently acetylated N-terminal amino acid residue of proteins. Yet we cannot ascertain that the N-terminal amino acid sequence is the same in the whole pool of nucleophosmin in HeLa cells, because only B23 extracted into solution during hypotonic treatment of the nuclei was analyzed; however, some part of the protein is not extracted and remains tightly bound to the nucleolus.

**Study of properties of extracted and nucleus-bound nucleophosmin.** The ability of truncated nucleophosmin forms found in the supernatant and the forms tightly bound to the nucleus to produce SDS-resistant oligomers was revealed in the experiments on estimation of possibility of oligomer stabilization using uni- and bivalent

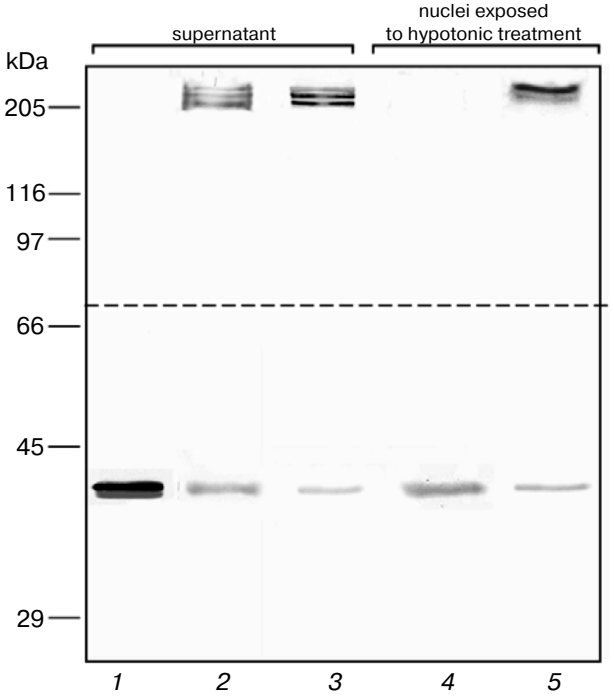


**Fig. 3.** Effect of introduction of Na and Mg ions on the monomer/oligomer state of nucleophosmin from supernatant fraction (*1-3*) and from nuclei exposed to hypotonic treatment (*4*, *5*). Samples were kept in 10 mM Tris-HCl (*1*, *4*), in 10 mM Tris-HCl containing 140 mM NaCl (*2*, *5*) or 7 mM $MgCl_2$ (*3*). Samples were treated for electrophoresis as described in the legend to Fig. 2, d and e (*3*, *4*). SDS-PAGE was performed by the Laemmli method in 7.5% polyacrylamide gel. Proteins were electrotransferred in two stages.

cations. Western-blot analysis (Fig. 3) showed that in the supernatant and nuclear pellet fractions obtained during the treatment with 10 mM Tris-HCl (pH 7.4) (lanes *1* and *4*) nucleophosmin is detected only as monomers. On addition of NaCl up to 140 mM (lanes *2* and *5*) and $MgCl_2$ up to 7 mM (lane *3*) to these fractions, different sets of oligomers are formed. Three and two major bands are detected in the oligomer zone of the supernatant and of the nuclear pellet fraction, respectively. In the supernatant, the most intense band corresponds to the low molecular weight oligomer, while the content of high molecular weight oligomer is significantly less. However, this latter oligomer dominates in the pellet of nuclei exposed to hypotonic treatment.

Thus, it has been shown that nucleophosmin truncated by nine amino acid residues really produces SDS-resistant oligomers, but their set does not correspond to the complete set of oligomers observed in the initial cell lysates. Most probably, the two forms of nucleophosmin (one extracted from the nuclei and the other tightly bound to the nucleoli) are structurally different. This fact is confirmed by MALDI-MS detection of an N-terminal tryptic peptide of nucleophosmin, beginning from Met1,

in the nucleoli of HeLa cells [39]. This will be ascertained by further structural studies of nucleophosmin tightly bound to the nucleolus and not washed out of the nuclei during the treatment with 10 mM Tris-HCl (pH 7.4).

It should be noted that our strategy for the analysis of B23 structural forms in HeLa cells can be used for the same analysis in other tumor cells.

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