Nuclear translocation and aggregate formation of heat shock cognate protein 70 (Hsc70) in oxidative stress and apoptosis

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SUMMARY

Recent evidence has shown a role for the heat shock cognate protein Hsc70 in the response to oxidative stress. We have investigated the subcellular distribution of Hsc70 by means of laser scanning confocal microscopy in neuroblastoma NB41A3 cells, in fibroblasts R6 cells and in R6-Bcl-2, an apoptosis-resistant cell line, and its function in oxidative stress and in apoptosis has been evaluated. Endogenous Hsc70 is localised predominantly in the cytoplasm in unstressed cells, whereas oxidative stress but not apoptosis induces its translocation into the nucleus. In transfected cells overexpressing Hsc70 increased nuclear translocation and aggregation of Hsc70 in intracellular speckles is observed after oxidative stress and, to a lesser degree, after exposure to apoptotic agents. Bcl-2 did not influence the movement of Hsc70 nor the formation of Hsc70-containing speckles. Nuclear translocation of Hsc70 can be modulated by the expression of components from a previously described plasma membrane oxidoreductase involved in the cellular response against oxidative stress. Our data may suggest a correlation between differential translocation of Hsc70 with specific functions in apoptosis and a potential role in the protection against reactive oxygen species.

Key words: Apoptosis, Confocal laser scanning microscopy, Heat shock protein 70, Nuclear import, Oxidative stress

INTRODUCTION

Heat shock protein Hsc70 and Hsp70 are molecular chaperones that participate in important cellular processes (Ellis, 1987; Gething and Sambrook, 1992; Hendrick and Hartl, 1993; Hartl, 1996; Hightower, 1980; Pelham, 1986; Rothman, 1989). Hsc70 plays an important role under non-stress conditions in stabilising the folding of newly synthesised polypeptides in the ER (Gething and Sambrook, 1992; Haas and Wabl, 1983) and for uptake into the mitochondrion and the ER (Chirico et al., 1988; Deshaies et al., 1988; Zimmermann et al., 1988). It interacts with nascent polypeptide chains in the cytosol by binding hydrophobic peptide segments in an ATP-dependent manner (Beckmann et al., 1990; Flynn et al., 1991). Evidence was given recently for a role of Hsc70 in the response to oxidative stress, anti-oxidants preventing gene expression in the focal regions (Turner et al., 1999). Also hypoxia induces Hsp/Hsc70 production by increased superoxide generation. Oxygen radicals and posthypoxic injury leads to the synthesis of Hsp’s (Gebhardt et al., 1999) and inhibitors of superoxide dismutase such as diethyldithiocarbamate induces whereas superoxide dismutase represses Hsp/Hsc70 synthesis. Hsc70 also modulates heme-regulated eIF-2α kinase (HRI) in hemin-supplemented rabbit reticulocyte lysate (RRL) in response to heat and oxidative stress (Thulasiraman et al., 1998) and Hsc70 may modulate the activation of HRI not only in heme-deficient RRL, but also in hemin-supplemented RRL in response to oxidative stress.

During embryonic neurulation Hsc70 levels depend on endogenous (pro)insulin which also prevents apoptosis. Apoptosis affects mainly cells with the lowest level of Hsc70, suggesting a role for Hsc70 in the control of apoptosis in embryogenesis (de la Rosa et al., 1998). Several studies have further shown Hsp70 and Hsc70 binding to the anti-apoptotic protein BAG-1 (Bimston et al., 1998; Hohfeld, 1998; Hohfeld and Jentsch, 1997; Stuart et al., 1998; Takayama et al., 1997, 1998, 1999). Overexpression of BAG-1 protected from heat shock-induced cell death whereas overexpression of Bcl-2 causes intracellular redistribution of BAG-1. Mechanistic basis for BAG-1 as a negative regulator of Hsc70/Hsp70 chaperones was given by Bimston et al. (1998). Three isoforms of human BAG-1 (BAG-1, BAG-1M, and BAG-1L) exist which all retain the ability to bind Hsc70 (Takayama et al., 1998). Overexpression of BAG-1 protected from heat shock-induced cell death whereas overexpression of Bcl-2 causes intracellular redistribution of BAG-1. Three isoforms of human BAG-1 (BAG-1, BAG-1M, and BAG-1L) exist which all retain the ability to bind Hsc70 (Takayama et al., 1998). This reveals an unexpected diversity in the regulation of Hsc70 and raises the possibility that the anti-apoptotic function of BAG-1 may be exerted through a modulation of the chaperone activity of Hsc70 on specific protein folding and maturation pathways.

In a previous study we have isolated a neuronal transplasmamembrane oxidoreductase (PMO) complex involved in the cellular response against oxidative stress. One major components of the complex was identified as Hsc70 (Bulliard et al., 1997). We therefore aimed in this study to define the role of Hsc70 in oxidative stress and in apoptosis with respect to the other components of the PMO complex, in both neuronal and non-neuronal cells. To clarify these we investigated the cellular distribution of Hsc70 by means of confocal laser
scanned by scanning microscopy and immunocytochemistry. We studied the effects of transient and stable overexpression of Hsc70-GFP in different cell types, either central nervous system (CNS)-derived cells (mouse neuroblastoma NB41A3), non-CNS derived cells (R6 fibroblast) or an apoptosis-resistant Bcl-2 expressing R6 cell line (R6-Bcl-2).

