The Chaperone Activity and Substrate Spectrum of Human Small Heat Shock Proteins*5

Received for publication, September 25, 2016, and in revised form, November 12, 2016 Published, JBC Papers in Press, November 30, 2016, DOI 10.1074/jbc.M116.760413

Evgeny V. Mymrikov, Marina Daake, Bettina Richter, Martin Haslbeck, and Johannes Buchner2

From the Center for Integrated Protein Science at the Department Chemie, Technische Universität München, Lichtenbergstrasse 4, 85748 Garching, Germany

Edited by Norma Allewell

Small heat shock proteins (sHsps) are a ubiquitous family of molecular chaperones that suppress the unspecific aggregation of miscellaneous proteins. Multicellular organisms contain a large number of different sHsps, raising questions as to whether they function redundantly or are specialized in terms of substrates and mechanism. To gain insight into this issue, we undertook a comparative analysis of the eight major human sHsps on the aggregation of both model proteins and cytosolic substrates and mechanism. We discovered that sHsps, which form large oligomers (HspB1/Hsp27, HspB3, HspB4/αA-crystallin, and HspB5/αB-crystallin) are promiscuous chaperones, whereas the chaperone activity of the other sHsps is more substrate-dependent. However, all human sHsps analyzed except HspB7 suppressed the aggregation of cytosolic proteins of HEK293 cells. We identified ~1100 heat-sensitive HEK293 proteins, 12% of which could be isolated in complexes with sHsps. Analysis of their biochemical properties revealed that most of the sHsp substrates have a molecular mass from 50 to 100 kDa and a slightly acidic pI (5.4–6.8). The potency of the sHsps to suppress aggregation of model substrates is correlated with their ability to form stable substrate complexes; especially HspB1 and HspB5, but also B3, bind tightly to a variety of proteins, whereas fewer substrates were detected in complex with the other sHsps, although these were also efficient in preventing the aggregation of cytosolic proteins.

Maintaining intracellular protein homeostasis is one of the most essential processes for life. Under both physiological and adverse conditions, molecular chaperones play a crucial role in this process (1). Upon different stress conditions protein unfolding is dramatically increased. This leads to increased levels of unfolded, aggregation-prone proteins (1). ATP-dependent chaperones, such as Hsp60, Hsp70, and Hsp90 can bind unfolded or improperly folded proteins and promote refolding using the energy of ATP hydrolysis (2). The group of small heat shock proteins (sHsps)3 represents a class of ATP-independent chaperones (3, 4), which bind unfolding substrate proteins supposedly in a promiscuous manner and keep them in a folding-competent state. The characteristic feature of all sHsps is the presence of a conserved α-crystallin domain that consists of two β-sheets flanked by a non-conserved long N-terminal extension and a short C-terminal segment (5). Another characteristic feature of sHsps is the formation of large heterogeneous oligomers; the size of such an oligomer can reach 1 MDa (6, 7). The human genome encodes 10 different sHsps (HspB1-B10) (8, 9) that exist as large and small oligomers (Table 1). Among them are the two α-crystallins (αA- and αB-crystallin, or HspB4 and HspB5), which represent up to 30% of total eye lens protein (10, 11) and play a crucial role in prevention of cataract (12). HspB1 (Hsp27) is the most ubiquitous human sHsp, which is expressed at a high level in different tissues (13). Two specific human sHsps, HspB9 and HspB10, are expressed in testis only (14, 15). The most extensively studied human sHsps are HspB1 (Hsp27), HspB5 (αB-crystallin), HspB6 (Hsp20), and HspB8 (Hsp22), whereas the available data on other human sHsps are poor. Differences in expression patterns and biochemical properties of human sHsps lead to the suggestion that they might have different substrate spectra and functions in vivo (16); however, detailed information on this issue is not yet available. Most of our knowledge on the chaperone function of sHsps is based on in vitro anti-aggregation assays using model substrate proteins, whereas in vivo data are rare (17, 18). In in vitro assays, the model substrate proteins are either thermally or chemically denatured, and the effects of an sHsp on their aggregation are assessed. Common model substrates are, among others, insulin (19), yeast alcohol dehydrogenase (ADH) (20), citrate synthase (CS) (21), and malate dehydrogenase (MDH) (22). These assays are excellent tools to determine whether a given protein has chaperone properties and to analyze specific features of the chaperone mechanism. In this context it is an important open question of how well these assays mimic the in vivo situation and reflect the cellular functions of sHsp and which (if any) of the model substrate assays indeed represents a good surrogate for the in vivo situation are yet unclear. These open questions seriously hamper the progress in understanding the cellular function of chaperones in general and that of sHsps in particular. There is indirect evidence for at least some sHsps that their

*5 The work was supported by the Deutsche Forschungsgemeinschaft (SFB 1035) and CIPSM. The authors declare that they have no conflicts of interest with the contents of this article.

1 This article contains supplemental Figs. 1–5 and Tables 1 and 2.

1 Recipient of a Peter und Traudl Engelhorn Stiftung post-doctoral fellowship.

2 Present address: Institute for Biochemistry and Molecular Biology, University of Freiburg, Stefan-Meier-Strasse 17, 79104 Freiburg, Germany.

2 To whom correspondence should be addressed. Tel.: 49-89-289-13340; Fax: 49-89-289-13345; E-mail: johannes.buchner@tum.de.

3 The abbreviations used are: sHsps, small heat shock protein; ADH, alcohol dehydrogenase; CS, citrate synthase; MDH, malate dehydrogenase. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
in vitro and in vivo chaperone activities do not correlate. As shown by Vos et al. (17), only three human sHsps (HspB1/ Hsp27, HspB4/α-crystallin, and HspB5/β-crystallin) were able to stabilize luciferase during heat shock and cooperate with ATP-dependent chaperones increasing its refolding in vivo. At the same time, in different studies other human sHsps (e.g. HspB6/Hsp20 and HspB8/Hsp22) were shown to be able to suppress the aggregation of amorphously aggregating model substrates in vitro (23–26). Recently, an assay using cell lysates and thermal unfolding was introduced to study human HspB1 (27) and HspB5 (28). This assay allows monitoring the effect of the sHsp on the aggregation of all soluble cytosolic proteins in biologically complex environment and thus might represent a format to compare chaperone activities in a quantitative manner. However, with this format no detailed mechanistic studies of the chaperones are possible.

Here, we set out to compare the chaperone activities of the eight major human sHsps using a panel of in vitro aggregation suppression assays employing different model substrates as well as lysates of two cell lines. This comprehensive study showed that, depending on the model substrate used, different activities toward the suppression of aggregation are observed for the sHsps studied. Interestingly, although most of the sHsps analyzed were able to suppress temperature-induced aggregation in cell lysate aggregation assays, some sHsps were found to be inactive in the in vitro chaperone activity assays, indicating a gap between in vitro and in vivo situations. Furthermore, we determined the substrate spectra of human sHsps by mass spectrometry after isolation of the sHsp-substrate complexes from heat-stressed cell lysates.

