Interaction of small heat shock proteins with light component of neurofilaments (NFL)

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Abstract The interaction of human small heat shock protein HspB1, its point mutants associated with distal hereditary motor neuropathy, and three other small heat shock proteins (HspB5, HspB6, HspB8) with the light component of neurofilaments (NFL) was analyzed by differential centrifugation, analytical ultracentrifugation, and fluorescent spectroscopy. The wild-type HspB1 decreased the quantity of NFL in pellets obtained after low- and high-speed centrifugation and increased the quantity of NFL remaining in the supernatant after high-speed centrifugation. Part of HspB1 was detected in the pellet of NFL after high-speed centrifugation, and at saturation, 1 mol of HspB1 monomer was bound per 2 mol of NFL. Point mutants of HspB1 associated with distal hereditary motor neuropathy (G84R, L99M, R140G, K141Q, and P182S) were almost as effective as the wild-type HspB1 in modulation of NFL assembly. At low ionic strength, HspB1 weakly interacted with NFL tetramers, and this interaction was increased upon salt-induced polymerization of NFL. HspB1 and HspB5 (α B-crystallin) decreased the rate of NFL polymerization measured by fluorescent spectroscopy. HspB6 (Hsp20) and HspB8 (Hsp22) were less effective than HspB1 (or HspB5) in modulation of NFL assembly. The data presented indicate that the small heat shock proteins affect NFL transition from tetramers to filaments, hydrodynamic properties of filaments, and their bundling and therefore probably modulate the formation of intermediate filament networks in neurons.

Keywords Small heat shock proteins · Neurofilaments · Cytoskeleton · Intermediate filaments · Neurodegenerative diseases

Abbreviations

DTT	Dithiothreitol
IFs	Intermediate filaments
IPTG	Isopropyl β -D-1-thiogalactopyranoside
ME	Mercaptoethanol
SEC	Size-exclusion chromatography
sHsp	Small heat shock proteins
TCA	Trichloroacetic acid

Introduction

Intermediate filaments (IFs) are important components of the cytoskeleton (Herrmann and Aebi 2004; Eriksson et al. 2009). The human genome contains about 70 genes encoding different proteins of intermediate filaments (Herrmann and Aebi 2004; Block et al. 2015). Intermediate filaments play an important role in mechanical stability, intracellular signaling, traffic, cellular motility, and many other vital processes (Eriksson et al. 2009). Since intermediate filaments are involved in diverse processes, their mutations are associated with more than 80 human diseases (Omary 2009). Six types of intermediate filaments are described in the literature (Eriksson et al. 2009; Kornreich et al. 2015). These types of filaments differ in tissue distribution, protein composition and mechanism of polymerization, mechanical properties, and their role in different cells. For instance, neurofilaments are the main cytoskeletal component of mature neurons of the central nervous system (Laser-Azogui et al. 2015). These filaments are composed of light, medium, and heavy molecular

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weight subunits (NFL, NFM, NFH, respectively) expressed at different levels in the course of ontogenesis and presented at an approximate molar ratio of 7:3:2 (Laser-Azogui et al. 2015; Scott et al. 1985). Light neurofilament (NFL) protein is predominantly responsible for polymerization of intermediate filaments (Heins et al. 1993; Lifshits and Williams 1984), whereas two other components predominantly participate in inter-filament interaction (Laser-Azogui et al. 2015). Mutations of NFH are associated with amyotrophic lateral sclerosis, whereas mutations of NFL are associated with different forms of Charcot-Marie-Tooth disease (Eriksson et al. 2009; Bucci et al. 2012).

Certain forms of distal hereditary motor neuropathy and Charcot-Marie-Tooth disease are also associated with mutations of small heat shock proteins (Benndorf et al. 2014; Datskevich et al. 2012). For instance, mutations G84R, L99M, R140G, K141Q, and P182S of HspB1 are associated with distal hereditary motor neuropathy of the second type (Benndorf et al. 2014; Datskevich et al. 2012). Molecular mechanisms underlying probable participation of small heat shock proteins in distal hereditary motor neuropathy remain poorly characterized; however, it is well accepted that the small heat shock proteins can be involved in the interaction with different proteins of intermediate filaments. For instance, HspB5 (α B-crystallin) interacts with desmin and prevents the entanglement and aggregation of desmin filaments (Houck et al. 2011). Mutation R120G of α B-crystallin, causing desmin-related myopathy, promotes desmin filament aggregation (Perng et al. 2004; Elliott et al. 2013). Pin array technique revealed several sites of α B-crystallin involved in its interaction with desmin (Ghosh et al. 2007), and swapping or deletion of certain parts of α B-crystallin confirmed that the β 3- and β 8-strands of crystallin domain participate in desmin binding (Houck et al. 2011). α B-crystallin interacts with glial fibrillary acidic protein (GFAP) and prevents non-covalent self-association of intermediate filaments formed by this protein (Perng et al. 1999), and the sites responsible for interaction of crystallin with GFAP partially overlap with the corresponding sites involved in the interaction of crystallin with desmin (Ghosh et al. 2007).

Less is known about the interaction of HspB1 (Hsp27) with intermediate filament proteins. Like α B-crystallin (HspB5), HspB1 interacts with GFAP and vimentin and prevents self-association of intermediate filaments (Perng et al. 1999). HspB1 interacts with desmin less effectively than α B-crystallin; however, at high concentrations, it also prevents self-association of desmin filaments (Elliott et al. 2013). HspB1 prevents keratin filament bundling and affects the assembly dynamics of intermediate filaments formed by keratin (Kayser et al. 2013). Cotransfection of NFL and S135F mutant (but not the wild type) HspB1 results in altered neurofilament assembly in cells devoid of cytoplasmic intermediate filaments (Evgrafov et al. 2004), whereas transient expression

of P182L mutant of HspB1 in cortical cells is accompanied by disruption of neurofilament assembly and accumulation of aggregates containing NFM (Ackerley et al. 2006). At the same time, to date, there are no detailed biochemical data concerning the direct interaction of different small heat shock proteins with NFL. This paper deals with a biochemical investigation of interaction of HspB1 and its mutants as well as HspB5, HspB6, and HspB8 with the low molecular weight component of NFL.

