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Analysis of Nucleolar Protein Fibrillarin Mobility and Functional State in Living HeLa Cells

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Abstract—Fibrillarin is an evolutionarily-conserved and obligatory protein component of eukaryotic cell nucleoli involved in pre-rRNA processing and methylation. In vertebrates the fibrillarin molecule contains two cysteine residues (Cys99 and Cys268) whose sulfhydryl groups are able to establish intramolecular –S–S– bridges. However, the functional state of fibrillarin with reduced or oxidized thiol groups is still practically unstudied. Besides, there are no data in the literature concerning existence of the –S–S– fibrillarin form in human cells. To answer these questions, we used plasmids encoding native human fibrillarin and its mutant form devoid of cysteine residues (fibrillarinC99/268S) fused with EGFP for temporary transfection of HeLa cells. The mobile fraction localizing the enzymatically active protein molecules and the fluorescence half-recovery time characterizing the rate of enzymatic reactions were determined by the FRAP technique using a confocal laser scanning microscope. Measurements were carried out at 37 and 27°C. The results show that the fibrillarin pool in HeLa cells includes two protein forms, with reduced SH groups and with oxidized SH groups forming intramolecular –S–S– bridges between Cys99 and Cys268. However, the absence of Cys99 and Cys268 has no effect on intracellular localization of fibrillarin and its main dynamic parameters. The human fibrillarin form without disulfide bridges is included into the mobile protein fraction and is consistent with its functionally active state.

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Fibrillarin is an evolutionarily conservative and vital nucleolar protein; human fibrillarin is 94.2% homologous to mouse fibrillarin, 82.9% homologous to amphibian fibrillarin, and 74% homologous to fibrillarin of baker's yeast Nop1p [1]. Fibrillarin homologs are also present in higher plants, the fungus *Physarum polycephalum*, and Archaebacteria [2, 3]. Fibrillarin is necessary for viability of pro- and eukaryotic cells [4, 5] and is an obligatory component of small nucleolar RNP (sncRNP) of C/D type—U3, U8, U13, and U16 [6-8].

Three structural domains are distinguished in human fibrillarin (321 amino acid residues (a.a.), p*I* 9.5) (Fig. 1) [1]. The N-terminal part of the molecule (a.a. 8-77) is formed by the GAR domain that is rich in glycine and arginine residues. The GAR domain contains the signal sequence providing for fibrillarin migration into nucleoli, is methylated at arginine residues, and is responsible for interaction with different proteins including factors of rRNA processing. The RNA-binding domain, characteristic of many proteins interacting with RNA and necessary for fibrillarin localization in nucleoli, is localized in the central part of the molecule (a.a. 133-222). A short α-helical domain (a.a. 274-306) is localized in the C-terminal region. There are two interdomain spacer regions whose roles are still poorly studied. The use of constructs encoding mutant fibrillarin forms fused with enhanced green fluorescent protein (EGFP) has shown that the absence of the first spacer does not prevent fibrillarin migration into nucleoli, which is indirect evidence against its functional significance. The second spacer is evidently responsible for fibrillarin localization in the pre-rRNA processing sites topologically associated with rDNA transcription foci [9, 10].

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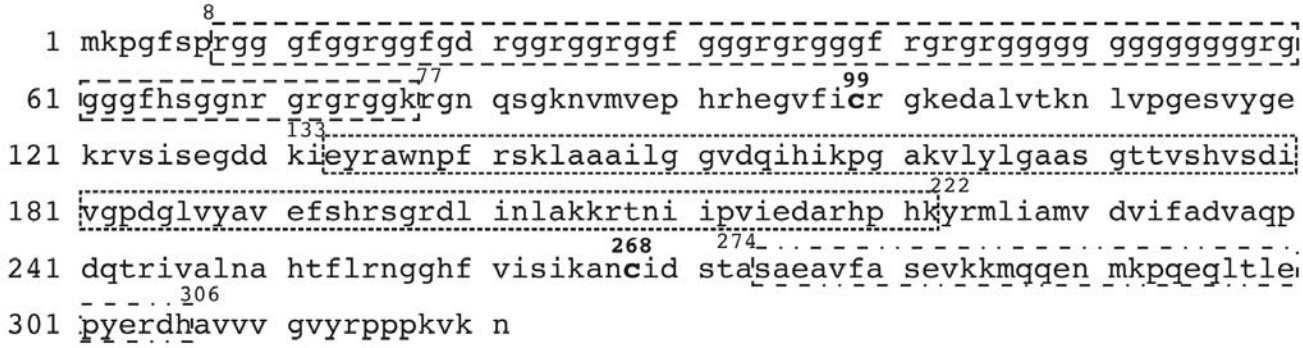

Fig. 1. Amino acid sequence of human fibrillarin. Three structural domains are distinguished in the molecule: glycine-arginine-rich domain (GAR domain) (a.a. 8-77), RNA-binding domain (a.a. 133-222), and α-helical domain (a.a. 274-306). The domains are separated by the first and second spacers containing Cys99 and Cys268, respectively. A methyl transferase domain supposedly includes the RNA-binding domain, the second spacer, and the α-helical domain.

Analysis of the crystal structure of fibrillarin of the Archaebacterium *Methanococcus jannaschii* has shown that its C-terminal sequence is analogous to that of catalytic domain of many S-adenosyl-L-methionine (AdoMet)-dependent methyl transferases [11-13]. In baker's yeast mutation in Nop1 protein (NOP1) completely blocked ribose methylation within rRNA [4]. These observations suggest that in other organisms fibrillarin is also the site-specific 2′-O-methyl transferase catalyzing methyl group transfer to different nucleophilic substrates, mainly nucleolar rRNA. It is believed that the methyl transferase domain in human fibrillarin includes the RNA-binding domain, the second spacer, and the α-helical domain [11] (Fig. 1). Another known function of fibrillarin is its involvement in processing of primary rRNA transcripts, which was convincingly shown using yeast mutants. Mutations in the *Nop1* gene disturbed normal rRNA maturation, especially processing of 5′-external transcribed spacer [4]. So far no knockdown of the fibrillarin gene in somatic cells of higher eukaryotes has been carried out. Also, no approaches have been elaborated for estimation of the intracellular functional state of fibrillarin *in vitro*.

A feature of fibrillarin primary structure in all vertebrates is the presence of two cysteine residues (Cys) with strictly fixed position. Thus, Cys99 and Cys268 present in human fibrillarin coincide by their position with cysteine residues in mouse, rat, and clawed frog fibrillarin. In humans Cys99 is incorporated into the first spacer, while Cys268 is localized in the second spacer and, respectively, in the methyl transferase domain (Fig. 1). Analysis of the electrophoretic mobility of *in vitro* translated human fibrillarin in the absence of reducing agent (2-mercaptoethanol) has shown that it migrates in the form of a duplet corresponding to two forms, with reduced SH groups of cysteine residues (~34 kDa) and with closed intramolecular –S–S– bond (~32 kDa) [1]. Similar results were obtained during investigation of mobility of fibrillarin of nuclear fraction of rat hepatocytes and total cell lysates of mouse macrophages [14]. However, the functional state of the two fibrillarin forms is still unstudied, and their presence in human cells has not been analyzed. In mice the fibrillarin form with reduced thiol groups is dominant [14].