MATERIALS AND METHODS

Cell culture

Neuroblastoma cells NB41A3 were obtained from ATCC (Bethesda, MD) and maintained as monolayers on tissue culture plastic flasks at 37°C in 5% CO2 as previously described (Augusti-Tocco and Sato, 1969). The growth medium was Dulbecco’s modification of Eagle’s medium (DMEM) at pH 7.4 containing 3.7 g/l NaHCO3, 4.5 g/l D-glucose, 0.11 g/l sodium pyruvate and 0.58 g/l glutamine, 10% foetal calf serum, 50 units/ml of penicillin and 50 µg/l of streptomycin. The rat 6 embryo fibroblast cell line (R6) and a derivative stably expressing R6 cell line (R6-Bcl-2) were kindly provided by Dr C. Borner, Institute of Biochemistry, Fribourg, Switzerland (Borner, 1996). They were grown under the same conditions as the neuroblastoma cells, except that the cell culture medium contained 5% instead of 10% foetal calf serum. For all cell lines the medium was changed every 3 days. Starving of the cultures and growth to post confluence were strictly avoided. The cells were trypsinised in the presence of 0.25% trypsin and 0.1% EDTA for splitting or harvested for RNA or protein extracts when they were subconfluent.

Construction of plasmids

The complete coding region of Hsc70 was amplified by PCR with the Advantage Klentaq polymerase mixture (CLONTECH) from mouse cDNA (mouse brain Marathon cDNA, CLONTECH) with 5’-AAA ACC CGG GGG TAC CAC CTC CA T CGA CCT CTT CAA TAG as upstream primer. The downstream primer was designed such as to replace the stop codon by nucleotides coding for three glycine residues. Additionally, two artificial restriction sites KpnI and Xmal were added to facilitate ligation (Tomassini et al., 1978). Frameshifted clones were produced either by using KpnI which produces a 4-base 3’ extension which is subsequently digested in a T4 DNA polymerase reaction or by using Asp718I (= Acc65I) that produces a 4-base 5’ extension which serves as a template to add the missing nucleotides by Klenow fragment. Blunt end re-ligation with the KpnI cut fragment(s) produces a +1 nucleotide frameshift, whereas the Asp718I cut fragment(s) produces a +1 nucleotide frameshift. The amplified products were subcloned into the pKS+ Bluescript (Stratagene, La Jolla, CA, USA) and the sequence confirmed by sequencing. Subsequently, the amplified DNA sequence was sequenced. Subsequently, the amplified DNA sequence was cloned in-frame with the sequence coding for green fluorescent protein (GFP) into expression vectors pEGFP-N1 or pEFPBFP-N1 (both Clontech, respectively).

Control plasmids that do not produce GFP, but only the Hsc70 protein, were prepared by disrupting the GFP sequence by a frameshift mutation at the artificial KpnI/Asp718 restriction site between the 3’-end of the Hsc70 gene and the start codon of GFP. Correct expression of these constructs was confirmed by immunofluorescence assays in transfected cells. All plasmids were purified on QIAGEN Midi columns.

Transfection

Cells grown on 12 mm glass coverslips in 6-well plates were transfected at 80-90% confluence using SuperFect (Qiagen). 1 µg of plasmid DNA suspended in 60 µl fresh DMEM without serum and 5 µl SuperFect were mixed, vortexed and incubated for 10 minutes. 600 µl of complete growth medium was then added and immediately transferred to PBS-washed cells. After 3-4 hours of incubation at 37°C/5% CO2, cells were washed once with PBS.

Induction of oxidative stress and apoptosis

Immediately after transient transfection, cells were trypsinised, split 1:3 and plated on glass coverslips placed in 6-well plates. After 24 hours the growth medium was replaced by new medium (control) or new medium containing either 300 µM FeCN, 100 µM H2O2, 1.5 µM MG132 or 200 nM staurosporine. Cells were exposed for 3 hours (to 300 µM FeCN or 100 µM H2O2) and for 6 hours or 24 hours (to 200 nM staurosporine or 1.5 µM MG132) and analysed under the microscope with and without prior fixation. The percentage of dead cells correlated logarithmically with increasing concentrations of H2O2 and FeCN.

