

# The Heat Shock Response Is Modulated by and Interferes with Toxic Effects of Scrapie Prion Protein and Amyloid $\beta$ \*

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Ulrike K. Resenberger<sup>‡</sup>, Veronika Müller<sup>‡</sup>, Lisa M. Munter<sup>§¶</sup>, Michael Baier<sup>||</sup>, Gerd Multhaup<sup>§¶</sup>, Mark R. Wilson<sup>\*\*</sup>, Konstanze F. Winklhofer<sup>‡‡</sup>, and Jörg Tatzelt<sup>‡‡¶¶1</sup>

From the <sup>‡</sup>Neurobiochemistry, Adolf-Butenandt-Institute, Ludwig-Maximilians-University Munich, D-80336 Munich, Germany,

<sup>§</sup>Institut für Chemie und Biochemie, Freie Universität, 14195 Berlin, Germany, the <sup>¶</sup>Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec H3A0G4, Canada, <sup>||</sup>Robert-Koch-Institut, 13302 Berlin, Germany, the <sup>\*\*</sup>School of Biological Sciences, University of Wollongong, Wollongong, New South Wales 2522, Australia, and the <sup>‡‡</sup>German Center for Neurodegenerative Diseases (DZNE), 80336 Munich, Germany

**Background:** The heat shock response (HSR) is a stress response pathway to counteract proteotoxic effects of aberrantly folded proteins.

**Results:** The HSR is deregulated by PrP<sup>Sc</sup> and A $\beta$  and can protect against toxic effects of PrP<sup>Sc</sup>, A $\beta$ , and a neurotoxic PrP mutant.

**Conclusion:** The toxicity of different pathogenic proteins is mediated via similar cellular pathways.

**Significance:** Identifying cellular pathways activated by neurotoxic proteins will help to develop therapeutic strategies.

The heat shock response (HSR) is an evolutionarily conserved pathway designed to maintain proteostasis and to ameliorate toxic effects of aberrant protein folding. We have studied the modulation of the HSR by the scrapie prion protein (PrP<sup>Sc</sup>) and amyloid  $\beta$  peptide (A $\beta$ ) and investigated whether an activated HSR or the ectopic expression of individual chaperones can interfere with PrP<sup>Sc</sup>- or A $\beta$ -induced toxicity. First, we observed different effects on the HSR under acute or chronic exposure of cells to PrP<sup>Sc</sup> or A $\beta$ . In chronically exposed cells the threshold to mount a stress response was significantly increased, evidenced by a decreased expression of Hsp72 after stress, whereas an acute exposure lowered the threshold for stress-induced expression of Hsp72. Next, we employed models of PrP<sup>Sc</sup>- and A $\beta$ -induced toxicity to demonstrate that the induction of the HSR ameliorates the toxic effects of both PrP<sup>Sc</sup> and A $\beta$ . Similarly, the ectopic expression of cytosolic Hsp72 or the extracellular chaperone clusterin protected against PrP<sup>Sc</sup>- or A $\beta$ -induced toxicity. However, toxic signaling induced by a pathogenic PrP mutant located at the plasma membrane was prevented by an activated HSR or Hsp72 but not by clusterin, indicating a distinct mode of action of this extracellular chaperone. Our study supports the notion that different pathological protein conformers mediate toxic effects via similar cellular pathways and emphasizes the possibility to exploit the heat shock response therapeutically.

Accumulation of misfolded and aggregated proteins is a hallmark of various neurodegenerative diseases. Prion diseases (for review, see Refs. 1–4) and Alzheimer disease (AD)<sup>2</sup> (for review,

see Refs. 5 and 6) are characterized by extracellular protein assemblies formed by the scrapie prion protein (PrP<sup>Sc</sup>) or amyloid  $\beta$  (A $\beta$ ) peptide, respectively. Whereas prion diseases and AD are clearly distinct disease entities, there appear to be commonalities concerning structural features of the pathogenic protein conformers as well as pathways implicated in their toxic effects (for review, see Refs. 7–10).

The protein deposits found in AD or prion diseases are associated with intra- and extracellular heat shock proteins (Hsps) (11–13), suggesting a role of Hsps in the pathogenic process. Hsps, many of which function as molecular chaperones, comprise a class of proteins that are induced under conditions of cellular stress when the concentration of aggregation-prone folding intermediates are increasing. However, Hsps exert fundamental functions also under physiological conditions as they are vitally engaged in protein folding, trafficking, and regulation of signaling pathways (for review, see Ref. 14). Hsps are found in all cellular compartments and organelles. In addition, clusterin is a secreted chaperone shown to be involved in the extracellular protein quality control system (15). Up-regulation of Hsps after acute or chronic proteotoxic damage is mediated by a highly conserved pathway denoted the heat shock response (HSR). At the molecular level, different stressors are integrated through the activation of a single transcription factor, the heat shock transcription factor 1 (HSF1), which binds to specific heat shock element (HSE) sequences present in the promoter region of inducible Hsp genes (for review, see Refs. 16 and 17). An increase in Hsp levels prevents protein aggregation and facilitates correct folding of non-native proteins after cellular stress. In addition, chaperones participate in anti-apoptotic pathways (for review, see Refs. 18–20). It is, therefore, not surprising that a deregulation of the HSR can contribute to the progression of various diseases. Consequently, the HSR represents a target for therapeutic intervention in a range of diseases

HSF1, heat shock transcription factor 1; HSE, heat shock element; HD, hydrophobic domain.

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<sup>1</sup> To whom correspondence should be addressed: Ludwig-Maximilians-University Munich, Schillerstrasse 44, D-80336 Munich, Germany. Tel.: 49-89-2180-75442; Fax: 49-89-2180-75415; E-mail: Joerg.Tatzelt@med.uni-muenchen.de.

<sup>2</sup> The abbreviations used are: AD, Alzheimer disease; PrP<sup>Sc</sup>, scrapie prion protein; A $\beta$ , amyloid  $\beta$ ; Hsp, heat shock protein; HSR, heat shock response;

(for review, see Refs. 21–26). For example, pharmacological induction of the HSR was shown to ameliorate disease progression and neuropathological alterations in mouse models of neurodegenerative diseases (27–29). Supporting a protective role of the HSR, deletion of HSF1 dramatically shortened the lifespan of scrapie-infected mice (30).

We have previously studied the HSR in scrapie-infected mouse neuroblastoma (ScN2a) cells, which offer a useful model to study certain aspects of prion diseases in cultured cells. Most importantly, ScN2a cells propagate partially protease-resistant PrP<sup>Sc</sup> and infectious prions (31, 32). The stress-induced expression of Hsp72 and Hsp28 is significantly impaired in ScN2a cells, whereas their uninfected counterparts are able to mount a normal stress response (33, 34). Notably, we found that the impaired HSR in ScN2a cells is caused by an accelerated deactivation of HSF1 after stress and can be restored by the Hsp90-binding drug geldanamycin (34).

In this study we characterized the impact of pathogenic protein conformers on the regulation of HSR by making use of cell culture models of PrP<sup>Sc</sup>- and A $\beta$ -induced toxicity. We demonstrate that PrP<sup>Sc</sup> and A $\beta$  have different effects on the HSR depending on whether they are applied in an acute or chronic manner to cells. Moreover, activation of the HSR or ectopic expression of individual chaperons is protective against PrP<sup>Sc</sup>- and A $\beta$ -induced cell death as well as the toxic activity of a pathogenic PrP mutant.