The problem of intramolecular interactions between SH groups of cysteine residues in mammalian fibrillarin is of principal significance not only for understanding their role in protein functioning, but also for elucidation of mechanisms of the increased sensitivity of fibrillarin to heavy metal ions, especially to mercury [15]. It has been supposed that Hg(II) binding to thiol groups stabilizes intramolecular disulfide bonds, which results in alteration of three-dimensional structure of fibrillarin, stimulates its cleavage by proteasomes, and induces generation of cryptic antigens initiating autoimmune reactions [15-17]. It is also well known that sulfhydryls are incorporated in active centers of many enzymes, and their blocking results in decrease or elimination of catalytic activities [18-20]. Since fibrillarin is a methyl transferase and its supposed methyl transferase domain contains Cys268 (Fig. 1), it is reasonable to suppose that the absence of cysteine residues would affect the main properties of the protein in living cells.

The main approach to *in vivo* analysis of proteins, including fibrillarin, is expression of their genes fused with genes of fluorescent marker proteins such as EGFP [21, 22]. In combination with confocal laser scanning microscopy and FRAP (fluorescence recovery after photobleaching), this approach makes it possible to study the localization and dynamic properties of the protein of interest in living cells [23, 24]. It is supposed that the mobile fibrillarin fraction is formed by the protein carrying out the enzymatic function, while stationary or low-mobility fraction corresponds to protein exhibiting structural function [9]. However, there have been no attempts to check these hypotheses in experiments. The FRAP

technique has shown that fibrillarin is among the fastest-moving nuclear proteins [9, 25, 26].

In this work we have estimated the role of Cys99 and Cys268 in specific localization of human fibrillarin in HeLa cells and the dynamic properties of the protein, including the mobile fraction and the fluorescence half-recovery time. To do this, we used plasmids encoding native human fibrillarin and its mutant form in which both cysteine residues were replaced by serine using site-specific mutagenesis (fibrillarinC99/268S-EGFP) [27]. Taking into account that decreasing temperature lowers the mobility of proteins exhibiting enzyme activity [28], the mobility of the native and mutant fibrillarin was studied both under physiological conditions (37°C) and at a lower temperature (27°C). For control, cells were transfected by plasmid encoding EGFP, which has no function in vertebrates and moves within cells due to free diffusion [25].

MATERIALS AND METHODS

HeLa cells were grown at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, glutamine, and antibiotics. The cells were transfected when they covered about 80% of the substrate surface and were in exponential growth phase. Lipofectamin™ 2000 reagent (Invitrogen, Great Britain) was used for transfection according to the manufacturer's recommendations.

Antibodies. Rabbit polyclonal antibodies to fibrillarin (Abcam, USA) and GFP (Abcam) were used as primary antibodies; antibodies to mouse and rabbit immunoglobulins conjugated with Texas red (Jackson Immunoresearch Lab., USA) or horseradish peroxidase (Sigma, USA) were used as second antibodies. For immunocytochemical staining, the antibodies were diluted in phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4); immunoblotting was carried out in TBS-T buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.6) containing 0.5% dry skim milk. Antibodies were used in optimal working concentrations.

Indirect immunocytochemistry. Cells were fixed with 3% para-formaldehyde in PBS for 20 min at room temperature and treated with 0.5% Triton X-100 for 10 min on ice. The cells were thoroughly washed with PBS and incubated with primary antibodies for 1 h at room temperature in a humid chamber. After washing with PBS, the cells were incubated with second antibodies for 45 min at room temperature, stained with 0.1 μg/ml DAPI (Sigma), and embedded in Moviol containing DABCO (1,4-diazobicyclo[2,2,2]octane) (Sigma). The preparations were studied in an Axiovert 200 epifluorescence microscope (Carl Zeiss, Germany) and LSM510 DuoScanMETA laser scanning microscope (Carl Zeiss) using 63×/NA 1.25 Plan-Neofluar objectives. Images were processed using the LSM510 DuoScanMETA program package of the microscope, as well as Adobe Photoshop CS (version 10.0) and Microsoft Office Excel software.

Immunoblotting. For electrophoresis under reducing conditions cells were lysed in standard Laemmli buffer (50 mM Tris-HCl, pH 6.9, 10% glycerol, 1.4% 2-mercaptoethanol (ME), 2% SDS, 0.01% bromphenol blue); for non-reducing conditions mercaptoethanol was not added to the Laemmli buffer [14]. Total protein concentration was determined according to Lowry using the Protein Assay Kit (Sigma) following the manufacturer's recommendations. For each sample about 100 μg protein was applied onto the lane and proteins were separated by electrophoresis in 12% SDS-polyacrylamide gel. After electroblotting in 50 mM Tris-HCl, pH 8.3-8.5, 40 mM glycine, 0.1% SDS, and 20% methanol, the nitrocellulose membrane was incubated with anti-fibrillarin or GFP antibodies, and then with appropriate antibodies to rabbit immunoglobulins conjugated with horseradish peroxidase. Immunoblots were developed using the ECL+Plus Detection Kit (AmershamPharmaciaBiotech, Great Britain) and X-ray film HyperFilm ECL (AmershamPharmaciaBiotech) according to manufacturer's recommendations.

Conditions of vital observations and FRAP technique. Protein mobility was measured by the FRAP technique in the LSM510 DuoScanMETA confocal laser scanning microscope (Carl Zeiss) equipped with an incubator for vital observations (Incubator XL LSM S; Carl Zeiss), heating element, and thermocontroller which maintained the required temperature. HeLa cells were plated for vital observations, and 24 h later they were transfected by plasmids encoding native fibrillarin (fibrillarin-EGFP), mutant fibrillarin (fibrillarinC99/268S-EGFP), or EGFP. Mobility of chimeric proteins was measured 36 h after transfection at pH 7.2-7.3 and at two temperatures (37 and 27°C). Conditions were identical for FRAP studies in cells transfected by different plasmids.

Before photobleaching, the whole nucleus was scanned 2-3 times using an argon laser (488 nm, 200 mW) and low (0.5%) irradiation power. Photobleaching of a chosen 0.9 μm^2 region, corresponding to the average size of a fibrillarin-positive focus in the nucleolus, was carried out for 100 msec at 50% laser power. It is known that under such conditions in the region of photobleaching, proteins are not exhausted and damaged and only fluorescence quenching of EGFP is achieved [5, 29]. Fluorescence recovery was registered at 0.5% laser power for 3 min, the interval between scanning operations being 2 sec for fibrillarin-EGFP and 100 msec for EGFP. The bleaching of the object during scanning did not exceed 6%.