Immunocytochemistry

Mouse anti-Hsc70 monoclonal antibody (Mab) (IgG) (Affinity BioReagents) and rabbit anti-GFP polyclonal antibody (IgG) (Clontech Laboratories, Inc., Palo Alto, CA, USA) were used as primary antibodies. As secondary antibodies Texas Red dye-conjugated goat anti-mouse IgG, Texas Red dye-conjugated goat anti-rabbit IgG, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rabbit IgG (all Jackson Immunoresearch Laboratories, Inc., Bar Harbor, ME, USA) were used for immunocytochemistry. When native GFP fluorescence was used to localise the GFP fusion proteins, the coverslip containing the adherent cells was directly placed on top of a drop of growth medium without Phenol Red on a microscope slide and immediately analysed. Otherwise cells were washed twice in PBS and fixed for 15 minutes in 4% p-formaldehyde in 0.1 M PIPES (pH 6.8) containing 0.1% Hoescht 33342. Cells were then rinsed twice in PBS, permeabilised for 5 minutes with 0.05% saponin in PIPES, again rinsed twice in PBS-EDTA and incubated for 10 minutes at ~20°C in acetone. After 2 washes with PBS, cells on the coverslip were incubated in 1% BSA in PBS for 2 hours with the first antibody, rinsed twice in PBS and further incubated for 2 hours with the fluorescent secondary anti-IgG before two washes in PBS. Fluorescence was extended by using SlowFade (Molecular Probes, Leiden, Netherlands). All incubations were performed in a light protected environment and, except for acetone, at room temperature.

Fluorescence microscopy

Cells were examined with a Nikon Eclipse E800 microscope under a ×100 oil immersion objective (Nikon Inc.) using a MRC-1024 Bio-Rad laser confocal microscope system equipped with an Krypton/Argon laser (Bio-Rad Labs, Hercules, CA, USA) and the LaserSharp acquisition software (Bio-Rad). Serial sections (at 0.5-1.5 µm intervals) in the z-axis of cells were collected in the slow scanning mode (approx. 160 Hz) with 1.5-4.5 mm diam. iris aperture by averaging pictures by Kalman (3 scan). Green fluorescence (GFP or FITC) and Texas Red were detected in parallel at 512×512 pixels. The pictures were processed with the LaserSharp processing software (Bio-Rad) or alternatively with ConfocalAssistant before picture files were transferred to Adobe Photoshop 4.0 and printed on a Kodak printer.

To visualise DNA staining with Hoescht 33342, a ×100 oil immersion Plan-Neofluar objective (Zeiss) with an inverted Zeiss Axiosvert 135 TV microscope equipped with a HBO 100-XBO 75 lamp was used.

Quantification

The serial sections through the z-axis of the cell were collected within a linear range of fluorescence intensity, superimposed to a 3-D stack and analysed as a 2-D projection. To compare GFP expression in different subcellular structures, the mean pixel intensity in a selected area was determined and normalised by subtracting the background. In short, all sections collected in the z-axis of the appropriate picture
by LSCM are stacked to a two-dimensional projection in which the intensities of the various sections are accumulated. A histogram is then prepared from the area under investigation in the 2-D-projection, evaluating the mean pixel intensity and the distribution of pixel intensities versus the standard deviation. A high mean pixel intensity correlates to high amount of expressed protein, whereas a high standard deviation indicates a patchy, irregular expression in the specific area. The final average value for protein expression levels was obtained by analysing each time at least 15 different cells of three independent experiments.

RESULTS

Subcellular translocation of endogenous Hsc70 from the cytoplasm into the nucleus after induction of oxidative stress

We determined the subcellular localisation of endogenous HSC70 in CNS-derived cells (neuroblastoma mNB41A3), in non-CNS derived cells (R6 fibroblast) and in an apoptosis-resistant Bcl-2 expressing cell line (R6-Bcl-2), using confocal laser scanning microscopy. Endogenous and transfected Hsc70 (or Hsc70-GFP) was monitored by means of immunocytochemistry using several antibodies, mainly clones 5A5 and 2A4 of Affinity Bioreagents, Inc (Milan TM), to exclude the possibility that the observed changes could be underestimated because of protein-protein interactions. The clone 5A5 recognises amino acids (AA) 122-264, which correspond to a sequence within the ATP binding domain of Hsc70, whereas clone 2A4 recognises AA 437-479, which are within the substrate-binding domain. Always both antibodies were used in separate experiments and yielded similar quantitative data, so the use of any antibody does actually not affect our data.

In unstressed NB41A3 cells about 70% of endogenous Hsc70 is expressed in the cytoplasm as shown by means of immunocytochemistry with anti-Hsc70 antibodies (Figs 1A,C, 2A). The ratio of the measured Texas Red fluorescence between cytoplasm and nucleus is constant in all NB41A3 cells. (H,P) Induction of apoptosis: Apoptotic cells display neither overexpression nor nuclear accumulation of endogenous Hsc70 (H,I) in NB41A3 cells after exposure to 200 nM staurosporine for 24 hours and in (J,K) in R6 cells or in (L) R6-Bcl-2 cells after exposure to 1.5 μM MG132 for 6 hours. The non-apoptotic cells express Hsc70 predominantly in the cytoplasm. (M, N) R6 cells form long processes after 6 hours induction of apoptosis by MG132. Most of them are not apoptotic. The arrow points to the only cell showing chromatin condensation. (O, P) Non-apoptotic R6-Bcl-2 cell with intact nucleus (blue) showing some nuclear translocation after exposure to 1.5 μM MG132 for 6 hours. (C,H,I,L,M,O) Visualisation of DNA by Hoechst blue staining. Apoptotic cells are marked with an arrow. (A,C,D,E,F,G,I,K,L,N,O) Detection of Hsc70 by TR staining of anti-Hsc70 Ab. (B,C) Visualisation of transfected Hsc70 by GFP tag. Bar, 50 μM.
In resting R6 fibroblasts endogenous Hsc70 is also predominantly in the cytoplasm and bound to the cytoskeleton (Fig. 1D). Its highest concentration is seen in the perinuclear area and at the plasma membrane. Cytoplasmic Hsc70 levels are higher in R6 cells and R6-Bcl-2 cells than in NB41A3 cells (Fig. 2A). However, 20% R6 cells are very flat and show a volcano-like Hsc70 distribution (Fig. 2A). Nuclear fragmentation was observed in 20% of R6-Bcl-2 cells after 24 hours of exposure. Therefore, staurosporine and MG132 were used at these concentrations to induce apoptosis.