Materials and methods

Proteins

Human small heat shock proteins HspB1, HspB5, HspB6, and HspB8 were expressed and purified from *Escherichia coli* as described earlier (Mymrikov et al. 2012). Point mutants of HspB1 associated with distal hereditary motor neuropathy G84R, L99M, R140G, K141Q, and P182S were produced by the earlier described methods (Nefedova et al. 2013a; Nefedova et al. 2013b; Chalova et al. 2014). Plasmid-encoding bovine NFL was kindly provided by Dr. Alexander Minin (Institute of Protein Research, Russian Academy of Sciences) and cloned in pET30 vector. Untagged recombinant NFL was expressed in BL21 (DE3) pLysS strain of *E. coli*. Expression was induced either by the addition of 0.5 mM IPTG or performed by autoinduction (Studier 2005). NFL was purified from inclusion bodies. Inclusion bodies were thoroughly washed two times in lysis buffer (50 mM MES (pH 6.25), 190 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 15 mM ME), two times by lysis buffer supplemented by 1% Triton X-100, two times in lysis buffer containing 0.5% Triton X-100 and 1.5 M NaCl, and finally two times in lysis buffer without any additives. Finally, the washed sample of inclusion bodies was stored at 4 °C. Inclusion bodies were dissolved in buffer Q (20 mM Tris-acetate pH 8.0, containing 8 M urea, 2 mM EGTA, 30 mM ME, 0.1 mM PMSF, 2 μ M pepstatin, 2 μ M leupeptin) and subjected to ultracentrifugation (30 min, 105,000g). Supernatant was collected and applied on a 5 ml HiTrap Q column equilibrated by buffer Q. The column was washed by buffer Q, and NFL were eluted by linear salt gradient (0–0.7 M NaCl, in eight column volumes). Fractions enriched in NFL were combined, and pH was adjusted to 4.2 by formic acid. This sample was loaded on a 5 ml HiTrap SP column equilibrated by SP buffer (10 mM Tris/formate pH 4.0, containing 8 M urea, 0.1 mM PMSF, 30 mM ME). After washing the column with SP buffer, the protein was eluted by linear salt gradient (0–0.35 M NaCl, in 10 column volumes). Fractions containing purified NFL were combined, and the pH was adjusted to 8.0 by 1 M Tris base. Samples thus obtained were subjected to ultrafiltration and after determination of protein

concentration (Bradford 1976) were aliquoted and stored at -70°C . The N-terminal fragment of NFL (residues 1–248) was expressed and purified in a similar manner. The purity of each protein sample estimated by Coomassie Blue-stained SDS polyacrylamide gel electrophoresis (Laemmli 1970) was above 92%.

Reassembly experiments

Three different approaches were used to investigate the interaction of NFL with small heat shock proteins. In the first case, small samples of NFL were mixed with fixed quantities of small heat shock proteins or their mutants in the presence of 8 M urea and then dialyzed against filament assembly buffer at 37°C overnight (Fig. 1a). The filament assembly buffer contained 20 mM HEPES/NaOH (pH 7.2), 0.19 M NaCl, 0.1 mM EGTA, 1 mM MgCl_2 , 0.1 mM PMSF, and 0.1 mM ME (buffer F). In the second case, the samples of NFL were preassembled as a tetramer by dialysis against low ionic strength buffer (5 mM Tris/acetate (pH 8.0) containing 1 mM EGTA (buffer T)). Then, the tetramers of NFL were mixed with small heat shock proteins, and filament assembly was initiated by salt addition (Fig. 1b). Finally, in the third scenario, a sample of NFL was dialyzed against high ionic strength buffer. Then, the assembled filaments were mixed with small heat shock proteins (Fig. 1c). In all cases, the samples of NFL, small heat shock proteins, and their mixes were subjected to low- and high-speed centrifugation. In the first case, 50–70 μl samples were centrifuged at 10,000g for 30 min at 37°C . In the second case, 50–100 μl was centrifuged in an LP40Ti rotor (Beckman) at 105,000g for 60 min at room temperature. Proportionally equal amounts of pellet and supernatant were subjected to SDS gel electrophoresis (Laemmli 1970), followed by quantitative densitometry using GelAnalyzer program.

NFL phosphorylation

NFL samples dialyzed against tetramer assembly buffer (buffer T) or filament assembly buffer (buffer F) were incubated in the absence or presence of HspB1 (NFL:HspB1 = 5:1 molar ratio) at 37°C in buffer P (20 mM Tris, 10 mM sodium phosphate (pH 7.5), 15 mM ME, 1 mM MgCl_2 , 0.1 mM PMSF) containing 50–70 μM ATP with trace amounts of γ - ^{32}P -ATP and catalytic subunit of cAMP-dependent protein kinase. The reaction was stopped by spotting the sample on Whatman 3MM filter or by mixing with an excess of EDTA and SDS-sample buffer. Excess unreacted radioactive ATP was removed by washing filters in 10% TCA containing 5 mM phosphate and 5 mM pyrophosphate and counted on a LKB Rack Beta 1219 scintillation counter. The same samples were separated on SDS gel electrophoresis, and radioactive bands were visualized by film autoradiography.