To determine the mobile fraction and the fluorescence half-recovery time ($t_{1/2}$), the data were first normalized using the generally accepted formula: $I_{norm,t} = (I_t -$

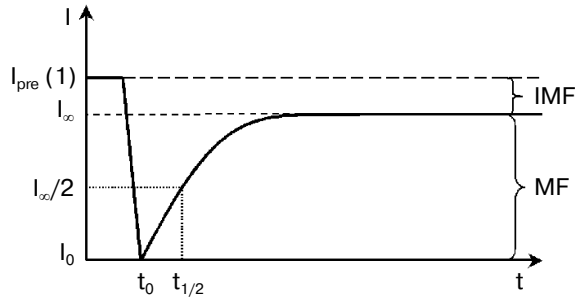

Fig. 2. Curve of fluorescence recovery after photobleaching that illustrates the principle of calculation of protein fractions and the fluorescence half-recovery time determined by the FRAP technique. The level of fluorescence in the ROI (Region Of Interest) before photobleaching (I_{pre}) is taken as 1. Immediately after photobleaching (t_0), the fluorescence intensity in the ROI is taken as zero (I_0). I_∞ is maximal fluorescence value in the ROI after photobleaching, $t_{1/2}$ is the fluorescence half-recovery time corresponding to $I_\infty/2$. MF is mobile fraction; IMF is immobile fraction.

$I_0)/(I_{pre} - I_0)$, where I_t is the fluorescence level at every moment; I_0 is fluorescence level immediately after photobleaching; I_{pre} is fluorescence level before photobleaching [24] (Fig. 2). The mobile fraction is expressed on a percentage basis. The immobile fraction was calculated using the formula: $(1 - I_\infty) \times 100\%$, where I_∞ is the fluorescence level at the plateau.

No less than 10 measurements were carried out for each experimental point (table). Differences between values were estimated using Student's criterion.

Plasmid constructs. The pEGFP vector encoded EGFP (Enhanced Green Fluorescent Protein). Vector pFibr-EGFP encoding native human fibrillarin fused with EGFP (fibrillarin-EGFP) was constructed on the basis of vector pEGFP-N3 (Invitrogen, USA). Both vectors were the courtesy of Dr. M. O. Olson (University of Mississippi, USA). Plasmid pFibrSS-EGFP encoding a mutant form of human fibrillarin devoid of cysteine residues (fibrillarinC99/268S-EGFP) was constructed by us earlier [27].

RESULTS

Localization and electrophoretic mobility of native fibrillarin in HeLa cells. In nucleoli of untransfected HeLa cells endogenous fibrillarin, revealed by specific antibodies, is localized in discrete foci corresponding to pre-rRNA processing sites that are structurally connected with rDNA transcription sites (Fig. 3a) [30]. On

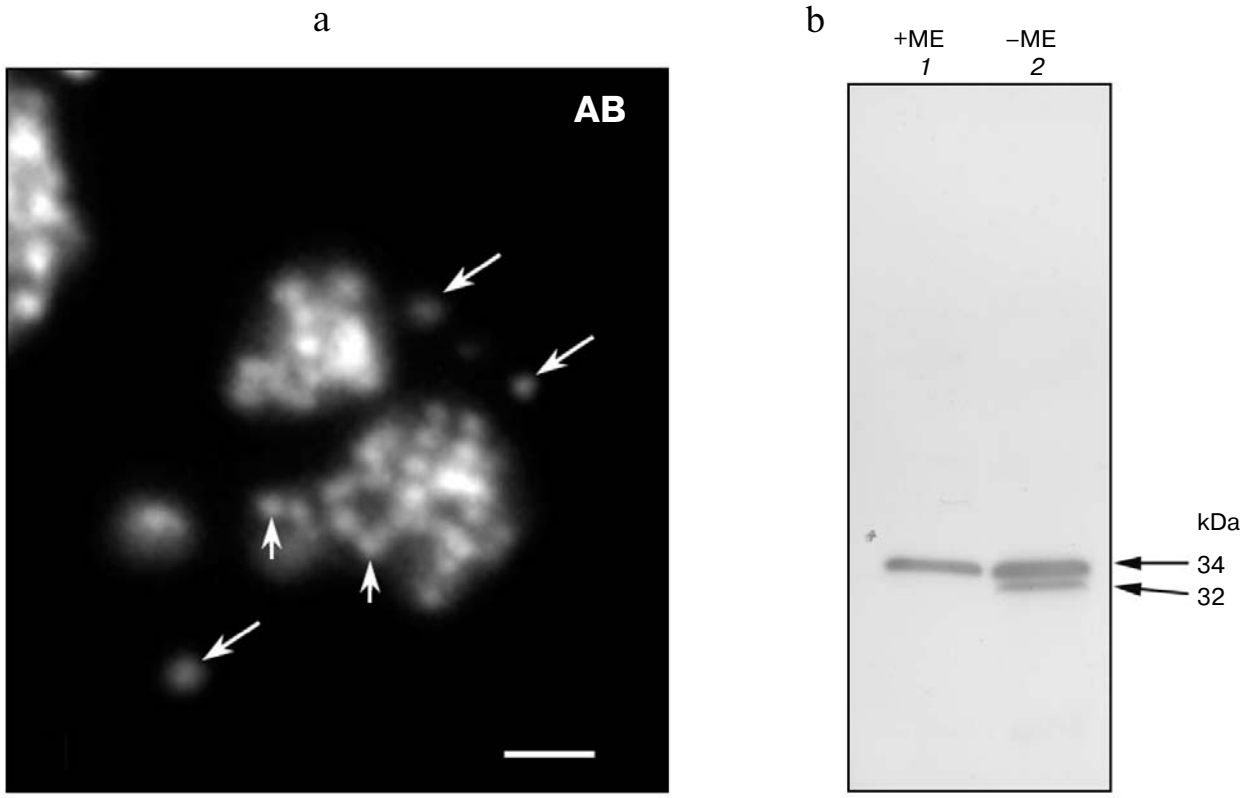

Fig. 3. Localization and electrophoretic mobility of endogenous fibrillarin. a) Immunocytochemical staining by antibodies (AB) to fibrillarin. Endogenous fibrillarin is revealed as discrete foci in the nucleolus (short arrows) and Kahal bodies (long arrows). b) Fibrillarin electrophoretic mobility in the presence of reducing agent 2-mercaptoethanol (ME) (lane *1*) and its absence (lane *2*). Scale 1 μm.

