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REGULATION OF STRESS-INDUCED INTRACELLULAR SORTING AND CHAPERONE FUNCTION OF HSP27 (HSPB1) IN MAMMALIAN CELLS THROUGH CHANGES IN OLIGOMERIZATION

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***In vitro*, small heat shock proteins were shown to have chaperone function capable of keeping unfolded proteins in a form competent for Hsp70-dependent refolding. However, this was never confirmed in living mammalian cells. In this study, we show that Hsp27 (HspB1) translocates into the nucleus upon heat shock, where it forms granules that co-localize with interchromatin granule clusters (IGCs). Although heat-induced changes in the oligomerization status of Hsp27 correlate with its phosphorylation and nuclear translocation, Hsp27 phosphorylation alone is not sufficient for effective nuclear translocation of HspB1. Using firefly luciferase as a heat sensitive reporter protein, it is demonstrated that HspB1 expression in HspB1-deficient fibroblasts enhances protein refolding after heat shock. The positive effect of HspB1 on refolding is completely diminished by overexpression of Bag-1, the negative regulator of Hsp70, consistent with the idea of HspB1 being the substrate holder for Hsp70. Although HspB1 and luciferase both accumulate in nuclear granules after heat shock, our data suggest that this is not related to the refolding activity of HspB1. Rather, granular accumulation may reflect a situation of failed refolding where the substrate is stored for subsequent degradation. Consistently, we found 20S proteasomes concentrated in nuclear granules of HspB1 after heat shock. We conclude that HspB1 contributes to an increased chaperone capacity of cells by binding unfolded proteins that are hereby kept competent for refolding by Hsp70 or that are sorted to nuclear granules if such refolding fails.**

List of abbreviations:
Bag-1: Bcl-2 associated athanogene; EGFP: enhanced green fluorescent protein; Hsp: Heat shock protein; HspB1: gene name for Hsp25 (murine) or Hsp27 (human); IGC: interchromatin granule clusters; MAPK: mitogen activated protein kinase; SAPK: stress-activated kinase

INTRODUCTION

The human small heat shock protein Hsp27 or HspB1 (also referred to as Hsp25 in rodents) confers resistance to a variety of stresses, especially heat shock [1]. The mechanism by which HspB1 protects cells have only been partially elucidated, but clearly the members of the small Hsp family are functionally implicated in the stabilization or/and restoration of the cytoskeletal elements in stressed cells [2, 3]. Besides that, *in vitro* data have indicated that small Hsps can act as ATP-independent molecular chaperones by binding to partially unfolded proteins, preventing their aggregation [4]. HspB1 needs to be dissociated from large oligomers into small oligomers (presumably dimers) [5] and these dimers are thought to bind the unfolded proteins. The client protein cannot be refolded by HspB1 alone, but can be presented to the ATP-dependent Hsp70 chaperone machinery for subsequent refolding [6]. In cells, the oligomeric size of HspB1 seems to be regulated by stress-dependent post-translational modifications. The most prominent modification is early stress-induced phosphorylation of HspB1 [5, 7] and HspB1 is known as a terminal substrate in the p38 stress- or mitogen-activated protein kinase (p38 MAPK) cascade [8]. Herein, serine residues Ser-15 and Ser-86 in rodent HspB1 or Ser-15, Ser-78 and Ser-82 in human HspB1 were identified as the sites of phosphorylation . Phosphorylation promotes dissociation of the large HspB1 oligomers to the smaller ones and eventually to tetramers and dimers [9, 10].

Direct proof for chaperone activity of HspB1 in living mammalian cells is yet lacking. Clearly, HspB1 can bind to the cytoskeletal elements, which seems associated with the stabilization or restoration of the cellular F-actin after stress [2, 3], which may depend on its chaperone-like activities. In addition, overexpressed HspB1 exhibited accelerated recovery from heat stress-induced nuclear protein aggregates [11]. The latter data, however, do not directly provide proof for the improved chaperone activity *in vivo* nor provide insight in the identity or possible fate (e.g. refolding, degradation) of putative HspB1-bound substrates.

The finding of an effect on recovery from nuclear protein aggregation [11] suggests a role for HspB1 in the cell nucleus. Indeed, in parallel with the well-known stress-induced nuclear translocation of components of the Hsp70 machine [12, 13], more recent reports describe that a fraction of HspB1 also enters the cell nucleus [3, 7, 14-17]. Heat or ATP-depletion stress causes detergent insolubility of HspB1 and formation of large (~10^6 kD) HspB1-containing structures inside the nucleus [7, 18] visible as granules [3, 14, 16] that also contain heat denatured proteins [3]. Neither the mechanism of (sub)nuclear redistribution nor the nuclear function(s) of HspB1 has been elucidated. It has been suggested that phosphorylation might induce the nuclear translocation [15] possibly via effects on the oligomeric size. On the other hand, however, indirect evidence suggested that HspB1 present in intranuclear granules is dephosphorylated [3, 16]. Thus, the role of phosphorylation of HspB1 in intranuclear sorting is yet unclear.

For Hsp70, the nuclear translocation was clearly demonstrated to promote resolubilization of heat-induced protein aggregates by reactivation of thermally denatured enzymes in the nuclei of recovering cells [11, 19]. More specifically, Hsp70 and Hsp40 accumulate into the nucleoli of heat-shocked cells [12, 13], which was associated with the refolding activity of the Hsp70 machine [20]. In contrast, the non-nucleolar granules in which HspB1 accumulates after cellular stress seem to be not associated with refolding [20], but do contain heat-unfolded proteins [3].

In the current report, we show a comprehensive analysis on the stress-induced HspB1 redistributions in relation to its presumed cellular chaperone functions *in vivo*. For the first time, it is demonstrated that HspB1 can prevent the irreversible denaturation/aggregation of heat-unfolded, non-cytoskeletal proteins in living mammalian cells. This activity, however, is not restricted to the nuclear compartment but also seen in the cytoplasm. The chaperone activity and nuclear translocation are influenced by phosphorylation, but HspB1 phosphorylation status *per se* is insufficient for both events. The appearance in nuclear granules, identified to be interchromatin granule clusters (IGCs), is also associated with HspB1 phosphorylation, but seems not linked to refolding of the HspB1-bound substrates. Rather, indirect evidence suggests that denatured substrate that accumulates with Hsp27 in nuclear granules is degraded by the proteasome.

EXPERIMENTAL PROCEDURES

Plasmids - pN-luc-EGFP expressing NLS-tagged luciferase fused to EGFP was described earlier [20]. pCyt-Luc-EGFP is a derivative of pN-luc-EGFP lacking its NLS, which results in a luciferase distributed to both

the cytoplasm and (partially) nucleus. Plasmids encoding the full-length gene of human Hsp27 and its phosphorylation mutants were created by subcloning the EcoRI - XbaI coding fragment from corresponding templates (pBC KS(+)27 WT/3A/3D/3G, kind gift from Dr. L.A. Weber, Reno, USA) into the pCMV5 mammalian expression vector to obtain pCMV-hum27 (WT), pCMV-hum27 (3A), pCMV-hum27 (3D), and pCMV-hum27 (3G). These constructs have been verified by sequencing and were used for overexpression of wild-type human HspB1 (WT), phosphorylation mutants HspB1 Ser15,78,82-> Ala15, 78,82 (3A), HspB1 Ser15,78,82-> Asp15, 78,82 (3D), and HspB1 Ser15,78,82-> Gly15, 78,82 (3G) in mammalian cells [21]. pCMV-70 and pCMV-Bag-1 were used to express Hsp70 and Bag-1 [22].

Cell lines and transfections - The rat cardiac fibroblasts Rat-1 and murine fibroblasts L929 were cultured at 37^{o}C in DMEM supplemented with 10% FBS in the presence of 5% of CO_2 in air. For transfection, cells were plated onto 6-well plates or 35-mm tissue dishes at $1x10^5$ cells/well. A day later the cells were incubated in Optimem (Gibco) containing pre-made complexes of DNA and a delivery reagent (GenePorter, Gene Therapy Systems) for 2-5 hours. After incubation the mixture was replaced with DMEM containing 20% (for Rat-1 cells) or 10% FBS (for L929 cells). After 18-24 hours cells were either fixed, immunostained and analyzed by fluorescent microscopy or further subcultured for biochemical assays. We use terms Hsp27 and Hsp25 to indicate the human or rodent orthologs.