Results

In Vitro Chaperone Activity Assays: Determining Standard Conditions—The chaperone function of sHsps has been investigated in a large number of studies. However, different model substrate proteins and different conditions were employed. Although insulin aggregation, one of the commonly used assays, can be performed in a wide range of buffers (19, 24, 27, 29, 30) without affecting the aggregation process (19), the impact of variations in the buffer composition on other model substrates and sHsp activity remains elusive. This makes the comparison of data from different studies for a given sHsp or between different sHsps difficult. Therefore, it is important to determine solvent conditions suitable for most of the model substrates and sHsps. We compared two buffer systems (PBS and 40 mM HEPES, pH 7.5) that are commonly used in aggregation assays. The aggregation kinetics of most of the analyzed substrates turned out to be highly dependent on the buffer selected (supplemental Fig. 1). We found that PBS is more suitable as a standard buffer because the aggregation of the model substrates (except for CS) was significantly faster in this buffer compared with HEPES buffer. Additionally, the composition and ionic strength of PBS are more similar to natural human buffer systems. We performed all assays in the presence of the reducing agent DTT, as most of the analyzed human sHsps contain cysteines and could become oxidized during the measurements (especially at high temperature) (24). This modification might affect chaperone activity (31). The conditions of all aggregation assays were identical to allow the comparison of data obtained on different model substrates. For detailed information on the standard procedure, see “Experimental Procedures.”

Oligomeric State of Human sHsps—As shown in Table 1, human sHsps vary significantly in their oligomeric state. We performed a size-exclusion chromatography for all sHsps utilized in the present study to directly compare their oligomeric state (supplemental Fig. 2). HspB1 (Hsp27), HspB4 (αA-crystallin), and HspB5 (αB-crystallin) form large oligomers with apparent molecular masses of ~450–500 kDa. This corresponds to the formation of predominantly 24-mers as was shown for HspB5 (32). Under the conditions used, HspB1 oligomers seem to be in equilibrium with a smaller species (indicated by a shoulder of the corresponding peak in the elution profile). The oligomers formed by HspB2 are significantly smaller, with an apparent molecular mass ~100 kDa, which correspond to a hexamer or an octamer. HspB3 forms extremely heterogeneous oligomeric species; their apparent molecular masses vary from ~50 kDa to ~500 kDa (dimers to 30-mers). Previously it was suggested that this protein forms only small species (33). However, incubation at 37 °C induces an association of HspB3 into larger oligomers (supplemental Fig. 2). The peaks of three other sHsps (HspB6/HspB7/HspB8)
correspond to small molecular weight species, presumably dimers.

Comparison of the Chaperone Activities of Human sHsps toward Model Substrates—To obtain a comprehensive picture of the chaperone activity of human sHsps, we compared the effect of the eight most important human sHsps on the aggregation of six model substrates. Two other human sHsps (HspB9 and HspB10/ODF1) are expressed only in testis (8). The effects of the respective human sHsps on the aggregation kinetics of the model substrate (Fig. 1) demonstrate a high variation in the abilities of different human sHsps at one selected ratio indicated by the red line in the graphs on the left side. The model substrates were incubated alone or with different sHsps at 45 °C (MDH, GAPDH, rhodanese, and CS), 42 °C (ADH), or 37 °C (insulin), and aggregation kinetics were followed by monitoring the apparent optical density at 360 nm. The mean of at least three independent replicates ± S.D. is shown in all graphs. a.u., arbitrary units.

FIGURE 1. The effect of human sHsps (HspB1-HspB8) on the aggregation of six different model substrates. Left panels, aggregation of model substrates was monitored in the presence of increasing concentrations of different sHsps. The levels of aggregation (apparent absorbance at 360 nm) at the end of the incubation period were normalized to the absorbance signal of spontaneous aggregation (without sHsp) and plotted against the sHsp concentration. Here and in all other figures the color code is as shown in the right bottom corner: black, HspB1; magenta, HspB2; orange, HspB3; blue, HspB4; cyan, HspB5; red, HspB6; brown, HspB7; green, HspB8; gray, without sHsp. Right panels, aggregation kinetics of six model substrates in the presence of different human sHsps at one selected ratio indicated by the red line in the graphs on the left side.
plotted the aggregation levels against the concentration of each sHsp (Fig. 1) and determined the potency of a given sHsp as reflected by the sHsp/substrate ratio needed for half-maximum suppression of aggregation ($IC_{50}$) (see Fig. 4). In this context it is important whether a given sHsp can suppress aggregation completely. For example, HspB4 and HspB5 (αA- and αB-crystallin) completely prevented the aggregation of MDH (Fig. 1), whereas HspB1 (Hsp27) was less active in this assay and reduced the aggregation only partially even when present in excess. For HspB6 (Hsp20) and HspB3 we did not observe any effect on MDH aggregation, and HspB2, HspB7, and HspB8 (Hsp22) even increased MDH aggregation (Fig. 1). The latter effect is presumably due to co-aggregation of the sHsp with the substrate.

The most active sHsps significantly reduced the aggregation of model substrates at a low sHsp/substrate ratio. Interestingly, the two α-crystallins (HspB4 and HspB5) showed similarly high activities toward the temperature-induced aggregation of all model substrates (MDH, GAPDH, rhodanese, and CS). However, the activity of another large oligomeric human sHsp, HspB1 (Hsp27), was dependent on the substrate used (see Fig. 1). Its activity was higher than those of the α-crystallins toward rhodanese aggregation, comparable in the case of CS and lower for MDH and GAPDH. Among the other human sHsps, only HspB3, which is also able to form large oligomers, effectively prevented the aggregation of CS and shows low activity in the GAPDH aggregation assays, but it did not affect the aggregation of MDH and rhodanese (Fig. 1). The remaining sHsps analyzed (HspB2, HspB6, HspB7, and HspB8), which form dimers or small oligomers, only slightly reduced the temperature-induced aggregation of specific model substrates; e.g., HspB2 was slightly active toward GAPDH aggregation, and HspB7 reduced CS aggregation to half-maximum values and had a moderate effect toward GAPDH aggregation, whereas HspB8 showed low chaperone activity in the rhodanese aggregation assay (see Fig. 1). Thus, these assays differentiate the respective sHsps mechanistically and especially suggest that the sHsps differ in their mode of interaction with specific model substrates (Fig. 1).