After 6 hours exposure of NB41A3 to either staurosporine or MG132, endogenous Hsc70 displays higher levels of expression in the nucleus, but the effect is much less pronounced than after oxidative stress. Even after 24 hours only small changes in subcellular localisation of endogenous Hsc70 were observed in non-apoptotic NB41A3 cells (Figs 1H,I, 2A). Nuclear fragmentation is observed in 20% of NB41A3 cells (Figs 1H, 3A), but these apoptotic cells do not accumulate or overexpress endogenous Hsc70 (Fig. 1I).

After exposure to staurosporine or MG132 most R6 cells have an unchanged morphology and display endogenous Hsc70 predominantly in the cytoplasm (Fig. 2A). However, a subset of R6 cells typically start to form long processes extending from their rounded-up cell body leading to a net-like appearance (Fig. 1N). Endogenous Hsc70 is displayed in the cytoplasm, including within the processes. 20% of these net-like cells concentrate endogenous Hsc70 in specific nuclear regions and are apoptotic (Fig. 1M). Other apoptotic R6 cells are mostly round and display endogenous Hsc70 in the cytoplasm or all over the cell, at levels close to endogenous Hsc70 expression in non-apoptotic control R6 cells (Fig. 1J,K). The overall percentage of R6 cells with fragmented nuclei after exposure to staurosporine or MG132 increases from 10% after 6 hours up to 60% after 24 hours (Fig. 3A).

Bcl-2 protects cells from the induction of apoptosis by MG132 or staurosporine (Fig. 3A). After 6 hours exposure to staurosporine, about 95% of the R6-Bcl-2 cells did not display nuclear fragmentation. Different patterns of Hsc70 localisation were observed: either (a) an unchanged cytoplasmic localisation in most cells with unchanged morphology, or (b) expression all over the cell (including the nucleus) within a network of cells with long processes that display endogenous Hsc70, or sometimes (c) localisation in a perinuclear ring and in the nucleus within flat volcano-like cells (Fig. 1O,P). After 24 hours of staurosporine treatment most R6-Bcl-2 cells had small rounded-up cell bodies and formed long processes (data not shown). After incubation with MG132 for 24 hours, in contrast, endogenous Hsc70 was predominantly in the cytoplasm or in the blebs of the cells, of which the majority did not have any processes at all (data not shown). After

![Subcellular expression of endogenous Hsc70 in untransfected cells](image1)

![Subcellular expression of Hsc70-GFP in transfected cells](image2)

**Fig. 2.** Subcellular expression of Hsc70 in untransfected and in Hsc70-GFP transfected cells. (A) Untransfected and (B) transiently transfected cells were analysed under unstressed conditions, after exposure to 100 μM H2O2 for 3 hours or after exposure to 200 nM staurosporine (STS) for 24 hours. The ratio between cytoplasmic and nuclear Hsc70 (or Hsc70-GFP) expression is indicated. Ratios above 1.0 display predominantly cytoplasmic expression. Nuclear translocation of Hsc70 is more pronounced after oxidative stress than after induction of apoptosis. Each experiment consisted of analysing 15 cells and was repeated three times. Thus, each data point indicates the average of 45 individual measurements.
induction of apoptosis the morphology of R6-Bcl-2 and R6 cells was similar, in both apoptotic and non-apoptotic cells (Fig. 1L).

**GFP-tag does not influence subcellular localisation of transfected HSC70-GFP**

Overexpression of Hsc70 was conducted by means of transfecting either Hsc70 or the Hsc70-GFP hybrid. Under our experimental conditions transfection efficiency is about 15% in all cell lines. Under resting conditions transfectected R6 and NB41A3 cells expressed in the average about 2.5-3 fold more Hsc70 than untransfected cells (Table 1).

**Table 1. Relative mean pixel intensity of total Hsc70 expression in NB41A3 cells**

<table>
<thead>
<tr>
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<th>Relative mean pixel intensity (%)</th>
<th>R6</th>
<th>NB41A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous</td>
<td>Non-apoptotic cells</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Hsc70</td>
<td>Apoptotic cells</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Transfected</td>
<td>Non-apoptotic cells</td>
<td>300</td>
<td>250</td>
</tr>
<tr>
<td>Hsc70-GFP</td>
<td>Apoptotic cells</td>
<td>300</td>
<td>250</td>
</tr>
</tbody>
</table>

*The relative mean pixel intensity was obtained by measuring total Hsc70 expression and mean pixel intensity in several apoptotic and non-apoptotic cells by means of histograms. The mean intensity of endogenous Hsc70 in non-apoptotic cells was defined as 100%. Each experiment consisted of analysing 15 cells and was repeated three times. Thus, each data point indicates the average of 45 individual measurements. s.d. is smaller than 5% in all tested conditions.