Electron microscopy

Filaments were formed in the presence or absence of sHsp after dialysis against high ionic strength assembly buffer (buffer F). Samples were applied to a carbon-coated electron microscopy grid, negatively stained with 1% uranyl acetate, and examined in an electron microscope JEOL 2100 TEM operated at 200 kV. The authors are grateful to Dr. O. S. Sokolova (Department of Bioinformatics and Bioengineering, School of Biology, Moscow State University) for her help in performing electron microscopy experiments.

Modification of NFL by pyrenyl-maleimide

Prior to each experiment, freshly prepared 10 M solution of urea was additionally purified by incubation (15 min at room temperature) with mixed ion exchanger. This urea solution was used immediately for the preparation of buffer U containing 8 M urea and 150 mM sodium phosphate pH 7.4. The sample of NFL (5–6 mg/ml) was reduced prior to the labeling. For that, sample dissolved in buffer N (8 M urea, 20 mM Tris/acetate pH 8.0) was incubated with DTT (10 mM) for 30 min at 30°C . To remove DTT, the sample was loaded on Nap10 column equilibrated by buffer U. The sample of reduced protein was mixed with 12 mM solution of pyrenyl-maleimide (Pyr-Mal) dissolved in dimethylformamide. The labeling was conducted in the dark for 3 h at room temperature at final molar ratio pyrenyl-maleimide:NFL 10:1. The reaction was stopped by adding DTT (5 mM), and the sample was centrifuged at 14,000g for 15 min. Labeled NFL was purified on Nap10 column equilibrated with buffer U. Modified protein was concentrated by ultrafiltration, and percent of modification was determined spectrophotometrically by measuring optical density at 280 and 344 nm. Under these conditions, the extent of modification was in the range of 0.8–1.2 mole of fluorescent label per mole of NFL. Modified protein was either frozen in buffer U and stored at -70°C or dialyzed overnight at 4°C against 5 mM Tris/acetate (pH 8.0) containing 1 mM EGTA (buffer T) and immediately used after that.

Kinetics of NFL polymerization followed by fluorescent spectroscopy

The sample of Pyr-Mal-modified NFL dialyzed against buffer T (5 mM Tris/acetate pH 8.0, containing 1 mM EGTA) was mixed with unmodified (dialyzed in the same conditions) NFL to satisfy the final rate of protein labeling in the range of 0.25–0.50 mol of fluorescent dye per mole of NFL. Protein samples were centrifuged at 4°C (14,000g, 15 min). The samples containing 0.5–0.7 mg/ml of NFL in buffer T were preincubated at 37°C for 5 min, and filament formation was initiated by addition of 10% volume of filament assembly buffer

Fig. 1 Experimental design for study of sHsp-NFL interaction. **a** Two proteins (NFL and small heat shock proteins) were mixed in 8 M urea, and the mixture was dialyzed against filament assembly buffer. **b** NFL were preassembled into tetramers by dialysis against low ionic strength

buffer. Then, sHsp were added and polymerization was initiated by salt addition. **c** NFL were assembled into filaments by dialysis against filament assembly buffer followed by addition of small heat shock proteins

(200 mM HEPES/NaOH (pH 7.0), 1.9 M NaCl, 10 mM MgCl_2). The ratio of fluorescence at 385 and 470 nm (F_{385}/F_{470}) (excited at 344 nm) was monitored for 90 min at 37 °C using Cary Eclipse spectrofluorometer. At the end of incubation, the samples were centrifuged (105,000g, 60 min, 4 °C), and the protein composition of supernatant and pellet was determined by SDS gel electrophoresis (Laemmli 1970).

Analytical ultracentrifugation

NFL were dialyzed against low ionic strength buffer T (5 mM Tris/acetate pH 8.0, containing 1 mM EGTA). Freshly prepared tetramers were mixed with equimolar quantities of HspB1 and subjected to analytical ultracentrifugation. Alternatively, isolated NFL or their equimolar mixture with HspB1 were dialyzed against filament assembly buffer (20 mM HEPES/NaOH (pH 7.0), 190 mM NaCl, 1 mM MgCl_2 , 0.1 mM EGTA, 0.1 mM PMSF). Samples thus obtained (400 μl) were subjected to analytical ultracentrifugation at 20 °C and 40,000 rpm in a six-hole AnJ-Ti rotor and 12-mm double-sector cells (Spinco model E analytical ultracentrifuge (Beckman)) equipped with absorbance optics, scanner, and computer online. All cells were scanned at 280 nm with 3-min interval. Differential distribution of sedimentation coefficient [$s(s)$ versus s] was estimated by using SEDFIT program (Schuck 2000).

Results

Interaction of the wild-type HspB1 with NFL

Different approaches were used for investigation of HspB1 interaction with NFL. In the first case, NFL in urea containing buffer were mixed with HspB1 and dialyzed against filament assembly buffer (Fig. 1). Samples thus obtained were subjected to low- and high-speed centrifugation, and protein composition of supernatant and pellet was determined by SDS gel electrophoresis.

Efficiency of NFL polymerization and the probability of filament bundling are strongly dependent on protein concentration (Angelides et al. 1989). Therefore, we analyzed the effect of HspB1 on polymerization and bundling of NFL filaments at different concentrations of NFL (Figs. 2 and 3). All experiments were performed at a fixed HspB1 concentration (2 μM) and variable concentrations of NFL (0–14 μM) so that the R value equal to the molar ratio of monomer of NFL to monomer of HspB1 varied in the range of 0–7. Under conditions used, isolated HspB1 predominantly remained in the supernatant after low-speed centrifugation (two left tracks in Fig. 2b). At low concentration, isolated NFL also predominantly remained in the supernatant after low-speed centrifugation. However, at high concentration (8–14 μM), a significant portion (about 20–30%) of isolated NFL was detected in the pellet, thus indicating that under these conditions, NFL