To test whether the effects observed are restricted to temperature-induced unfolding, we employed model substrates that aggregate in response to reduction-induced unfolding. In these assays, where aggregation of ADH and insulin was studied, almost all sHsps analyzed (except HspB7) were able to prevent aggregation completely or partially (Fig. 1). Indeed, in the ADH aggregation assays, six of eight sHsps (except HspB3 and HspB7) reduced aggregation almost completely at equimolar ratios (Fig. 1). In this assay, HspB1, HspB4, and HspB6 were more active than HspB2, HspB5, and HspB8, and HspB3 was significantly less active than all the above-mentioned sHsps (Fig. 1). In the insulin aggregation assay, the sHsp with the highest activity was HspB1. Almost all other sHsps analyzed were able to suppress aggregation of this substrate to a similar degree; only HspB3 and HspB4 were slightly less active (Fig. 1). Interestingly, in both reduction-induced aggregation assays, the chaperone activities of the two closely related sHsps, αA- and αB-crystallins (HspB4 and HspB5), were significantly different, which is in contrast to their quite similar behavior in temperature-induced aggregation assays. In contrast to all other sHsps, HspB7, which forms mostly dimers according to size-exclusion chromatography (supplemental Fig. 2), was completely inactive toward both ADH and insulin aggregation (Fig. 1).

**Chaperone Activity of sHsps on Cytosolic Proteins**—Because the aggregation suppression of the different sHsps analyzed turned out to be highly dependent on the model substrate used, we decided to determine the chaperone activities of human sHsps in a biologically complex environment. To do that we used cytosolic extracts of human cell lines as substrates in the chaperone assay. Recently, this method was successfully employed to compare the chaperone activities of wild type HspB1 (Hsp27) and HspB5 (αB-crystallin) and their phospho-mimicking mutants (27, 28). Initially, we analyzed sHsps expression in three commonly used cell lines (HEK293, HeLa, and MCF7). In HeLa and MCF7, we detected expression of HspB1 (Hsp27), whereas no sHsp was detected in the HEK293 cell line by Western blotting (supplemental Fig. 4). Furthermore, we could detect HspB1 in HEK293 by mass spectrometry, indicating a very low expression level.

Similar to the assays described above, aggregation of cell lysate proteins was induced by incubation at 45 °C. Because of the high amount of different proteins aggregating at the same time, monitoring aggregation by light scattering in this case did not provide a representative read-out of the reaction. We, therefore, separated soluble and insoluble fractions after incubation and analyzed the difference in the protein amount in the insoluble fractions with SDS-PAGE (Fig. 2A). Interestingly, for the MCF7 cell line, the total amount of insoluble proteins after incubation at 45 °C was significantly reduced compared with HeLa and HEK293 cell lines (Fig. 2A, lower panel). This could be due to the high expression level of endogenous HspB1 of >1% (0.4 μM under conditions of the aggregation assay). At the same time the addition of 0.5 μM HspB1 (~1.3% of cell lysate proteins) reduced aggregation of HEK293 and HeLa lysate proteins significantly (Fig. 2C). For this reason we excluded MCF7 from further analysis. Of note, the sHsp concentration in skeletal and smooth muscles can reach similar values of >1.0% of total protein (34, 35).

The addition of any sHsp except HspB7 to the cell lysate significantly reduced the aggregation of all cellular proteins (Fig. 2B). To measure the chaperone activity quantitatively in the cell lysate assay, we determined the total amount of insoluble proteins after SDS-PAGE (Fig. 2B) for each lane by densitometry. The amount of insoluble proteins after incubation in the presence of a specific sHsp was normalized to that of the control sample (no sHsp added). The resulting values were plotted against the concentration of sHsp in the sample (Fig. 2C). This allowed us to quantify the sHsp concentration required for half-maximum reduction of aggregation ($IC_{50}$) for every sHsp analyzed (see Fig. 4). According to the data presented in Fig. 2C, HspB1 (Hsp27) possesses the highest chaperone activity for the HEK293 cell lysate among the sHsps analyzed. However, both α-crystallins (HspB4 and HspB5) are only slightly less effective than HspB1. In the HEK293 lysate, the three sHsps reduced the amount of insoluble proteins by ~60% (Fig. 2C). Interestingly, almost all
human sHsps (except HspB7) showed significant chaperone activity in the lysate-based assay. The chaperone activities of the human sHsps decrease in the order HspB1/HspB4/HspB5/HspB6/HspB8/HspB3/HspB2/HspB7, where HspB7 is completely inactive (Fig. 2C). The addition of GFP as a control protein at the same concentrations did not affect the aggregation of lysate proteins (Fig. 2C). Similar results were obtained for HeLa cell lysates (Fig. 2C). However, we observed that several sHsps (namely, HspB2, HspB6, HspB7, and HspB8) were proteolytically degraded during incubation with HeLa lysate at elevated temperature even in the presence of protease inhibitors (supplemental Fig. 5A). In HEK293 lysates we detected degradation of only HspB8 (supplemental Fig. 5B).

Thus, although the comparison of the chaperone activities of the full set of human sHsps was not possible using HeLa lysates, we were able to assess that also here the three oligomeric sHsps (HspB1, HspB4, and HspB5) suppressed aggregation most efficiently (Fig. 2C).

Identification and Characterization of sHsp Interactors—Based on the results of the aggregation assays for model substrates, we concluded that human sHsps demonstrate certain preferences in their substrate specificity (Fig. 1). For example, HspB1 (Hsp27) was highly active toward rhodanese and CS aggregation but only partially suppressed aggregation of MDH and GAPDH. On the other hand, in cell lysate assays, we detected a promiscuous reduction of the aggregation of lysate proteins (Fig. 2B). To investigate this issue in more detail, we analyzed the substrate specificity of human sHsps by isolating sHsp-substrate complexes from cell lysates followed by their identification via mass spectrometry. As a reference, we analyzed the so-called heat-sensitive fraction of HEK293 lysates, containing proteins that precipitate upon incubation at ele-
vated temperatures. Mass spectrometric analysis of this fraction revealed \( \sim 1100 \) different proteins (supplemental Table 1). Because almost all human sHsps analyzed promiscuously suppressed the aggregation of HEK293 lysate proteins (see Fig. 2C), we can assume that most of the detected proteins might be potential sHsp substrates; however, the effect of different sHsps on individual proteins may vary significantly. To identify which lysate proteins are stably bound to a single sHsp under heat shock conditions, we performed immunoprecipitation studies for HspB1-HspB7 (HspB8 was unstable under the conditions used). In total, \( \sim 12\% \) (130 unique hits; Fig. 3A) of all heat-sensitive proteins were found as potential sHsp substrates in the pulldown experiments. The number of hits detected varied substantially among different sHsps. HspB1 and HspB5 bound the highest number of different substrates (109 and 88, respectively) followed by HspB3 (54 hits), HspB4 (24 hits), and HspB2 (19 hits), whereas for HspB6 and HspB7 almost no interactors were detected (Fig. 3A). When we compared the interactors of the three sHsps with the highest number of detected substrates, HspB1, HspB3, and HspB5, a significant overlap was detected (Fig. 3B). The complete list of potential substrates of human sHsps analyzed (HspB1-HspB7) is presented in supplemental Table 2.