Immunofluorescence - Cells were plated onto glass coverslips in a 24-well plate at density $8x10^4$ cells per well and grown for 1-2 days. When the cells reached a sub-confluent monolayer stage they were fixed with 4% formaldehyde, prepared in PBS with 0.1% Triton X-100, for 5-10 minutes and processed for immunostaining. To visualize rat Hsp25 and human Hsp27, appropriate rabbit and mouse antibodies from StressGen have been used (cat. numbers SPA801 and SPA800, respectively). Secondary antibodies were Cy3-conjugated goat anti-rabbit and FITC-labeled goat anti-mouse (Jackson Immunoresearch). Localization of luciferase was visualized by its fluorescent EGFP tag without additional immunolabeling. DNA was co-stained with DAPI (0.5 μg/ml).

To reveal 20S proteasome localization, the PW8155 rabbit polyclonal antibody (Affinity Research) was used. For double staining of 20S proteasomes along with Hsp25 cells were first incubated with anti-20S antibody, washed with PBS-T (PBS, 0.05% Tween20), and subsequently incubated with secondary Cy3-labeled F_{ab} fragments of goat anti-rabbit antibody (Jackson Immunoresearch). After this incubation and an additional washing step anti-Hsp25 antibody (SPA801) was applied following by incubation with goat anti-rabbit FITC-labeled antibody (Jackson Immunoresearch). Absence of cross-staining was verified by appropriate controls omitting primary antibodies at different steps of staining. Reference cells were stained with either antibody alone. Alternatively, cells transiently expressing human Hsp27 were used for conventional double labeling procedure with primary rabbit anti-20S PW8155 and mouse anti-Hsp27 SPA800 antibodies and the corresponding secondary antibodies. This approach produced very similar staining patterns to the method described above thus confirming its reliability. In addition, cells were transiently transfected with a GFP-tagged proteasomal subunit LMP-2 [23] and subsequently immunostained for endogenous Hsp25.

Electron microscopy - To reveal intranuclear compartments, cells were briefly (45 seconds) permeabilized in TEA/Mg buffer (20mM TEA, 1% Triton X-100, 4mM $MgCl_2$) before fixation [24]. After lysis cells were fixed with 3.7% paraformaldehyde in TEA/Mg buffer for 20 minutes, blocked sequentially with 20 mM glycine and 1% BSA in the same buffer and immunolabeled with primary anti-Hsp25 antibody (StressGen, SPA801) and secondary goat anti-rabbit 5 nm gold-labeled antibodies diluted in TEA/Mg buffer. To enlarge the gold particles the technique of silver enhancement was applied as described [25, 26]. Immunolabeled samples were routinely dehydrated and embedded in Epon 812. A 2% uranyl-acetate solution in 70% ethanol was applied for one hour in the course of embedding procedure. Embedded specimens were sliced for ultrathin sections and mounted on formvar-coated copper grids. Specimens were analyzed and photographed in transmission electron microscope HU-11 (Hitachi Corp, Japan).

Luciferase in vivo refolding assay - Luciferase inactivation and refolding was measured as described before with slight modifications. One day after transfection with pN-luc-EGFP (nuclear luciferase) or pC-luc-EGFP (cytosolic luciferase) cells were plated onto 24-well plates in quadruplicates and allowed to grow for 24 hours. Before heat shock (30min at 43°C), the growth medium was refreshed with the one containing 20 mM MOPS pH 7.0. Immediately after heat shock, an equal volume (0.5 ml/well) of growth medium containing double concentration of cycloheximide (40 μg/ml) was added to block *de novo* protein synthesis. Cells were lysed and scraped prior to and 0-180 minutes after heat shock in BLUC (25mM Tris/H_3PO_4, pH 7.8, 10 mM

$MgCl_2$, 1% Triton X-100, 15% glycerol, 1 mM EDTA). Luciferase activity in samples was measured for 10 integrated seconds after injecting the substrate buffer (BLUC, 1.25 mM ATP, 0.087 mg/ml D-luciferin) in a Berthhold Lumat 9501.

Isoelectrofocusing - Separation of Hsp25 isoforms was done essentially as described earlier [3]. Briefly, cells were lysed with IEF buffer (9M urea, 1% Nonidet P40, 2% β-mercaptoethanol, protease and phosphatase inhibitor cocktails (Roche)) and run in 7% acrylamide gel containing 4% ampholytes (pH range 5-7) in IEF apparatus (LKB). The focusing gels were stripped off from underlying gel bonds and processed for Western blotting.

Size pore exclusion gel electrophoresis - To estimate oligomeric size of HspB1 complexes, the native size pore exclusion pore electrophoresis was performed as described earlier [27] with minor modifications. Rat-1 cells were lysed in PELE sample buffer (20 mM Tris HCl, pH 7.4, 5 mM $MgCl_2$, 0.5% Triton X-100, 10% glycerol, 10 mM NaF, 1 mM dithiothreitol, anti-protease cocktail) before and after heat shock, vortexed for 30 seconds, and then centrifuged for 10 minutes at maximum speed in a desktop centrifuge (Eppendorff). Aliquots of the supernatants were applied on discontinuous 2-18 % acrylamide gradient gel. Samples in the gels were run in large electrophoresis chambers at constant 120 V, usually for 18 hours in a cold box. Electrode buffer consisted of 90 mM Tris HCl, pH 8.4, 80 mM boric acid, 2 mM EDTA. Separated protein complexes in gels were soaked in SDS-containing Tris-glycin buffer (25 mM Tris, pH 8.3, 192 mM glycine, 0.25% SDS) and then electroblotted onto nitrocellulose in Tris-glycin buffer without SDS.

Western blotting - Transfected cells, growing on 35-mm tissue dishes, were washed with PBS and harvested using a cell scraper on ice into BLUC buffer (100µl/dish) supplemented with anti-protease cocktail (Complete, Roche). The samples were re-dissolved in 50 µl of BLUC and along with the supernatants mixed with 50 µl 2x PAGE sample buffer, sonicated at 50 W for 5 seconds and boiled for 5 minutes. Prepared samples were run in 10% polyacrylamide gels and blotted onto nitrocellulose membrane. After blocking with 5% non-fat milk membranes were probed with a primary rabbit anti-Hsp27 antibody (StressGen, SPA803). Antibody binding was detected using peroxidase-conjugated secondary antibody (Pierce) and ECL kit (Amersham Pharmacia).

RESULTS

Stress-inducible nuclear redistribution of HspB1

First, the intracellular localization of the endogenous rodent HspB1 (here referred to as Hsp25) was evaluated in Rat-1 cardiac fibroblasts since these cells express sufficient amounts of this protein. Under normal conditions Hsp25 was diffusely distributed throughout the cytoplasm of Rat-1 cells, with minimal nuclear staining (Fig. 1A, *CNTR*). After a mild heat shock (43^{o}C for 30 minutes), a significant proportion of Hsp25 accumulated in the nucleus and partially reorganized into nuclear granules (Fig. 1A, *HS*). Nuclear accumulation of Hsp25 and its subnuclear patterns were totally reversible and after 3 hours of recovery most of the protein was back in the cytoplasm (Fig. 1A, *HS+rec180`*).

To precisely identify the different nuclear compartments in which Hsp25 resides after heat shock, we employed immunoelectron microscopy. A special protocol was used for this purpose. Prior to fixation, the cells were briefly lysed in TEA buffer containing Mg^{2+}. This treatment liberates the nuclei from the soluble (nucleosolic) components and condenses chromatin [28]. Other architectural features like nucleolus, interchromatin granule clusters (IGCs), and perichromatin material remain intact (for review see [29]) and become more visible [30, 31]. At 30 minutes after heat shock, Hsp25 was detected throughout the nucleus as well as in IGCs of various sizes (Fig. 2A, B) clearly without labeling inside of condensed chromatin blocks. Interestingly, IGCs also contain splicing factors (e.g. SC35) and are identified at the light microscopy level as nuclear speckles [32]. Consistent with this finding, others [14, 33] have revealed that small Hsps co-localize with the SC35 in nuclear speckles (see also below; figure 6). However, unlike alpha-B crystallin that localizes to nuclear speckles under non-stress conditions [33], we never found Hsp25 present in nuclear patterns in control cells. Our electron microscopy data also revealed that Hsp25 was decorating perichromatin fibers (Fig. 2C) corresponding to detergent-insoluble diffuse pattern seen by immunofluorescent staining (Fig. 2A, *inset)*.