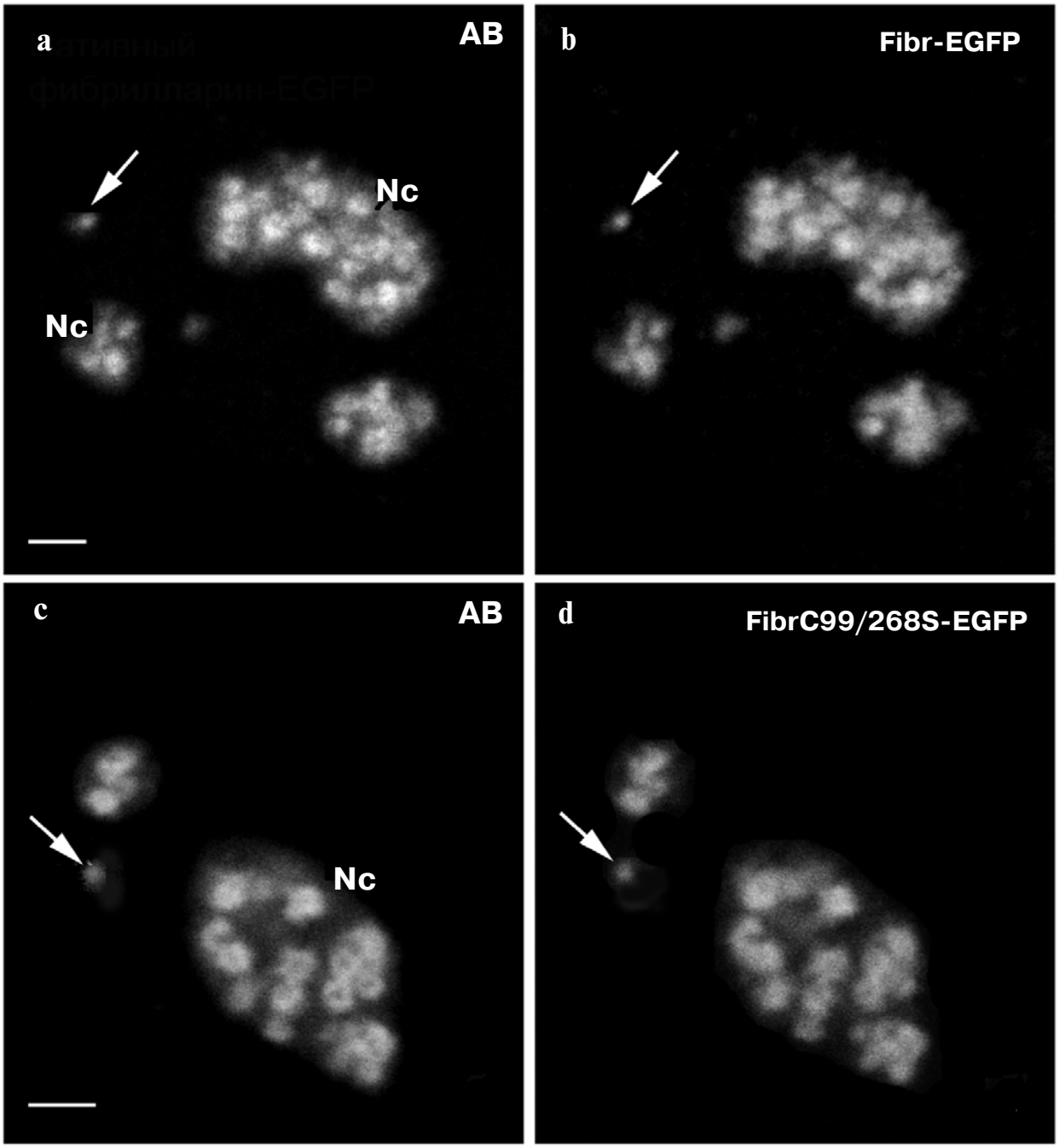

Fig. 4. Co-localization of ectopic wild-type fibrillarin-EGFP (b) and its mutant variant (fibrillarinC99/268S-EGFP) (d) with endogenous fibrillarin (a, c) in HeLa cell nuclei. Nucleoli in the same nucleus are shown for each cell. a, c) Immunocytochemical staining by antibodies (AB) to fibrillarin. b, d) Localization of chimeric protein. Nc, nucleoli; arrows point to Kahal bodies. Confocal laser scanning microscopy. Scale 1 μm.

immunoblots under conditions of reducing electrophoresis (in the presence of 2-mercaptoethanol) fibrillarin was revealed as a single band in the region of 34 kDa (Fig. 3b, lane *1*). Under conditions of non-reducing electrophoresis there are two distinct bands corresponding to fibrillarin with reduced (~34 kDa) and oxidized (~32 kDa) thiol groups (Fig. 3b, lane *2*).

In 36 h after transfection by the plasmid encoding native fibrillarin, no less than 80% of the HeLa cells expressed fibrillarin-EGFP. Fibrillarin-EGFP was detected in nucleoli and Kahal bodies, where it was colocalized with endogenous fibrillarin (Fig. 4, a and b). These observations show that fusion of fibrillarin with EGFP does not change its specific intracellular localization.

Electrophoresis of total lysates of HeLa cells transfected by the fibrillarin-EGFP plasmid was carried out under reducing and non-reducing conditions. The lanes were revealed by antibodies to fibrillarin or GFP. Under reducing conditions endogenous fibrillarin migrated as a single band in the region of 34 kDa, while fibrillarin-EGFP moved in the region of 62 kDa (Fig. 5, lane *1*), which corresponds to total molecular mass of fibrillarin (34 kDa) and EGFP (27 kDa) proteins.

In electrophoresis without mercaptoethanol both endogenous fibrillarin and fibrillarin-EGFP were revealed as two bands. The position of bands corresponding to endogenous fibrillarin did not differ from that in untransfected cells (Fig. 5, lane *2*, and Fig. 3, lane *2*). Fibrillarin-EGFP was revealed as a duplet of minor (upper) band with probable mass of 66-68 kDa and major (lower) band in the region of 62 kDa (Fig. 5, lane *2*). Taking into account that under reducing conditions the upper band is absent (Fig. 5, lane *1*), it can be supposed that it corresponds to the fibrillarin fraction with intramolecular –S–S– bridges. The lower band, independent of mercaptoethanol, is represents the protein fraction with reduced thiol groups.

Thus, the above-presented results show that fibrillarin is present in HeLa cells in two forms, with SH groups and with intramolecular –S–S– bridges and that chimeric protein fibrillarin-EGFP is able to form intramolecular –S–S– bonds similarly to endogenous fibrillarin.