To assess the common features of sHsp substrates, we performed a comparative analysis of the size, pI, and total hydrophobicity distribution of HspB1-HspB5 substrates (Fig. 3, C–E) using both the fraction of heat-sensitive lysate proteins and the total human proteome as references. Most of the heat-sensitive proteins have monomer molecular masses of 31–93 kDa (50% of hits between the 1st and the 3rd quartiles) with a median at
Activity and Specificity of Human sHsps

50 kDa. Compared with the whole human proteome, the $M_r$ distribution in the heat-sensitive fraction is slightly broader because of a shift toward larger proteins (compare the two last bars in Fig. 3C). The distributions of sHsp substrates are significantly shifted to larger proteins in a more narrow range than that of the heat-sensitive fraction (Fig. 3C). Therefore, the 1st quartile for sHsp substrates is shifted to 48 kDa for HspB1 and HspB5 and even higher for HspB3 and HspB4 (56 and 58 kDa, respectively). Correspondingly, the medians are also shifted to higher values (Fig. 3C). This indicates an increased binding of the sHsp analyzed to larger, aggregation-prone proteins in the range of 50–100 kDa. Analysis of the pI distribution also revealed a difference of sHsp substrate spectra compared with the heat-sensitive fraction and the total human proteome. In the total proteome, the pI distribution is extremely broad, with a median $\sim$7.0 (Fig. 3D, the last bar), whereas in the heat-sensitive fraction the distribution is narrower with 50% of the hits located in the neutral region between pI of 5.6 and 7.7 (the median is at 6.5) (Fig. 3D). Interestingly, most of the sHsp substrates detected have a pI in the range between 5.4 and 6.8. Moreover, there were almost no interactors detected with a pI above 9.0 (except for two proteins, which were bound to HspB1) and <4.3 (Fig. 3D). This indicates that human sHsps preferentially bind to proteins with a moderate negative charge.

It is generally accepted that sHsps bind unfolded substrate proteins via interactions with hydrophobic patches, which become accessible upon unfolding (36, 37). Therefore, we also analyzed the distribution of total hydrophobicity using the GRAVY index (38) (Fig. 3E). This index is calculated based on the amino acid composition, and positive values indicate a high percentage of hydrophobic residues in a given protein. We did not observe a significant shift of the GRAVY index toward positive values for sHsp substrates. It varies from $-0.6$ to $-0.2$ for most of the heat-sensitive proteins as well as for proteins bound to sHsps and in a slightly broader range also in the total human proteome (Fig. 3E).

Using data from the PDB the PaxDb database (39) for protein abundance in HEK293 (40), we determined how abundant the proteins detected in the heat-sensitive fraction and the sHsp substrates are (Fig. 3F). In the heat-sensitive fraction, we observed an under-representation of low abundant proteins ($\leq$0.1 ppm), which might be due to detection limits. We could detect proteins with abundances in HEK293 cells between 0.1 and 10,000 ppm (supplemental Table 1). However, among the proteins detected there were several with a very low abundance $<0.1$ ppm (for example, dual specificity mitogen-activated protein kinase kinase 2, integrator complex subunit 3, EH domain-binding protein 1). We would further like to note that $\sim$18% of the proteins present in our heat-sensitive fraction were not detected in the analysis of the total HEK293 proteome (40) and that their abundances are, therefore, not annotated (supplemental Table 1). Potential sHsp substrates were also found to be relatively highly abundant compared with both the total HEK293 proteome and the heat-sensitive fraction (Fig. 3F).

However, some high abundant proteins, e.g. heterogeneous nuclear ribonucleoproteins A2/B1, peroxiredoxin-1, and cyclophilin A, which were found in the heat-sensitive protein fraction (see supplemental Table 1), were absent among the sHsp interactors.

Along with the analysis of the biochemical properties of sHsp substrates, we performed a functional overrepresentation test using the PANTHER online tool (41) for the five sHsps (HspB1-HspB5) with significant numbers of interactors identified (Fig. 3G). We used default settings and the total human proteome as a reference (20,814 unique hits). An overrepresentation test in “GO Biological functions” revealed a relatively high amount of proteins involved in different “metabolic processes” (RNA, DNA, protein, etc.), in classes related to translation (“regulation of translation,” “translation,” “tRNA metabolic processes”), and in protein turnover (classes of “protein folding,” “protein complex assembly,” “protein complex biogenesis”) (Fig. 3G). In the class of tRNA metabolic process, we mostly found aminoacyl-tRNA ligases (14 of 20 in total). Different proteins involved in the regulation and functioning of the translation machinery seem to be one among the most important targets of sHsps. We also found several chaperones from different groups (e.g. CCT3, CCT8, HSPA4/Hsp70R/Y/APG2, DNAJB1/Hsp40). Most likely they are not sHsp substrates but bind the same unfolded proteins simultaneously with sHsps at elevated temperature.

Discussion

Small heat shock proteins play a key role in protecting the cell from irreversible protein aggregation under diverse stress conditions (42, 43) as well as aging (44). Members of the sHsp family are found in almost all living organisms, and the number of different sHsps is substantially increased in higher eukaryotes compared with prokaryotes and lower eukaryotes (5, 45). One reason for this phenomenon is their specialization in terms of localization, function, and expression. The human sHsps seem to show not only tissue and developmental specificity but also functional diversity (46, 47). The reasons of this diversity are still unclear, and comparative functional studies are rare.

In previous studies on sHsps (as well as on other molecular chaperones), often only a single model substrate protein was used to determine their anti-aggregation properties in vitro. Recently, a novel technique based on the prevention of enzyme inactivation by ATP-dependent chaperones was suggested (48). This technique is not applicable for sHsps because in their case inactivation of enzymes is not a good readout for chaperone activity due to the possibility of transient or stable interactions (4). Thus there is still no standardized, commonly accepted protocol to determine and compare chaperone activities of sHsps, and experimental conditions vary a lot, sometimes even in the same study when different substrates are applied. As a result, despite a wealth of experimental data that compare chaperone activities of sHsps, the data obtained in different studies are incomparable. In our analysis, we utilized a panel of six different model substrates and lysates of two cell lines to compare the chaperone activities of eight human sHsps. To reduce variations in the experimental setup, we standardized the conditions of the aggregation assays. This allowed for the first time a comparative analysis of the chaperone activity of sHsps as summarized in Fig. 4. This analysis shows distinct chaperone activities for the sHsp depending on the model sub-
were proteolyzed during the cell lysate assays. This indicates that the respective sHsp is not active or co-aggregates with the substrate. The results of aggregation assays for single model substrates cannot adequately describe the chaperone activity of human sHsps in a biologically complex protein mixture (cell lysate) to determine general chaperone activity. However, this correlation is not always true; e.g. some in vitro chaperone assays HspB1 (Hsp27), HspB4, and HspB5 (αA- and αB-crystallins), which form large oligomers. HspB6, HspB7, and HspB8 were inactive or showed only moderate activity. As they do not form large oligomers, it is tempting to speculate that this property correlates with the strength of chaperone activity. However, this correlation is not always true; e.g. in some in vitro chaperone assays HspB1 (Hsp27) is only weakly active. For HspB3, we show that it forms large heterogeneous oligomers that are able to reduce the thermal aggregation of only two of four model substrates. Moreover, for all sHsps studied, the ability to form large oligomers did not seem to play a role for the chaperone activity toward reduction-induced aggregation (Fig. 4). Seemingly, the mechanisms of reduction- and temperature-induced unfolding are different. Two sHsps (HspB6 and HspB8) that form only small oligomeric species were unable to suppress the thermal aggregation of single model substrates but could significantly reduce the reduction-induced protein aggregation as well as the aggregation of cellular proteins (Fig. 2C and 4). The comparison of the results of aggregation assays for single model substrates with data obtained on cell lysates leads us to the conclusion that model substrates cannot adequately describe the chaperone activity of a human sHsp in a biologically complex protein mixture such as a cell lysate (Fig. 4).