In order to test whether the subcellular localisation or expression level of proteins is unchanged after cell fixation and immunostaining we analysed the green fluorescence in living cells transfected by Hsc70-GFP and compared the results with immunofluorescent analysis where Hsc70-GFP was indirectly

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**Fig. 3.** Apoptosis and formation of intracellular Hsc70-positive speckles in untransfected and in Hsc70-GFP transfected cells. (A, B) Apoptosis in (A) untransfected and (B) transfected cells after exposure to 200 nM staurosporine (STS) or to 1.5 μM MG132. The percentage of apoptotic cells showing nuclear fragmentation or chromatin condensation as detected by Hoechst blue staining in cells expressing endogenous Hsc70 or transfected Hsc70-GFP is indicated after 0, 6 and 24 hours of exposure time. Bcl-2 overexpressing R6 cells are resistant to apoptosis induced by MG132 or staurosporine. Each data point is an average of three individual analyses of different populations, each containing at least 50 cells. (C) Percentage of transfected cells exposed for different time (from 0-6 hours) to 100 μM H2O2 or to 300 μM FeCN forming Hsc70-GFP positive speckles. Additionally, a set of cells (24+3) was first stressed for 6 hours, incubated further for 18 hours in normal growth medium and then stressed again for 3 hours. The increase in formation of speckles correlates with longer exposure times to stressing agents. Each data point is an average of three individual analyses of different populations, each containing at least 50 cells.

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**Fig. 4.** RGB-images of R6, R6-Bcl-2, and NB41A3 cells stained with Hoechst blue. (A) Representative single optical sections of expressing endogenous Hsc70 in untransfected cells. (B) Representative single optical sections of Hsc70-GFP transfected cells. (C) Representative single optical sections of transfected cells expressing Hsc70-GFP positive speckles. (D) Representative single optical sections of transfected cells expressing Hsc70-GFP positive speckles. (E) Representative single optical sections of transfected cells expressing Hsc70-GFP positive speckles. (F) Representative single optical sections of transfected cells expressing Hsc70-GFP positive speckles. (G) Representative single optical sections of transfected cells expressing Hsc70-GFP positive speckles. (H) Representative single optical sections of transfected cells expressing Hsc70-GFP positive speckles. (I) Representative single optical sections of transfected cells expressing Hsc70-GFP positive speckles. (J) Representative single optical sections of transfected cells expressing Hsc70-GFP positive speckles.

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**Table 2.** Percentage of transfected cells expressing Hsc70-GFP positive speckles. Additionally, a set of cells (24+3) was first stressed for 6 hours, incubated further for 18 hours in normal growth medium and then stressed again for 3 hours. The increase in formation of speckles correlates with longer exposure times to stressing agents. Each data point is an average of three individual analyses of different populations, each containing at least 50 cells.
detected by anti-GFP antibodies in fixed cells. Our experiments
demonstrated neither qualitative nor quantitative differences
between fixed and unfixed cells.

The effect of the GFP-tag on protein localisation was tested
by transfection with either the Hsc70-GFP or the Hsc70
(without tag) expression vector. In both cell lines the
subcellular distribution of transfected Hsc70 (red) (Fig. 4C,D)
was similar to that of transfected Hsc70-GFP (green) (Fig.
4E,F) irrespective of whether cells were unstressed, or exposed
to either H2O2, FeCN, staurosporine or MG132 (data not
shown). Also the number of cells undergoing apoptosis in
response to staurosporine or MG132 were comparable. These
observations indicate that the GFP tag does not influence the
expression and localisation of Hsc70.
Increased nuclear translocation and accumulation of Hsc70-GFP in intracellular speckles after induction of oxidative stress is not prevented by Bcl-2

After transient transfection with Hsc70-GFP NB41A3 cells contained in the average about 65% GFP fluorescence in the cytoplasm, 1.7 times more than in the nucleus (Figs 4E, 2B). This ratio was constant in all cells and similar to ratios in untransfected cells (Fig. 1B,C). Hsc70 was displayed at different intensities in the cytoplasm in a patchy pattern. The nucleolus was devoid of Hsc70-GFP and signs of apoptosis were not detected (Figs 1C, 3B). The distribution pattern of Hsc70-GFP was very similar among all unstressed NB41A3 cells and did not depend on the shape of the cells. In R6 and R6-Bcl-2 cells most transiently transfected Hsc70-GFP is localised in the perinuclear area of the cytoplasm, but their Hsc70-GFP levels in the nucleus are higher than those of untransfected fibroblasts (Fig. 4G). No change in shape or cell viability was observed compared to untransfected R6 and R6-Bcl-2 cells.

