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ORIGINAL PAPER

Interplay between recombinant Hsp70 and proteasomes: proteasome activity modulation and ubiquitin-independent cleavage of Hsp70

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Abstract The heat shock protein 70 (Hsp70, human HSPA1A) plays indispensable roles in cellular stress responses and protein quality control (PQC). In the framework of PQC, it cooperates with the ubiquitin-proteasome system (UPS) to clear damaged and dysfunctional proteins in the cell. Moreover, Hsp70 itself is rapidly degraded following the recovery from stress. It was demonstrated that its fast turnover is mediated via ubiquitination and subsequent degradation by the 26S proteasome. At the same time, the effect of Hsp70 on the functional state of proteasomes has been insufficiently investigated. Here, we characterized the direct effect of recombinant Hsp70 on the activity of 20S and 26S proteasomes and studied Hsp70 degradation by the 20S proteasome in vitro. We have shown that the activity of purified 20S proteasomes is decreased following incubation with recombinant human Hsp70. On the other hand, high concentrations of Hsp70 activated 26S proteasomes. Finally, we obtained evidence that in addition to previously reported ubiquitin-dependent degradation, Hsp70 could be cleaved independent of ubiquitination by the 20S proteasome. The results obtained reveal novel aspects of the interplay between Hsp70 and proteasomes.

Keywords Heat shock proteins · Hsp70 · Proteasome · Proteasome regulators · Ubiquitin-independent degradation

Introduction

Heat shock proteins (HSPs) constitute evolutionarily conserved cellular machinery for protein folding and maintenance of protein homeostasis (Richter et al. 2010). Hsp70 is one of the most abundant HSPs found in all organisms. Induction of endogenous Hsp70 is a biomarker of cell adaptation to adverse environmental conditions (Evgen'ev 2014). Hsp70 protects cells from toxic reactive oxygen species (ROS) (Rozhkova et al. 2010; Yurinskaya et al. 2015b), stimulates antioxidant enzyme activity, binds and sequesters damaged and aggregation-prone proteins (Evgen'ev 2014). Endogenous Hsp70 prevents formation of toxic protein aggregates (Wacker et al. 2004) and modulates apoptosis (Li et al. 2000; Samali and Cotter 1996). As a contributor to the protein homeostasis of the cells, Hsp70 performs protein quality control (PQC) and acts in a tight cooperation with several co-chaperones and the cellular protein degradation machinery (Ciechanover and Kwon 2015; Shiber and Ravid 2014; Westhoff et al. 2005). In association with co-chaperones, it determines the fate of damaged proteins. If the client protein cannot be repaired, it is tagged with ubiquitin and escorted for degradation (Alberti et al. 2003; Ballinger et al. 1999; Murata et al. 2001; Westhoff et al. 2005).

Many intracellular proteins are degraded by the ubiquitin-proteasome system (UPS). The principal element of the UPS is a proteasome. The 20S proteasome represents a 700 kDa barrel-like structure, composed of four stacked

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heptomerical rings. Two outer rings are made of seven alpha subunits and two inner rings—of seven beta subunits. N-termini of the alpha subunits form gates that sequester the proteasome proteolytic chamber where protein degradation is performed by beta subunits (Abramova et al. 2002). Three of seven beta subunits of the constitutive 20S proteasome display three proteolytic activities: β1—caspase-like (cleave after acidic amino acids), β2—trypsin-like (cleave after basic amino acids), and β5—chymotrypsin-like (cleaves after hydrophobic residues) (Schmidt and Finley 2014). Under certain conditions, including oxidative stress and inflammation, constitutive catalytic beta subunits may be replaced by immune subunits β1i, β2i, and β5i, respectively, forming the immunoproteasome. Immunoproteasomes effectively generate major histocompatibility complex class I-compatible peptides, play important roles in cellular stress adaptation and immune reactions (Ferrington and Gregerson 2012). The 20S proteasome can bind regulator particles that open alpha-subunit gates and determine the substrate specificity of the complexes (Jung and Grune 2013). Free 20S proteasomes and proteasomes bound to different regulators (19S, 11S, PA200) represent different forms of proteasomes. Despite initially closed alpha-gates, free 20S proteasomes are still able to degrade peptides as well as oxidized and damaged proteins, which are also substrates of 20S proteasomes bound to 11S regulators (Pickering et al. 2010) (Pickering and Davies 2012). When the 20S proteasome binds 19S regulators, it becomes 26S proteasome, a complex that recognizes ubiquitinated substrates and cleaves them in an ATP-dependent manner (Jung and Grune 2013).

UPS and Hsp70 are involved in the PQC system serving the maintenance of protein homeostasis, and both participate in modulation of cellular stress and immune responses. Considering the apparent links between the two systems, their crosstalk is of special interest and mutual influence could be anticipated. Indeed, experimental data indicate that Hsp70 has a therapeutic effect on diseases or in states where UPS is compromised or attenuated (Bobkova et al. 2015; Bobkova et al. 2014; Evans et al. 2006; Gifondorwa et al. 2007). Moreover, during adaptation to oxidative stress, Hsp70 was shown to participate in the detachment of 19S regulators from the 26S proteasome by binding and preserving the regulator complexes (Grune et al. 2011). On the other hand following the stress relief, Hsp70 is itself ubiquitinated by co-chaperone CHIP and rapidly degraded in the 26S proteasome (Qian et al. 2006; Soss et al. 2015). Taken together, a growing body of evidence indicates that Hsp70 can influence activity, substrate specificity, and subunit composition of the intracellular proteasomes (Bobkova et al. 2015; Grune et al. 2011; Lyupina et al. 2013; Morozov 2017).

Here, to investigate the effects of human recombinant Hsp70 on the functional state of proteasomes, we used several proteasome forms of different purity and obtained from a number of sources.