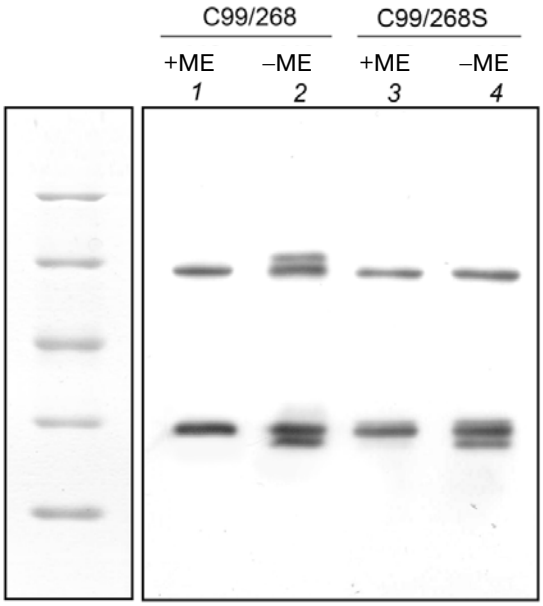

Fig. 5. Electrophoretic mobility of endogenous fibrillarin (lanes *1-4*, at the bottom), native fibrillarin-EGFP (lanes *1* and *2*, at the top), and mutant fibrillarinC99/268S-EGFP (lanes *3* and *4*, at the top) in the presence of 2-mercaptoethanol (ME) (lanes *1* and *3*) (reducing conditions) and without 2-mercaptoethanol (lanes *2* and *4*) (non-reducing conditions). Marker positions are shown to the left (from top to bottom): phosphorylase B (97 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carboanhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa).

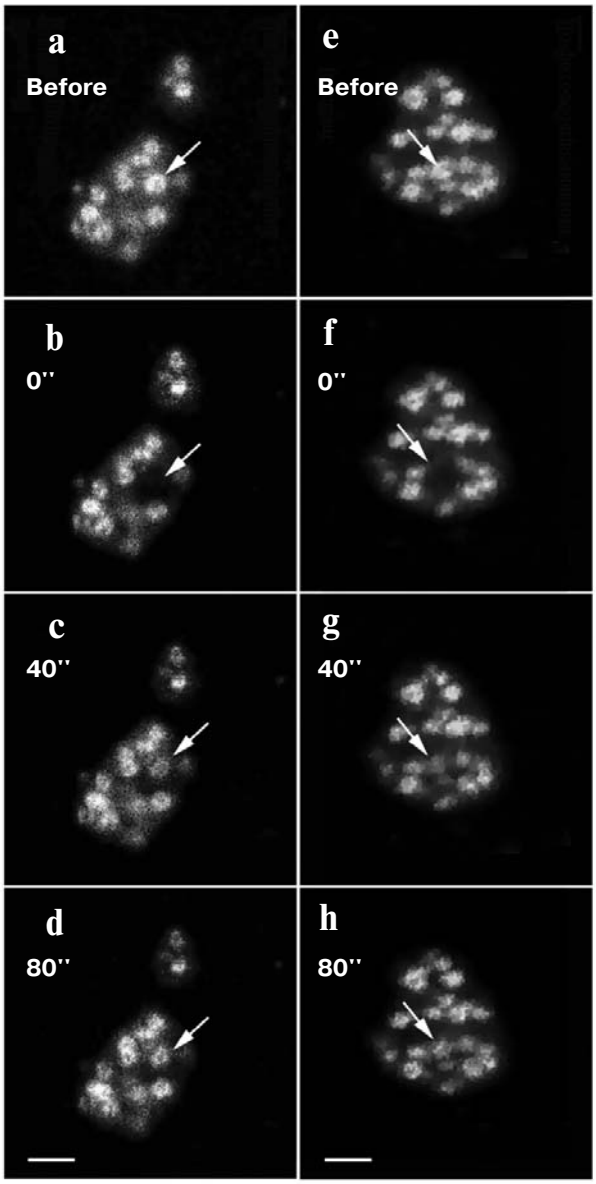

Fig. 6. Mobility of native fibrillarin-EGFP (a-d) and mutant fibrillarinC99/C268S-EGFP (e-h) revealed by the FRAP technique in HeLa cells. Nucleoli in the same nucleus are shown for each cell. In each nucleus one nucleolar focus of fibrillarin (area about 0.9 μm^2) was photobleached (b, f); fluorescence intensity in 40 sec (c, g) and in 80 sec (d, h) after photobleaching. Scale 1 μm.

Localization and electrophoretic mobility of mutant fibrillarinC99/268S-EGFP in HeLa cells. The level of HeLa cell transfection by the plasmid encoding the mutant fibrillarinC99/268S-EGFP was 70-80%. Transfection by this plasmid had no noticeable effect on the cell viability and proliferative activity.

Analysis of fibrillarinC99/268S-EGFP by confocal laser microscopy showed unique sites of the mutant pro-