## EXPERIMENTAL PROCEDURES

**Plasmids, Antibodies, and Reagents**—Expression constructs have been described previously: PrP<sup>C</sup> (35); HSE-luc (36); Hsp72 (37);  $\Delta$ HSE, wtHSF (38); PrP $\Delta$ H $\Delta$ D (35); pRc/VMV-clusterin (39). Amino acid numbers refer to mouse prion protein sequence (GenBank<sup>TM</sup> accession number NP 035300). As the transfection marker, the EYFP-C1 vector (Clontech) was used. The following antibodies were used: mouse monoclonal anti-PrP 3F4 antibody (40), rabbit polyclonal anti-PrP antibody A7 (41), mouse monoclonal anti-Hsp72 antibody C92 (42), mouse monoclonal anti-clusterin antibody 41D (43), mouse monoclonal anti- $\beta$ -actin antibody (Sigma), rabbit polyclonal anti-active caspase-3 antibody (Promega), fluorescent dye-labeled anti-rabbit IgG antibody Alexa Fluor<sup>®</sup> 555 (Invitrogen), fluorescent dye-labeled anti-mouse IgG antibody Alexa Fluor<sup>®</sup> 555 (Invitrogen), horseradish peroxidase-conjugated anti-mouse IgG antibody (Amersham Biosciences, Promega), horseradish peroxidase-conjugated anti-rabbit IgG antibody (Promega), horseradish peroxidase-conjugated anti-rat IgG antibody (Santa Cruz Biotechnology), rat monoclonal anti-A $\beta$  antibody 2D8 (44), rabbit polyclonal anti-A $\beta$  antibody 3552 (45). The following reagents were used: TO-PRO<sup>®</sup>-3 iodide (642/661) (Invitrogen) and DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester). The mounting medium Mowiol (Calbiochem) was supplemented with DAPI (4',6-diamidino-2-phenylindole; Sigma).

**Cell Culture, Transfection, Co-culture**—Cells were cultured and transfected as described earlier (35). The human SH-SY5Y cell line (DSMZ number ACC 209) is a sub-line of bone marrow biopsy-derived SK-N-SH cells. Stably transfected Chinese hamster ovary cells (CHO-7PA2) that express the familial AD

mutation V717F in the amyloid precursor protein APP<sub>751</sub> and secrete A $\beta$  were described earlier (46). Cells cultured in 3.5-cm dishes were transfected with DNA by a liposome-mediated method using LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's instructions. For co-culture experiments, SH-SY5Y cells were grown on glass coverslips. 2 h after transfection coverslips were transferred into dishes containing a 90% confluent cell layer of either ScN2a or N2a or CHO-7PA2 or CHO cells (47, 48). After 16 or 24 h of co-culture, either apoptotic cell death or luciferase activity was analyzed (see below). For stable transfection, SH-SY5Y cells were transfected with the plasmid pCEP4 containing the coding sequence for APP695 using Transfectin (Bio-Rad) according to the manufacturer's instructions. Stably transfected cells were selected with hygromycin (250  $\mu$ g/ml). The empty vector was used as control (mock-transfected).

**Cell Lysis, Immunoprecipitation, and Western Blot Analysis**—As described earlier (49), cells were washed twice with cold phosphate-buffered saline (PBS), scraped off the plate, and lysed in cold detergent buffer A (0.5% Triton X-100, 0.5% sodium deoxycholate in PBS). Total lysates or secreted and trichloroacetic acid (TCA)-precipitated proteins were boiled with Laemmli sample buffer and analyzed by Western blotting as described previously (50). For proteolysis experiments, lysates of ScN2a or N2a cells were digested with Proteinase K for 30 min at 37 °C (final concentration 10  $\mu$ g/ml). Reaction was stopped by the addition of PMSF (final concentration 2 mM), and PrP was analyzed by Western blotting using the polyclonal anti-PrP antibody A7. A $\beta$  in conditioned medium of CHO-7PA2 cells or stably transfected SH-SY5Y cells were analyzed by immunoprecipitation with the polyclonal antibody 3552 followed by Western blotting using the monoclonal antibody 2D8. To block A $\beta$  generation, CHO-7PA2 cells were treated for 24 h with DAPT before immunoprecipitation. To interfere with PrP<sup>Sc</sup>-induced toxicity, transfected cells were pretreated for 1 h with the monoclonal anti-PrP antibody 3F4 (1  $\mu$ g/ml) before co-culture. The antibody was also present during co-cultivation.

For quantification of Hsp72, total lysates were analyzed by Western blotting using the monoclonal anti-Hsp72 antibody C92. Chemiluminescence was determined using a Fujifilm LAS-4000 ChemiDot imager and the Multi Gauge V3.0 software and normalized to  $\beta$ -actin. Values of CHO-7PA2 cells or SH-SY5Y cells overexpressing wild type APP were compared with either CHO cells or mock-transfected SH-SY5Y cells subjected to the same heat shock. Quantifications were based on at least three independent experiments.

**Exosome Isolation**—Conditioned media of ScN2a or N2a cells were centrifuged for 10 min at 3,000  $\times$  *g* and ultracentrifuged for 30 min at 10,000  $\times$  *g* and for 1 h at 100,000  $\times$  *g* as described earlier (55). Pellets were resuspended in cold detergent buffer A (0.5% Triton X-100, 0.5% sodium deoxycholate in PBS) and digested with Proteinase K for 30 min at 37 °C (final concentration 10  $\mu$ g/ml). The reaction was stopped by the addition of PMSF (final concentration 2 mM), and PrP was analyzed by Western blotting using the polyclonal anti-PrP antibody A7.

**Luciferase Assays**—Co-cultivated SH-SY5Y cells or SH-SY5Y cells cultured in 3.5-cm dishes were transiently transfected with firefly luciferase reporter plasmid (HSE-luc) and subjected to the stress treatment indicated. After 8 h of incubation at 37 °C, cells were lysed in Reporter Lysis Buffer (Promega). Luciferase activity was analyzed luminometrically using the luciferase assay system (Promega) and a LB96V or Mithras LB 940 luminometer (Berthold Technologies, Bad Wildbad, Germany) according to the manufacturer's instruction. The measured values were analyzed using a WinGlow Software (Berthold Technologies). Quantifications were based on at least three independent experiments.

**Apoptosis Assay and Immunofluorescence**—For quantification of apoptotic cell death, SH-SY5Y cells were fixed on glass coverslips with 3.7% paraformaldehyde for 20 min, washed, and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. Fixed cells were incubated with an anti-active caspase-3 antibody overnight at 4 °C followed by an incubation with the secondary antibody fluorescently labeled with Alexa Fluor® 555 for 1 h at room temperature. Cells were then mounted onto glass slides and examined by fluorescence microscopy using a Zeiss AxioScope 2 plus microscope (Carl Zeiss). The number of cells positive for activated caspase-3 from at least 1000 transfected cells was determined in a blinded manner. All quantifications were based on at least three independent experiments. For immunofluorescence analysis of the stress-inducible Hsp72 in N2a or ScN2a or CHO or CHO-7PA2 cells, cells were grown on glass coverslips. At day 2 (CHO/CHO-7PA2) or day 4 (N2a/ScN2a) in culture, cells were subjected to the heat shock indicated, returned to 37 °C, and analyzed after an additional 8 or 16 h, respectively. After incubation, cells were fixed, permeabilized, and stained for Hsp72 using the monoclonal anti-Hsp72 antibody C92. Nuclei were stained with ToPro. Cells were examined by confocal fluorescence microscopy using a Zeiss Axiovert 200M microscope (Carl Zeiss).