Stress-inducible HspB1 phosphorylation and its nuclear redistribution

Heat shock inducible redistribution of Hsp25 in Rat-1 cells was paralleled by changes in its phosphorylation status. Under non-stress conditions, Hsp25 is only partly phosphorylated (Fig 1B, *lane 1*) correlating to its cytoplasmic localization (Fig 1A, *CNTR*). Hsp25 promptly became hyperphosphorylated during heat shock (Fig 1B, *lane 2*) coinciding with nuclear translocation. The peak of heat shock-inducible phosphorylation coincided with nuclear accumulation of Hsp25 (Fig. 1A, *HS*). When the phosphorylation status of Hsp25 was back to control levels after longer recovery periods (Fig. 1B, *lane 4*) all Hsp25 had returned to the cytoplasm (Fig. 1A, *HS+rec180*`).

To test whether phosphorylation is required for nuclear translocation of Hsp25, cells were pre-treated with SB202190, an inhibitor of stress-induced Hsp25 phosphorylation [3]. Under such conditions Hsp25 was indeed not hyperphosphorylated after heating (Fig. 1B, *lane3*), but yet still able to enter the nucleus and form nuclear granules (Fig. 1A, *HS+SB*). So, although phosphorylation of Hsp25 positively correlates with the changes in the subcellular distribution, it is not a prerequisite for nuclear translocation under heat shock conditions.

Phosphorylation mutants of HspB1 exhibit different properties for nuclear entry

To further elucidate the role of phosphorylation in the nuclear translocation of Hsp25, we transiently expressed human wild type HspB1 (further referred to as Hsp27) and three of its phosphorylation mutants in Rat-1 cells. First, *in situ* double-labeling using species-specific antibodies was used to test whether the ectopically expressed human Hsp27 is localized in the nuclei of heat-shocked cells in the same manner as the endogenous rodent Hsp25. After heat shock, a fraction of the human Hsp27 was found in the cell nucleus and it concentrated into nuclear granules (Fig. 3A), demonstrating that this protein behaves exactly like the endogenous Hsp25 after heat stress.

Next, Hsp27 phosphorylation mutants were used in which all three known phosphorylatable serines at positions 15, 78, and 82 were mutated either for alanines (Hsp27-3A, a non-phosphorylatable mutant) or aspartic acids (Hsp27-3D, a pseudophosphorylated mutant). Since phosphorylation also dramatically changes the oligomerization state of Hsp27, we also used another non-phosphorylatable mutant, Hsp27-3G, obtained with appropriate substitutions for glycines, which results in impaired oligomerization ability. All mutants formed oligomers of different sizes that varied from mutant to mutant, but that did not change in response to heat shock (Fig. 3B). In contrast, wild type proteins (ectopically expressed Hsp27 and endogenous Hsp25) did change their oligomers to smaller sizes after heat shock (Fig. 3B).

Surprisingly, under normal growth conditions all mutants, regardless of their phosphorylation or oligomerization status, mostly remained in the cytoplasm (Fig 3C, *CNTR*). However, heat shock resulted in nuclear accumulation of the smaller oligomers of the Hsp27-3D and Hsp27-3G mutants, whereas the larger multimeric Hsp27-3A did not relocalize into the nucleus (Fig. 3C, *HS*). Since the two non-phosphorylatable mutants (Hsp27-3G and Hsp27-3A) behaved differently in terms of relocalization, nuclear translocation of Hsp27 seems not to depend on phosphorylation *per se*. Rather, nuclear entry was associated with reduced oligomeric size. However, since none of the Hsp27 mutants showed nuclear staining under normal growth conditions, oligomeric size alone appears to be insufficient to trigger nuclear translocation of Hsp27. It must thus be concluded that additional heat-induced events along with the de-oligomerization are required for nuclear accumulation of HspB1.

Overexpression of HspB1 is associated with enhanced refolding of a heat-inactivated reporter protein

To investigate the biological significance of the Hsp27 redistribution after heat shock and whether this may be related to the chaperone function that small Hsps seem to have in cell-free systems [4], the heat sensitive, nuclear targeted luciferase reporter (N-luc-EGFP) was expressed in Rat-1 cells. The luciferase domain of this chimeric protein is highly thermosensitive whereas EGFP is thermoresistant. Tracing the signal from EGFP allows to reveal localization of N-luc-EGFP in the nucleus regardless of its luciferase activity [20]. Under normal conditions N-luc-EGFP is localized diffusely throughout the nucleosol (Fig. 4A, *CNTR*). Heat shock at 43°C for 30 minutes resulted in insolubilization (data not shown) and redistribution of N-luc-EGFP to the nucleoli and some small foci throughout the nucleus (Fig. 4A, *HS)*, similar to previous observations made in other cell lines [3, 20]. Interestingly, there was always a fraction of N-luc-EGFP that was dispersed diffusely throughout the nucleus (Fig. 4A, *HS*), despite the finding that N-luc-EGFP completely lost Triton-X 100 solubility and became enzymatically inactive upon such heat shock (not shown). Upon recovery (Fig. 4A, *HS+rec180'),* a partial restoration of the N-luc-EGFP staining towards a more diffuse, control-like pattern was observed. The heat shock-induced N-luc-EGFP re-allocation patterns in and out nuclear foci closely resembled those of HspB1 (Fig 1, 2). Indeed, confocal analysis revealed that non-nucleolar N-luc-EGFP distribution after heat shock substantially overlapped with that of the endogenous

Hsp25 (Fig. 4B). These data suggest that HspB1 may associate with some heat-unfolded nuclear proteins. However, the subnuclear compartment of such interaction seems not exclusively restricted to that in the granules only, since fractions of both luciferase and Hsp25 were also found outside the granules in the diffuse pattern. Yet the biological significance of these interactions needs further investigation.

To test for a possible role of Hsp27 in the post-stress fate of denatured proteins, we examined whether HspB1 overexpression had an effect on the renaturation kinetics of N-luc-EGFP and whether this may be associated with the presence of HspB1 in nuclear granules or not. However, when wild type human Hsp27 was overexpressed in Rat-1 cells, it had only a minor effect on luciferase reactivation rates (data not shown). Since Rat-1 cells already constitutively express rather high levels of endogenous Hsp25, we reasoned that these levels might already be optimal for refolding. Therefore, we turned to the murine fibroblasts L929, which are deprived of constitutive Hsp25 expression. Human Hsp27 was overexpressed in L929 cells and its *in situ* behavior after heat shock at 43^{o}C for 30 minutes resembled that of the endogenous Hsp25 seen in Rat-1 cells, i.e. it was nuclear and partially present in granules. Also, the various phosphorylation mutants behaved similarly in L929 and in Rat-1 cells: after heat shock, Hsp27-3A showed no nuclear entry whereas the Hsp27-3D and Hsp27-3G mutants accumulated in nuclei and nuclear granules (not shown).

The expression of wild type Hsp27 in L929 cells (Fig. 5A) resulted in a clear enhancement of refolding of heat-denatured luciferase as compared to control cells transfected with an empty vector (Fig. 5B). Equal expression of the non-phosphorylatable Hsp27-3A, incapable of entering the nucleus upon heat shock, had no significant effects on refolding of N-luc-EGFP (Fig. 5B). Interestingly, expression of Hsp27-3D to similar levels as wild type Hsp27 (Fig. 5A) resulted in the largest, 2-fold enhancement of N-luc-EGFP refolding. Finally, Hsp27-3G expression enhanced refolding to similar levels as wild type Hsp27 (Fig. 5B), which could be explained by relatively poor phosphorylation of human Hsp27 in rodent cells (Bryantsev, unpublished observations). These data for the first time show that Hsp27 contributes to the chaperone activity in living mammalian cells and link this activity to the smallest (phosphorylated) oligomers of HspB1 (see also fig. 3B).