Fig. 2 Low-speed centrifugation of isolated NFL (a) or NFL mixed with the wild-type HspB1 (b). HspB1 at fixed concentration (2 μ M) was added to NFL in the buffer containing 8 M urea. The mix was dialyzed against filament forming buffer (as indicated in Fig. 1a) at 37 °C. Protein ratio was varied by different concentration of NFL (0 and 14 μ M) so that the molar ratio of NFL to a monomer of HspB1 (R value indicated at the

bottom) was between 1 and 7. The samples were centrifuged at low speed. Two left tracks on b correspond to low-speed centrifugation of isolated HspB1. Positions of HspB1 and NFL are labeled by horizontal lines. The *s* and *p* are the supernatants and pellets, respectively. The picture is a representative result of six independent experiments

Fig. 3 High-speed centrifugation of isolated NFL or NFL mixed with the wild-type HspB1. HspB1 was added to NFL in the buffer containing 8 M urea. The mix was dialyzed against filament-forming buffer (as indicated in Fig. 1a) at 37 °C and subjected to high-speed centrifugation. **a** Percentage of NFL remaining in the supernatant after high-speed centrifugation. Concentration of HspB1 was fixed and equal to 2 μ M, and concentration of NFL varied between 0 and 8 μ M so that the R value indicated at the bottom and equal to the molar ratio of NFL monomer to HspB1 monomer varied between 1 and 4. Mean values of seven experiments with standard deviation are presented. **b** Protein composition of supernatants (*s*) and pellets (*p*) obtained after high-speed centrifugation of isolated HspB1 (2 μ M) (left panel), isolated NFL (2, 6, and 8 μ M) (middle panel), and of the mixture of HspB1 (2 μ M) and NFL (2, 6, and 8 μ M) (right panel). The positions of HspB1 and NFL are labeled by horizontal lines

filaments tend to aggregate and to form bundles pelleted at low-speed centrifugation (Fig. 2a). HspB1 partially prevented NFL filament bundling leading to a decrease of NFL quantity in low-speed pellet (compare Fig. 2a, b).

After high-speed centrifugation, isolated HspB1 again predominantly remained in the supernatant and only small amounts of HspB1 were detected in the pellet (two left tracks in Fig. 3b). In contrast, NFL was predominantly detected in the pellet and the quantity of protein detected in the supernatant decreased at high concentrations of NFL (Fig. 3b). For instance, at low concentration of NFL (2 μ M), only about 30% of NFL remained in the supernatant after high-speed centrifugation. At the same time, the addition of equimolar quantities of HspB1 ($R = 1$) under these conditions increased the quantity of NFL in supernatant up to 66–65% (Fig. 3a). Qualitatively similar results were obtained at practically all R values; however, increased NFL concentration (increase of R value) was accompanied by a decrease of NFL in the supernatant (Fig. 3a). It is worth mentioning that at high NFL concentration (high R value), a significant part of HspB1 was detected in the pellet (Fig. 3b). These data indicate that HspB1 somehow decreases pelleting of NFL filaments, and at the same time, part of HspB1 was bound to NFL filaments and was sedimented at high-speed centrifugation.

In order to determine the stoichiometry of the complex formed by pelleted NFL and HspB1, we dialyzed NFL against filament assembly buffer and mixed the filaments thus obtained (11 μ M per monomer) with variable quantities (0–15 μ M per monomer) of wild-type HspB1. The scheme of this experiment is presented in Fig. 1c. After high-speed centrifugation, we determined the quantity of HspB1 bound to NFL filaments (Fig. 4). Under these conditions, the apparent dissociation constant of HspB1-NFL complex was in the range of 3–5 μ M and saturation was achieved when 1 mole of HspB1 monomer was bound per 2 moles of NFL monomers.

Fig. 4 Titration of NFL filaments by HspB1. NFL filaments (11 μ M per monomer) obtained as indicated on the scheme of Fig. 1c were mixed with increasing quantities of HspB1 and subjected to high-speed centrifugation. Protein composition of supernatants (*s*) and pellets (*p*) was determined by quantitative SDS gel electrophoresis. On saturation, about 6 μ M of HspB1 monomer was bound to 11 μ M of NFL monomers. Mean values with standard deviation of four independent experiments are presented

Electron microscopy was used for investigation of HspB1 interaction with NFL. On electron micrographs, isolated HspB1 was detected in the form of small spheres (Fig. 5a). At the concentration used (2 μ M), HspB1 remained in the supernatant and was not practically precipitated at high-speed centrifugation (Fig. 5a, insert). Isolated NFL at 2 μ M concentration was present in the form of short kinky filaments with diameter of about 10 nm (Fig. 5b), i.e., in the form described earlier in the literature (Heins et al. 1993). Under these conditions, NFL was predominantly detected in the pellet of high-speed centrifugation and only small quantities of NFL remained in the supernatant (Fig. 5b, insert). After high-speed centrifugation of the mixture of NFL and HspB1 (1:2 molar ratio), we detected NFL predominantly in the supernatant (Fig. 5c, insert). The data presented might indicate that the

wild-type HspB1 either affects the hydrodynamic properties of NFL filaments or inhibits NFL assembly. Special attempts were made to obtain standard intermediate filaments instead of short kinky filaments presented in Fig. 5. Unfortunately, these attempts were unsuccessful since under conditions suitable for formation of long standard intermediate filaments (pH 6.25), HspB1 tends to aggregate and precipitate. Therefore, all experiments were performed under “near-physiological” conditions, i.e., at pH 7.2 when only short kinky filaments of NFL were formed.