Material and methods

Hsp70 preparations

In our research, we used wild-type human recombinant Hsp70 (HSPA1A) and modified human Hsp70 with mutated five putative N-glycosylation sites ("Hsp70 128") described in (Gurskiy et al. 2016). Sequences encoding both proteins (modified and wild type) were cloned into pET-14b plasmid to provide the N-terminal polyhistidine tag (MGSSHHHHHHSSGLVPRGSH). The Hsp70 proteins with 6× His were purified by affinity chromatography using Ni-NTA resin (Qiagen, Germany) according to the manufacturer's protocol. Wild-type Hsp70 purity was assessed by the SDS-PAGE in 10% gel. Of commercial Hsp70 (Sigma, USA) and of obtained protein, 0.2 μg was loaded onto the gel. The gel was stained with Roti blue quick solution (Roth, Germany) (Fig. 1a). Purity check of Hsp70 128 is described in (Gurskiy et al. 2016). Additionally, protein purity was tested by Western blot (data not shown). Protein concentration was measured according to Bradford's protocol (Bradford 1976). The specific activity of both proteins was measured in vitro. The ability of proteins to reduce endotoxin-induced ROS production was estimated; substrate-binding test and protein substrate refolding test were performed as described in (Gurskiy et al. 2016). According to the experiments, both proteins were effective in all the tests described above (data not shown).

Cells

Human myeloblast Kasumi-1 (ATCC® CRL-2724™) cell line was grown in RPMI medium, supplemented with 10% fetal bovine serum, L-glutamine, and the antibiotics (all from Invitrogen, USA) at 37 °C, 5% CO_2, and 95% humidity.

Preparation of cellular lysates

Kasumi-1 cells were washed and resuspended in homogenization buffer (HB) (50 mM Tris-HCl buffer (pH 7.5), containing 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol, 10 mM $Na_2S_2O_5$, and 2 mM ATP), added in the ratio 100 μl/4 × 10^6 of cells. Cells were lysed using a homogenization pestle (Bio Plas Inc., USA). Samples were thoroughly mixed and left on ice for 10 min. Lysates were centrifuged at 16,100×*g* for 30 min, and cell supernatants were collected. Obtained lysates were aliquoted. Total protein concentration in the lysates was measured by the Lowry method (Lowry et al. 1951).

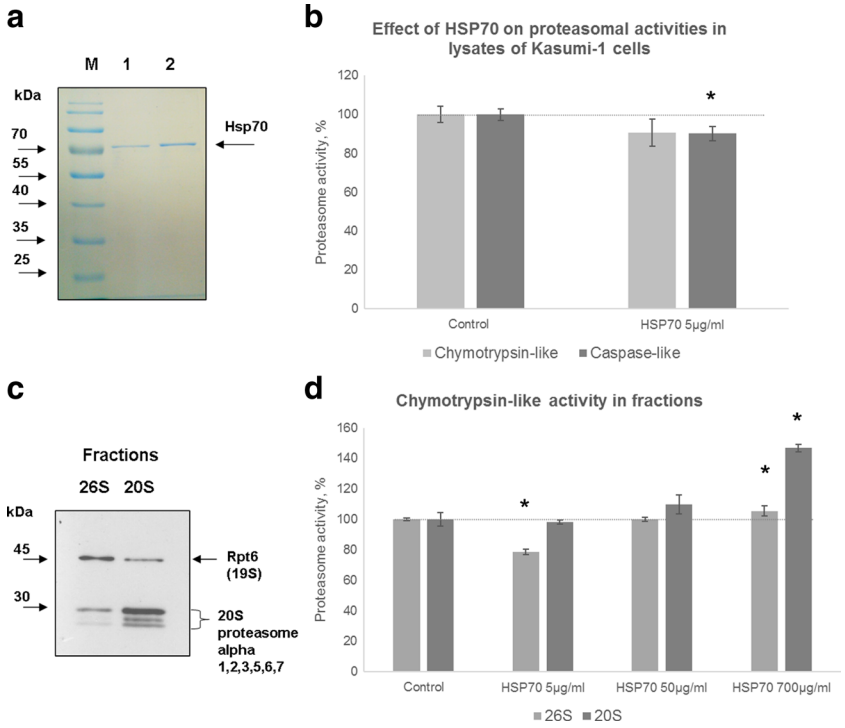

Fig. 1 Hsp70 influences proteasome activity in lysates of Kasumi-1 cells and in the proteasome-enriched fractions. **a** Obtained recombinant Hsp70 is as pure as commercial protein. Of commercial (track 1) and obtained Hsp70 (track 2), 0.2 μg was separated in SDS-PAGE followed by staining with Roti blue quick. Prestained protein ladder (Fermentas, Lithuania) (*M*). **b** Chymotrypsin-like and caspase-like activity in crude Kasumi-1 cellular lysates incubated with 5 μg/ml of Hsp70 for 40 min. Chymotrypsin-like activity is shown in *gray* and caspase-like activity in *dark gray*. Proteolytic activity in untreated lysates was set as control. The data are mean values from three experiments ± SD. Statistically significant differences versus control are indicated by asterisks: $*p < 0.05$, calculated by two-tailed Student's *t* test. *Dotted line* indicates proteasome activity in control samples. **c** Western blot of 26S and 20S proteasome-enriched protein fractions, obtained from Kasumi-1 cells. α-Subunits (1, 2, 3, 5, 6, 7 (∑α)) of the 20S proteasomes and Rpt6 subunits of 19S regulators were revealed in both fractions and indicated by *arrowhead* and a *curly brace*. The prevalence of the 26S proteasomes in the 26S fraction and domination of 20S proteasomes in the 20S fraction were confirmed: 26S fraction contained 5 times less α-subunits but 2.27 times more of Rpt6 subunits than the 20S fraction (estimated using ImageJ software). **d** Chymotrypsin-like proteasome activity in 26S proteasome-enriched (*gray*) and 20S proteasome-enriched (*dark gray*) fractions from Kasumi-1 cells after incubation with Hsp70. Aliquots of fractions (~8 μg of total protein) were incubated with three different concentrations of Hsp70: 5 μg/ml (0.07 μM), 50 μg/ml (0.7 μM), and 700 μg/ml (10 μM). Proteolytic activity in untreated fractions was set as control. The data are mean values from three experiments ± SD. Statistically significant differences versus control are indicated by asterisks: $*p < 0.05$, calculated by two-tailed Student's *t* test. *Dotted line* indicates proteasome activity in control samples