After exposure to 100 μM H2O2 slightly over 50% of Hsc70-GFP was localised in the nucleus of stressed NB41A3 cells (Figs 4J, 2B). This stress resulted in formation of speckles accumulating Hsc70-GFP in 25% of NB41A3 cells. The remaining NB41A3 cells were mostly of round shape, had short processes and their nuclear Hsc70-GFP level was increased. A correlation between the exposure time to 100 μM H2O2 and the amount of speckles formation was observed (Fig. 3C). Between 15 and 35% of NB41A3 cells accumulated Hsc70-GFP fluorescence in speckles depending upon the incubation time. Besides NB41A3 cells expressing Hsc70-GFP in speckles only, neither in the nucleus nor in the cytoplasm, we also observed cells at intermediate stages, where speckle formation is observed in particular regions of the cell (Fig. 4K). With both stress agents, H2O2 and FeCN, the ratio of nuclear translocation increases in parallel with exposure times.

Oxidative stress induced by 300 μM FeCN for 4 hours raised the level of Hsc70-GFP in the nucleus in most cells (80%; data not shown). As a consequence the Hsc70-GFP level was about equal in the nucleus and in the cytoplasm (Fig. 4H). Nuclear Hsc70-GFP fluorescence was strongest in patches around the nucleolus (which is devoid of Hsc70-GFP) and weaker in other parts of the nucleus. Formation of Hsc70-GFP positive speckled spots were sometimes observed (5% of NB41A3 cells; Fig. 4I). The percentage of such cells expressing Hsc70-GFP in small speckles in response to 300 μM FeCN was time and dose dependent and increased up to 15% after 6 hours (Fig. 3C).

The response of R6 and R6-Bcl-2 cells to induction of oxidative stress by either H2O2 or FeCN was very similar. In both cases increased nuclear localisation of Hsc70-GFP was observed (Figs 4L, 2B), but it was less pronounced than in transfected NB41A3 cells. R6-Bcl-2 did not influence the cellular response to oxidative stress, in particular it did not inhibit translocation of Hsc70-GFP into the nucleus.

Hsc70-GFP overexpression does not inhibit apoptosis but protects from cell body shrinkage

After 6 hours exposure to staurosporine or MG132 the majority of NB41A3 cells had not changed their morphology and displayed Hsc70-GFP at 1.7 times higher levels in the cytoplasm than in the nucleus. After 24 hours, however, in contrast to untransfected cells, this ratio decreases up to 1.3 (Fig. 2B), because of nuclear translocation of Hsc70-GFP in 30% of non-apoptotic NB41A3 cells (Fig. 4M). The percentage of apoptotic NB41A3 cells was comparable to untransfected cells, i.e. 2-5% after 6 hours, 20% after 24 hours exposure to staurosporine or MG132 (Fig. 3B). Apoptotic NB41A3 cells did not significantly change their morphology and the expression of Hsc70-GFP was identical to the one in non-apoptotic cells.

In contrast to untransfected R6 cells, Hsc70-GFP transfected R6 cells exposed to MG132 or staurosporine displayed only rarely long processes branching off from rounded-up cell bodies were observed (Table 2), indicating a protective role of Hsc70-GFP overexpression against these effects in response to induction of apoptosis. The number of apoptotic R6 cells after 6 hours and after 24 hours exposure was similar to untransfected R6 cells (Fig. 3B). Localisation and expression level of Hsc70-GFP were not increased and similar to non-apoptotic R6 cells. In contrast most R6-Bcl-2 cells transfected with Hsc70-GFP and exposed to staurosporine for 6 hours had no sign of apoptosis (Fig. 3B) and displayed Hsc70-GFP along intracellular fibres within the entire cell (Fig. 4N,O). Also 24 hours after induction of apoptosis with staurosporine the cell viability of Hsc70-GFP transfected R6-Bcl-2 cells was about 80%, not significantly altered compared to untransfected R6-Bcl-2 cells, but significantly higher than in Hsc70-GFP transfected R6 cells (Fig. 3B). 24 hours incubation with either MG132 or staurosporine yielded a comparable percentage of nuclear fragmentation, but the number of R6-Bcl-2 cells with altered shape was much higher with MG132. Most cells were devoid of processes and the cell body had shrunk and was rounded up.

Table 2. Hsc70-GFP overexpression partially prevents formation of shrunk cell bodies with long processes after exposure to MG132

<table>
<thead>
<tr>
<th></th>
<th>Shrunken cell bodies with long processes (%)</th>
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<tbody>
<tr>
<td>Untransfected R6</td>
<td>30</td>
</tr>
<tr>
<td>Hsc70-GFP transfected R6</td>
<td>15</td>
</tr>
<tr>
<td>Untransfected R6-Bcl-2</td>
<td>20</td>
</tr>
<tr>
<td>Hsc70-GFP transfected R6-Bcl-2</td>
<td>10</td>
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</table>

Percentage of cells with shrunk cell body and long processes after exposure to 1.5 μM MG132 for 24 hours decreased in both R6 and R6-Bcl-2 cells when Hsc70-GFP was overexpressed. Each data point is an average of three individual analyses of different populations, each containing at least 50 cells. s.d. is smaller than 5% in all tested conditions.