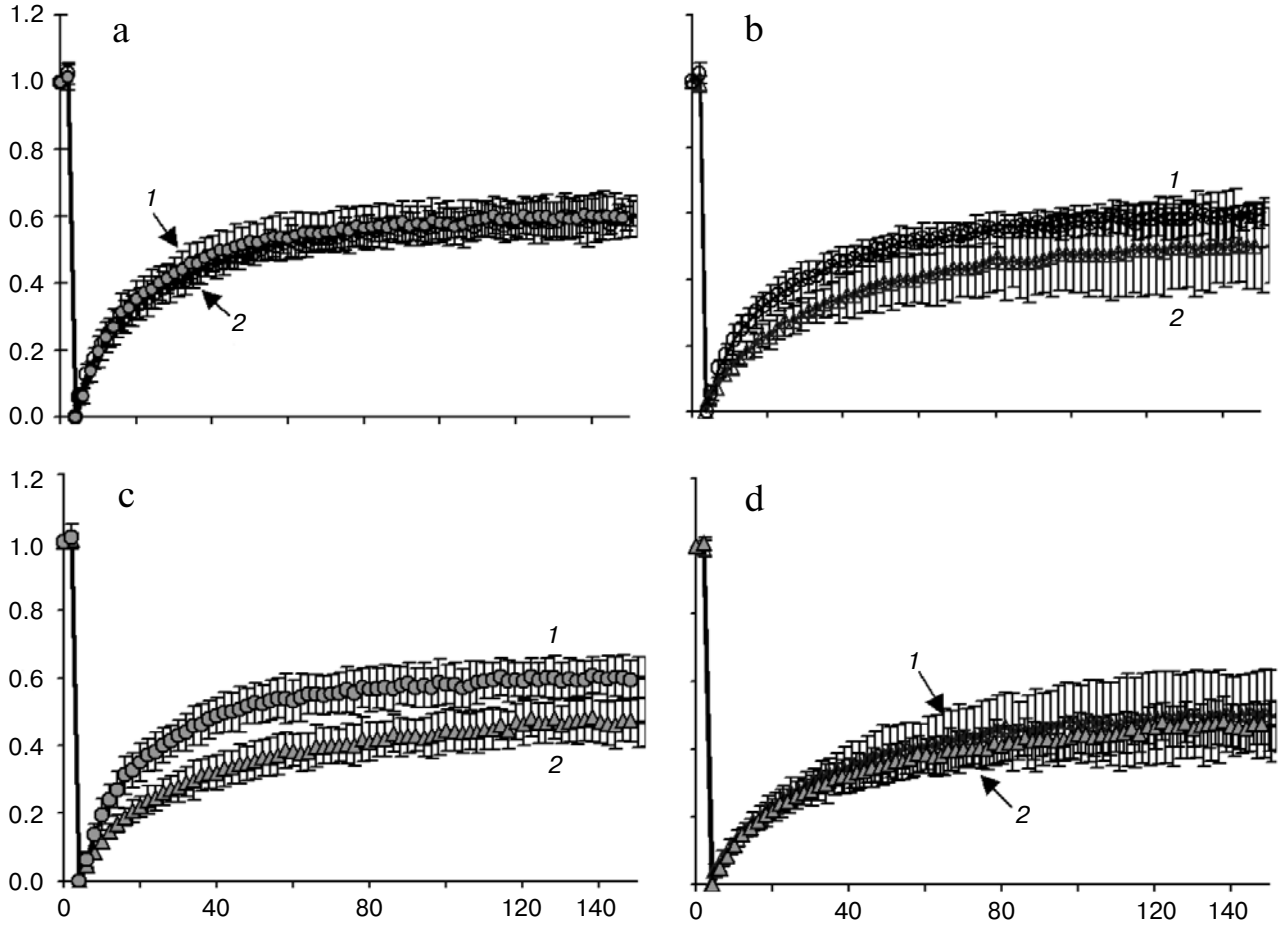

Fig. 7. Comparison of dynamic properties of native fibrillarin-EGFP and mutant fibrillarinC99/268S-EGFP at different temperatures. a) Recovery of fibrillarin-EGFP (*1*) and fibrillarinC99/268S-EGFP (*2*) fluorescence at 37°C. b) Recovery of fibrillarin-EGFP fluorescence at 37°C (*1*) and at 27°C (*2*). c) Recovery of fibrillarinC99/268S-EGFP fluorescence at 37°C (*1*) and at 27°C (*2*). d) Recovery of fibrillarin-EGFP (*1*) and fibrillarinC99/268S-EGFP (*2*) fluorescence at 27°C. Horizontal axis shows time in seconds, vertical axis shows fluorescence intensity; initial level of fluorescence is taken as 1. Vertical dashes designate root-mean-square deviation in each time point corresponding to 2 sec scanning interval. The number of measurements for different experimental conditions is shown in the table.

tein localization in nucleoli and Kahal bodies, where it is completely co-localized with endogenous fibrillarin (Fig. 4, c and d). On immunoblots of total lysates of cells transfected by fibrillarinC99/268S-EGFP plasmid, in the presence of ME fibrillarin is revealed in the form of two bands in the region of 34 and 62 kDa (Fig. 5, lane *3*). The lower band corresponds to reduced (with SH groups) form of endogenous fibrillarin, and the upper corresponds to the mutant fibrillarin-EGFP. Also, in the absence of ME there also appeared the endogenous fibrillarin fraction with disulfide bridges (~32 kDa), but fibrillarinC99/268S-EGFP continued to migrate in the form of a single band (~62 kDa) corresponding to its reduced form (Fig. 5, lane *4*). These results confirm that no –S–S– bonds are formed in the fibrillarin molecule devoid of cysteine residues.

Mobility of native fibrillarin-EGFP in living HeLa cells at different temperatures. Typical examples of the fibrillarin-EGFP fluorescence recovery are shown in Figs. 6 (a-d) and 7a, curve *1*. Calculations show that the fibrillarin-EGFP mobile fraction in nucleoli is 60 ± 4% and that of the immobile fraction is 40 ± 4%. The half-recovery time of fibrillarin-EGFP fluorescence level is 16 sec (table).

Incubation of cells for 1.5-2.5 h at 27°C caused no major changes in fibrillarin localization, only enlargement of separate fibrillarin-positive foci in nucleoli being observed (data not shown). However, the dynamic properties of fibrillarin-EGFP at 27°C noticeably changed compared to the analogous parameters at physiological temperature. As seen in Fig. 7b and the table, the chimeric protein mobile fraction decreases from 60 ± 4 to 51 ± 8% and the fluorescence half-recovery time increases from 16 to 22 sec. Comparison of distinctions by Student's criterion showed that they are statistically reliable ($p < 0.01$).

Dynamic parameters of native (C99/268) and mutant (C99/268S) fibrillarin-EGFP in HeLa cells

	Native fibrillarin		Mutant (C99/268S) fibrillarin		pEGFP	
T, °C	37	27	37	27	37	27
Mobile fraction, %	60 ± 4**	51 ± 8**	61 ± 5***	48 ± 5***	100	100
Immobile fraction, %	40 ± 4	49 ± 8	39 ± 5	52 ± 5	0	0
$t_{1/2}$, sec*	16	22	16	24	$60 \cdot 10^{-3}$	$60 \cdot 10^{-3}$
Number of measurements	11	12	10	11	5	5

* $t_{1/2}$, fluorescence half-recovery time after photobleaching (in seconds).
** Pairs of values that differ by Student's criterion with reliability $p < 0.01$.
*** Pairs of values that differ by Student's criterion with reliability $p < 0.001$.

Mobility of mutant fibrillarinC99/268S-EGFP in living HeLa cells at different temperatures. Dynamic characteristics of the mutant fibrillarin-EGFP were measured under the same conditions as for native fibrillarin-EGFP. According to visual estimation, the mobility of the mutant fibrillarinC99/268S-EGFP was similar to that of native fibrillarin-EGFP (Fig. 6, e-h). Calculations showed that the portion of fibrillarinC99/268S-EGFP mobile fraction was 61 ± 5% and the fluorescence half-recovery time was 16 sec, which did not differ from $t_{1/2}$ of native fibrillarin-EGFP (16 sec) (Fig. 7a, curve *2*, and the table). These results show that replacement of cysteine residues by serine in the fibrillarin molecule had practically no effect on the dynamic properties of the protein.

Temperature decrease did not noticeably alter localization of mutant fibrillarin. Measurements of fibrillarinC99/268S-EGFP at 27°C showed that the mutant protein reacts to the temperature decrease similarly to the native fibrillarin (Fig. 7c, curve *2*): the mobile fraction decreases (from 61 ± 5 to 48 ± 5%) and the fluorescence half-recovery time increases from 16 to 24 sec (Fig. 7c and the table). For clarity, Fig. 7d shows curves of fluorescence recovery for native (curve *1*) and mutant (curve *2*) fibrillarin. It is seen that, like at 37°C (Fig. 7a), these curves practically coincide.

Analysis of EGFP mobility in living HeLa cells. It is known that decrease in temperature can influence free diffusion of proteins in living cells [25, 28]. To clarify the free diffusion contribution to fibrillarin mobility, HeLa cells were transfected by the EGFP-encoding plasmid. Observations were carried out 24 h after transfection. It is seen in Fig. 8a that EGFP is practically uniformly distributed within the nucleus and cytoplasm and is present only in trace amounts in nucleoli. The standard region in the nucleus underwent photobleaching (Fig. 8a). Taking into account high intracellular mobility of pEGFP [25], the scanning interval after photobleaching was decreased to 10 msec. As seen in Fig. 8b, the EGFP fluorescence half-recovery time was ~60 msec both at 37 and at 27°C (Fig. 8b and the table). Thus, incubation of HeLa cells at 27°C did not change the dynamic properties of EGFP.