Treatment of cellular lysates with Hsp70 and estimation of proteasome activities

To characterize the chymotrypsin-like and peptidylglutamyl-peptide hydrolyzing (caspase-like) activities of proteasomes in lysates, we used two fluorogenic substrates Suc-LLVY-AMC (Enzo, USA) and Z-LLE-AMC (Enzo, USA), respectively. The tests were performed at least in triplicates. Aliquots (~8 μg) of Kasumi-1 cellular lysates were treated with 5 μg/ml of Hsp70 in 90 μl of the reaction buffer (RB), containing 40 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM $MgCl_2$, and 1 mM ATP for 40 min at 37 °C. After that, either Suc-LLVY-AMC or Z-LLE-AMC was added to the samples to reach the final concentration of 30 μM. Samples were mixed and incubated for 20 min at 37 °C. Control reactions with 10 μM of the proteasome inhibitor MG132 (Tocris, UK) were performed to test nonspecific degradation of substrates. Reactions were stopped with 2% SDS solution (in ddH_2O). Fluorescence at the excitation wavelength 380 nm and emission wavelength 440 nm was measured using VersaFluor Fluorometer (Bio-Rad, USA). To calculate relative activity levels, the activity levels in probes with MG132 were subtracted from the values detected in samples, containing cell lysates. Then, the average activity in 1 μl of the homogenate was calculated and normalized using estimated total protein concentration for every sample.

SDS-PAGE and Western blot

Proteins and protein fractions, obtained from cell lysates, were analyzed in 10% or 12% Tris-glycine polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were stained with Ponceau S (Sigma, USA) to monitor transfer efficacy. Primary antibodies (ESM File 1 Table 1) were used to detect proteasome subunits, subunits of regulators, and Hsp70. Blots were then incubated with appropriate secondary

antibodies (ESM File 1 Table 1) and developed using ECLPrime kit (GE Healthcare, UK).

Quantification of signal intensities using ImageJ software

X-ray films were examined using ImageJ software (https://imagej.nih.gov/ij/). In brief, rectangular sections were placed over the detected protein bands, and histograms indicating the intensity of each of the bands were obtained. The peaks were separated from the background, and the peak area values, indicating the relative optical density of the bands, were calculated.

Fractionation of proteasomes

Fractionation of the proteasomes from Kasumi-1 cells was performed as described (Morozov 2016) on the basis of the technique published by Abramova et al. (2004). In brief, cells were lysed and centrifuged at 16,100×*g* for 30 min. Cellular supernatants were collected. Ammonium sulfate was gradually added to the supernatant to reach 40% saturation. Then, the supernatant was collected and centrifuged at 16,100×*g* for 20 min. The pellet, containing protein fraction enriched by the 26S proteasomes (26S fraction), was dissolved in buffer composed of 20 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 20% glycerol, and 1 mM ATP. Next, ammonium sulfate was slowly added to the resting supernatant to reach 70% saturation. After that, it was centrifuged at 16,100×*g* for 20 min. The supernatant was discarded, and a pellet, containing protein fraction enriched by the 20S proteasomes (20S fraction), was dissolved in 150 μl of 20 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1 mM DTT, and 20% glycerol and aliquoted. Samples were stored at −80 °C before use. Protein concentration was estimated by the Lowry method (Lowry et al. 1951). The quality of the fractions was assessed using PAGE and Western blot with antibodies (ESM File 1 Table 1) targeting the 20S proteasome α-subunits, Rpt6 subunit of the 19S regulator, and α-subunit of the 11S regulator. Control chymotrypsin-like activity estimations in the 26S and 20S fractions were performed as described above in the RB supplemented with 30 μM of fluorogenic substrate in the absence or the presence of 0.02% SDS (Kisselev et al. 1999) (data not shown). In case of the 20S fraction, ATP and $MgCl_2$ were not added to the RB. Reactions were performed in triplicates.

Estimation of proteolytic activities in proteasome-enriched fractions and of purified 20S and 26S proteasomes treated with Hsp70

Modulation of chymotrypsin-like and caspase-like proteasome activities by Hsp70 was characterized in the obtained proteasome-enriched fractions and using highly purified human constitutive 20S (Enzo, USA), immune 20S (Enzo, USA), and 26S proteasomes (Enzo, USA). Aliquots of the proteasome-enriched fractions (~8 μg of total protein), 0.67 μg of the 26S, 0.33 μg of the 20S, and 0.5 μg of the immune 20S proteasomes, were mixed with 90 μl of the RB, containing various concentrations of Hsp70 and incubated for 40 min at 37 °C. After that, either Suc-LLVY-AMC or Z-LLE-AMC was added to the samples to reach the final concentration of 30 μM. Samples were mixed and incubated for 20 min at 37 °C. When proteasome activities in the 20S fraction or of the purified 20S proteasomes were studied, the RB was free of 5 mM $MgCl_2$ and 1 mM ATP. Control reactions with the 10 μM of the proteasome inhibitor MG132 (Tocris, UK) were performed to test unspecific degradation of substrates. Reactions were stopped, and the activities were measured as described above. Tests were performed in triplicates.