Overexpression of other proteins of the neuronal PMO complex can modulate the subcellular localisation of Hsc70-GFP

Because Hsp70 is a component of the neuronal PMO complex, we investigated whether its nuclear translocation is affected by other components of the complex, e.g. glyceraldehyde-3-phosphate dehydrogenase (GAPDH), aldolase C, enolase-γ and Ulip-2. These were prepared as blue fluorescent protein (BFP) fusion proteins into expression vectors and co-transfected with Hsc70-GFP vectors.

When Hsc70-GFP was transfected together with enolase-
Table 3. Influence of co-transfection of components from the neuronal PMO complex on the subcellular localization of Hsc70-GFP

<table>
<thead>
<tr>
<th>Protein co-expressed with Hsc70-GFP</th>
<th>Cells accumulating Hsc70-GFP in speckles*</th>
<th>Nuclear Hsc70-GFP*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control H2O2</td>
<td>H2O2</td>
</tr>
<tr>
<td>Hsc70-GFP only</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Enolase-BFP</td>
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<tr>
<td>Aldolase-BFP</td>
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<td>20</td>
</tr>
<tr>
<td>Ulip-BFP</td>
<td>0</td>
<td>20</td>
</tr>
</tbody>
</table>

*The percentage of cells accumulating Hsc70-GFP in intracellular speckles and the percentage of Hsc70-GFP expressed in the nucleus is indicated.

BFP in neuroblastoma cells, no difference in Hsc70-GFP distribution was observed compared to cells transfected with Hsc70-GFP alone. However, after oxidative stress with 100 μM H2O2 the percentage of cells forming intracellular Hsc70-GFP-specules augmented from 35% to 50%. This effect was not observed with 300 μM FeCN. Nuclear translocation of Hsc70-GFP was 10% higher under both conditions (Table 3).

Hsc70-GFP localisation is not influenced by GAPDH-BFP co-overexpression in resting cells. But after exposure to either 100 μM H2O2 or 300 μM FeCN elevated nuclear Hsc70 localisation was observed, i.e. 40% more in double-transfected cells (85%) than in cells overexpressing Hsc70-GFP alone (Table 3). On the other hand, concomitant overexpression of Hsc70 with GAPDH increased the cytoplasmic level of GAPDH in both stressed and unstressed cells (unpublished data). These observations indicate that overexpression of Hsc70 partially prevents nuclear translocation of GAPDH, which has been reported to induce apoptosis.

Aldolase-BFP or Ulip-BFP co-transfected with Hsc70-GFP had no influence on Hsc70-GFP localisation neither before nor after oxidative stress (Table 3).

These observations show that co-expression of two components of the neuronal PMO complex (GAPDH and enolase-γ) can modulate the expression of Hsc70-GFP. Vice versa, overexpression of Hsc70 also has an impact on the subcellular localisation of components of the PMO complex.

DISCUSSION

Translocation of endogenous Hsc70 into the nucleus is pronounced during oxidative stress but not during apoptosis

Nuclear translocation of endogenous Hsc70 is only observed during oxidative stress and much less during apoptosis. In all conditions Hsc70-GFP behaved like the untagged Hsc70 protein, as detected by immunofluorescence, enabling us to follow the translocation of Hsc70 by monitoring the fluorescence of GFP. Apoptotic cells did not display increased levels of either endogenous Hsc70 or overexpressed Hsc70-GFP. Under similar experimental conditions, in contrast, GAPDH shows nuclear translocation under oxidative stress and during apoptosis and is increased in apoptotic cells (unpublished data). In control experiments with enolase-γ-GFP and GFP alone no changes in the subcellular localisation of these proteins was observed under the same conditions, clearly indicating that nuclear translocation after oxidative stress and apoptosis is a specific phenomenon for Hsc70. It is possible that the nuclear translocation mechanisms of Hsc70 for reactive oxygen species (ROS)-induced oxidative stress and for MG132 or staurosporine-induced apoptosis are different. Hsc70 functions as a molecular chaperone in the correct refolding of proteins. When oxidative stress is induced, nuclear proteins become damaged by oxygen radicals and Hsc70 may probably be imported into the nucleus in order to catalyze refolding them into a suitable conformation.

The shuttling of Hsc70 between the nucleus and the cytoplasm observed in this report confirms previous observations (Lamian et al., 1996). These studies established that the nuclear import of purified Hsc70 is not inhibited by a nuclear localisation signal peptide conjugated with BSA. A basic domain (246KKHKDISENKRAVRR262) of Hsc70 acts as a nuclear localisation signal and promotes nuclear import. However, inactivation of this signal by deletion of the first six amino acids has no effect on Hsc70 import, so Hsc70 could use a different import signal and enter the nucleus by a mechanism different from classical nuclear localisation signals (Lamian et al., 1996). Nuclear translocation of Hsc70, but not of Hsp70, was observed during early S phase of the cell cycle, indicating a role for Hsc70 in the process of S phase entry (Zeise et al., 1998). From our data nuclear translocation of Hsc70 plays also a role in oxidative stress.