**Statistical Analysis**—Quantifications were based on at least three independent experiments. Data were shown as the means  $\pm$  S.E. Statistical analysis was performed using Student's *t* test. *p* values are as follows: \*, *p* < 0.05; \*\*, *p* < 0.005; \*\*\*, *p* < 0.0005.

## RESULTS

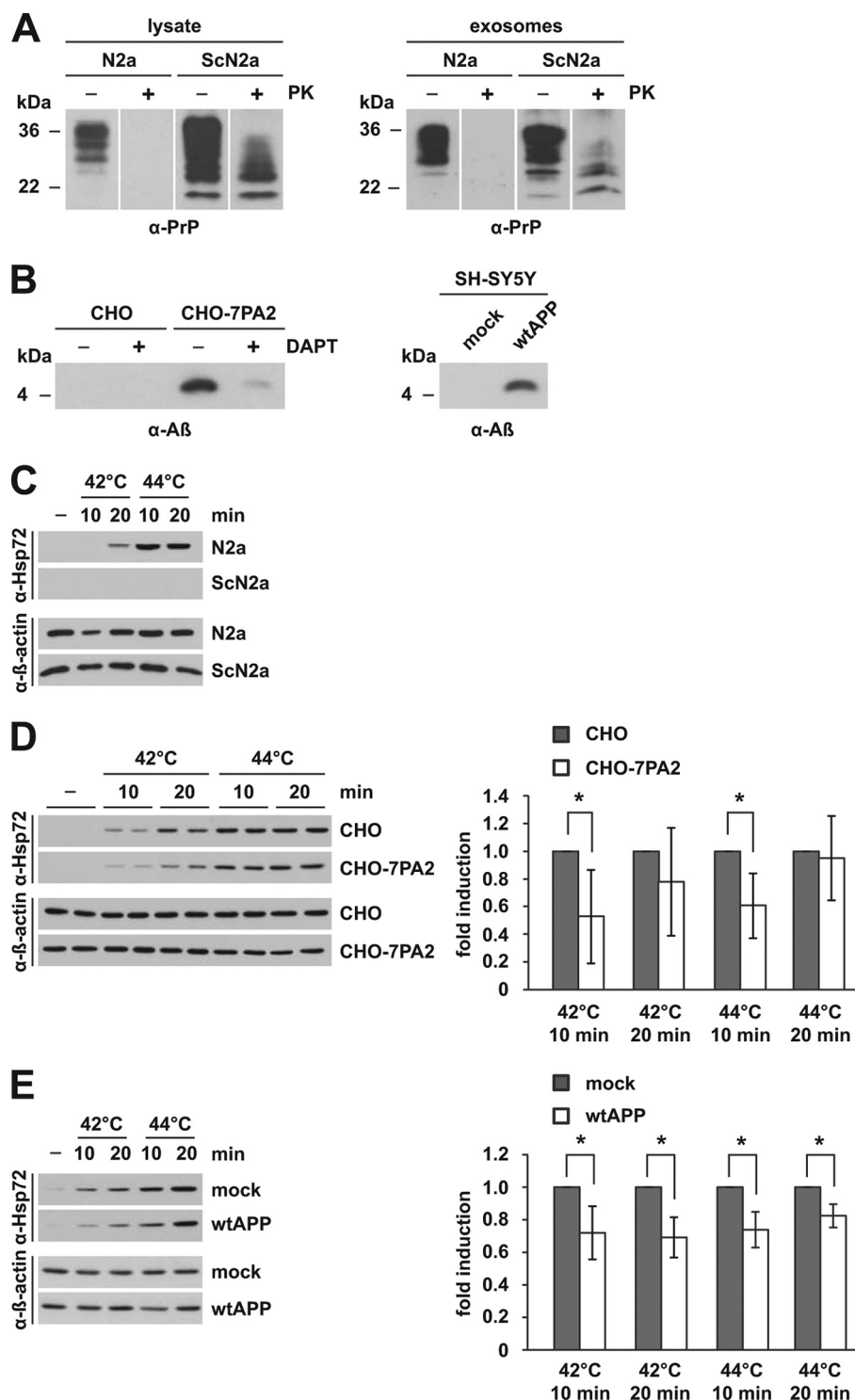
**The Heat Shock Response Is Impaired in Cell Lines Chronically Exposed to PrP<sup>Sc</sup> or A $\beta$** —We previously showed that the HSR in scrapie-infected mouse neuroblastoma (ScN2a) cells, which propagate proteinase K-resistant PrP<sup>Sc</sup> and infectious prions (Fig. 1A), is significantly impaired (33, 34). The amount of Hsp72, which is expressed at high levels only after heat shock or other forms of metabolic stress (51), is greatly increased in uninfected N2a cells after a heat shock of 10 or 20 min (42 or 44 °C), whereas ScN2a cells do not express Hsp72 after being subjected to the same stress conditions. This phenomenon is illustrated by Western blotting (Fig. 1C) and indirect immunofluorescence (Fig. 2A, left panel). Prompted by these results, we asked whether chronic exposure to another pathogenic protein assembly would also modulate the HSR. To experimentally address this possibility, we made use of a stably transfected

Chinese hamster ovary cell line (CHO-7PA2) that expresses the familial AD mutant V717F of the human amyloid precursor protein APP<sub>751</sub> and secretes A $\beta$  (46) (Fig. 1B, left panel). Importantly, secreted A $\beta$  from CHO-7PA2 cells is neurotoxic, demonstrated by its ability to potently inhibit long term potentiation *in vivo* and to interfere with neuronal viability (48, 52, 53). In addition, we generated a stably transfected SH-SY5Y cell line expressing wild type human APP. Similarly to the CHO-7PA2 cells, SH-SY5Y-wtAPP cells secreted significantly increased levels of A $\beta$  when compared with the mock-transfected control (Fig. 1B, right panel).