The N-terminal head domain plays an important role in the regulation of NFL polymerization (Laser-Azogui et al. 2015), and phosphorylation of certain sites in the N-terminal head domain (Cleverley et al. 1998) inhibits NFL polymerization (Nakamura et al. 1990; Yates et al. 2009). We analyzed the effect of the wild-type HspB1 on phosphorylation of NFL. For this purpose, we either dialyzed NFL against low ionic strength buffer (–NaCl) and obtained NFL tetramers (Fig. 1b) or dialyzed NFL against high ionic strength filament assembly buffer (+NaCl) (Fig. 1c) and obtained polymerized NFL filaments (Fig. 6). NFL tetramers and NFL filaments were mixed with the wild-type HspB1 (1:5 monomer molar ratio) and isolated proteins, or their mixture was subjected to phosphorylation by catalytic subunit of cAMP-dependent protein kinase (Fig. 6). Under these conditions, we found that isolated HspB1, isolated NFL filaments, or their mixture were poorly phosphorylated by cAMP-dependent protein kinase (Fig. 6b, c). NFL tetramers or their mixture with HspB1 were much more effectively phosphorylated by cAMP-dependent protein kinase (Fig. 6). These data agree with earlier published results (Nakamura et al. 1990). The wild-type HspB1 had practically no effect either on the rate or on extent of NFL tetramer phosphorylation and was not itself phosphorylated by cAMP-dependent protein kinase (Fig. 6a, b). Similar results were obtained with the N-terminal fragment (residues 1–248) of NFL (data not presented), although the extent and the rate of phosphorylation of the N-terminal fragment of NFL were smaller than that of intact NFL. The data presented

Fig. 5 Electron microscopy of HspB1 (a), NFL (b), and NFL mixed with HspB1 (1:2 molar ratio) (c). On insert, the aliquot of the same sample was centrifuged at high speed and pellet (*p*) and supernatant (*s*) were subjected to SDS gel electrophoresis. The scheme of experiment is presented in Fig. 1a

Fig. 6 Effect of HspB1 on NFL phosphorylation. Filaments of NFL prepared by dialysis against buffer containing NaCl (+NaCl) (as indicated on the scheme of Fig. 1c) or tetramers of NFL prepared in low ionic strength buffer (−NaCl) (as indicated on the scheme of Fig. 1b) were mixed with the wild-type HspB1 (NFL:HspB1 = 5:1 molar ratio). The samples were incubated with cAMP-dependent protein kinase in the presence of ^{32}P - γ -ATP followed by SDS gel electrophoresis with Coomassie Blue staining and

autoradiography (a, b). Kinetics of phosphorylation of filaments (*open triangles*), their mixture with the wild-type HspB1 (*filled triangles*), or isolated HspB1 (*filled squares*) was followed by incorporation of radioactive phosphate (c). Similarly, kinetics of phosphorylation of isolated NFL tetramers (*open circles*), their mixture with the wild-type HspB1 (*filled circles*), or isolated HspB1 (*filled squares*) were also followed by incorporation of radioactive phosphate (c). The results are representative of three independent experiments

indicate that HspB1 either does not interact with unassembled NFL or that the sites of interaction of HspB1 with NFL are located far from the sites of NFL phosphorylation.

Analytical ultracentrifugation was used for further analysis of interaction of HspB1 with NFL. In this case, we used another experimental setup (Fig. 1). Isolated NFL were dialyzed against low ionic strength buffer leading to formation of NFL tetramers (Fig. 1b) followed by the addition of equimolar quantities of HspB1. Samples thus obtained were subjected to analytical ultracentrifugation. Isolated tetramers of NFL were heterogeneous; however, as a rule, we detected species with sedimentation coefficient in the range of 4–7 s (Fig. 7a). In agreement with earlier published data (Lelj-Garolla and Mauk 2006), under low ionic strength conditions, HspB1 was sedimented as a peak with sedimentation coefficient of ~10 s. Sedimentogram of an equimolar mixture of NFL

tetramers and HspB1 was practically indistinguishable from the sum of corresponding sedimentograms of isolated proteins (Fig. 7a). Neither new peaks nor significant changes in the size of corresponding peaks were detected when the mixture of NFL tetramers and HspB1 was subjected to ultracentrifugation.

Alternatively, isolated NFL or equimolar mixture of NFL and HspB1 was dialyzed against high salt buffer resulting in the formation of NFL polymers (Fig. 1a). Polymerized isolated NFL filaments were very large and heterogeneous, and therefore, we did not detect separate peaks in the range of 10–120 s (Fig. 7b). Under these conditions, isolated HspB1 was sedimented as a sharp peak with a sedimentation coefficient of about 15–17 s (Fig. 7b) in agreement with our earlier published results (Nefedova et al. 2013b). Sedimentation of equimolar mixture of polymerized NFL and HspB1 revealed

Fig. 7 Analytical ultracentrifugation of NFL and HspB1. **a** Ultracentrifugation of NFL tetramers (1), isolated HspB1 (3), or of their equimolar mixture (2) obtained as indicated on the scheme of Fig. 1b. **b** Ultracentrifugation of isolated NFL filaments (1), isolated HspB1 (3), or NFL polymerized in the presence of equimolar quantities of HspB1 (2) and obtained as indicated on the scheme of Fig. 1a

decrease of the amplitude of the peak corresponding to isolated HspB1 and appearance of a broad peak in the range of 60–90 s, probably corresponding to comparably small size polymers of NFL (Fig. 7b), thus indicating the formation of complexes of HspB1 and polymerized NFL.