In both cell lysate assays the relative activities of the human sHsps are similar (Fig. 4). Consequently, the use of a complex protein mixture (cell lysate) to determine general chaperone activity in terms of aggregation suppression seems to represent an integral method summarizing the effects of the chaperones on the natural proteome, thus reflecting more closely the cellular situation. However, it is worth mentioning that such an approach restricts mechanistic studies of chaperone action. The use of different in vitro model substrates allowed us to conclude that the human sHsps demonstrate specificity in protecting substrates from amorphous aggregation. Therefore, for comprehensive comparative studies we suggest the use of a panel of model and authentic substrates as well as cell lysates. The first approach allows the analysis of sHsp chaperone specificity and mechanistic studies, whereas cell lysates are suitable to address chaperone action in a complex cellular milieu. Recently, a comprehensive comparative study of sHsp chaperone activities toward polyQ aggregation and reactivation after heat-induced unfolding (but not amorphous aggregation) of luciferase in a human cell line was reported (17). Co-expression analysis of different human sHsps and firefly luciferase showed that HspB1 (Hsp27) and both α-crystallins (HspB4 and HspB5) provide a higher level of luciferase reactivation after heat shock compared with all other human sHsps. However, they were much less active or completely inactive toward the suppression of the amyloid-like aggregation of polyQ proteins (17). In our study we found that HspB1 (Hsp27) and the two α-crystallins (HspB4 and HspB5) are the most active human sHsps in suppressing the thermal aggregation of cytosolic proteins, whereas the other human sHsps showed lower activity. This additionally supports our assumption that cell lysates are a good proxy for the overall chaperone action of sHsps against amorphous aggregation in the cell. Our study together with previous evidence shows that among human sHsps HspB7 is special. It was inactive toward both model substrate and cell lysate aggregation in vitro (this study) as well as toward refolding of luciferase in vivo (17). Thus, it does not seem to bind unfolded proteins and suppress their aggregation; however, it is able to protect cells from polyQ aggregation, seemingly indirectly through affecting the autophagy pathway (17, 46). Such a specific function of HspB7 might be due to its unusual N-terminal domain containing a long polyserine linker (49), which is predicted to be largely disordered (50).

Using co-immunoprecipitation to identify substrates that are tightly bound to human sHsps upon heat shock (supplemental Table 2), we found the highest number of interactors (~100) for HspB1 (Hsp27) and HspB5 (αB-crystallin) in agreement with their higher activity in the aggregation assays (Fig. 4). Noteworthy, these two sHsp are the most ubiquitously expressed in man, and in contrast to other sHsps their expression is strongly up-regulated upon stress conditions (51). 54 hits were detected for

| sHsp | Temperature-induced aggregation | Reduction-induced aggregation | Cell lysate aggregation | CoIP substrates |
|------|--------------------------------|--------------------------------|------------------------|----------------|
|      | MDH | GAPDH | Rhodanese | CS | ADH | Insulin | HEK | HeLa |      |
| HspB1 | 2.00 | 4.00 | 0.11 | 4.35 | 0.12 | 0.04 | 1.2 | 1.1 | 109 |
| HspB2 | n.a. | >8 | n.a. | >8 | 0.39 | 0.07 | >10 | >10* | 19  |
| HspB3 | n.a. | >8 | n.a. | 6.54 | 0.84 | 0.20 | >10 | >10 | 54  |
| HspB4 | 0.12 | 0.75 | 0.25 | 2.74 | 0.19 | 0.20 | 2.0 | 1.6 | 24  |
| HspB5 | 0.07 | 0.67 | 0.23 | 2.95 | 0.37 | 0.12 | 1.9 | 1.8 | 88  |
| HspB6 | n.a. | n.a. | n.a. | n.a. | 0.17 | 0.13 | >10 | 8.7* | 2   |
| HspB7 | n.a. | >8 | n.a. | 8.0 | n.a. | n.a. | n.a. | * | 2   |
| HspB8 | n.a. | n.a. | n.a. | n.a. | 0.41 | 0.12 | >10* | n.a. | n.d. |
Activity and Specificity of Human sHsps

HspB3, the sHsp that was less active toward model substrates aggregation. Interestingly, the substrates of these three sHsps overlap to a high extent (Fig. 3B). Only 24 unique interactors were detected for HspB4 (αA-crystallin), which might be due to its adaptation to the specific environment of the eye lens. Our isolation procedure may be biased toward detecting strong binders, which share similar properties. In this context, one should note that stable substrate binding and prevention of aggregation are two different and probably independent processes; some sHsps can tightly bind certain proteins and do not suppress their aggregation. However, specific sHsps seem to bind substrates transiently and still prevent aggregation efficiently. This might be the case for HspB4, which in a non-natural environment binds significantly less substrates compared with HspB1 and HspB5. Interestingly, only a few proteins were detected stably bound to the two non-oligomeric sHsps (HspB6 and HspB7). Taking into account that HspB6 could effectively suppress the aggregation of reduced proteins and cell lysates, we speculate that this sHsp might recognize substrates promiscuously and bind them transiently without forming stable sHsp-substrate complexes. Thus, HspB6 shares functional properties with the previously described sHsp from Deinococcus radiodurans (Hsp17.7) (52). Recently, it was also shown that even classical sHsps (HspB1/Hsp27 and HspB5/αB-crystallin), for which we detect stable complexes here, can prevent the amyloid-like aggregation of α-synuclein via transient interactions (53).

The comparison of the biochemical properties of the identified substrates with the heat-sensitive fraction of HEK293 showed that all 5 sHsps analyzed (HspB1-HspB5) preferentially bind relatively large (50–100 kDa) and slightly acidic (pI from 5.4 to 6.8) proteins. The indicated molecular mass corresponds to a monomer, whereas many substrate proteins detected (e.g. aminoacyl-tRNA ligases of class II, d-3-phosphoglycerate dehydrogenase) form different oligomeric species. If we consider this, the average size of interactors should be even higher. This observation is consistent with the previously described preference for substrate binding of lbpB from Escherichia coli, Hsp20.2 from D. radiodurans, and Caenorhabditis elegans Sip1 and Hsp16.2 (54, 55), suggesting that this is a common and conserved feature of the sHsp family. Interestingly, we did not detect a preferential binding to hydrophobic proteins. As molecular chaperones bind to hydrophobic segments, which become exposed upon unfolding, the overall hydrophobicity of a protein does not seem to be important for chaperone recognition.