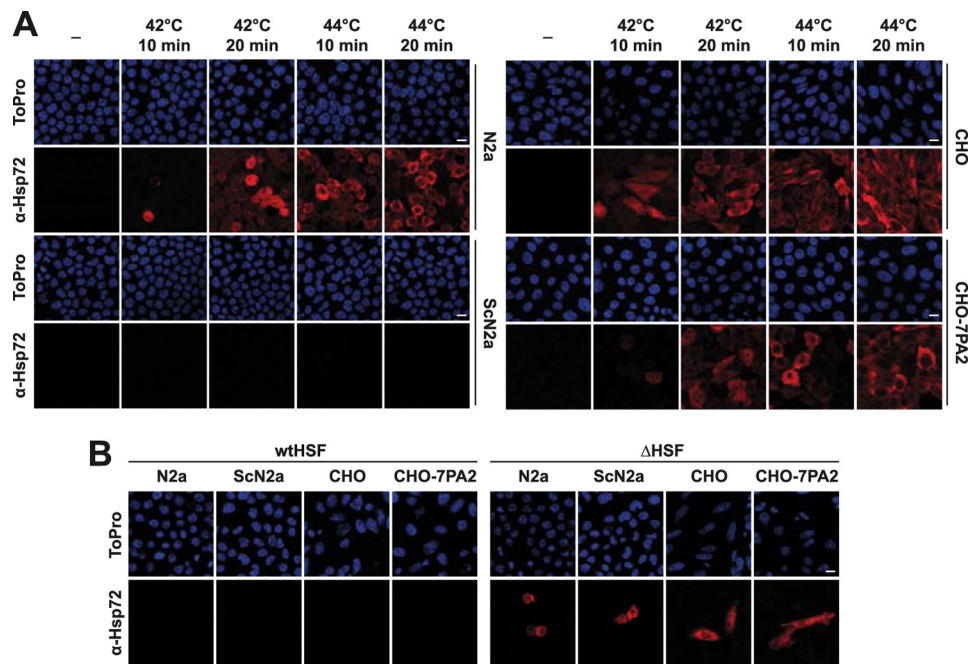
To analyze the HSR, we subjected CHO-7PA2 and SH-SY5Y-wtAPP cells to different heat shock conditions and analyzed expression of Hsp72 after the cells had been cultivated for another 8 h at 37 °C. The Western blot (Fig. 1, D and E) and immunofluorescence analysis (Fig. 2A, right panel) revealed that A $\beta$ -overexpressing CHO-7PA2 and SH-SY5Y-wtAPP are able to mount a stress response; however, the amount of Hsp72 in stressed CHO-7PA2 and SH-SY5Y-wtAPP was lower when compared with CHO or mock transfected SH-SY5Y cells, respectively, subjected to the same stress conditions. These differences were significant under all stress conditions tested for the SH-SY5Y cell lines (Fig. 1E), whereas after more severe stress (42 or 44 °C for 20 min) Hsp72 levels were comparable in CHO and CHO-7PA2 cells (Fig. 1D). Ectopic expression of a mutant of the heat shock transcription factor 1 ( $\Delta$ HSF), which contains a deletion in the regulatory domain ( $\Delta$ 202–316) and is constitutively active (38), induced the up-regulation of Hsp72 in both ScN2a and CHO-7PA2 cells (Fig. 2B). These findings suggest that the impaired Hsp72 expression after stress is obviously caused by a deregulated HSF1 activation/inactivation pathway and not by mutations in the promoter regions of stress-regulated genes (34). These results demonstrate that cells chronically exposed to A $\beta$  or PrP<sup>Sc</sup> have a higher threshold to mount a HSR.

**Acute Exposure of Cells to PrP<sup>Sc</sup> Lowers the Threshold for a Heat Shock Response**—ScN2a cells had been established from a population of cells acutely infected with prions. Thus, it might well be that an impaired stress response was a selection advantage to counteract adverse effects of PrP<sup>Sc</sup> on cell viability. We, therefore, wanted to analyze possible acute effects of PrP<sup>Sc</sup> on the HSR by employing a novel cell culture assay, which is based on the co-culture of SH-SY5Y cells with N2a or ScN2a cells (47, 48). In this context it is important to note that scrapie-infected cells release PrP<sup>Sc</sup> and infectious prions into the extracellular environment (Fig. 1A, right panel) (54, 55). Our experimental set-up allows us to study the HSR in SH-SY5Y cells after transient exposure to PrP<sup>Sc</sup> present in the cell culture medium (Fig. 3A). To assess the HSR in a quantitative manner, we used a reporter gene construct (HSE-luc) expressing firefly luciferase under the control of the highly heat-inducible promoter of the human Hsp70B gene (36). After a brief heat shock, transcription of the luciferase gene is induced, and luciferase activity can be determined luminometrically (Fig. 3B). First, we examined whether PrP<sup>Sc</sup> released by ScN2a cells would induce an HSR in co-cultured SH-SY5Y cells. Luciferase activities in SH-SY5Y cells co-cultured with ScN2a cells for 24 h were comparable to





**FIGURE 1. Impaired heat shock response in cell lines chronically exposed to PrP<sup>Sc</sup> or Aβ.** *A*, chronically scrapie-infected N2a cells (ScN2a) are characterized by the formation of Proteinase K (PK)-resistant scrapie prion protein (PrP). Total cell lysates and isolated exosomes prepared from N2a or ScN2a cells were treated with proteinase K or left untreated and then analyzed by Western blotting using the polyclonal anti-PrP antibody A7. *B*, stably transfected CHO cells (CHO-7PA2) or SH-SY5Y cells generate amyloid β (Aβ). Aβ present in conditioned medium of CHO or CHO-7PA2 cells and stably transfected SH-SY5Y cells was analyzed by immunoprecipitation with the polyclonal antibody 3552 followed by Western blotting using the monoclonal antibody 2D8. To block Aβ generation, CHO-7PA2 cells were treated for 24 h with DAPT before immunoprecipitation. *C–E*, ScN2a, CHO-7PA2, and stably transfected SH-SY5Y cells exhibit an impaired heat shock response. *C*, N2a and ScN2a cells were subjected to heat shock conditions as indicated. The stress-inducible heat shock protein Hsp72 was analyzed by Western blotting using the monoclonal anti-Hsp72 antibody C92. *D*, CHO and CHO-7PA2 cells were subjected to heat shock conditions as indicated. The stress-inducible heat shock protein Hsp72 was analyzed by Western blotting using the monoclonal anti-Hsp72 antibody C92. Band intensities of the Hsp72 signals from CHO and CHO-7PA2 cells were quantified and normalized to β-actin. The fold induction of Hsp72 in CHO-7PA2 cells in response to various stresses, relative to CHO cells, is shown in the *right panel*. *E*, mock- and wild type APP-transfected SH-SY5Y cells were subjected to heat shock conditions as indicated. The stress-inducible heat shock protein Hsp72 was analyzed as described under Fig. 1*D*. The relative amounts of Hsp72 are represented as the mean ± S.E. of three to four independent experiments. \*, *p* < 0.05.



**FIGURE 2. Cells chronically exposed to PrP<sup>Sc</sup> or A $\beta$  exhibit a higher threshold to mount a heat shock response.** A, ScN2a and CHO-7PA2 cells have an impaired heat shock response. N2a, ScN2a, CHO, and CHO-7PA2 cells were subjected to the heat shock conditions as indicated. Hsp72 was analyzed by indirect immunofluorescence using the monoclonal anti-Hsp72 antibody C92. B, expression of a constitutively active mutant of the heat shock transcription factor 1 ( $\Delta$ HSF) induces expression of Hsp72 in both ScN2a and CHO-7PA2 cells. N2a, ScN2a, CHO, and CHO-7PA2 cells were transiently transfected with wild type HSF (wtHSF) or the constitutively active  $\Delta$ HSF mutant. 24 h after transfection expression of Hsp72 was analyzed by indirect immunofluorescence as described under Fig. 2A. Nuclei were stained with ToPro. Scale bars, 10  $\mu$ m.

those in cells co-cultured with N2a cells, indicating that acute exposure to PrP<sup>Sc</sup> did apparently not induce the HSR (Fig. 3C).