Using cell-free experiments, it has been demonstrated that HspB1 alone can prevent irreversible aggregation of proteins but cannot enable their refolding unless the Hsp70 machine is added . To test whether the effects of HspB1 on *in vivo* refolding are also dependent on the action of Hsp70, we used co-expression of Hsp70 modulator Bag-1. We previously showed that overexpression of Bag-1 inhibits Hsp70-dependent refolding in hamster O23 cells [22]. The same was found to be true in L929 cells (Fig. 5C). Next, Bag-1 was co-expressed with Hsp27-3D and this completely negated the effect of the small Hsp on refolding (Fig 5C). A similar effect was seen for the wild type Hsp27 (data not shown). These data clearly indicate that Hsp27-mediated effects on refolding were completely dependent on the activity of the Hsp70 machine.

Since large fractions of HspB1 remain cytoplasmic during and after heat shock, we next tested whether the observed chaperone-like function was confined to the cell nucleus only. Using a cytosolic luciferase, we found that this was not the case. The overexpression of, especially, Hsp27-3D enhanced refolding of heat damaged cytosolic luciferase (Fig. 5D); the wild type and Hsp27-3G had some effects on refolding of heat-denatured cytosolic luciferase, whereas Hsp27-3A had no effect at all (Fig. 5D). Again, the action of Hsp27 depended on a functional Hsp70 machine as shown in Figure 5E for Hsp27-3D. Similar data were obtained for the wild type and Hsp27-3G (data not shown). So, the best chaperone activity of Hsp27-3D seems to be not necessarily linked to its localization in the nucleus and nuclear granules after heat shock but rather is linked to its presence in both compartments in a (pseudo)phosphorylated form.

Nuclear granules of HspB1 co-localize with components of the proteasomal machine.

Although we demonstrated that denatured luciferase does partially co-localize to nuclear granules (Fig. 4, [3]), we previously showed that for more severe heat shock conditions than used here granules with luciferase were not associated with refolding [20]. Since the effects of Hsp27 on luciferase refolding were also not exclusively linked to its presence in nuclear granules, we wondered what else could be the functional significance of the presence of HspB1 in nuclear granules. Recently, the group of Boelens has found that the small Hsp alpha-B crystallin can bind to FBX4 [33], a F-box protein implicated in protein degradation [34], and can recruit this protein to nuclear speckles [33]. Moreover, a direct association between alpha-B crystallin and one of the proteasomal subunits has also been observed [35]. To test whether HspB1 in nuclear granules may be related to degradation of heat denatured proteins, we probed heat-treated cells with a specific antibody to the 20S proteasomal complex. Confocal analysis revealed the presence of 20S proteasomal complexes diffusely throughout the nucleus of unheated cells (Fig. 6A, *CNTR*). Heat shock promoted the partial redistribution of these proteasomal complexes into granular structures (Fig. 6A, *HS*). This re-localization was also seen when using transient transfection with the GFP-tagged proteasomal subunit LMP-2 [23] (Fig 6B).

These data furthermore revealed that LMP-2 colocalized with the splicing factor SC-35 consistent with these structures being IGCs (figure 2) or nuclear speckles [32]. Clearly, nuclear speckles are also present under control conditions whereas the proteasomal subunit LMP-2 or 20S complexes only co-associate with these speckles after heat shock (Fig 6 A,B). Also, only after heat shock Hsp25 is co-localized in these nuclear speckles with the 20S complexes (Fig. 6C) or with LMP-2 (data not shown).

DISCUSSION

The stress-induced nuclear accumulation of several Hsps, including HspB1, suggests the need for their activity in the cell nucleus. Whereas Hsp70/40 translocation to the nucleus has been clearly demonstrated to function in enhanced protein refolding [20, 36], the role of nuclear translocation of HspB1 had remained unclear. Here, we show that in 2 different cell lines (Rat-1 and L929) HspB1 is translocated into the nucleus after heat shock. Although the nuclear translocation does not absolutely require HspB1 phosphorylation, the intracellular distribution after heating is associated with the phosphorylation status of HspB1 that is correlated with a reduction in its oligomeric size. Moreover, we show for the first time that ectopically expressed human HspB1 (Hsp27) shows a similar translocation upon heat shock as the endogenously expressed rodent HspB1 (Hsp25) and results in the enhanced capacity of the transfected cells to refold heat denatured nuclear proteins. Also, overexpression of Hsp27 enhanced refolding in the cytoplasm. This enhanced refolding in both the cytoplasm and the nucleus was depended on a functional Hsp70 machine. Interestingly, enhanced refolding in the nucleus was not related to the association with nuclear granules as such. Rather, the co-localization of the 20S proteasome with the nuclear granules and the findings that inhibitors of proteasomal degradation reduced the reversibility of Hsp27 in granules (Bryantsev, unpublished observations) suggest an association of Hsp27 in these granules with degradation of those protein substrates that could not be refolded by the Hsp70 machine.

Nuclear entry and speckle localization of HspB1

HspB1 is known to undergo phosphorylation-sensitive changes in its oligomerization state [9, 10]. We now show that these correlate with the presence or absence of HspB1in the nucleus (this report) since the ectopically expressed human non-phosphorylatable Hsp27-3A mutant does not translocate into the nucleus upon heating, whereas the pseudophosphorylated mutant Hsp27-3D does. In some cell lines, HspB1 was found to be present in nuclei of non-heated cells in a phosphorylation-dependent manner [15, 37]. Although we can only speculate why, we (this report, [3]) and others [14] did not find nuclear HspB1 in non-heated cells. It is interesting to note that the stress-activated kinases p38 MAPK and SAPK/JNK were already active in non-heated hippocampal progenitor cells used in the work by Geum and colleagues [15]. Maybe the viral infection used in the latter study to deliver Hsp27 or the sensitivity of some cell types to the 37°C incubation temperature was sufficient to activate the phosphorylation and consequential oligomeric changes needed to drive HspB1 into the nucleus.

As no apparent nuclear localization signals are found in mammalian HspB1, the most likely model for nuclear translocation could be that upon phosphorylation, HspB1 oligomers become reduced in size, partially into dimers of 54 kD, that are small enough to enter passively through the nuclear pore complex. Such an idea is consistent with findings that EGFP-tagged wild type HspB1 (114 kD, if reduced to dimers) was not detected in the cell nucleus after heating (data not shown, [38]). Although stress-induced phosphorylation may drive nuclear translocation of wild type HspB1, the oligomeric size seems to be the most critical prerequisite for nuclear entry rather than phosphorylation *per se*. This is supported by the notions that (i) inhibition of phosphorylation did not prohibit nuclear entry and that (ii) the smaller oligomers of non-phosphorylatable Hsp27-3G mutant also entered the nucleus after heating. Even more so, irrespective of size and/or phosphorylation, additional stress-inducible events seem to be required for nuclear accumulation of HspB1 since none of the smaller oligomeric mutants accumulated in the nucleus under non-stress situations. Perhaps heat stress-inducible but phosphorylation-independent reductions in the HspB1 oligomerization may play a role. However, we see no changes in the size distribution of the Hsp-3G or Hsp-3D mutant after heat shock and yet they do accumulate in the nucleus upon heating. If entry occurs via passive diffusion, one could envision that nuclear retention of HspB1 due to binding to unfolded nuclear substrates is responsible for the accumulation of HspB1 in the nucleus after heat shock. Alternatively, HspB1 nuclear export could be inhibited under heat shock conditions.

Secondary to the nuclear translocation, we found that HspB1 accumulated in nuclear granules that were identified as interchromatin granules, or nuclear speckles. In line with the data on alpha-B crystallin

[39], it was found that endogenous and transfected HspB1 accumulated in nuclear speckles after heat shock. However, HspB1 behaves differently from its family member alpha-B crystallin that is already found in nuclear speckles of unheated cells [14, 39], whereas no evidence for accumulation in nuclear speckles of HspB1 is apparent under non-stress conditions (this report, [14]).