DISCUSSION

A characteristic feature of fibrillarin in vertebrates is its high conservatism and the presence of two cysteine residues at fixed position (Fig. 1). Thiol groups in Cys99 and Cys268 of mouse and human fibrillarin, able to establish intramolecular –S–S– bridges, play the key role in maintenance of their three-dimensional structure [19, 20] and are responsible for increased binding by fibrillarin of heavy metal ions including Hg(II) [31-33]. However, the problem of *in vivo* existence of –S–S– bonds is poorly studied, and the situation in human cells was not previously analyzed.

Results of this work show that in HeLa cells endogenous fibrillarin exists in two forms, with reduced and oxidized cysteine thiol groups capable of formation of intramolecular –S–S– bonds. Fusion of native fibrillarin with EGFP had no effect on its ability to form both the oxidized and reduced forms. In combination with data of the literature [1, 14], these observations indicate that the "reduced" and "oxidized" fibrillarin forms are characteristic of different mammalian cell types, including human tumor cells.

As is known, two cysteine residues are present in the EGFP molecule [21]. It can be assumed that within the fibrillarin-EGFP chimeric protein, thiol groups of EGFP cysteines establish disulfide bonds with SH groups of fibrillarin cysteines. To test this assumption, total lysates were prepared from HeLa cells transfected by plasmids encoding mutant fibrillarin-EGFP with single serine substitutions for Cys99 and Cys268 [27]. However, elec-

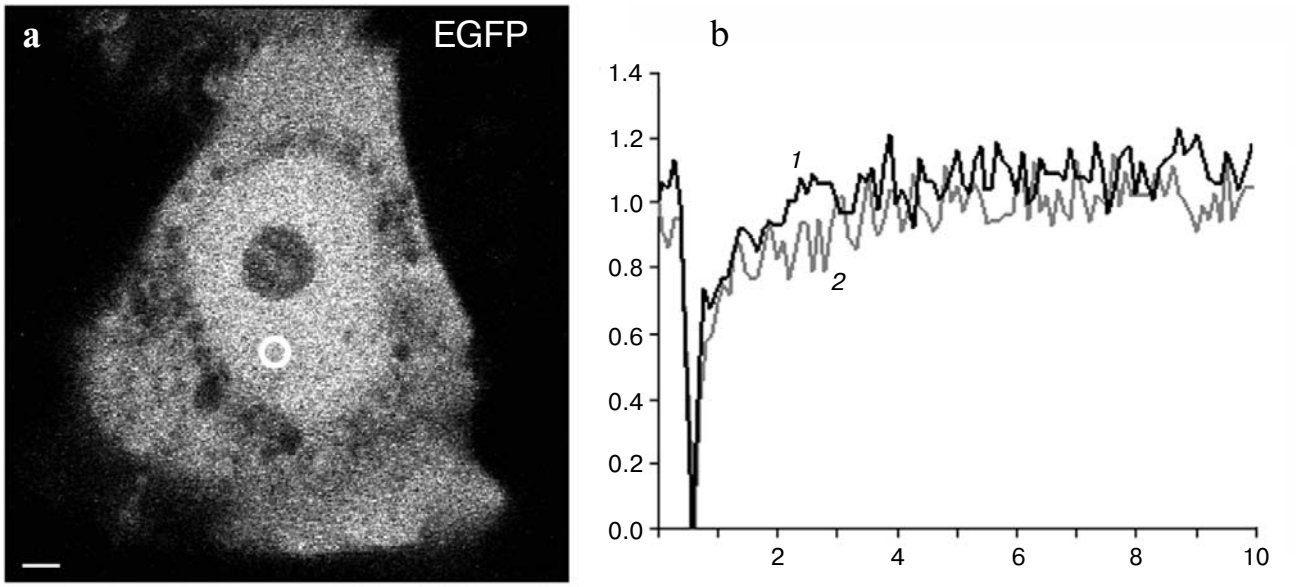

Fig. 8. EGFP localization (a) and mobility in HeLa cells at 37 and 27°C (b). a) A small circle marks photobleaching of a standard nuclear region. b) Recovery of EGFP fluorescence at 37 (*1*) and at 27°C (*2*). Horizontal axis shows time in seconds, vertical axis shows fluorescence intensity. Scanning interval is 100 msec.

trophoresis without mercaptoethanol revealed in both cases only a single band corresponding to the reduced form of the chimeric protein (our unpublished data). Together with results of this work these observations show that in native fibrillarin-EGFP no disulfide bridges are formed between cysteine residues of fibrillarin and EGFP.

Recent mutational analysis of human fibrillarin has shown that deletions or inversions in different regions of the protein molecule prevent its specific localization in nuclei [9, 10]. Unlike these mutant forms, fibrillarinC99/268S-EGFP was completely co-localized with endogenous fibrillarin: in nucleoli it was localized in discrete foci corresponding to the pre-rRNA processing sites and in nuclei in Kahal bodies (Fig. 4). These observations show that cysteine residues no more than slightly influence the specific localization of fibrillarin in living HeLa cells.

To analyze the functional state of native and mutant fibrillarin, we studied their dynamic properties in living cells at two temperatures, 37 and 27°C. In this case two main parameters were compared: mobile fraction which, according to data from the literature, corresponds to the portion of rapidly metabolized (enzymatically active) protein molecules, and the time of fluorescence half-recovery after photobleaching ($t_{1/2}$) that correlates with the rate of reactions involving enzymes [25, 28]. Since up to the present time there is no unique formula for calculation of diffusion coefficient (Deff) for non-membrane proteins, and Deff of human fibrillarin, cited by different authors, differs by more than an order of magnitude [9, 25], migration rates for native and mutant fibrillarin were compared by $t_{1/2}$.

Analysis of dynamic parameters of native and mutant fibrillarin (fused with EGFP) in HeLa cells at physiological temperature (37°C) showed that both forms contain mobile and immobile fractions. In both cases the mobile fraction was dominant, being about 60% of the total protein pool (table).

Temperature decrease from 37 to 27°C reduces the mobile fraction in both native (from 60 ± 4 to 51 ± 8%) and mutant (from 61 ± 5 to 48 ± 5%) fibrillarin forms. In both cases there is a significant increase in $t_{1/2}$ (from 16 to 22 sec in native fibrillarin and from 16 to 24 sec in its mutant variant). It is significant that distinctions for each form are statistically reliable, while in the case of mutant fibrillarin-EGFP they reach the third threshold of reliability ($p < 0.001$) by Student's criterion.