Interaction of HspB1 mutants associated with distal hereditary motor neuropathy with NFL

Data in the literature (Evgrafov et al. 2004; Ackerley et al. 2006) indicate that HspB1 mutations might affect its interaction with neurofilaments. Therefore, it seemed reasonable to analyze the interaction of HspB1 mutants associated with distal hereditary motor neuropathy or Charcot-Marie-Tooth disease with NFL. In order to analyze this interaction, we first dialyzed NFL against low ionic strength buffer and obtained NFL tetramers. Afterwards, we mixed NFL tetramers with different mutants of HspB1 associated with distal hereditary

motor neuropathy at molar ratio NFL/HspB1 equal to 1 and induced NFL polymerization by increasing ionic strength (see scheme in Fig. 1b). The mixture thus obtained was subjected to high-speed centrifugation, and the protein composition of supernatants and pellets was analyzed by SDS gel electrophoresis (Fig. 8). As expected, under these conditions, isolated wild-type HspB1 and all analyzed mutants were detected in the supernatant and only negligible low quantities of HspB1 species were detected in the pellet after high-speed centrifugation (Fig. 8a). At the same time, the addition of salt was accompanied by polymerization of isolated NFL, and therefore, NFL were detected only in the pellet (Fig. 8b, first two tracks). Addition of the wild-type HspB1 or its mutants G84R, L99M, R140G, K141Q, or P182S were accompanied by redistribution of NFL, and part of NFL was detected in the supernatant, thus indicating that the wild-type HspB1 and its mutants affect polymerization or hydrodynamic properties of NFL filaments. All analyzed mutants were as effective as the wild-type HspB1 in affecting NFL pelleting at high-speed centrifugation. As already mentioned, part of HspB1 was coprecipitated with polymerized NFL and therefore was detected in the pellet (Fig. 8b). All analyzed mutants of HspB1 were also coprecipitated with NFL, and their quantity in the pellet was slightly but insignificantly larger than the quantities of the wild-type HspB1 (see Fig. 8b). These data indicate that the wild-type HspB1 and the largest part of its analyzed mutants more or less equally prevent pelleting of NFL at high-speed centrifugation and at the same time are able to interact with NFL and are coprecipitated with NFL after high-speed centrifugation.

Interaction of different small heat shock proteins with NFL

Four human small heat shock proteins (HspB1, HspB5, HspB6, and HspB8) are expressed ubiquitously (Taylor and Benjamin 2005; Vos et al. 2009). However, to our knowledge, only two ubiquitously expressed proteins, namely, HspB1 and HspB5, were analyzed for their ability to interact with intermediate filament proteins. Therefore, it seemed reasonable to analyze the interaction of two other ubiquitously expressed small heat shock proteins, HspB6 and HspB8, with NFL. We mixed NFL (10 μ M) and different small heat shock proteins (10 μ M) and dialyzed this mixture against filament assembly buffer (Fig. 1a). Samples thus obtained were subjected to low- and high-speed centrifugation (Fig. 9). Under these conditions, about 30–40% of isolated NFL formed bundles and were precipitated after low-speed centrifugation (Fig. 9a). The addition of any small heat shock proteins prevented the formation of NFL bundles, and about 90% of NFL remained in the supernatant (Fig. 9a). All small heat shock proteins were equally effective in preventing NFL bundling. High-speed centrifugation was accompanied by

Fig. 8 Effect of HspB1 mutations on interaction with NFL filaments. NFL were dialyzed against low ionic strength buffer (see scheme of Fig. 1b). Thus obtained NFL tetramers were mixed with different species of HspB1, dialyzed against polymerization buffer, and subjected to high-speed centrifugation. SDS gel electrophoresis of supernatants (s) and

pellets (p) obtained after centrifugation of isolated HspB1 and its mutants (a) or of the mixture of HspB1 and its mutants (11 μ M) and polymerized NFL (11 μ M) (b). Positions of NFL and HspB1 species are indicated by lines. Representative results of three independent experiments are presented

practically complete sedimentation of isolated NFL filaments, and only about 10% of NFL remained in the supernatant (Fig. 9b). Small heat shock proteins differently affected distribution of NFL after high-speed centrifugation. The addition of

HspB1 and HspB5 was accompanied by a significant increase of NFL in the supernatant and a concomitant decrease of NFL in the pellet after high-speed centrifugation (Fig. 9b). HspB6 and HspB8 also increased the quantity of NFL in the

Fig. 9 Distribution of NFL in supernatants and pellets after low-speed (a) and high-speed (b) centrifugation in the absence and in the presence of different small heat shock proteins and protein composition of supernatants and pellets obtained after high-speed centrifugation (c). a, b NFL (10 μ M) and different small heat shock proteins (10 μ M) were mixed and dialyzed against polymerization buffer (see scheme in Fig. 1a) and subjected to low- and high-speed centrifugation. Mean values with standard deviation of four independent experiments are presented on the diagram.

c NFL (18 μ M) was mixed with each of four different small heat shock proteins (HspB1, HspB5, HspB6, HspB8, 2 μ M), dialyzed against polymerization buffer as indicated on the scheme of Fig. 1a, and subjected to high-speed centrifugation followed by SDS electrophoresis of supernatants (s) and pellets (p). Representative results of three independent experiments are presented. Positions of NFL and small heat shock proteins are marked by lines

supernatant and decreased its content in the pellet; however, in this case, the quantity of NFL in the pellet was either equal or larger than its quantity in the supernatant. Thus, HspB8 and especially HspB6 were much less effective than HspB1 or HspB5 in decreasing precipitation of NFL filaments after high-speed centrifugation.

It is worth mentioning that at high NFL concentration (18 μ M), significant quantities of HspB1 and HspB5 and somewhat smaller quantities of HspB8 were coprecipitated with NFL filaments (Fig. 9c). At the same time, only negligible quantities of HspB6 were coprecipitated with NFL filaments (Fig. 9c). These data indicate that HspB1, HspB5, and probably HspB8 tightly interact and effectively modulate NFL assembling, whereas HspB6 only weakly interacts with polymerized NFL.