Chaperone activity of HspB1: Hsp70 dependent refolding or proteasomal degradation

Our current data present the first evidence that overexpression of HspB1 indeed can enhance the capacity of living mammalian cells to refold heat denatured non-cytoskeletal proteins in both the cytoplasm and the nucleus. By inhibiting the activity of the Hsp70 machine with Bag-1 [40], we could demonstrate that this HspB1 positive effect on refolding is lost, consistent with the models based on cell free data [4, 6] that HspB1 can keep unfolded substrates in a form competent for refolding by the Hsp70 machine.
Regarding to the role of phosphorylation and oligomeric size, *in vitro* models have suggested that small HspB1 oligomers (as small as dimers) appear to be the active, substrate binding species [6, 41, 42]. Whilst Hsp70 (or the DnaK system in E. coli) appears to be able to refold small, soluble HspB1-substrate complexes, this system alone cannot efficiently refold substrates from large, insoluble HspB1-substrate aggregates [43]. This is all consistent with our *in vivo* findings where the pseudophosphorylated Hsp27-3D (smallest oligomers) shows the best chaperone activity both in the nucleus and cytosol. Because Hsp27 size distributions were measured in total lysates, we can only speculate about the oligomeric size of Hsp27 complexes in the nucleus or nuclear speckles. However, our data could be consistent with the following model. As long as these smaller oligomeric HspB1-substrate complexes are not accumulating into the larger oligomeric insoluble complexes, such as the nuclear granules, in which luciferase indeed is insoluble [20], Hsp70 can take over the HspB1-bound substrates. If the latter fails and/or with time after heat shock, HspB1 accumulates in the nuclear granules with unfolded luciferase still associated to it [3]. Here, Hsp70 can no longer act on the damaged reporter as evident from our previous findings [20]. Yet, the reversibility of the speckle-associated Hsp27 and luciferase suggests that there is subsequent processing. It is known that the AAA+ proteins ClpB (E. coli) and Hsp104 (yeast) can rescue large insoluble HspB1-protein aggregates [43, 44]. No functional equivalents for ClbB/Hsp104 in mammals have been described yet. Therefore, we would like to speculate here that in case HspB1-bound substrates have accumulated into large aggregates in nuclear granules (formed at sites of regular subnuclear domains – nuclear speckles), they are substrates for degradation by the proteasome. Several (indirect) lines of evidence support this hypothesis. <text deleted> First, nuclear speckles (with accumulated HspB1 and luciferase) stain positively for the 20S proteasomal complex (this study). Second, accumulation of heat-denatured luciferase in speckles is no longer reversible after more severe heat treatments [20] that inhibit activity of the proteasome.

Third, HspB1 overexpression in various cell types was shown to enhance the degradation of ubiquitinated proteins by the proteasome [45]. Fourth, HspB1 binds to ubiquitin [33, 46] and alpha-B crystallin associates with FBX4, an adapter protein for a ubiquitin ligase and a proteasomal subunit [33] also localizes in SC35-positive nuclear speckles [33]. Fifth, recent data have clearly shown that nuclear speckles are sites for proteasomal activity [47]. Finally, nuclear alpha-B crystallin also can enhance refolding of heat-denatured luciferase (data not shown), but this protein that is associated with nuclear speckles before heat shock actually is released from the speckles during and is present in the nucleosol upon recovery after heating during the time period of refolding (den Engelsman and Boelens, personal communication). However, although the data strongly suggest that HspB1 accumulation in granules is not related to refolding, we realize that none of these data are conclusive evidence for a role of HspB1 and nuclear speckles in the degradation of stress-induced unfolded proteins. Since heat denatured luciferase is a poor substrate for degradation by the proteasome [22], this could not be tested directly. A possible alternative explanation could be that nuclear granules of HspB1 are the nuclear equivalents of the cytosolic aggresomes [48] in which damaged proteins accumulate after failure of the protein quality control system to refold or degrade them. Irrespective of their function, nuclear speckles (or nuclear granules of HspB1) seem to be ideal sites for storage of unfolded or non-essential proteins as being interchromosomal domains that are relatively less crowded regions outside the expressed chromosomal territories: storage in nuclear speckles will thus not interfere with normal chromatin functioning [49, 50].

In summary, we propose that phosphorylation-dependent de-oligomerization of HspB1 provides the cells with a rapid increase in the chaperone capacity to bind unfolded proteins that are hereby kept competent for refolding by Hsp70 or that are sorted to nuclear granules if such refolding fails.

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FIGURE LEGENDS

Figure 1: Distribution and phosphorylation patterns of endogenous HspB1 (Hsp25) in Rat-1 fibroblasts before, during and after heat shock
Localization of Hsp25 in cells (panel A) or phosphorylation status of Hsp25 in cell lysates (panel B) at normal conditions (CNTR, lane *1*), after heat shock at 43°C for 30 min without (HS, lane *2*) or with the kinase inhibitor SB202190 (HS+SB, lane *3*), or after heat shock followed by a recovery for 180 minutes at 37°C (HS+rec180`, lane *4*).
A: Confocal images of cells immunostained with the antibody against rodent Hsp25. Bar = 30μm.
B: Cells were lysed and run in 7% acrylamide isoelectrofocussing gels and processed for Western blotting using the antibody against rodent Hsp25. Positions of non-phosphorylated (*a*), monophosphorylated (*b*), and diphosphorylated (*c*) isoforms are indicated.

Figure 2: Ultrastructural immunolocalization of Hsp25 in the nuclei of heat-shocked Rat-1 fibroblasts.
To identify the intranuclear localization of rodent Hsp25 after heating at the ultrastructural level, heated (30 minutes at 43°C) Rat-1 cells were briefly lysed in TEA buffer containing Mg^{2+} prior to standard fixation in paraformaldehyde. After immunolabeling with primary anti-Hsp25 antibody and secondary goat anti-rabbit 5 nm gold-labeled antibody, ultrathin sections of the cells were processed for transmission electron microscopy.
A: Entire cell nucleus of a cell heat shocked for 30 min. Arrows indicate interchromatin granule clusters and the arrowhead indicates the nucleolus. The inset represents a typical nucleus of a similarly treated cell, stained for immunofluorescence. Bar = 1μm.
B: Enlarged image of an interchromatin granule cluster (IGC) clearly enriched with the Hsp27 label. Bar = 1μm.
C: Enlarged fragment of a perichromatin fiber, containing the label along its periphery. Bar = 1μm.

Figure 3: Intracellular distribution and oligomeric states of endogenous Hsp25 and ectopically expressed the wild type and mutants of human Hsp27 in Rat-1 fibroblasts.
A: Rat-1 cells were transiently transfected with a plasmid encoding human Hsp27, heat shocked and processed for immunofluorescence analysis using confocal microscopy. Endogenous rat Hsp25 (green) and ectopically expressed human Hsp27 (red) and the merged image show their excellent co-localization (overlay: yellow). Bar = 10 μm.
B: Rat-1 cells were transiently transfected with plasmids encoding wild type human Hsp27 (WT) or its mutants Hsp27-3A (3A), Hsp27-3G (3G), and Hsp27-3D (3D). The oligomeric size distribution of the endogenous rodent Hsp25 and ectopically expressed Hsp27 variants was analyzed in cells before (-) and after (+) heat shock at 43°C for 30 min (HS). Cell lysates were run by native non-reducing pore size-exclusion electrophoresis, blotted onto nitrocellulose membrane and probed with species-specific anti-Hsp antibodies, recognizing either Hsp25 or Hsp27. Arrows show three major intermediates in oligomers corresponding to their large (*L*), medium (*M*) and small (*S*) size. For details on the various Hsp27 phosphorylation mutants see the text.
C: Rat-1 cells were transiently transfected with plasmids encoding wild type human Hsp27 (WT) or its mutants Hsp27-3A (3A), Hsp27-3G (3G), and Hsp27-3D (3D) and their intracellular localization was analyzed using confocal microscopy as described under **A**, before (*CNTR*) and after heat shock (*HS*). Bar = 10 μm.