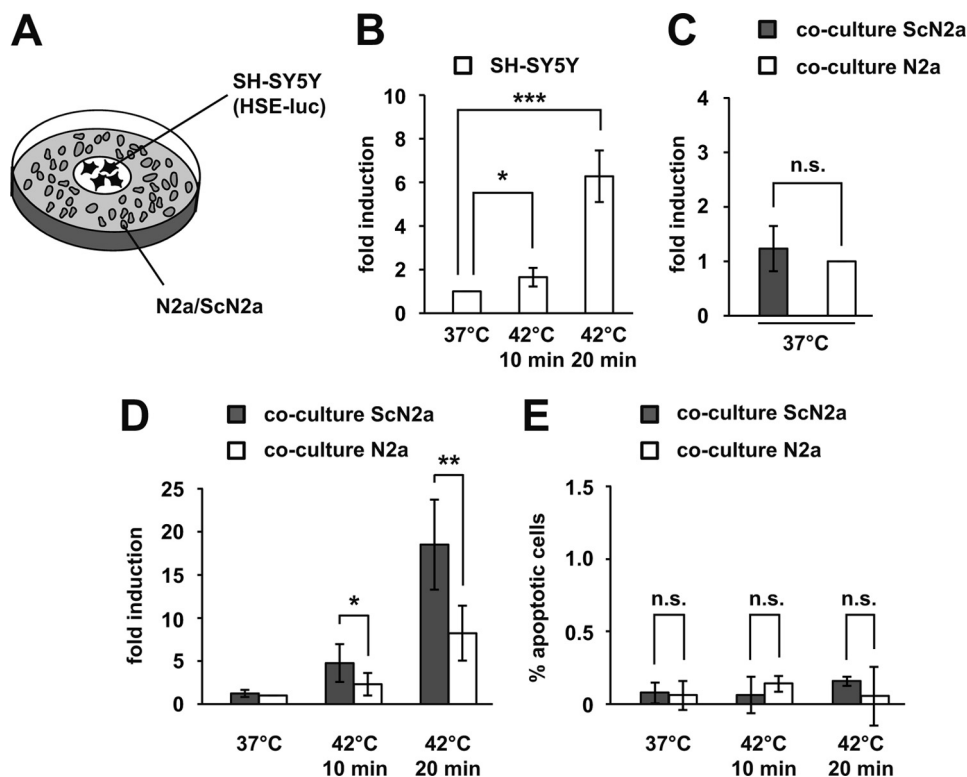
Next we tested whether acute exposure to PrP<sup>Sc</sup> modulates the HSR. To this end we co-cultured HSE-luc-expressing SH-SY5Y cells with ScN2a cells and then subjected them to a brief heat shock (Fig. 3D). SH-SY5Y cells co-cultured with ScN2a cells showed significantly higher luciferase activities after a heat shock than cells co-cultured with N2a cells. For example, a 20-min heat shock at 42 °C led to an 8-fold induction of luciferase in SH-SY5Y cells co-cultured with N2a cells, whereas the same heat shock condition led to a 18.5-fold induction in cells pre-exposed to PrP<sup>Sc</sup> (Fig. 3D). Of note, there was no increase in cell death of co-cultured SH-SY5Y cells under the heat shock conditions applied (Fig. 3E).

**Induction of the HSR or Increased Expression of Hsp72 or Clusterin Protects against PrP<sup>Sc</sup>- or A $\beta$ -induced Toxicity**—To address the possibility that an induction of the HSR can protect cells from the toxic activity of PrP<sup>Sc</sup> or A $\beta$ , we employed a previously established cell culture model (47, 48). As illustrated in Fig. 4A, *left panel*, PrP<sup>Sc</sup> induces cell death in co-cultured SH-SY5Y cells expressing the cellular prion protein (PrP<sup>C</sup>). Similarly, expression of PrP<sup>C</sup> sensitizes cells to the toxic effects of A $\beta$  (Fig. 4B). Toxicity of PrP<sup>Sc</sup> could be suppressed by performing the co-culture in the presence of the monoclonal anti-PrP antibody 3F4 (Fig. 4A, *right panel*). Likewise, co-cultivation with CHO-7PA2 cells pretreated with the  $\gamma$ -secretase inhibitor DAPT did not induce apoptotic cell death in PrP<sup>C</sup>-expressing SH-SY5Y cells, indicating that the toxic effect of CHO-7PA2 cells was dependent on the generation of A $\beta$  (48).

To induce the HSR without a stress treatment, we expressed the constitutively active  $\Delta$ HSF mutant, which increases expres-

sion of many heat shock proteins, for example of Hsp72 (Fig. 2B). SH-SY5Y cells transiently co-transfected with PrP<sup>C</sup> and  $\Delta$ HSF or GFP as a control were co-cultured with ScN2a or CHO-7PA2 cells, and apoptotic cell death was analyzed after 16 h of co-culturing. ScN2a or CHO-7PA2 cells induced cell death in co-cultured SH-SY5Y cells expressing PrP<sup>C</sup> and GFP, whereas the co-expression of  $\Delta$ HSF protected the cells from PrP<sup>Sc</sup>- or A $\beta$ -induced cell death (Fig. 4C). In a next step we tested whether it is sufficient to express individual chaperones to block PrP<sup>Sc</sup>- or A $\beta$ -induced toxicity. To analyze chaperones located in different cellular compartments, we chose Hsp72, a cytoplasmic chaperone, and clusterin, an extracellular chaperone that has recently been genetically associated with AD (56, 57). Indeed, expression of either Hsp72 or clusterin was sufficient to inhibit PrP<sup>Sc</sup>- or A $\beta$ -induced cell death (Figs. 4D and 5).

**Hsp72 and  $\Delta$ HSF but Not Clusterin Protect against a Neurotoxic PrP Mutant**—Several PrP mutants can induce neuronal cell death in the absence of infectious prion propagation (for review, see Ref. 8). PrP<sup>C</sup> can acquire a neurotoxic potential by deleting the internal hydrophobic domain (HD) (58, 59). Similar to PrP<sup>C</sup>, PrP $\Delta$ HD is glycosylated with complex sugars and linked to the outer leaflet of the plasma membrane via a glycosylphosphatidylinositol anchor (35). To assess whether an activated HSR and the expression of chaperones can also interfere with the toxic effects of a pathogenic PrP mutant located at the plasma membrane, we used a cell culture model previously established in our group (47, 60). Upon ectopic expression of PrP $\Delta$ HD, apoptotic cell death is induced in SH-SY5Y cells. The toxic effects of PrP $\Delta$ HD are abrogated by co-expression of PrP<sup>C</sup> (Fig. 6A). This activity of PrP<sup>C</sup> has been conclusively documented in various transgenic mouse models and cultured cells;



**FIGURE 3. Acute exposure to PrP<sup>Sc</sup> lowers the threshold for a heat shock response.** A, shown is a schematic model of the co-culture assay. SH-SY5Y cells were plated on glass coverslips. 2 h after transfection coverslips were transferred into dishes containing a 90% confluent layer of either ScN2a or N2a cells. After 24 h of co-culture, the coverslips were removed, and the SH-SY5Y cells were analyzed. Either luciferase activity was determined in cell lysates (B, C, and D), or SH-SY5Y cells were fixed, permeabilized, and stained for active caspase-3 to assess apoptotic cell death (E). All quantifications were based on at least three independent experiments. B, heat shock induces expression of luciferase. SH-SY5Y cells were transiently transfected with a reporter gene construct (HSE-luc) expressing firefly luciferase under the control of the highly heat-inducible promoter of the human Hsp70B gene. 18 h after transfection cells were subjected to a heat shock (42 °C) for the time indicated or held at 37 °C. After additional 8 h at 37 °C luciferase activity in total cell lysates were determined luminometrically and plotted as fold induction relative to cells held at 37 °C. C, acute exposure to PrP<sup>Sc</sup> does not induce a heat shock response. SH-SY5Y cells transiently transfected with HSE-luc were co-cultured with ScN2a or N2a cells for 24 h at 37 °C, and then luciferase activity was analyzed; -fold induction relative to cells co-cultured with N2a cells at 37 °C is plotted. D, acute exposure to PrP<sup>Sc</sup> lowers the threshold for a stress response. SH-SY5Y cells transiently transfected with HSE-luc were co-cultured with ScN2a or N2a cells for 16 h at 37 °C. Cells were subjected to a heat shock (42 °C) for the time indicated or held at 37 °C. After an additional 8 h at 37 °C, luciferase activity was analyzed as described above. The fold induction relative to cells co-cultured with N2a cells at 37 °C is plotted. E, apoptotic cell death was not increased by the heat shock conditions tested. SH-SY5Y cells were co-cultured with ScN2a or N2a cells and heat shocked as described under D. For quantification of apoptotic cell death, SH-SY5Y cells were fixed, permeabilized, and stained for active caspase-3. n.s., not significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.005$ ; \*\*\*,  $p < 0.0005$ .