Effect of small heat shock proteins on the kinetics of NFL polymerization

Fluorescent spectroscopy was earlier successfully used to follow the kinetics of intermediate filament polymerization (Nakamura et al. 1990; Angelides et al. 1989; Ip and Fellows 1990). We tried to use this approach to follow the kinetics of Pyr-Mal NFL polymerization. For this purpose, we used ratiometric measurement of Pyr-Mal fluorescence, simultaneously recording fluorescence at 385 nm (fluorescence of Pyr-Mal monomer) and 470 nm (fluorescence of Pyr-Mal excimer). Modified by Pyr-Mal NFL tetramers had a comparatively low ratio of fluorescence at 365 and 470 nm (Fig. 10a) and remained in the supernatant after high-speed centrifugation (Fig. 10a, insert). The addition of filament assembly buffer was accompanied by the formation of NFL filaments that were precipitated after high-speed centrifugation (Fig. 10a, insert) and a significant increase in the ratio of fluorescence at 385 and 470 nm. Polymerization of modified NFL was accompanied by more than 1.5 increase of F_{385}/F_{470} ratio (Fig. 10a). This effect seems to be due to a polymerization-induced decrease of excimer and an increase of monomer fluorescence of Pyr-Mal attached to the single SH group (Cys323) of NFL. In NFL, tetramers formed upon dialysis of NFL against low ionic buffer (buffer T) Cys of two parallel neighbor NFL molecules are close to each other, making possible the formation of a pyrene excimer. Upon polymerization, the orientation or distance between two pyrenes is changed, leading to a decrease of the excimer and an increase of monomer fluorescence. Dissociation of filaments induced by the addition of 1% of SDS is accompanied by a complete disappearance of the excimer and a significant increase of monomer fluorescence (Fig. 10a).

We used fluorescent spectroscopy to investigate the effect of different small heat shock proteins on the kinetics of filament assembly. At molar ratio, NFL/sHsp equal to 2 HspB1 decreased both the rate of increase and the amplitude of the

fluorescent signal (Fig. 10b). The rate of increase of the fluorescent signal upon NFL polymerization in the presence of HspB5 was rather high; however, the maximal amplitude of fluorescence estimated by exponential fitting was comparable with that observed in the presence of HspB1 and significantly lower than that observed in the case of isolated NFL (Fig. 10b). For instance, in all experiments, initial F_{385}/F_{470} ratio was close to 2.87 and according to approximation was increased up to 3.91 in the absence of sHsp and only up to 3.49 in the case of HspB1 and up to 3.57 in the case of HspB5. The addition of HspB6 or HspB8 decreased the rate of

Fig. 10 Kinetics of NFL polymerization recorded by fluorescence spectroscopy. **a** Fluorescence spectra of NFL modified by Pyr-Mal. Fluorescence spectra of Pyr-Mal labeled NFL before (1) and after (2) polymerization. Curve 3 represents the fluorescence spectrum after addition of SDS (final concentration 1%) to polymerized filaments. Fluorescence was excited at 344 nm and recorded in the range of 350–600 nm. *Insert*, protein composition of supernatants (s) and pellets (p) obtained after high-speed centrifugation of unpolymerized (1) and polymerized (2) Pyr-Mal NFL. Position of NFL is labeled by line. **b** Effect of different small heat shock proteins on kinetics of NFL polymerization. Kinetics of NFL polymerization in the absence of small heat shock proteins or in the presence of HspB1, HspB5, HspB6, or HspB8. Molar ratio NFL/small heat shock proteins was equal to 2:1. Representative data of five independent experiments are presented

increase of the fluorescent signal, whereas the approximated maximal value of F_{385}/F_{470} ratio was equal to 3.67, indicating that HspB6 and HspB8 were less effective than the two other small heat shock proteins.

Discussion

Small heat shock proteins interact and affect the polymerization of different intermediate filament proteins. For instance, HspB5 (α B-crystallin) interacts and affects assembly and self-association of desmin (Houck et al. 2011; Ghosh et al. 2007; Perng et al. 2004; Elliott et al. 2013), GFAP (Ghosh et al. 2007; Perng et al. 1999), vimentin and peripherin (Djabali et al. 1997), and keratin (Elliott et al. 2013). HspB1 interacts with glial fibrillary acidic protein (Perng et al. 1999), desmin (Perng et al. 1999; Elliott et al. 2013), and keratin (Kayser et al. 2013). The data of cell biology (Evgrafov et al. 2004; Ackerley et al. 2006; Holmgren et al. 2013) indicate that point mutants of HspB1 associated with distal hereditary motor neuropathy (or Charcot-Marie-Tooth disease) can affect the structure and intracellular transportation of neurofilaments. However, to our knowledge, the literature is lacking any data concerning the direct interaction of different small heat shock proteins and their mutants with individual components of neurofilaments.

The wild-type HspB1 decreased the quantity of NFL detected in low-speed centrifugation pellet (Fig. 2). This effect is similar to an analogous effect described for the wild-type HspB5 on desmin. Indeed, HspB5 prevented the formation of desmin bundles and by this means decreased the quantity of desmin in low-speed centrifugation pellet and prevented the formation of gel formed by desmin filaments (Houck et al. 2011; Elliott et al. 2013). Similar results were recently described for HspB1 and keratin (Kayser et al. 2013). In this case, the addition of HspB1 was accompanied by a decrease in the rate of keratin filament bundling and the formation of a fainter network of keratin filaments.