Hsp70 degradation assay

One microgram of recombinant human Hsp70 was mixed either with 1 μg of 20S proteasomes (Enzo, USA) or with the mixture of 1 μg of 20S proteasomes (Enzo, USA) and 10 μM of MG132 in buffer containing 40 mM Tris-HCl (pH 7.5), 1 mM DTT, and 0.02% SDS. Control mixture contained only Hsp70 dissolved in the buffer described above. Mixtures were made in duplicates. The first set of samples was left for 6 h and the second for 24 h at 37 °C. The same set of reactions was performed without 0.02% SDS and with increased concentration of MG132 (1 mM); however, samples were kept 24 and 48 h at 37 °C. In the latter case, Hsp70 128 was used as an additional control. Following the incubation, samples were immediately mixed with SDS PAGE sample buffer, boiled, and analyzed in 10% Tris-glycine polyacrylamide gel followed by wet transfer. Membranes were stained with anti-Hsp70 and anti-20S proteasome alpha-subunit IgGs (ESM File 1 Table 1) as in the procedure described above. Differences among the optical densities of revealed bands were estimated using ImageJ software. Experiment was performed two times in addition. In one setting Hsp70 128 was omitted instead wild-type Hsp70 was mixed with 1 μg of immune 20S proteasome in parallel with constitutive 20S proteasome. In both additional experiments, no control reactions with MG132 were performed.

Statistics

Statistical significance of obtained results was assessed using two-tailed Student's *t* test. Online *t* test calculator (http://www.socscistatistics.com/tests/studentttest/) was used to estimate *p* values. Differences between groups were considered significant if $p < 0.05$.

Results

The major functional parameter of cellular proteasomes is their proteolytic activity. To test if Hsp70 can directly modulate proteasome activity, we evaluated its effect on different proteasome preparations. To begin with, we estimated how addition of recombinant Hsp70 (Fig. 1a) influences proteasome activity in lysates obtained from a cell line with low constitutive endogenous Hsp70 expression level (Nijhuis et al. 2008). Kasumi-1 cells were lysed, and the crude lysates were incubated with 5 μg/ml (0.07 μM) of recombinant Hsp70. Chymotrypsin-like activity demonstrated near significant decrease by 9%, while caspase-like activity was statistically significantly suppressed by 10% (Fig. 1b). These results indicate that Hsp70 can directly affect proteasome activity. At the same time, crude lysate contains various forms of proteasomes (Fabre et al. 2014; Tanahashi et al. 2000), and hence, different effects of Hsp70 on different forms of proteasomes cannot be excluded. Therefore, as a next step, we evaluated the modulation of proteasome activity by Hsp70 in protein fractions enriched by different forms of proteasomes.

Fractionation of proteasomes from Kasumi-1 cellular lysate was performed as described in "Material and methods." Protein fractions enriched in 26S (26S fraction) and 20S (20S fraction) proteasomes were obtained and analyzed by Western blot (Fig. 1c). Aliquots of fractions were incubated with three different concentrations of Hsp70: 5 μg/ml (0.07 μM), 50 μg/ml (0.7 μM), and 700 μg/ml (10 μM). In the 26S proteasome-enriched fraction, Hsp70 induced opposite effects depending on the concentration (Fig. 1d). The chymotrypsin-like activity in the 26S fraction was decreased by 21% compared to untreated fraction following the incubation with 5 μg/ml of Hsp70, did not change following incubation with 50 μg/ml of Hsp70, and demonstrated a slight, but statistically significant (6%) increase following treatment with 700 μg/ml of Hsp70 (Fig. 1d). In the 20S fraction, 50 and 700 μg/ml of Hsp70 induced 10 and 47% increase of proteolytic activity of proteasomes, respectively (Fig. 1d). These results indicated that indeed, opposite effects of Hsp70 treatment might be revealed when various protein concentrations and different forms of proteasomes are used.

According to our estimation, the proteasome-enriched fractions contained only ~10% of proteasomes and 90% of other proteins by mass. It cannot be ruled out that some of these proteins can participate in proteasome activity modulation in the presence of Hsp70. To test this proposition, we incubated Hsp70 with highly pure commercial 26S, 20S, and immune 20S proteasomes. In the previous experiment, proteasome activity modulation was most prominent when 5 and 700 μg/ml of Hsp70 were used. Thus, subsequently, we used these two concentrations of Hsp70. It was demonstrated that 700 μg/ml of Hsp70 induces a 126% increase of chymotrypsin-like and a near significant ($p = 0.08$) 11% increase of caspase-like activity of purified 26S proteasomes (Fig. 2a). Lower concentration of protein (5 μg/ml), while displaying a similar tendency, had no statistically significant effect, confirming that Hsp70-associated 26S proteasome activation is strongly concentration dependent (Fig. 2a). In contrast, surprisingly, Hsp70 induced significant decreases of both activities of purified 20S proteasomes. Compared to nontreated 20S proteasomes, chymotrypsin-like activity was decreased by 52 and 43%, and the caspase-like activity was suppressed by 47 and 59% in the presence of 5 and 700 μg/ml of Hsp70, respectively (Fig. 2b). Furthermore, we confirmed that the decrease of 20S proteasome activity in the presence of Hsp70 is dose dependent and evident even when equimolar quantities of the protein and proteasomes are present in the test mixtures. In the presence of 1 μg/ml (1/3 proteasome to Hsp70 molecule ratio) and 0.5 μg/ml (1/1.5 molecule ratio) of Hsp70, chymotrypsin-like activity of 20S proteasomes was decreased by 39 and 32%, respectively (Fig. 2c). 20S immunoproteasome activity was also suppressed by Hsp70 although to a lesser extent (especially caspase-like activity) (Fig. 2d). The reduced inhibiting effect of Hsp70 on the caspase-like activity is likely due to a shift of catalytic activity of the immune 20S proteasomes to chymotrypsin-like and overall attenuation of caspase-like activity of these proteasomes (Ferrington and Gregerson 2012).