Figure 4: Localization of the nuclear stress-sensitive luciferase reporter in relation to Hsp25 localization and enzymatic activity before and after heating Rat-1 cells.
A: Rat-1 cells were transiently transfected with plasmids encoding EGFP-tagged nuclear luciferase (N-luc-EGFP) and either left unheated (*CNTR*), or heated for 30 minutes at 43°C (*HS*) or heated and allowed to recover for 180 minutes at 37°C (*HS+rec180'*) before processing to confocal microscopy using EGFP as a tracer. Arrows indicate the position of the nucleoli. Note the similarity in kinetic behavior after heat shock between nuclear luciferase and Hsp25 (Figure 1). Bar = 10 μm.
B: Rat-1 cells transiently expressing N-luc-EGFP were heat shocked before processing to confocal microscopy using EGFP as a tracer for luciferase localization (green) or anti-Hsp25 immunostaining (red). The merged image clearly demonstrates an excellent co-localization (overlay: yellow). DNA was conterstained with DAPI in blue.

Figure 5: Hsp27 overexpression enhances refolding of heat-inactivated luciferase reporter in L929 cells.
L929 cells lacking endogenous Hsp25 were transiently transfected with plasmids encoding EGFP-tagged nuclear luciferase (A-C) or cytosolic luciferase (D) and co-transfected with an empty plasmid (CNTR) or with plasmids encoding wild type human Hsp27 (WT) or its mutants Hsp27-3A (3A), Hsp27-3G (3G), or Hsp27-3D (3D).
A: Expression levels of wild type Hsp27 and its mutants in L929 upon transient transfection as detected by Western blotting (upper lanes). Transfection efficiencies were equal for all plasmids used. Extracts of HeLa cells, naturally expressing moderate levels of human Hsp27, are provided as a positive control. Immunostaining for GAPDH was used to demonstrate equal loading between lanes (lower panel).
B: L929 cells expressing nuclear luciferase were heated (30 minutes at 43°C) and cells were lysed 0-180 minutes after heating. Cycloheximide (CHX, 20 μg/ml) was added just prior to heating to prevent new luciferase synthesis. Recovery of luciferase activity is indicated for L929 cells co-transfected with an empty vector (closed circles) or co-transfected with wild type human Hsp27 (open squares), Hsp27-3A (closed squares), Hsp27-3G (crosses), and Hsp27-3D (closed triangles). Activity was calculated as percentage of the initial luciferase activity before heat shock.
C: Same as panel B, but here recovery of nuclear luciferase activity is depicted for cells co-transfected with an empty vector (closed circles), Hsp27-3D alone (closed triangles), Hsp27-3D and Bag-1 (open triangles), Hsp70 alone (closed squares), and Hsp70 and Bag-1 (open squares).
D: Same as panel B, now showing recovery of cytosolic luciferase activity (*CYT-LUC*)
E: Same as panel C, now showing recovery of cytosolic luciferase activity (*CYT-LUC*)

Figure 6: Association between nuclear granules of Hsp25, nuclear speckles, and the 20-S proteasomal machine.
A: Confocal images of cell nuclei decorated with anti-20S proteasomal antibodies in the nuclei of cells before (*CNTR*) and 60 minutes after a 30 minute heat shock at 43°C (*HS*). Bar = 10 μm.
B: Multicolor channel images of nuclei, expressing GFP-labeled 20S core subunit LMP2 before (panel CNTR) and after a 30-minute heat shock at 43°C (panel HS). Each horizontal series represent the same nucleus in which epigenetical LMP2-GFP (LMP: green) and endogenous SC35 (SC35: red) are revealed and overlaid (Overlay: colocalization is evident by presence of yellow color). 20S proteasomes (LMP) become concentrated in nuclear speckles (SC35) after heat shock. Bar = 5 μm.
C: Confocal images of heat shock cells immunostained with the anti-Hsp25 antibody (*Hsp25*: green) and the anti-20S proteasome antibody (*20S*: red). Co-localization is evident in the overlay image (*Overlay*: yellow). On the overlaid images nuclear outline is indicated with blue (DAPI staining). Bar = 10 μm.

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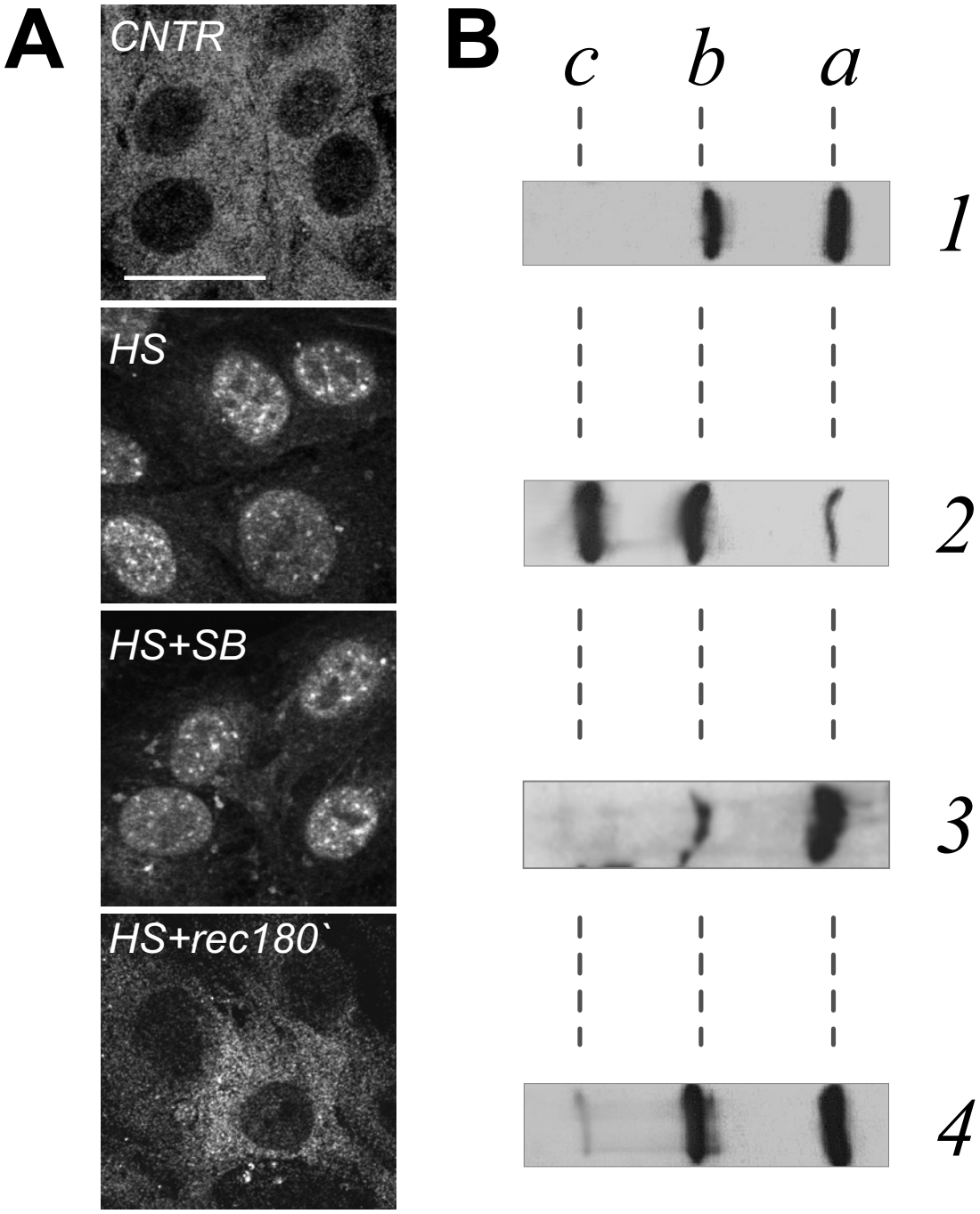

Figure 1.