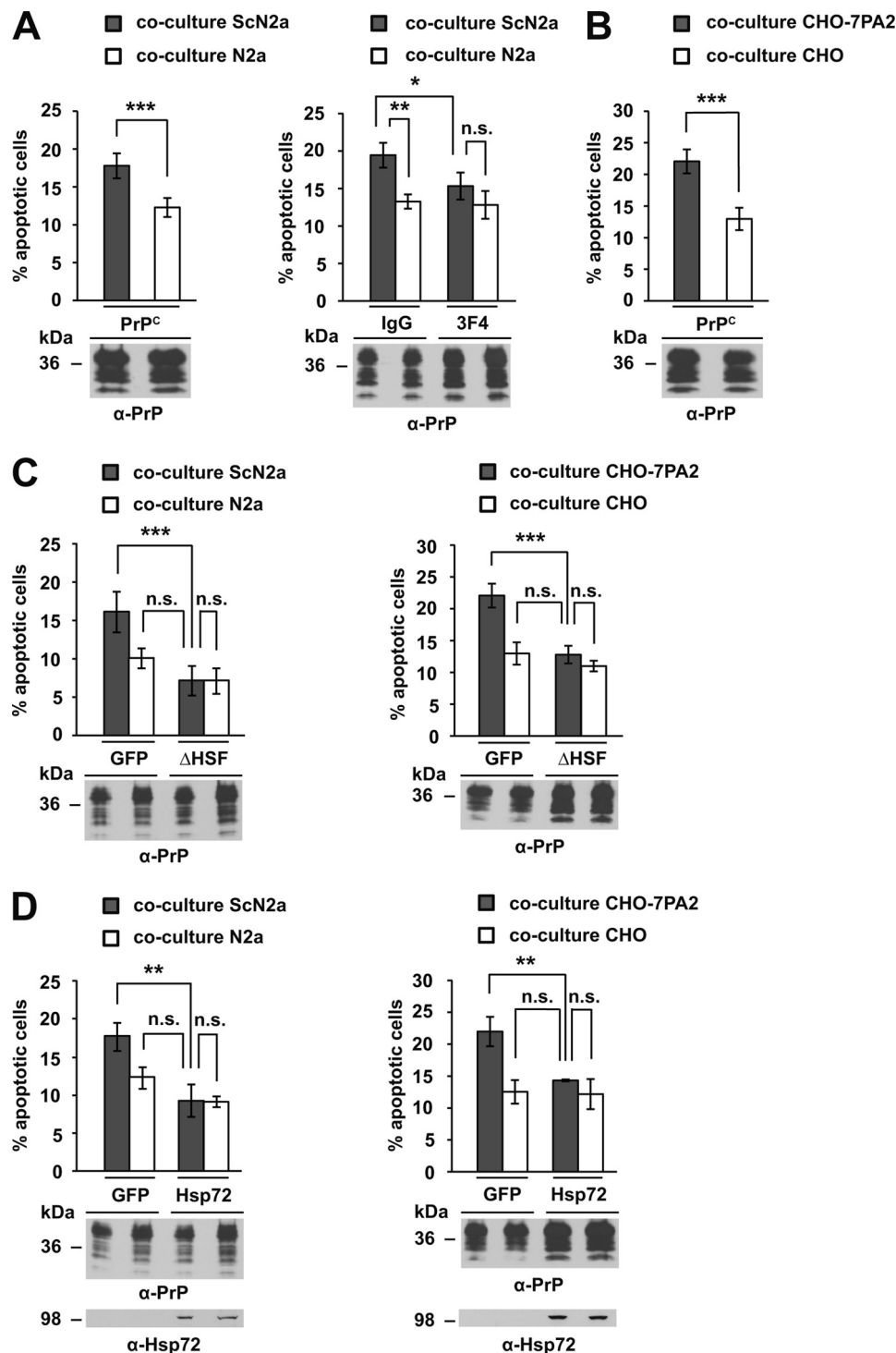
however, the underlying mechanisms are elusive (47, 58–63). To test a possible protective effect of an activated HSR or of individual chaperones, we co-expressed PrP<sup>ΔHD</sup> alternatively with ΔHSF or Hsp72 or clusterin. Indeed, co-expression of either ΔHSF or Hsp72 protected cells against PrP<sup>ΔHD</sup>-induced toxicity (Fig. 6, B and C). In contrast, clusterin, which efficiently interfered with PrP<sup>Sc</sup>- or Aβ-induced cell death, could not prevent toxic effects mediated by PrP<sup>ΔHD</sup> (Fig. 6D). Importantly, ΔHSF or Hsp72 expression did not reduce PrP<sup>ΔHD</sup> protein levels, nor did expression of PrP<sup>ΔHD</sup> prevent secretion of clusterin.

## DISCUSSION

Regulation of the cellular stress response is critical to maintain cellular homeostasis and to protect cells from proteotoxicity. Our results indicate that pathogenic oligomers made from different proteins deregulate the HSR; in particular, they can modify the threshold for the stress-induced expression of heat shock proteins. Furthermore, we present evidence that the toxic effects of three different neurotoxic protein conformers (PrP<sup>Sc</sup>, Aβ, and PrP<sup>ΔHD</sup>) can be ameliorated by activating the HSR or by increasing the expression of individual chaperones.

*The HSR is Modulated by Different Pathogenic Protein Assemblies; Distinct Effects of Acute and Chronic Exposure—*PrP<sup>Sc</sup> and Aβ form pathogenic protein assemblies within the secretory/endosomal pathway and/or at the plasma membrane. Both protein species are released into the extracellular space where they can form amyloid plaques. To study how chronic exposure of neuronal cells to these aberrantly folded proteins might modulate the HSR, we made use of previously established cell lines generating neurotoxic PrP<sup>Sc</sup> or Aβ. ScN2a cells represent a well characterized cell culture model to study pathomechanistic pathways linked to prion diseases. Notably, proteinase K-resistant PrP<sup>Sc</sup> and infectious prions are released into the cell culture medium. Generation of Aβ is a physiological process; however, it was previously shown that Aβ secreted into the medium of CHO-7PA2 cells is neurotoxic, demonstrated by its ability to potently inhibit long term potentiation *in vivo* and to interfere with neuronal viability (48, 52, 53).