Changes in protein mobility at lower temperature were not the result of retardation of their free diffusion rate. This follows from stability of dynamic properties of control EGFP under different temperature conditions (table). Therefore, it can be assumed that Cys99 and Cys268 do not play any immediate role in maintenance of fibrillarin functional activity in living HeLa cells. As was said above, the main known functions of fibrillarin in mammals are rRNA methylation and processing [1, 4, 11].

The increase in the low-mobility fibrillarin fraction upon inhibition of ribosome synthesis by actinomycin D (our unpublished data) shows that this fraction might correspond to the protein fraction playing a structural role. The possibility that fibrillarin can play the role of structural protein is also supported by its presence in inactive nucleoli of erythrocytes of clawed frog and hen [34, 35].

On the whole, results of this work allow us to draw the following conclusions. In human tumor HeLa cells the fibrillarin pool includes molecules with reduced (-SH) and oxidized (–S–S– bridge) thiol groups of Cys99 and Cys268. The absence of cysteine residues does not influence the specific localization and dynamic properties of fibrillarin in living cells. The fibrillarin pool is subdivided by dynamic properties to mobile and immobile (low-mobility) protein fractions. Under physiological conditions the mobile fraction is larger and includes protein molecules devoid of intramolecular disulfide bonds. Results of this work favor the enzymic activity of fibrillarin in living mammalian cells, but they do not confirm the direct involvement of Cys99 and Cys268 in its maintenance.

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REFERENCES

1. Aris, J. P., and Blobel, G. (1991) *Cell Biol.*, **88**, 931-935.
2. Pearson, D. L., Reimonenq, R. D., and Pollard, K. M. (1999) *Protein Expres. Purif.*, **17**, 49-56.
3. Aittaleb, M., Visone, T., Fenley, M. O., and Li, H. (2004) *J. Biol. Chem.*, **279**, 41822-41829.
4. Tollervey, D. (1993) *Cell*, **72**, 443-457.
5. Newton, K., Petfalski, E., Tollervey, D., and Caceres, J. F. (2003) *Mol. Cell. Biol.*, **23**, 8519-8527.
6. Fatica, A., Galardi, S., Altieri, F., and Bozzoni, I. (2000) *RNA*, **6**, 88-95.
7. Gerbi, S. A., and Borovjagin, A. (1997) *Chromosoma*, **105**, 401-406.
8. Tran, E. J., Zhang, X., and Maxwell, E. S. (2003) *EMBO J.*, **22**, 3930-3940.
9. Snaar, S., Wiesmeijer, K., Jochemsen, A. G., Tanke, H. J., and Dirks, R. W. (2000) *J. Cell Biol.*, **151**, 653-662.
10. Levitsky, S. A., Mukhar'yamova, K. Sh., Veiko, V. P., and Zatsepina, O. V. (2004) *Mol. Biol. (Moscow)*, **38**, 1-10.
11. Wang, H., Boisvert, D., Kim, K. K., Kim, R., and Kim, S.-H. (2000) *EMBO J.*, **19**, 317-323.
12. Martin, J. L., and McMillan, F. M. (2002) *Curr. Opin. Struct. Biol.*, **12**, 783-793.
13. Reichow, S. L., Hamma, T., Ferre-D'Amare, A. R., and Varani1, G. (2007) *Nucleic Acids Res.*, **35**, 1452-1464.
14. Pollard, K. M., Lee, D. K., Casiano, C. A., Bluthner, M., Johnston, M. M., and Tan, E. M. (1997) *J. Immunol.*, **158**, 3521-3528.
15. Takeuchi, K., Turley, S. J., Tan, E. M., and Pollard, K. M. (1995) *J. Immunol.*, **154**, 961-971.
16. Chen, M., Rockel, T., Steinweger, G., Hemmerich, P., Risch, J., and von Mikecz, A. (2002) *Mol. Biol. Cell*, **13**, 3576-3587.
17. Chen, M., and von Mikecz, A. (2005) *Ann. N. Y. Acad. Sci.*, **1051**, 382-389.
18. Torchinsky, Yu. M. (1971) *Sulfhydryl and Disulfide Groups in Proteins* [in Russian], Nauka, Moscow.
19. Jocelyn, P. C. (1972) *Biochemistry of the SH Group*, Academic Press, London-New York.
20. Friedman, M. (1973) *The Chemistry and Biochemistry of the Sulfhydryl Group in Amino Acids, Peptides, and Proteins*, Oxford, New York.
21. Gurskaya, N. G., Verkhusha, V. V., Shcheglov, A. S., Staroverov, D. B., Chepurnykh, T. V., Fradkov, A. F., Lukyanov, S., and Lukyanov, K. A. (2006) *Nat. Biotechnol.*, **24**, 461-465.
22. Trinkle-Mulcahy, L., and Lamond, A. I. (2007) *Science*, **318**, 1402-1407.
23. Houtsmuller, A. B. (2005) *Adv. Biochem. Eng. Biotechnol.*, **95**, 177-199.
24. Van Royen, M. E., Farla, P., Mattern, K. A., Geverts, B., Trapman, J., and Houtsmuller, A. B. (2009) in *The Nucleus* (Hancock, R., ed.) Humana Press, New York, pp. 363-385.
25. Phair, R. D., and Misteli, T. (2000) *Nature*, **404**, 604-609.
26. Fradkov, A. F., Verkhusha, V. V., Staroverov, D. B., Bulina, M. E., Yanushevich, Y. G., Martynov, V. I., Lukyanov, S., and Lukyanov, K. A. (2002) *Biochem. J.*, **368**, 17-21.
27. Levitsky, S. A., Mukhar'yamova, K. Sh., Zatsepina, O. V., and Veiko, V. P. (2004) *Biotekhnologiya*, **6**, 19-26.
28. Hoogstraten, D., Nigg, A. L., Heath, H., Mullenders, L. H., van Driel, R., Hoeijmakers, J. H., Vermeulen, W., and Houtsmuller, A. B. (2002) *Mol. Cell.*, **10**, 1163-1174.
29. Dobrucki, J. W., Feret, D., and Noatynska, A. (2007) *Biophys. J.*, **93**, 1778-1786.
30. Mukhar'yamova, K. Sh., and Zatsepina, O. V. (2001) *Tsitologiya*, **43**, 792-795.
31. Ershov, Yu. A., and Pletneva, T. V. (1989) *Mechanisms of Toxic Effects of Inorganic Compounds* [in Russian], Meditsina, Moscow.
32. Kutsenko, S. A. (2004) *Bases of Toxicology* [in Russian], Foliant Publishing House, St. Petersburg.
33. Zalups, R. K., and Ahmad, S. (2004) *J. Am. Soc. Nephrol.*, **15**, 2023-2031.
34. Verheggen, C., le Panse, S., Almouzni, G., and Hernandez-Verdun, D. (2001) *Exp. Cell Res.*, **269**, 23-34.
35. Ochs, R. L., and Smetana, K. (1991) *Exp. Cell Res.*, **197**, 183-190.