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A

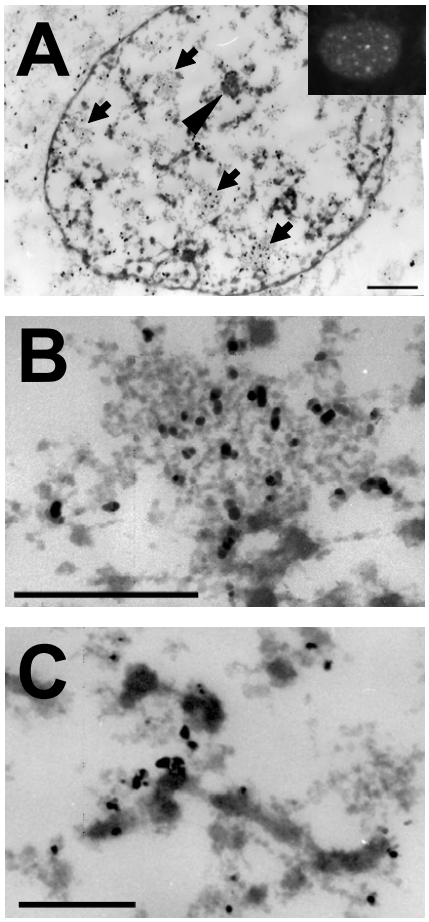

Figure 2.

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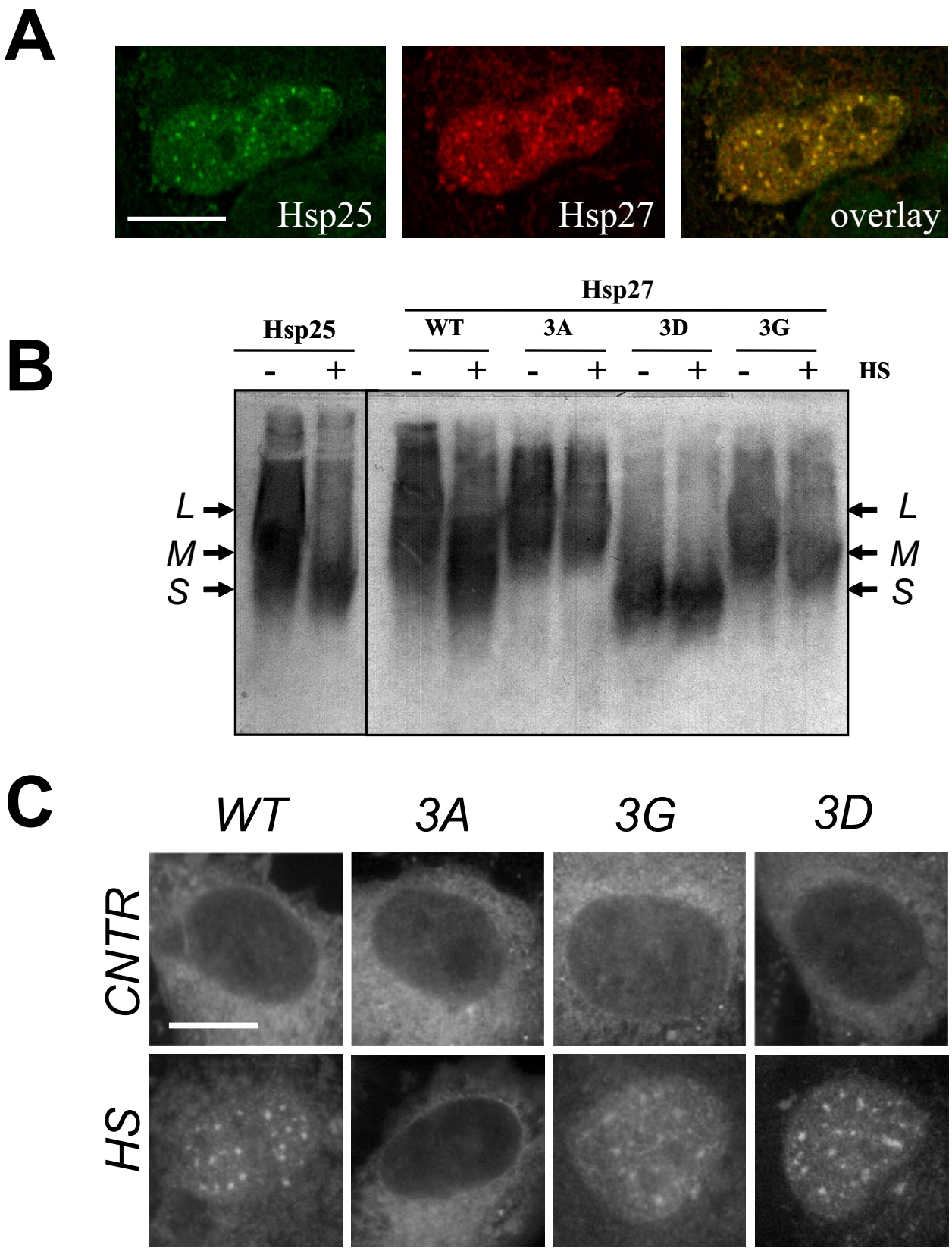

Figure 3.

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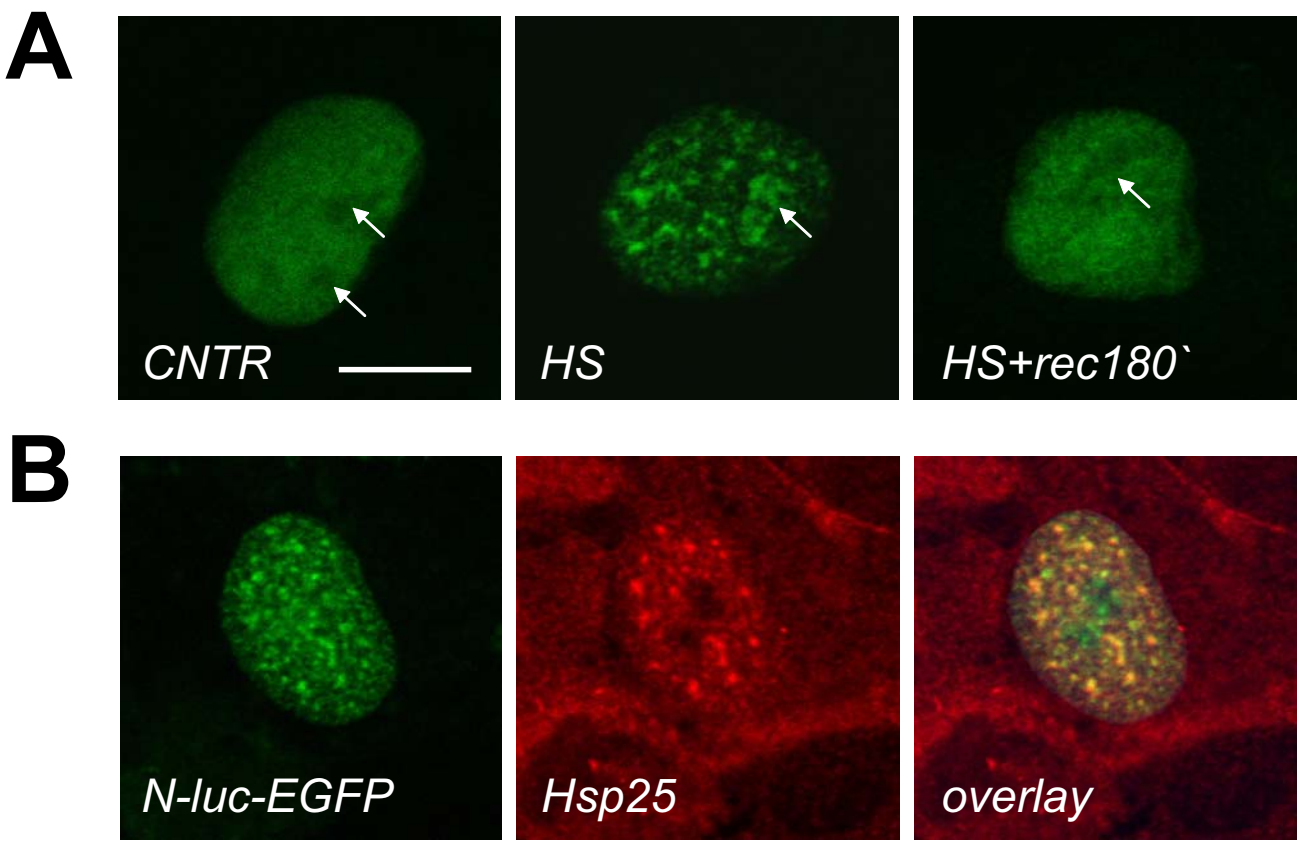

Figure 4.