To assess the nature of the 20S proteasome activity decrease, induced by Hsp70, we performed Hsp70 degradation assay. First, the protein was incubated with 20S proteasomes, activated by 0.02% SDS. The addition of SDS into the reaction was shown to induce alpha-subunit gate opening stimulating the access of the substrates into the catalytic chamber of the proteasome (Kisselev et al. 1999). Noticeably, Hsp70 was efficiently degraded: 93% of the protein was degraded after 24 h, and the addition of proteasome inhibitor MG132 partially abrogated this process (Fig. 3a, b). Since MG132 inhibits predominantly chymotrypsin-like activity of the proteasome while the other two activities are less affected (Alexandrova et al. 2008), it is reasonable that Hsp70 degradation is attenuated, but not completely blocked. This is additionally illustrated by the lower part of the X-ray film obtained with a longer exposure, where additional bands are seen (Fig. 3b). As a next step, we investigated if the 20S proteasome can degrade Hsp70 without activation. Here along with wild-type protein, we used Hsp70 128 as an additional control. Hsp70 128 has mutations in five putative N-glycosylation sites. However, the protein shows intact chaperone activities and as a wild-type Hsp70 effectively protects cells from ROS (Gurskiy et al. 2016). We demonstrated that although much slower than the activated, nonactivated 20S proteasome degraded Hsp70 (Fig. 3c–f, ESM File 1 Fig. 1). Only 18–22% of wild-type Hsp70 and surprisingly 39–40% of Hsp70 128 were cleaved after 48-h incubation with the proteasome (Fig. 3c). Thus,

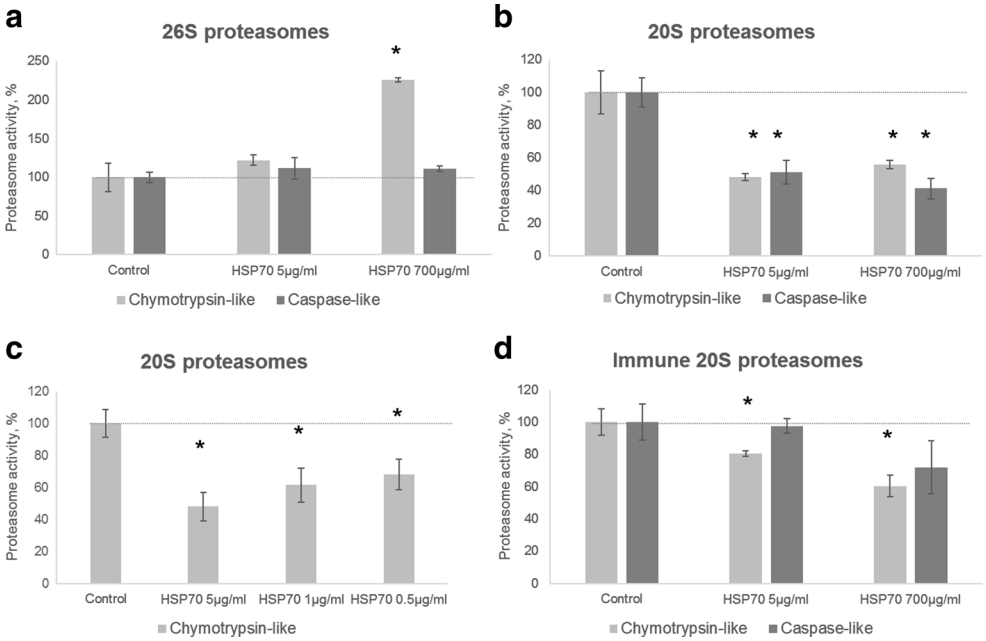

Fig. 2 The effect of Hsp70 on proteolytic activity of highly purified proteasomes. The effect of Hsp70 on proteolytic activity of 26S (**a**), constitutive 20S (**b**, **c**), and immune 20S (**d**) proteasomes. Proteasomal activity was measured after treatment with 5 and 700 μg/ml of the Hsp70 (**a**, **b**, **d**). Chymotrypsin-like activity is shown in *gray* and caspase-like activity in *dark gray*. **c** Dose-dependent effect of Hsp70 on chymotrypsin-like activity of 20S proteasomes. The activity was measured in samples incubated with 5, 1, and 0.5 μg/ml of Hsp70. Proteolytic activity in protein-free samples was set as control. The data are mean values from three experiments ± SD. Statistically significant differences versus control are indicated by asterisks *$p < 0.05$, calculated by two-tailed Student's *t* test. *Dotted line* indicates proteasome activity in control samples

observed decreased activity of the 20S proteasomes is likely a result of competition of a fluorogenic substrate with Hsp70 for the active site of the proteasome.

Interestingly, Hsp70 degradation was accompanied by production of a distinct cleavage product with a molecular weight around 30 kDa (Fig. 3d, f, ESM File 1 Fig. 1). Smaller specific degradation product/s were also detected (Fig. 3d, f). It should be mentioned that almost no 30 kDa distinct cleavage product was detected following the incubation of Hsp70 with the SDS-activated 20S proteasome (Fig. 3a, b). In order to investigate which part of the protein does the 30 kDa fragment represents, we repeated the degradation experiment and used monoclonal antibodies (C92F3A-5) raised against C-terminal Hsp70 fragment a.a. 436–503 in parallel with rabbit polyclonal serum. Monoclonal antibodies detected only full-length Hsp70 (Fig. 3f), indicating that the observed Hsp70 cleavage product is either a part of the N-terminal domain or a part of C-terminal domain with the linker region between domains. Noticeably, 48-h incubation of Hsp70 with immune 20S proteasome resulted in more efficient degradation (22% of full-length Hsp70 was degraded by constitutive 20S proteasome and 47%—by the immune 20S proteasome) and ensured generation of the same 30 kDa protein fragment (Fig. 3f).

