HSP70 Kinetics Study by Continuous Observation of HSP–GFP Fusion Protein Expression on a Perfusion Heating Stage

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ABSTRACT: The direct correlation between levels of heat shock protein expression and efficiency of its tissue protection function motivates this study of how thermal doses can be used for an optimal stress protocol design. Heat shock protein 70 (HSP70) expression kinetics were visualized continuously in cultured bovine aortic endothelial cells (BAECs) on a microscope heating stage using green fluorescent protein (GFP) as a reporter. BAECs were transfected with a DNA vector, HSPp–HSP70–GFP which expresses an HSP70–GFP fusion protein under control of the HSP70 promoter. Expression levels were validated by western blot analysis. Transfected cells were heated on a controlled temperature microscope stage at 42°C for a defined period, then shifted to 37°C for varied post-heating times. The expression of HSP70–GFP and its sub-cellular localization were visualized via fluorescence microscopy. The progressive expression kinetics were measured by quantitative analysis of serial fluorescence images captured during heating protocols from 1 to 2 h and post-heating times from 0 to 20 h. The results show two sequential peaks in HSP70 expression at approximately 3 and 12 h post-heat shock. A progressive translocation of HSP70 from the cytoplasm to the nucleus was observed from 6 to 16 h. We conclude that we have successfully combined molecular cloning and optical imaging to study HSP70 expression kinetics. The kinetic profile for HSP70–GFP fusion protein is consistent with the endogenous HSP70. Furthermore, information on dynamic intracellular translocation of HSP70 was extracted from the same experimental data.

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KEYWORDS: HSP70; fusion protein expression; kinetics; GFP; heat shock

Introduction

Heat shock protein 70 (HSP70) is an essential molecular chaperone of primary importance to all mammalian cells. It has been demonstrated to protect cells, tissues, and organs from stress (Kiang and Tsokos, 1998) by promoting the folding of nascent polypeptides and by correcting the misfolding of denatured proteins. The cytoprotective benefits of over-expressed HSP70 have been realized, for example, in terms of reducing ischemia injury in the heart (Gray et al., 1999) and brain (Yenari et al., 1999). Heat shock induced HSP70 expression also plays a role in the pathway of anti-apoptosis (Sreedhar and Csermely, 2004). The level of HSP70 expression is critical in achieving effective tissue protection (Donnelly et al., 1992). Thus, an understanding of how the temperature and time of stress can be manipulated to control the HSP70 expression process is necessary to design thermal protocols to elicit HSP70-derived protection.

We have conducted a prior study (Wang et al., 2003) of endogenous HSP70 expression kinetics demonstrating dependence on stress duration (heating time), stress level (temperature), and recovery process (post-heating time). A temperature of 42–43°C was effective in inducing an enhanced level of HSP70 expression. A 90 min heating duration at 42°C followed by 12 h recovery at 37°C produced a...
peak endogenous HSP70 expression that exceeded basal levels by more than ninefold as determined by Western blot analysis (Wang et al., 2003). However, conventional Western blot analysis is time-consuming and cannot provide continuous expression data for a single specimen. Therefore, we designed and implemented a new technique to facilitate real time assessment of HSP70 expression kinetics.

The complete nucleotide sequence of the human HSP70 gene and its 5′ promoter region was determined by Hunt and Morimoto (1985). For our purpose, a DNA vector in which the HSP70 promoter drives expression of an HSP70 gene fused in frame to a fluorescence reporter, green fluorescent protein (GFP), was constructed and introduced into cells. Thereby, whenever and wherever HSP70 expression occurs, it is detected by fluorescence imaging for GFP. A controlled temperature perfusion stage was used on an inverted fluorescence microscope to stress cells transiently in a tissue culture environment and to visualize HSP70 expression kinetics directly. In this manner it was possible to follow the full expression process for single cells, including observing the intracellular migration of HSPs. Here we present the results of investigations conducted with this system in the context of our foregoing studies.