We also observed that the wild-type HspB1 decreased the quantity of NFL detected in high-speed centrifugation pellet and increased the quantity of NFL in the supernatant (Fig. 3). This effect seems to be different from an analogous effect observed in the case of HspB5 and desmin. HspB5 affected only low-speed pelleting of desmin without any pronounced effect on sedimentation of desmin at high-speed centrifugation (Houck et al. 2011). We suppose that this apparent difference can be explained in the following manner. Desmin like many other intermediate filament proteins forms long filaments tending to bundling at high protein concentration (Houck et al. 2011). On the contrary, under our experimental conditions at pH 7.2–7.5, NFL formed very short kinky filaments (Fig. 5). In this respect, our results agree with the earlier published data (Heins et al. 1993; Angelides et al. 1989). These

short kinky filaments will not be precipitated at low-speed centrifugation, and these filaments will move to the pellet only at high protein concentration and high-speed centrifugation. Interacting with short NFL filaments, HspB1 affects their hydrodynamic properties and/or prevents their association and by this means decreases the quantity of NFL precipitated at high-speed centrifugation.

Phosphorylation of NFL affects the assembly and stability of neurofilaments and can be involved in the regulation of axonal transport (Yates et al. 2009; Nakamura et al. 1990). Therefore, it seemed advisable to analyze the effect of HspB1 on NFL phosphorylation by cAMP-dependent protein kinase. In good agreement with earlier published data (Nakamura et al. 1990), we found that NFL tetramers are phosphorylated more rapidly than assembled filaments. The addition of the wild-type HspB1 did not affect phosphorylation of intact NFL tetramers or its N-terminal fragment carrying all sites phosphorylated by cAMP-dependent protein kinase (Fig. 6). This means that either HspB1 is unable to interact with unassembled filaments or that the sites of interaction with HspB1 are far from the sites phosphorylated by cAMP-dependent protein kinase in NFL. The data of analytical ultracentrifugation indicate that indeed HspB1 only weakly interacted with NFL tetramers (Fig. 7) and probably therefore did not affect their phosphorylation by cAMP-dependent protein kinase.

Damage to intracellular traffic is supposed to be one of the most important factors leading to different neuronal diseases, among them distal hereditary motor neuropathy and/or Charcot-Marie-Tooth disease (Bucci et al. 2012). The system of intermediate filaments plays an important role in the intracellular traffic (Laser-Azogui et al. 2015). Therefore, it seemed reasonable to analyze the interaction of HspB1 mutants associated with distal hereditary motor neuropathy and/or Charcot-Marie-Tooth disease with NFL. By using differential centrifugation, we compared the interaction of the wild-type HspB1 and its point mutants with NFL (Fig. 8). Running these experiments under different conditions, we found that five-point mutants of HspB1 associated with distal hereditary motor neuropathy (G84R, L99M, R140G, K141Q, P182S) like the wild-type HspB1 decreased the quantity of NFL in high-speed pellet, and the effect induced by these mutants was not significantly different from the corresponding effect induced by the wild-type protein (Fig. 8). Although we failed to detect a direct effect for the analyzed mutations of HspB1 on NFL assembly, this does not necessarily mean that in vivo these mutations will not affect formation of neurofilaments. Indeed, it is well known that the small heat shock proteins can form heterooligomers and the properties of these heterooligomers can be different from the corresponding properties of homooligomers (Mymrikov et al. 2012; Arrigo 2013). Point mutations of HspB1 associated with distal hereditary motor neuropathy and/or Charcot-Marie-Tooth disease

affect interaction of HspB1 and HspB6 (Nefedova et al. 2013a; Nefedova et al. 2013b; Muranova et al. 2015). This can affect concentration of HspB1 homooligomers in the cell and by this means indirectly affect its interaction with NFL modulating assembly, composition, and the structure of neurofilaments.

As already mentioned, there are many data on the interaction of HspB1 and HspB5 with different intermediate filament proteins. However, the effect of other ubiquitously expressed small heat shock proteins (HspB6 and HspB8) on the intermediate filament proteins was not analyzed in detail. By using differential centrifugation, we compared the effect of four different small heat shock proteins on NFL assembly (Fig. 9). HspB1 and HspB5 were almost identically effective in decreasing NFL accumulation in low- and high-speed pellets (Fig. 9). Similarly, the quantities of HspB1 and HspB5 coprecipitated with NFL in high-speed pellets were also similar (Fig. 9c). These data indicate that HspB1 and HspB5 similarly affected assembly of NFL. HspB8 inhibited NFL bundling as effectively as HspB1 or HspB5 (Fig. 9a); however, it was less effective than HspB1 and/or HspB5 in inhibiting pelleting of NFL at high-speed centrifugation (Fig. 9b). HspB6 effectively prevented pelleting of NFL at low-speed centrifugation (Fig. 9a) but was rather ineffective in decreasing the quantity of NFL in high-speed pellets (Fig. 9b). Moreover, the quantities of HspB8 and especially of HspB6 coprecipitated with NFL under conditions of high-speed centrifugation were smaller than the corresponding quantities of HspB1 or HspB5 (Fig. 9c). In addition, both HspB8 and HspB6 were less effective in the inhibition of NFL assembly recorded by fluorescent spectroscopy (Fig. 10). A comparison of the effect of different small heat shock proteins on NFL assembly is presented in Table 1. We might conclude therefore that HspB8 and especially HspB6 interacted with NFL much less effectively than their HspB1 and HspB5 counterparts. This conclusion agrees with the earlier reported data indicating that HspB6 in contrast to HspB5 is unable to prevent desmin gel formation (Elliott et al. 2013). This means that there is strict specificity in the interaction of different small heat shock proteins with intermediate filament proteins and

requires further detailed investigation of the effect of different small heat shock proteins and their heterooligomeric complexes on cytoskeleton.

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Table 1 Effect of different small heat shock proteins on NFL assembly

Small heat shock proteins	Inhibition of NFL bundling	Inhibition of polymerization after joint dialysis from urea	Inhibition of polymerization from NFL tetramers	Binding to polymerized NFL
HspB1	+	+++	++	+++
HspB5	+	+++	++	+++
HspB6	+	+	+	+
HspB8	+	++	+	++

The number of crosses qualitatively corresponds to the strength of effect

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