Intriguingly, Western blot analysis of Hsp70 preparations with rabbit polyclonal antibodies revealed bands of approximately 60–65 and 45–47 kDa (Fig. 3a, c, f, ESM File 1 Figs. 1 and 3). Both fragments were more evident in the wild type than in Hsp70 128 preparation (Fig. 3c, ESM File 1 Fig. 1). When Hsp70 samples were incubated with 20S proteasomes, the quantity of 60–65 and 45–47 kDa fragments was affected similarly to the full-length protein (Fig. 3c, f, ESM File 1 Fig. 1). These fragments may represent a result of possible self-proteolysis of Hsp70 (Mitchell et al. 1985), which is likely attenuated in the case of Hsp70 128 due to the presence of mutations that can affect protein secondary structure (ESM File 1 Fig. 2). Specificity of the bands was confirmed by Western blot of obtained wild-type Hsp70 in line with differently prepared *Escherichia coli* lysates (ESM File 1 Fig. 3). After standard in gel tryptic digest procedure (Shevchenko et al. 2006), proteins with molecular weight 45–47 kDa (Fig. 3f) were analyzed by MALDI MS. Mascot search files (ESM File 1 Fig. 4, ESM Files 2 and 3) show the presence of Hsp70 peptides on the MALDI MS tryptic maps. The map of 45–47 kDa fragment from Hsp70 sample that was not incubated with the proteasome shows the presence of Hsp70 peptides from a.a. 37 to 526, indicating probably a mixture of C-terminally and N-terminally truncated forms (ESM File 1 Fig. 4A, ESM File 2). Incubation with proteasome facilitated primarily disappearance of N-terminally truncated variant from the mixture (ESM File 1 Fig. 4B, ESM File 3); in this case, the MALDI MS tryptic map demonstrated peptides spanning a.a. 57 to 361 of the Hsp70.

Finally, using MS, we additionally confirmed the degradation of Hsp70 by the 20S proteasome. The peptides covering the entire Hsp70 were revealed in the band corresponding to

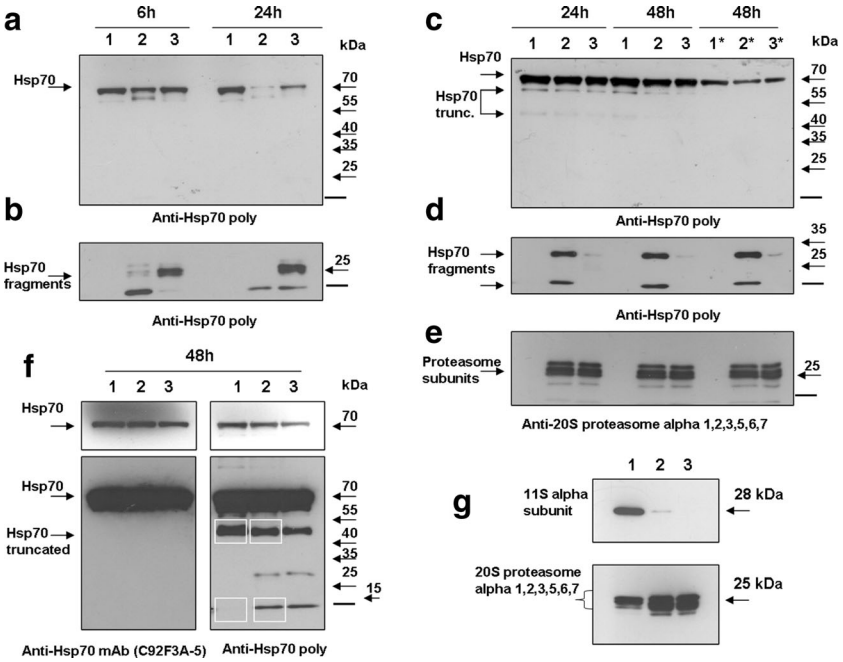

Fig. 3 Hsp70 is cleaved without ubiquitination by the 20S proteasomes. **a** Western blot analysis of Hsp70 and SDS-activated constitutive 20S proteasome mixtures. Samples in order: 1 μg of Hsp70 (*1*), 1 μg of Hsp70 and 1 μg of 20S proteasome (*2*), 1 μg of Hsp70, 1 μg of 20S proteasome, and 10 μM of MG132 (*3*). Samples were incubated 6 (*left panel*) or 24 (*right panel*) h. Membrane was stained with polyclonal anti-Hsp70 antibodies. In samples containing activated 20S proteasomes, 47 and 93% of the Hsp70 were degraded following 6 and 24 h, respectively. Addition of chymotrypsin-like activity inhibitor MG132 partially attenuated the degradation. Optical density of the protein bands was quantified using ImageJ software. Proteins and molecular weight markers are indicated by *arrowheads*. *Bar* represents electrophoresis front. **b** Lower part of the X-ray film **a** obtained at longer exposure. **c** Western blot analysis of Hsp70 and constitutive 20S proteasome mixtures incubated without SDS. Samples in order: 1 μg of Hsp70 (*1*), 1 μg of Hsp70 and 1 μg of 20S proteasome (*2*), 1 μg of Hsp70, 1 μg of 20S proteasome, and 1 mM of MG132 (*3*). *Asterisks* indicate that in the reaction mixtures, the Hsp70 128 was used. Membrane was stained with anti-Hsp70 antibodies. Samples were incubated 24 or 48 h. Samples, containing 20S proteasomes approximately from 10 and 20–40% (depending on Hsp70 used) of the Hsp70, were degraded following 24 and 48 h, respectively. Addition of chymotrypsin-like activity inhibitor MG132 partially attenuated the degradation. Optical density of the protein bands was quantified using ImageJ software. Proteins and molecular weight markers are indicated by *arrowheads*. *Bar* represents electrophoresis front. **d** Lower part of the X-ray film **c** obtained at longer exposure. **e** The membrane depicted in **c** and **d** stripped and stained with antibodies to 20S proteasome alpha subunits α 1, 2, 3, 5, 6, and 7. **f** Western blot analysis of Hsp70 and mixtures of Hsp70 with constitutive and immune 20S proteasomes incubated in SDS-free conditions. Samples in order: 1 μg of Hsp70 (*1*), 1 μg of Hsp70 and 1 μg of 20S proteasome (*2*), 1 μg of Hsp70 and 1 μg of immune 20S proteasome (*3*). Samples were incubated 48 h. Reactions were performed in triplicates. Three gels were run. Two were used for blotting and subsequent staining with antibodies. One membrane was stained with mouse monoclonal (C92F3A-5) and another with rabbit polyclonal anti-Hsp70 antibodies. *Upper part of the image* represents short, while *lower* a longer exposure of the film. Third gel was stained with Coomassie brilliant blue, and the white frames indicate corresponding gel slices that were excised and analyzed by mass spectrometry (ESM File 1 Fig. 4, ESM Files 2–4). Proteins and molecular weight markers are indicated by *arrowheads*. *Bar* represents electrophoresis front. **g** Comparative analysis of 20S fraction obtained from Kasumi-1 cells and commercial highly pure 20S and immune 20S proteasome preparations. Samples were analyzed by SDS-PAGE in the 12% gel. Ten micrograms of 20S fraction (track 1) and 1 μg of commercial 20S (track 2) and immune 20S (track 3) proteasomes were loaded onto the gel. Western blot with antibodies to the α-subunit of 11S regulator and α-subunits (1, 2, 3, 5, 6, 7 (∑α)) of the 20S proteasomes was performed