The functional overrepresentation test revealed several protein classes preferentially associated with sHsps. These are proteins involved in tRNA metabolism, the translation machinery (initiation factors and others) and in nucleic and amino acid metabolism as well as different classes of molecular chaperones. The preferential binding of proteins involved in the translation and transcription machinery allows protecting regulatory proteins under unfavorable conditions and their transfer to ATP-dependent chaperones for refolding immediately after stress (4). We hypothesize that by this, the translation process might be restored faster, in agreement with previous suggestions for heat shock in yeast (56). The chaperone proteins co-immunoprecipitated with sHsps could be bound to the same substrate proteins. Among them are two subunits of the TRiC/CCT complex, which strikingly interact with all (CCT8) or six of seven (CCT3) sHsps analyzed (supplemental Table 2). Also we detected one Hsp70 protein (HSPA4) interacting with 4 sHsps, whereas the most ubiquitous member of the Hsp40 family, DNAJ1B1 (9), was found only as an HspB4 interactor. These observations indicate an involvement of sHsps in a network of chaperones working on aggregation-prone proteins. It has been shown that in the eukaryotic cytosol, sHsps seem to bind cytoskeletal proteins under stress conditions and protect them (57, 58). In our mass spectrometry analysis, we found several cytoskeletal proteins, among them, α-tubulin, α-centrinactin, dynein 1, and kinesin 1. However, these were also bound unspecifically in the controls. Therefore, we had to exclude them from further analysis to be consistent.

Summing up, we conclude that most of the human sHsps possess chaperone activity and suppress the aggregation of substrate proteins. However, the interaction with single model substrates varies significantly. The cell lysate aggregation assay provides a new view on the anti-aggregation properties of sHsps in a biological context as the complex protein mixture better mimics the cellular milieu. Here, surprisingly, all sHsps except HspB7 were active. Interestingly, the formation of stable sHsp-substrate complexes does not always correlate with the ability of a given sHsp to prevent the aggregation of cellular proteins. This changes our view concerning the interactions of sHsps with their substrates and leads to the conclusion that two modes of interaction, stable and transient, are important for sHsp function in the cellular context.

Experimental Procedures

Proteins—Human small heat shock proteins (HspB1–HspB6, HspB8) were expressed in E. coli BL21(DE3) and purified as described elsewhere (23, 24, 33, 59–61). HspB7 was purified according to the procedure described for purification of human HspB3 (33). GFP was expressed and purified according to Dashvets et al. (62). MDH (from pig heart), GAPDH (from rabbit muscle), rhodanese (from bovine liver), CS (from pig heart), and insulin (from bovine pancreas) were from Sigma, ADH (from yeast) was purchased from Roche Applied Science. Before use proteins were dissolved or diluted in an appropriate buffer and dialyzed if necessary. The concentration was determined using a NanoDrop2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA). Extinction coefficients for all proteins were obtained from the UniProt database. The concentration of HspB7 was determined by Bradford assay (63) using BSA as a standard because its extinction coefficient at 280 nm is very low (1490 m⁻¹ cm⁻¹).

Size-exclusion Chromatography—Analysis of sHsps oligomeric state was done by size-exclusion chromatography using Superdex 200 HR 10/30 column (GE Healthcare). 20 μl sHsp was incubated at 37 °C in PBS (the standard Dulbecco’s formulation; Ref. 64) containing 1 mM DTT for 1 h. 50 μl of the sample were loaded on a pre-equilibrated Superdex 200 HR 10/30 column (GE Healthcare) connected to Shimadzu HPLC system. A flow rate was 0.5 ml/min. Because of the high difference in the extinction coefficients, elution profiles of human sHsps were
normalized to the total area. Carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (141 kDa), β-amylose (200 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa) (all were from Sigma) were used as protein markers.

Model Substrate Aggregation Assays—The thermal aggregation of MDH (2 μM) (22), GAPDH (1 μM) (65), CS (1.0 μM) (59), and rhodanese (2 μM) (66) was tested in PBS containing 1 mM DTT and in 40 mM HEPES-NaOH, pH 7.5, 1 mM DTT at 45 °C. Aggregation of ADH and insulin was induced chemically by reduction of their intrinsic disulfide bonds (20, 67, 68), and the same buffers but without addition of DTT were tested for these substrates. ADH (4 μM) aggregation was initiated by the addition of 20 mM DTT and 2 mM EDTA at 42 °C (67, 68). Insulin (40 μM) aggregation assays were performed at 37 °C in the presence of 20 mM DTT (19). The PBS was selected for chaperone activity measurements as the most suitable for all model substrates. The concentration of the model substrate was in the micromolar range, which resulted in similar values of the apparent optical density at 360 nm. To analyze the effect on the aggregation of model substrates, sHsps were added at different concentrations to a reaction mix and preincubated 5 min before adding the substrate protein. The spontaneous aggregation of the model substrate in the absence of sHsps was monitored in every case as a control. The concentration of sHsps tested varied from 0.125 μM to 2.0 μM (per monomer) in MDH and rhodanese aggregation assays (corresponding to a molar ratio of substrate/shsp in the range of 8:1 to 1:1). In the case of the GAPDH aggregation assay, the concentration was varied from 0.5 μM to 8.0 μM (corresponding to molar ratios of 2:1 to 1:8), and in the CS aggregation assay from 0.25 μM to 8.0 μM (corresponding to molar ratios of 4:1 to 1:8). In reduction-induced assays, the concentrations of sHsps were 0.25–4.0 μM in the ADH assay (corresponding to molar ratios of 8:1 to 1:1) and 0.5–8.0 μM in the insulin assay. Concentrations of all proteins refer to a monomer.

Aggregation processes were monitored for 45–90 min (until reaching a plateau) following the increase in light scattering by recording the absorbance at 360 nm. All measurements were performed using a Cary 50 UV-visible spectrophotometer (Varian Inc., Palo Alto, CA) equipped with a temperature-adjustable cell holder. All measurements were repeated at least 3 times, and the mean values of apparent absorbance and standard deviations (S.D.) were calculated. To further analyze the effect of sHsps on substrate aggregation, the light scattering values at the end point (45, 60, or 90 min depending on the substrate) were normalized to the level of spontaneous aggregation of the model substrate in the absence of sHsp. IC$_{50}$ values were calculated as a ratio of sHsp/substrate required for a half-maximum reduction of apparent absorbance.