It has been shown that stress factors result in increased hsp70 expression in human monocytes (Mariethoz et al., 1997), but the subcellular distribution pattern is different depending on the type of stress. Heat-shock induces a rapid translocation of hsp70 into the nucleus, but not phorbol ester nor erythropagocytosis, consistent with our findings showing a different type of subcellular localisation after oxidative stress or apoptosis. This differential expression probably relates to distinct regulation and functions of Hsc70 in apoptosis or oxidative stress.

Programmed cell death involves a shut down of protein expression and protein breakdown. To show that the observed changes in the ratio between cytosolic and nuclear Hsc70 is not influenced by this fact we performed controls with enolase-GFP and GFP alone and did not observe changes in such a ratio, indicating that nuclear translocation is specific for Hsc70.

Overexpression of Hsc70 renders cells more resistant to oxidative stress

Untransfected cells exposed to oxidative stress displayed often a shrivelled cell body and long processes, an effect that was rarely observed in Hsc70-GFP-transfected cells. Probably overexpression of Hsc70-GFP protects cells from damages induced by oxidative stress, and the formation of long processes might result from decreasing attachment of stressed cells to the growth surface. Despite the fact that Bcl-2 efficiently protects cells from apoptosis, Bcl-2 does not influence nuclear translocation and speckle formation that arise in response to oxidative stress.
After induction of oxidative stress and apoptosis

Hsc70 accumulates in aggregates similar to those formed by polyglutamine-expanded proteins

We observed that formation of Hsc70-positive intracellular speckles does not occur in resting cells, but only in stressed cells and correlates with increasing concentrations of stressing agents. These speckles are very similar to aggregates formed by polyglutamine-expanded proteins in pathological cells. Polyglutamine expansion encoded by CAG repeats is now recognised to be a major cause of inherited neurodegenerative disease including Huntington’s disease, spinobulbar muscular atrophy and spinocerebellar ataxia (Koshy and Zoghbi, 1997; Perutz, 1999). The toxicity of the disease protein (e.g. ataxin or androgen receptor) with expanded polyglutamine seems linked to its tendency to assume an abnormal conformation, which promotes aggregation. Recent studies have now shown that pathological cells that express expanded polyglutamine protein in aggregates elicit a stress response manifested by marked induction of Hsp70 and Hsc70 and that these two co-localise with the aggregates (Chai et al., 1999; Stenoien et al., 1999). A common hypothesis is that polyglutamine aggregation may be necessary, but not sufficient, to elicit a stress response. However, our observations indicate that aggregation may be a secondary phenomenon and not responsible for the stress response induction. Induction and accumulation of Hsc70 in aggregates may represent an effort by the cell to increase refolding, disaggregation or elimination of misfolded (polyglutamine) proteins. This view is supported by the fact that overexpression of other heat shock proteins, Hsp40 chaperones, can suppress aggregation of polyglutamine protein (Cummings et al., 1998; Chai et al., 1999; Stenoien et al., 1999).

Hsc70 may also have a role in hypoxia and apoptosis

Besides Hsc70 expression, the expression of two other major components of the neuronal PMO complex, GAPDH and enolase-γ, is also significantly changed after hypoxia (Graven and Farber, 1995, 1998; Graven et al., 1994, 1998). Enolase-γ is up-regulated in the cytoplasm of hypoxic cells, GAPDH is up-regulated both in the cytoplasm and in the nucleus and its glycolytic activity is decreased. Therefore GAPDH, and perhaps enolase-γ, exert functions during hypoxia aside from their catalytic function in glycolysis. This is an interesting observation since we isolated a multi-enzyme complex containing GAPDH and enolase-γ tightly bound to Hsc70 (Bulliard et al., 1997).

It is well established that overexpression of both inducible (hsp70i) and constitutive (hsp70c) isoforms confers resistance to oxidative challenges generated by ROS, again showing an antioxidant role for these proteins (Chong et al., 1998). Lee also showed that glucose deprivation induces metabolic oxidative stress via a stress-activated protein kinase, accompanied by an increase in Hsp70 mRNA which is suppressed by overexpression of Bel-2 (Lee and Corry, 1998). ROS production is common to apoptosis induced by agents such as e.g. camptothecin and actinomycin D, against which Hsps conferred protection, but not by Fas-mediated apoptosis, against which Hsps showed no protective effect (Creagh and Cotter, 1999). Selective protection against these agents is mimicked by pre-treatment with antioxidant compounds, and occurs downstream of ROS production. Consistent with our results, Creagh found no correlation between Bel-2- and Hsp70-mediated protection. Similarly Hsc70 protection against hypoxia-induced apoptosis has been described in hypoxia-induced apoptosis-resistant macrophages (HARMs; Yun et al., 1997; Kim et al., 1997). A significant increase in TNFα production in HARM but a decrease in other macrophages was observed after hypoxic treatment, indicating that a selective population of macrophages can adapt to hypoxic conditions by overcoming the apoptotic signal, in agreement with our observations.

Together, our findings demonstrate that the differential translocation of Hsc70 observed after induction of oxidative stress or apoptosis is in direct correlation with specific actions of Hsc70.

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