the mixture of proteins with the molecular weight equal and below 15 kDa (Fig. 3f, ESM File 1 Fig. 4C, ESM File 4); these were not detected in Hsp70 samples incubated without the proteasome.

Discussion

HSPs are in a large family of proteins some of which are constitutively expressed in cells and others are rapidly upregulated in response to various stress conditions (Evgen'ev 2014; Hartl et al. 2011; Radons 2016). HSP family members are classified upon their molecular mass and constitute subfamilies designated Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small Hsp families (Li and Srivastava 2004). Among these families, Hsp70 is one of the most evolutionary conserved. Hsp70 family members play central roles in the cellular PQC network. PQC represents an elaborate cellular system that monitors the integrity of cellular proteins and encompasses pathways that facilitate folding and either refolding, degradation, or sequestration of misfolded polypeptides (Chen et al. 2011; Ciechanover and Kwon 2015; Gestwicki and Garza 2012). Incorrectly folded proteins are recognized by molecular chaperones, and if the substrate protein could

not be repaired, it is delivered for degradation by UPS, chaperone-mediated autophagy (CMA), or macroautophagy depending on properties of the protein (Ciechanover and Kwon 2015). In the framework of PQC, Hsp70 is tightly linked with the UPS, and experimental evidences favor the intensive interplay and mutual effects of the UPS components and Hsp70 (Bobkova et al. 2015; Grune et al. 2011; Morozov 2017; Qian et al. 2006; Yurinskaya et al. 2015a). The deeper insight into the mechanisms of modulation of proteasomes by Hsp70 can help explain beneficial effects of Hsp70-based therapy on neurodegenerative diseases (Bobkova et al. 2014; Kalmar et al. 2012; Turturici et al. 2011; Wang et al. 2013), which are characterized by attenuated or compromised PQC and UPS (Bendotti et al. 2012; Bentea et al. 2016; Gestwicki and Garza 2012; Ross and Poirier 2004; Tseng et al. 2008).

Here using various proteasome preparations, we have shown that recombinant Hsp70 can directly modulate the activity of different forms of proteasomes in a different way (Fig. 2). This effect is dependent on Hsp70 concentration. It was revealed that high concentration of Hsp70 increases the activity of 26S proteasomes (Figs. 1d and 2a), while low concentration had almost no effect (Fig. 2a). On the contrary, even equimolar concentrations of Hsp70 induced significant decrease of the highly pure 20S proteasome activity (Fig. 2b, c).

The activation of 26S proteasomes by Hsp70 is in line with our previous findings. Recently, we demonstrated that amyloid-beta (Aβ) peptide induces a concentration-dependent increase of 26S proteasome activity (at least regarding fluorogenic peptide degradation) (Morozov 2016). The effect was attributed to the possible interaction of the peptide with the 19S proteasome regulator. It was shown that Hsp70 interacts with the 19S regulator (Grune et al. 2011). Thus, we propose that Hsp70 modulates 26S proteasome activity by a similar regulator-dependent and concentration-dependent mechanism (Fig. 2a). This explains changes of the 26S proteasome activity in the 26S fraction following incubation with different concentrations of Hsp70 (Fig. 1d). The decreased activity observed in 26S fraction after incubation with 5 μg/ml of the Hsp70 could be a result of an inhibition of a small number of 20S proteasomes (Fig. 2b, c) that are likely present in the fraction. This is apparently overwhelmed by the 26S proteasome activation when the Hsp70 concentration is increased (Figs. 1d and 2a).

The role of proteasome regulator in modulation of the proteasome activity following Hsp70 treatment from a first sight is challenged by increased proteasome activity in the 20S fraction (Fig. 1d). Indeed, 20S proteasome is free of regulators and in accordance with data obtained using purified 20S proteasomes (Fig. 2b, c); the inhibition of these complexes by Hsp70 could be anticipated. On the other hand, the 20S fraction contains large amount of proteasomes with 11S regulators (Fig. 3g) (Morozov 2016), which represent the most catalytically active form of proteasome in this fraction (Morozov 2016). Keeping in mind very low amount of 26S proteasomes in the 20S fraction (Fig. 1c) (Morozov 2016) and decrease of the activity of purified 20S proteasomes following Hsp70 treatment (Fig. 2b, c), it is very likely that the increased proteasome activity in this fraction is mediated via another form of proteasome: 20S proteasomes with 11S regulators.