Cell Lysate Preparation and Western Blotting—HeLa, HEK293, and MCF7 cells (obtained from ATCC, Manassas, VA) were grown in DMEM, supplemented with 4.5 g/liter glucose, 2 mM glutamine (Gibco, Waltham, MA), and 10% fetal calf serum (FCS). Cell lysates were prepared according to the previously described procedure (28) with minor changes. At 90–95% confluence, cells were washed with sterile DPBS (Gibco), detached from the flask by incubation with trypsin/EDTA (Serva Electrophoresis GmbH, Heidelberg, Germany), pelleted, and mechanically lysed in PBS containing 1 mM DTT and protein inhibitor mix M (Serva Electrophoresis GmbH) by passing them several times through a 0.6-mm needle. Cell debris was removed by centrifugation at 16,000 × g for 10 min at 8 °C. Cleared cell lysate was depleted for ATP by incubation for 20 min at 20 °C with 30 units/ml hexokinase (Roche Applied Science) and 2 mM MgCl$_2$, flash-frozen in liquid nitrogen, and stored at −80 °C. The total protein concentrations were determined by Bradford assay using BSA as a standard (63).

To analyze sHsp expression in HeLa, HEK293, and MCF7 cell lines, 40 μg of total proteins of each lysate and recombinant purified sHsps were loaded on an SDS-PAGE followed by Western blotting. After SDS-PAGE, proteins were transferred to nitrocellulose membranes. Membranes were blocked in 5% milk-PBST (PBS containing 0.1% Tween 20), incubated with primary antibodies (mouse anti-HSP27/B1, mouse anti-α-crystallin/HSPB5, mouse anti-αB-crystallin/HSPB4 (all from StressMarq Biosciences Inc., Victoria, Canada; catalogue no. SMC-161, SMC-142, and SMC-165), monoclonal mouse anti-Hsp20/HSPB6, monoclonal mouse anti-Hsp22/HSPB8 (both kindly provided by Prof. A. Katrukha, Moscow State University, Moscow, Russia), monoclonal mouse anti-HSPB2 and monoclonal rabbit anti-HSPB7 (both from Abcam, Cambridge, UK; catalogue no. ab204994 and ab150390), polyclonal rabbit anti-HSPB3 and monoclonal mouse anti-GAPDH for a loading control (both from Sigma, catalogue no. SAB1100972 and G8795)), then washed 5 times with PBST and incubated with the secondary anti-mouse or anti-rabbit IgG antibodies conjugated with HRP (Sigma, catalogue no. A2304 and AP307P). Most of the antibodies were used at a 1:10,000 dilution, anti-HSPB2 and anti-HSPB3 at a 1:1000 dilution. After additional washing steps, bands were visualized with WesternBright ECL Spray (Advansta, Menlo Park, CA) and the ImageQuant LAS4000 chemiluminescence detection system (GE Healthcare).

Prevention of Protein Aggregation in Cell Lysates—Before use, the cell lysate was cleared by centrifugation for 10 min at 16,900 × g. The lysate was incubated for 90 min at 45 °C in a water bath at a concentration 0.8 mg/ml without or with the addition of sHsp (the final concentration of sHsp varied in between of 0.25 μM to 8.0 μM) in PBS containing 1 mM DTT. As a control, we analyzed the aggregation of cell lysate proteins in the presence of the same amounts of GFP. After incubation at 45 °C, the samples were centrifuged for 10 min at 8,600 × g to separate insoluble proteins. Pellets were washed twice with ice-cold PBS, dissolved in 1-fold Laemmli buffer, and analyzed by SDS-PAGE. Gels were scanned with ImageScanner III (GE Healthcare), and the amount of insoluble proteins was quantified by densitometry using the one-dimensional gel analysis tool in the ImageQuantTL software (GE Healthcare) as follows. The complete lane was selected for analysis, then a background was subtracted with the function “Image rectangle,” and band borders were adjusted to the top and the bottom of the analyzed lane. This way we obtained the total integral densities, and then the integral densities of bands corresponding to sHsps or GFP added (measured accordingly) were subtracted from these values. Each assay was repeated at least three times, and the mean ± S.D. was calculated. Resulting values were plotted
Activity and Specificity of Human sHsps

against the sHsp concentration. The IC_{50} values were calculated as sHsp concentration (in μM) required for half-maximum aggregation suppression normalized to the concentration of total cell lysate proteins in mg/ml (resulting values in μM/(mg/ml)).

Substrate Spectra Analysis—0.8 mg/ml of cell lysate (HeLa or HEK293) was incubated with 10 μM sHsp in PBS containing 1 mM DTT for 90 min at 45 °C in a water bath; the total sample volume was 50 μl. After heat shock, samples were chilled on ice, and sequentially the corresponding primary antibodies and Protein G-Sepharose (GE Healthcare) were added. Samples were incubated for 1 h (with antibodies) and 2 h (with Protein G-Sepharose) at 4 °C at constant mixing. After incubation, unbound proteins were removed by three to four washing steps with ice-cold PBST (PBS containing 0.1% Tween 20), and bound shsp-substrate complexes were eluted with 0.1 M glycine buffer, pH 2.5. Every sample was prepared in triplicate, and two independent experiments (CoIP1 and CoIP2) were done with the minor variations in experimental conditions. As a reference, we prepared the total HEK293 heat-sensitive protein fraction. HEK293 lysate at a final concentration of 0.8 mg/ml was incubated at 45 °C for 90 min, then soluble and insoluble proteins were separated as described above (centrifuged for 10 min at 8600 × g), and the pellet was washed twice with ice-cold PBS and dissolved in 1-fold Laemmli buffer. Samples were analyzed by 15% (CoIP1) or 4–20% (pre-casted from Serva Electrophoresis GmbH; CoIP2) SDS-PAGE followed by mass spectrometry. The Coomassie-stained gel lanes were cut and prepared for liquid chromatography-mass spectrometry analysis as described previously (52). Lanes were sliced into five parts and treated as individual samples. Proteins were reduced, alkylated, and digested overnight with trypsin. Peptides were extracted in 5 steps by adding sequentially 200 μl of buffer A (0.1% formic acid in water), acetonitrile (ACN), buffer A, ACN, and ACN. After each step samples were treated for 15 min by sonication. After steps 2, 4, and 5, the supernatant was removed from the gel slices and collected for further processing. The collected supernatants were pooled, evaporated to dryness in a SpeedVac (DNA 120, ThermoFisher Scientific), and stored at −80 °C. For the MS measurements, the samples were dissolved by adding 24 μl of formic acid and sonification for 15 min. The samples were then filtered through a 0.22-μm centrifuge filter (Merck Millipore, Darmstadt, Germany). Peptides were loaded onto an Acclaim PepMap RSLC C18 trap column (Trap Column, NanoViper, 75 μm × 20 mm, C18, 3 μm, 100 Å; ThermoFisher Scientific) with a flow rate of 5 μl/min and separated on a PepMap RSLC C18 column (CoIP1: 75 μm × 150 mm; CoIP2: 75 μm × 500 mm; C18, 2 μm, 100 Å, ThermoFisher Scientific) at a flow rate of 0.2 μl/min. A linear gradient from 4% (v/v) to 35% (v/v) buffer B (acetonitrile with 0.1% formic acid and 5% DMSO) eluted the peptides in 80 min (CoIP1) or 135 min (CoIP2) to an LTQ Orbitrap XL (ThermoFisher Scientific). Full scans and five dependent collision-induced dissociation MS^2 scans were recorded in each cycle.