Based on the finding that the HSR response is significantly impaired in ScN2a cells (33, 34), we first compared the HSR of CHO to that of CHO-7PA2 cells by analyzing expression of Hsp72, the stress-inducible Hsp70 variant, after moderate,

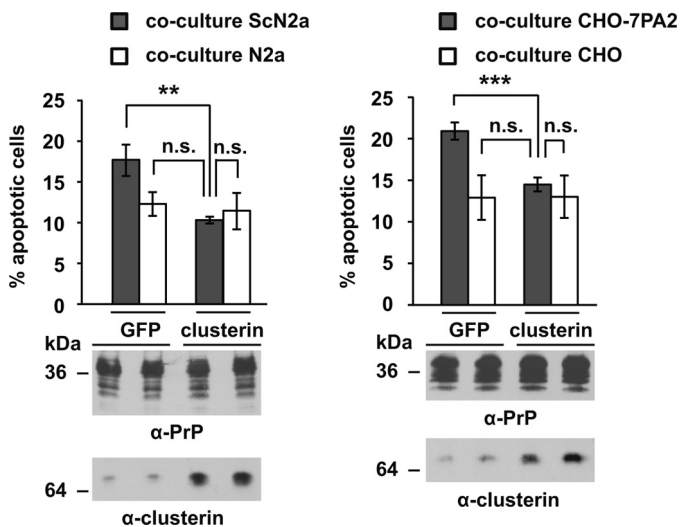


**FIGURE 4. Induction of the heat shock response or increased expression of Hsp72 protects against PrP<sup>Sc</sup>- and A $\beta$ -induced toxicity.** A, scrapie prions induce apoptosis in SH-SY5Y cells expressing PrP<sup>C</sup>. SH-SY5Y cells expressing the cellular prion protein (PrP<sup>C</sup>) were co-cultured with ScN2a or N2a cells in the presence or absence of the monoclonal anti-PrP antibody 3F4. B, A $\beta$  secreted by stably transfected cells is toxic to cells expressing PrP<sup>C</sup>. SH-SY5Y cells expressing PrP<sup>C</sup> were co-cultured with the indicated cell lines. C, expression of a constitutively active HSF1 mutant ( $\Delta$ HSF) protects against PrP<sup>Sc</sup>- and A $\beta$ -induced toxicity is shown. SH-SY5Y cells co-expressing PrP<sup>C</sup> and  $\Delta$ HSF were co-cultivated with the indicated cell lines. D, expression of a Hsp70 variant protects against PrP<sup>Sc</sup>- and A $\beta$ -induced toxicity. SH-SY5Y cells co-expressing PrP<sup>C</sup> and Hsp72 were co-cultivated with the indicated cell lines. In A–D, after 16 h of co-culture, apoptotic cell death in SH-SY5Y cells was determined as described under “Experimental Procedures”. Expression of PrP and Hsp72 were analyzed by Western blotting using the monoclonal anti-PrP antibody 3F4 or the monoclonal anti-Hsp72 antibody C92, respectively. n.s., not significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.005$ ; \*\*\*,  $p < 0.0005$ .

non-lethal heat shock conditions. In contrast to ScN2a cells, CHO-7PA2 cells are able to increase expression of Hsp72 in response to heat shock; however, their efficiency to mount a

heat shock response is reduced, which is most evident under mild heat shock conditions. To exclude the possibility that the observed effect is specific for CHO-7PA2 cells or the mutant



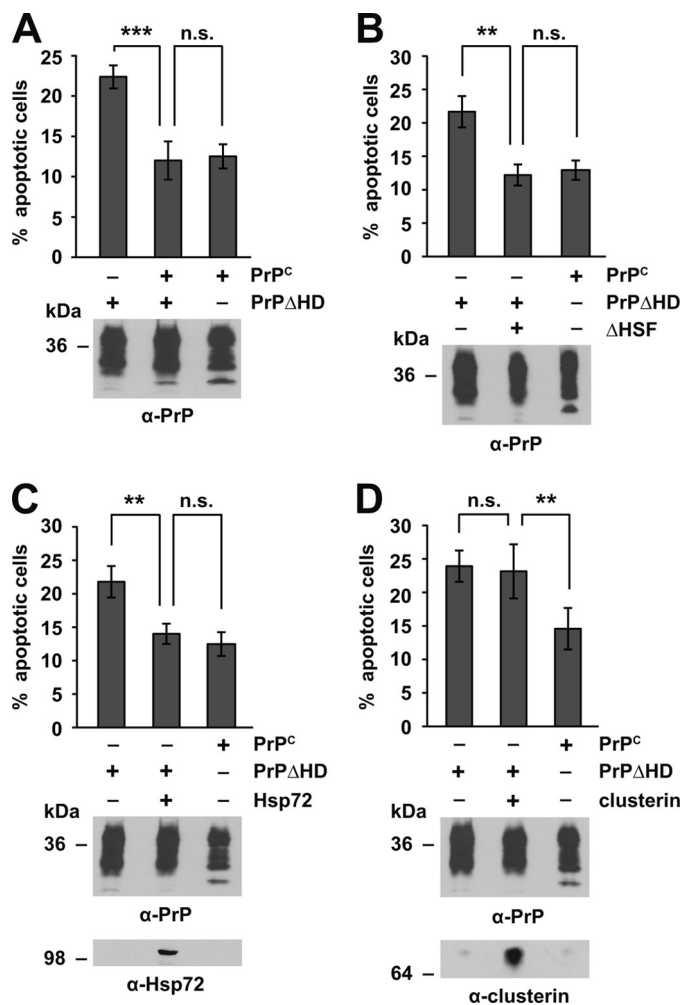


**FIGURE 5. Expression of the extracellular chaperone clusterin protects against PrP<sup>Sc</sup> and A $\beta$ -induced toxicity.** Expression of the extracellular chaperone clusterin prevents PrP<sup>Sc</sup>- and A $\beta$ -induced toxicity. SH-SY5Y cells co-expressing PrP<sup>Sc</sup> and clusterin were co-cultivated with the indicated cell lines. After 16 h of co-culture, apoptotic cell death in SH-SY5Y cells was determined as described under "Experimental Procedures". Expression of PrP was analyzed by Western blotting using the monoclonal anti-PrP antibody 3F4. Secretion of clusterin in conditioned media was determined by TCA precipitation followed by Western blotting using the monoclonal anti-clusterin antibody 41D. n.s., not significant; \*\*,  $p < 0.005$ ; \*\*\*,  $p < 0.0005$ .

human APP expressed in this line, we show an impaired HSR also in stably transfected SH-SY5Y cell lines overexpressing human wild type APP. Similarly to what we observed in ScN2a cells, forced expression of a constitutively active mutant of HSF1 ( $\Delta$ HSF) efficiently induced Hsp72 expression in CHO-7PA2 cells. These data agreed that the reduced levels of Hsp72 in CHO-7PA2 cells are not due to mutations in the promoter region of the Hsp72 gene but rather to a modulation of the activation/deactivation pathway of HSF1. Such a scenario is in line with our previous finding that the impaired HSR in ScN2a cells is caused by an accelerated deactivation of HSF1 after stress (34).

With the help of a co-culture model we were able to study acute effects of pathogenic protein conformers on the HSR. Exposure of SH-SY5Y cells to PrP<sup>Sc</sup> *per se* did not induce Hsp72 expression but increased Hsp72 expression in response to heat shock conditions. Mechanistically, it is conceivable that the acute exposure of cells to PrP<sup>Sc</sup> sensitizes the HSF1 activation pathway, thereby lowering the threshold for efficient Hsp72 expression in response to additional stress.