Decreased activity of highly pure 20S proteasomes (both constitutive and immune) in the presence of Hsp70 additionally confirms the determinative role of proteasome regulator in the Hsp70-induced proteasome activity modulation (Fig. 2b–d). These data are also in line with our and previous findings by others where decrease of the pure 20S proteasome activity following incubation with different Aβ peptides was demonstrated (Morozov 2016; Tseng et al. 2008; Zhao and Yang 2010). However, Zhao and Yang have shown that Aβ is a substrate of 20S proteasome and observed proteasome inhibition results from protein and fluorogenic peptide competition for the active sites of the proteasome (Zhao and Yang 2010). We hypothesized that this could hold true for Hsp70. Indeed, analogously, we demonstrated that at least in vitro Hsp70 is degraded in ubiquitin-independent manner by the 20S proteasome (Fig. 3).

Although the majority of proteasome substrates are ubiquitinated and degraded by the 26S proteasome, it became clear recently that proteins are frequently degraded by the proteasomes without being tagged by ubiquitin (reviewed in Ben-Nissan and Sharon 2014). Moreover, ubiquitin-independent degradation of oxidized and otherwise damaged proteins is thought to be the main function of 20S proteasomes (Pickering and Davies 2012). This is facilitated by stress-induced exposure of hydrophobic patches on the surface of the substrate protein that are recognized by 20S proteasomes and can induce an alpha-subunit gate opening (Kisselev et al. 2002). Furthermore, Baugh et al. has demonstrated that 20S proteasomes cleave approximately 22% of all cellular proteins. These intrinsic proteasome substrates (IPSs) are often cleaved within extended disordered regions including flexible linker regions representing a universal structural degradation signal (Baugh et al. 2009). Importantly, the cleavage often results in generation of distinct cleavage products (Baugh et al. 2009). Generally, the presence of an unstructured region in the protein is the primary requirement for the ubiquitin-independent degradation (Baugh et al. 2009; Ben-Nissan and Sharon 2014).

Hsp70 has all the necessary elements of the intrinsic proteasome substrate. It has a flexible linker region between nucleotide binding and substrate binding domains (Bardwell and Jakob 2012; Turturici et al. 2011; Zhang et al. 2014). Moreover, Hsp70 was shown to have disordered C-terminus (Smock et al. 2011; Uversky 2011; Zhang et al. 2014) (ESM File 1 Fig. 5). Indeed, following incubation of Hsp70 with the constitutive and immune 20S proteasome, the amount of full-length protein was decreased and the distinct cleavage

product with molecular weight around 30 kDa was observed by using polyclonal serum (Fig. 3d, f, ESM File 1 Fig. 1). This fragment was not recognized by monoclonal antibodies directed towards Hsp70 a.a. 436–503 (Fig. 3f), indicating that 30 kDa fragment contains N-terminal part of the protein and that Hsp70 degradation by the 20S proteasome likely starts from the disordered C-terminus. This was confirmed by mass spectrometry results, which demonstrated disappearance of 45–47 kDa form of Hsp70 containing C-terminal part following the incubation with the 20S proteasome (ESM File 1 Fig. 4A, B, ESM Files 2, 3).

All this indicates that Hsp70 is cleaved by the 20S proteasomes. Here, it should be mentioned that both ubiquitin dependent and independent degradation of the same substrates was demonstrated (Asher et al. 2005; Ben-Nissan and Sharon 2014). Previously, the degradation of Hsp70 was shown to be 26S proteasome-dependent and induced by ubiquitination of the protein by its co-chaperone CHIP (Qian et al. 2006). Our results demonstrate that although it seems to be the most important and efficient way for Hsp70 turnover, a ubiquitin-independent and ATP-independent hydrolysis of Hsp70 can also take place and that the C-terminus of the protein likely serves as a degradation initiation site. This is supported by our previous results; exogenous recombinant radiolabeled Hsp70 efficiently penetrated cells and was rapidly degraded without ubiquitination (Yurinskaya et al. 2015a).

Cleavage of some IPS by the 20S proteasome was shown to keep structured domains intact and to produce functional proteins (Baugh et al. 2009; Moorthy et al. 2006). In this regard, the identification of truncated variant of Hsp70 isoform in several neuroendocrine tumors is of special interest (Zierhut et al. 2004). Authors proposed that generation of this truncated form can result from specific protease cleavage (Zierhut et al. 2004). Taking our results into consideration, one cannot exclude that the 20S proteasomes contribute to the cleavage. Furthermore, many tumors have increased proteasome activity (Arlt et al. 2009), elevated levels, and different localization of Hsp70 in comparison with normal cells (Multhoff and Hightower 2011); thus, one can expect a broader repertoire of truncated forms of the protein in the transformed cells. All this indicates that a spectrum of Hsp70 proteins could be additionally broadened by truncated forms that can have biological activity and perform certain functions under various conditions.

Finally, it should be noted that Hsp70 128 was degraded by 20S proteasome slightly more efficient than the wild-type protein (Fig. 3c, ESM File 1 Fig. 1). This could be associated with introduced mutations which can alter the secondary structure of the protein towards increased disorder (ESM File 1 Fig. 2). To check this possibility and since no crystal structure of Hsp70 128 is available, we used five-protein disorder prediction software and compared wild-type and mutated protein sequences (ESM File 1 Fig. 5A–E). Although no significant differences were detected, minor increase of disorder of Hsp70 128 was predicted by PrDOS and DisEMBL software (ESM File 1 Fig. 5B, C).

Conclusions

In summary, we have shown that Hsp70 modulates the activity of different forms of proteasomes in a different way: increasing the activity of 26S proteasomes and decreasing the activity of 20S proteasomes. This highlights the role of proteasome regulators in the interplay between Hsp70 and proteasomes. The decreased activity of 20S proteasomes is likely a result of competition of a fluorogenic substrate with the Hsp70 for the active site of the proteasome, since Hsp70 is itself a substrate of 20S proteasomes and at least in vitro could be cleaved in a ubiquitin-independent manner. These results disclose novel aspects of the interplay between Hsp70 and proteasomes in the cell.

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Compliance with ethical standards

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