The mass spectrometry data derived from every single sample were searched against the Swiss-Prot Homo sapiens Database downloaded from UniProt (15.02.2016 edition) using the Sequest HT Algorithm implemented into the “Proteome Discoverer 1.4” software (ThermoFisher Scientific). The search was limited to tryptic peptides containing a maximum of two missed cleavage sites and a peptide tolerance of 10 ppm for precursors and 0.6 Da for fragment masses. Proteins were identified with two distinct peptides with a target false discovery rate for peptides <1% according to the decoy search. Proteins detected in the control experiments were reasoned to bind unspecifically to the Protein G-Sepharose and thus were subtracted from the respective hit-lists. Keratin hits were manually removed as it was not possible to distinguish actual hits from contaminations. sHsps were removed from hit lists as well. For further evaluation two independent datasets (CoIP1 and CoIP2) were combined. Only hits that were observed in both datasets were taken into account. Furthermore, hits that appeared in two replicates in one dataset but only once in the other dataset were excluded. A statistical comparison (box plots) of M_r, pI, hydrophobic score (GRAVY), and abundance distributions for substrates of five sHsps were done using OriginPro 8.6 software (OriginLab, Northampton, MA). The same analysis was performed for HEK293 heat-sensitive fraction and the complete human proteome (20195 unique proteins, downloaded from UniProt) or, in the case of the abundance analysis, for HEK293 proteome (4278 unique proteins, downloaded from PaxDb database) (39, 40). The functional classification of substrate proteins was performed using PANTHER online tool (41). The PANTHER binomial statistics overrepresentation test with default settings was employed to determine enrichment of certain protein categories (GO Biological Functions) in sHsps-bound substrates in comparison to the complete human proteome.

Author Contributions—E. V. M., M. H., and J. B. designed the study. E. V. M. planned and performed the experiments shown on Figs. 1 and 2. E. V. M., M. D., and B. R. planned and performed the experiments shown on Fig. 3. E. V. M., M. D., M. H., and J. B. analyzed the data and contributed to writing the paper. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We thank to Prof. N. B. Gusev (Moscow State University, Moscow, Russia) and Prof. S. Weinkauf (Technische Universität München, Munich, Germany) for a critical discussion of the manuscript.

References

1. Tyedmers, J., Mogk, A., and Bukau, B. (2010) Cellular strategies for controlling protein aggregation. Nat. Rev. Mol. Cell Biol. 11, 777–788
2. Richter, K., Haslbeck, M., and Buchner, J. (2010) The heat shock response: life on the verge of death. Mol. Cell 40, 253–266
3. Jakob, U., Gaestel, M., Engel, K., and Buchner, J. (1993) Small heat shock proteins are molecular chaperones. J. Biol. Chem. 268, 1517–1520
4. Haslbeck, M., and Vierling, E. (2015) A first line of stress defense: small heat shock proteins and their function in protein homeostasis. J. Mol. Biol. 427, 1537–1548
5. Kriechhuber, T., Rattei, T., Weinmaier, T., Bepperling, A., Haslbeck, M., and Buchner, J. (2010) Independent evolution of the core domain and its flanking sequences in small heat shock proteins. FASEB J. 24, 3633–3642
6. Haley, D. A., Horwitz, J., and Stewart, P. L. (1998) The small heat-shock protein, αB-crystallin, has a variable quaternary structure. J. Mol. Biol. 277, 27–35
48. Hristozova, N., Tompa, P., and Kovacs, D. (2016) A novel method for assessing the chaperone activity of Proteins. *PLoS ONE* **11**, e0161970
49. Huntley, M. A., and Golding, G. B. (2006) Selection and slippage creating serine homopolymers. *Mol. Biol. Evol.* **23**, 2017–2025
50. Dunker, A. K., Brown, C. I., Lawson, J. D., Iakoucheva, L. M., and Obradovic, Z. (2002) Intrinsic disorder and protein function. *Biochemistry* **41**, 6573–6582
51. Mymrikov, E. V., Seit-Nebi, A. S., and Gusev, N. B. (2011) Large potentials of small heat shock proteins. *Physiol. Rev.* **91**, 1123–1159
52. Bepperling, A., Alte, F., Kriehuber, T., Braun, N., Weinkauf, S., Groll, M., Haslbeck, M., and Buchner, J. (2012) Alternative bacterial two-component small heat shock protein systems. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 20407–20412
53. Cox, D., Selig, E., Griffin, M. D., Carver, J. A., and Ecroyd, H. (2016) Small Heat-shock proteins prevent Hsp25, Hsp20, and cvHsp in heart and skeletal muscle. *Histochem. Cell Biol.* **122**, 415–425
54. Ehrnsperger, M., Gaestel, M., and Buchner, J. (2000) Analysis of chaperone properties of small Hsp's. *Methods Mol. Biol.* **99**, 421–429
55. Peschek, J., Braun, N., Franzmann, T. M., Georgalis, Y., Haslbeck, M., Weinkauf, S., and Buchner, J. (2009) The eye lens chaperone α-crystallin forms defined globular assemblies. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 13272–13277
56. Prabhu, S., Raman, B., Ramakrishna, T., and Rao, Ch. M (2012) HspB2/myotonic dystrophy protein kinase binding protein (MKBP) as a novel molecular chaperone: structural and functional aspects. *PLoS ONE* **7**, e29810
57. Dashivets, T., Wood, N., Hergersberg, C., Buchner, J., and Haslbeck, M. (2009) Rapid matrix-assisted refolding of histidine-tagged proteins. *Chembiochem* **10**, 869–876
58. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
59. Dulbecco, R., and Vogt, M. (1954) Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* **99**, 167–182
60. Khanova, H. A., Markossian, K. A., Kleimenov, S. Y., Levitsky, D. I., Chebotareva, N. A., Golub, N. V., Asryants, R. A., Muronetz, V. I., Saso, L., Yudin, I. K., Muranov, K. O., Ostrovsky, M. A., and Kurganov, B. I. (2007) Effect of α-crystallin on thermal denaturation and aggregation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. *Biophys. Chem.* **125**, 521–531
61. Stromer, T., Ehrnsperger, M., Gaestel, M., and Buchner, J. (2003) Analysis of the interaction of small heat shock proteins with unfolding proteins. *J. Biol. Chem.* **278**, 18015–18021
62. Farahbakhsh, Z. T., Huang, Q. L., Ding, L. L., Altenbach, C., Steinhoff, H. J., Horwitz, J., and Hubbell, W. L. (1995) Interaction of α-crystallin with spin-labeled peptides. *Biochemistry* **34**, 509–516
63. Panasenko, O. O., Seit Nebi, A., Bukach, O. V., Marston, S. B., and Gusev, N. B. (2002) Structure and properties of avian small heat shock protein with molecular weight 25 kDa. *Biochim. Biophys. Acta* **1601**, 64–74