HSF activation/deactivation is regulated in the cytoplasmic and nuclear compartment at multiple steps via the interaction with chaperones and by different posttranslational modifications (for review, see Ref. 64). It is difficult to discriminate whether PrP<sup>Sc</sup> or A $\beta$  modulates any of these steps directly by interacting with any of the HSF1 modulators or indirectly via disruption of the proteostasis. Both PrP<sup>Sc</sup> and A $\beta$  have been found in the cytoplasmic compartment where they could interact with either HSF1 or chaperones implicated in HSF1 regulation. On the other hand, it has also been shown that accumulation of PrP<sup>Sc</sup> or A $\beta$  disrupts the proteostasis network. For example, cytosolic PrP<sup>Sc</sup> inhibits proteasomal activity (65), and



**FIGURE 6. Hsp72 and  $\Delta$ HSF but not clusterin protect against a neurotoxic PrP mutant.** A, expression of PrP<sup>Sc</sup> protects against PrP $\Delta$ HD-induced toxicity. B and C, Hsp72 or  $\Delta$ HSF interferes with PrP $\Delta$ HD-induced toxicity. D, the extracellular chaperone clusterin does not prevent toxic effects of PrP $\Delta$ HD. In A–D, apoptotic cell death in SH-SY5Y cells expressing the indicated proteins was determined as described under "Experimental Procedures". Expression of PrP and PrP $\Delta$ HD or Hsp72 was analyzed by Western blotting using the monoclonal anti-PrP antibody 3F4 or the monoclonal anti-Hsp72 antibody C92. The presence of clusterin in conditioned media was determined by TCA precipitation followed by Western blotting using the monoclonal anti-clusterin antibody 41D. n.s., not significant; \*\*,  $p < 0.005$ ; \*\*\*,  $p < 0.0005$ .

A $\beta$  interferes with mitochondria function (for review, see Ref. 66).

**Activation of the HSR or Expression of Cytosolic Hsp72 Protects against Toxic Effects of A $\beta$ , PrP<sup>Sc</sup>, and a Neurotoxic PrP Mutant**—The possibility to harness the stress response therapeutically have been demonstrated in various misfolding disease models previously (22, 26, 64, 67, 68). New in our study are the approaches to study cell ability to mount a HSR under conditions of acute and chronic exposure to PrP<sup>Sc</sup> and A $\beta$  and to analyze three different neurotoxic proteins under comparable experimental conditions. Moreover, we evaluated the protective effect of individual chaperones located in different cellular compartments. Although the exact mechanisms of how PrP<sup>Sc</sup>, A $\beta$ , or other pathogenic protein conformers interfere with neuronal function are largely unknown, there appear to be common features. In particular, there is increasing experimental evidence that different toxic protein assemblies are structurally



related and can activate similar cellular signaling pathways (6–10, 69, 70). Notably, it has been shown that the cellular prion protein can serve as a cell surface receptor to mediate toxic signaling of both PrP<sup>Sc</sup> and A $\beta$  (47, 48, 71–85). We cannot exclude the possibility that cytosolic chaperones directly interact with PrP<sup>Sc</sup> or A $\beta$ . For example, studies in yeast demonstrated that chaperones can interact with and modulate maintenance and propagation of prions (for review, see Refs. 86–91). Similarly, employing *Caenorhabditis elegans* and yeast as models of polyglutamine-induced toxicity, it was shown that cytosolic chaperones can ameliorate toxic effects of aberrantly folded protein conformers (92–96). However, it is also plausible that the protective activity of  $\Delta$ HSP and Hsp72 expression is based on a modulation of PrP<sup>Sc</sup>- and A $\beta$ -induced signaling pathways by cytosolic chaperones. A potential candidate for such an intracellular signaling molecule is the stress kinase JNK as Hsp72 can alleviate toxic effects of various stressors by suppression of JNK signaling (for review, see Ref. 97). In support of such a scenario are data showing that a JNK inhibitor suppressed toxic effects of PrP<sup>Sc</sup> (47). A different activity of Hsp72 was recently described in a mouse model of severe muscular dystrophy. This study indicated that Hsp72 can slow progression of disease by interacting with the sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) (98). In this context it is important to note that PrP<sup>C</sup> can restrict Ca<sup>2+</sup>-influx into the cell by limiting excessive N-methyl-D-aspartate (NMDA) receptor activity. Notably, this inhibitory activity of PrP<sup>C</sup> is lost upon interaction with A $\beta$  (82, 84, 99).

Interestingly, an activated HSR and increased Hsp72 expression also efficiently prevented toxic effects of the pathogenic PrP mutant PrP $\Delta$ HD. PrP $\Delta$ HD is located at the plasma membrane and does not form protein assemblies related to PrP<sup>Sc</sup> or A $\beta$ . Different models have been proposed to explain the toxic activity of PrP $\Delta$ HD, including the interaction with a yet unidentified receptor or a channel-forming activity of PrP $\Delta$ HD (for review, see Refs. 70 and 100). Irrespective of the exact mechanism, our results indicate that structurally unrelated pathogenic proteins can activate similar cellular pathways and that PrP $\Delta$ HD toxicity might be related to that of PrP<sup>Sc</sup> and A $\beta$ .

*An Extracellular Chaperone Interferes with PrP<sup>Sc</sup>- and A $\beta$ -induced Cell Death but Not with Neurotoxic Signaling of a PrP Mutant*—Our study on clusterin revealed interesting activities of this extracellular chaperone. Similarly to Hsp72, clusterin protected against PrP<sup>Sc</sup>- and A $\beta$ -induced toxicity; however, it could not interfere with toxic effects of PrP $\Delta$ HD expression.

A variety of activities has been reported for clusterin, including modulation of amyloid formation by interacting with prefibrillar structures (101), clearance of extracellular misfolded proteins (102), and sequestration of oligomeric forms of A $\beta$  (103). Thus, we suggest that despite a similar protective activity against PrP<sup>Sc</sup>- and A $\beta$ -induced toxicity, Hsp72 and clusterin exert different modes of action. Although Hsp72 seems to modulate intracellular pathways induced by PrP<sup>Sc</sup> or A $\beta$  (see above), clusterin obviously interferes with PrP<sup>Sc</sup>- and A $\beta$ -induced toxicity by a direct interaction with the toxic protein assemblies, most likely in the extracellular compartment. As a consequence, PrP<sup>Sc</sup> or A $\beta$  no longer interacts with PrP<sup>C</sup> at the plasma membrane, which in our cell culture model is the major cell

surface receptor of PrP<sup>Sc</sup>- or A $\beta$ -induced toxicity. The failure of clusterin to interfere with PrP $\Delta$ HD-induced toxicity indirectly supports such a mode of action, as PrP $\Delta$ HD-mediated toxicity seems not to be linked to the formation of  $\beta$ -sheet-rich protein assemblies (for review, see Refs. 70 and 100).

Our findings emphasize complex interrelations between the HSR and neurotoxic proteins. For example, toxic oligomers can both sensitize and desensitize the HSR in a time-dependent manner. As a consequence, it might be beneficial to interfere with the HSR at an early phase of the disease, whereas HSR stimulation is a possible strategy at later time points. Indeed, the protective effect of Hsp72 and clusterin supports the concept to use forced expression of individual chaperones or pharmacological induction of the HSR to delay progression of neurodegenerative disease. In addition, a combination of chaperones promises additive or synergistic effects as different chaperones can target distinct steps in neurotoxic signaling pathways.

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**The Heat Shock Response Is Modulated by and Interferes with Toxic Effects of  
Scrapie Prion Protein and Amyloid  $\beta$**

Ulrike K. Resenberger, Veronika Müller, Lisa M. Munter, Michael Baier, Gerd  
Multhaup, Mark R. Wilson, Konstanze F. Winklhofer and Jörg Tatzelt

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