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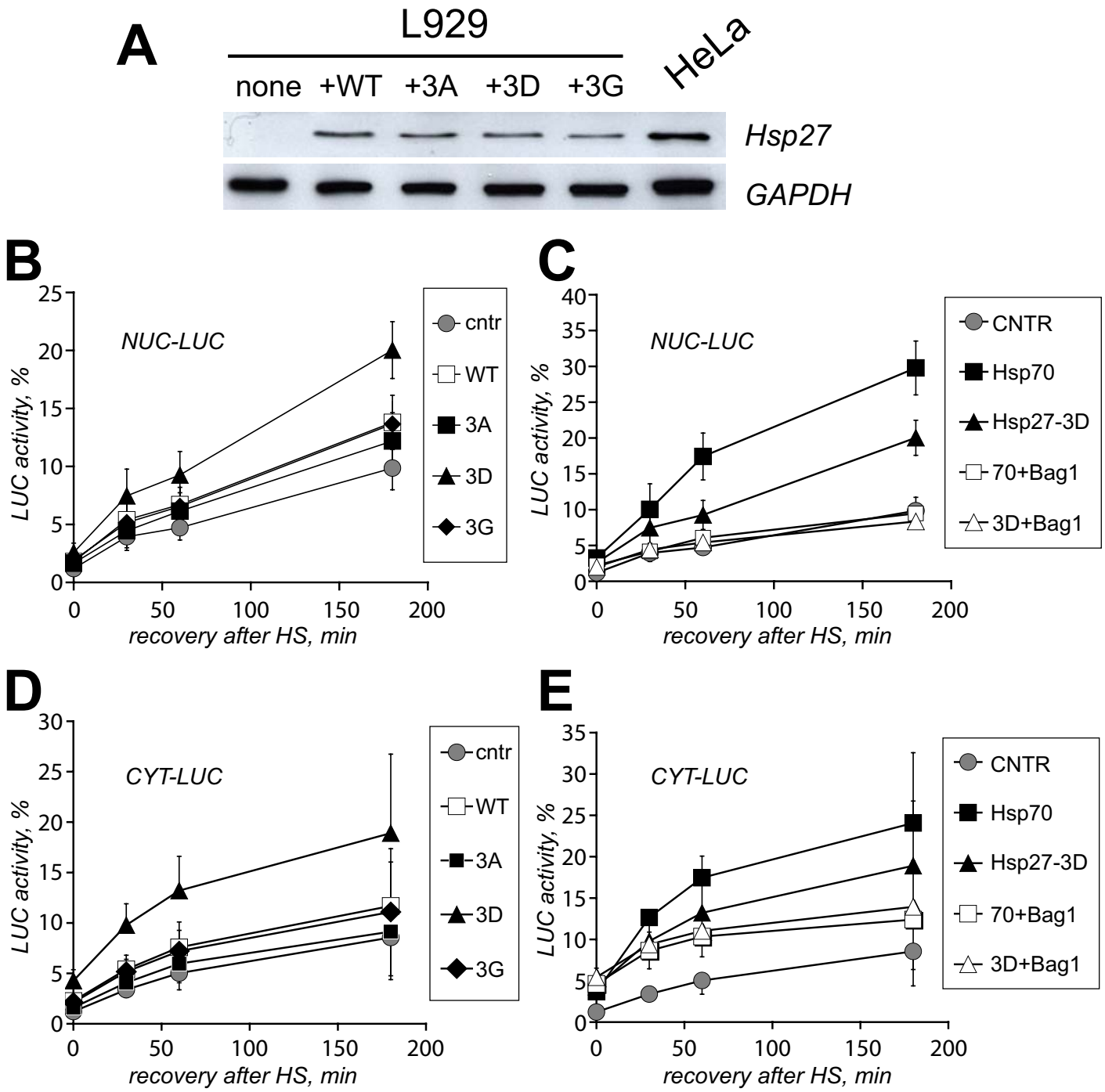

Figure 5.

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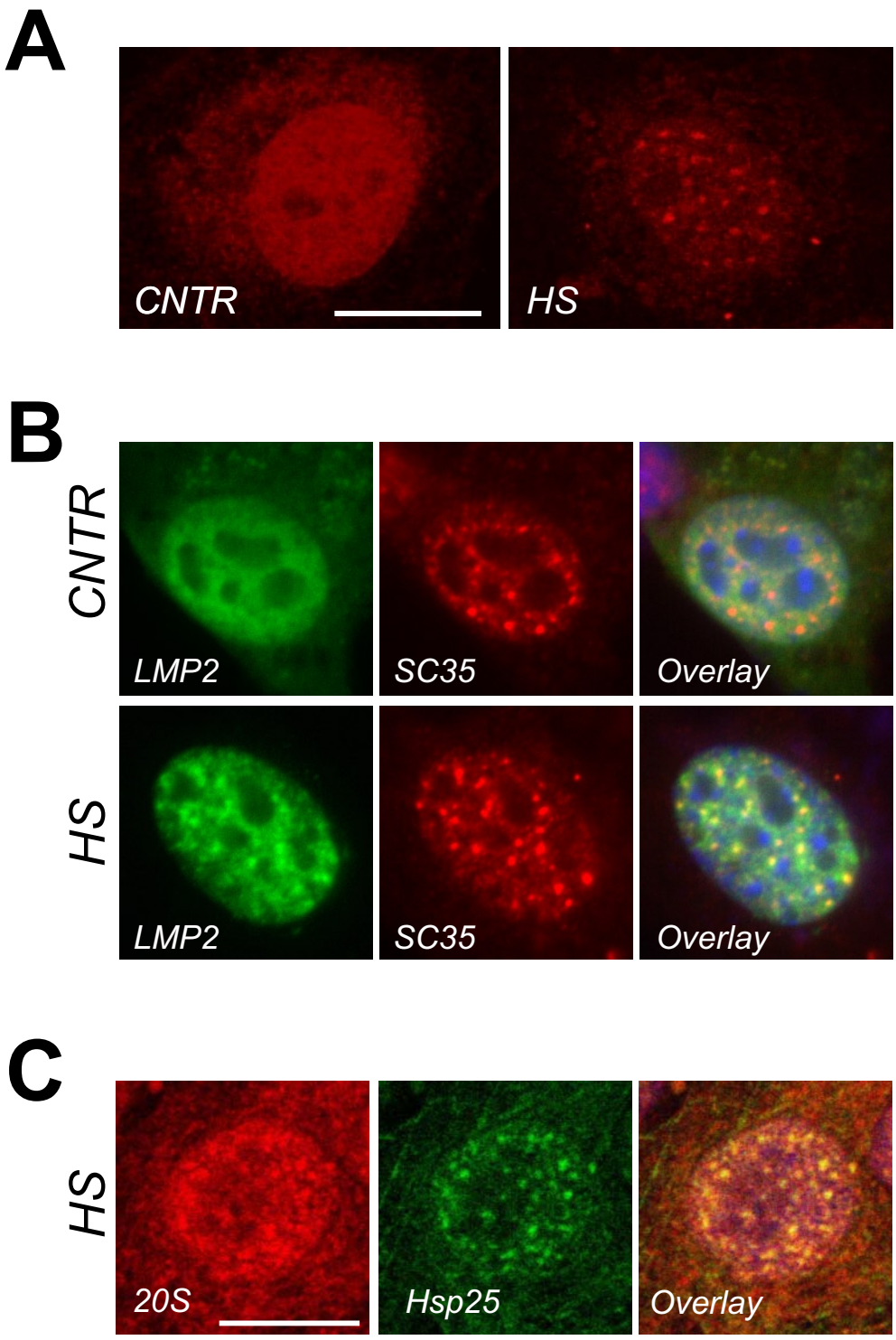

Figure 6.