Materials and Methods

Construction of the HSP70–GFP Fusion Protein Vector

The fusion protein vector was generated from pEGFP-N2 (Clontech, Mountain View, CA) by replacing the CMV promoter with the human HSP70 promoter (HSP70) to produce pHSP70–GFP. The HSP70 open reading frame (ORF) excluding its stop codon was amplified by polymerase chain reaction (PCR) from pH2.3 (a plasmid having no HSP70 promoter, as kindly provided by Dr. Richard Morimoto, Northwestern University) and BamHI restriction sites were created on both ends. The primers (Invitrogen, Carlsbad, CA) for the PCR were designed to ensure that the HSP70 ORF was followed in-frame by GFP such that a fusion protein, HSP70–GFP, was created. To ensure that the HSP70 promoter was followed in-frame by GFP such that a fusion protein, HSP70–GFP, was constructed, the HSP70 promoter was first ligated to the HSP70–GFP vector. The HSP70 open reading frame (ORF) excluding its stop codon was amplified by polymerase chain reaction (PCR) from pH2.3 (a plasmid having no HSP70 promoter, as kindly provided by Dr. Richard Morimoto, Northwestern University) and BamHI restriction sites were created on both ends. The primers (Invitrogen, Carlsbad, CA) for the PCR were designed to ensure that the HSP70 ORF was followed in-frame by GFP such that a fusion protein, HSP70–GFP, was constructed, the HSP70 promoter was first ligated to the HSP70–GFP vector.

Chaperon Function Verification of HSP70–GFP Fusion Protein

In order to check whether the HSP70–GFP fusion protein still maintains HSP70 chaperon function, the effects of ~2 kb of the inducible HSP70–EGFP fusion. The total length of the plasmid is 6.5 kb. The large quantity of pHSP70–GFP was purified with QIAGEN Plasmid Maxi kits (QIAGEN, Cat#12163).

Selection of Clonal Cell Lines With Heat Inducible HSP70–GFP Expression

Bovine aortic endothelial cells (BAECs, GM07372A) were purchased from Coriell Cell Repositories (Camden, NJ) and cultured with Eagle’s MEM (Sigma-Aldrich, St. Louis, MO, M-0643) with 10% FBS, MEM vitamins and penicillin-streptomycin. Cells were grown in three 10 cm culture dishes until they were 70% confluent. For stable transfection, cells at a density of 5 × 10^6 cells/mL were electroporated with a Gene Pulser (BioRad, Hercules, CA, #165–2106) in 0.5 mL–0.4 cm cuvettes at a voltage of 280 V and a capacitance of 960 μF with 10 μg of linearized pHSP70–GFP encoding resistance to the neomycin analogue drug, G418. Following electroporation, surviving cells were transferred to 10 cm culture dishes and grown for 2 weeks in a medium containing 0.7 mg/mL G418 freshly supplemented every 3 days. Well-separated, drug-surviving single colonies from each dish were harvested with sterile self-sealing barrier tips (Molecular BioProducts, San Diego, CA, ART 200, #2069) inside a Biosafety hood and placed in 24-well plates with selective medium including 0.35 mg/mL G418.

Clones were initially screened by fluorescence activated cell sorting (FACS; FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, #34012430) to eliminate those that had constitutively high GFP expression without heating (negative screening). Such clones likely derived from random insertion of pHSP70–GFP DNA into an active chromatin region which by-passed the need for induction of the ectopic heat shock promoter. Clones demonstrating low GFP expression by FACS were subjected to Western blotting with anti-HSP70 (Santa Cruz Biotechnology, Santa Cruz, CA, sc-24) to select those expressing low to modest levels of the fusion protein. Clonal cell lines then were heated for 2 h at 42°C in an incubator followed by 12–16 h recovery at 37°C. Only cell lines expressing both endogenous HSP70/HSC70 (70 kDa) and fusion protein HSP70–GFP (98 kDa), were chosen for further analysis. Western blotting with anti-GFP monoclonal antibody (Santa Cruz Biotechnology, sc-5385) was also performed to check whether the fusion protein degraded into HSP70 and GFP after heating. Cell lines that met these criteria were further selected by FACS. Only those lines which demonstrated at least a twofold fluorescence intensity increases following heating were chosen for continuous HSP70 expression monitoring on the heating stage (positive screening).
HSP70–GFP on heat inactivation of luciferase activity were examined (Michels et al., 1997). The same stable transfection with pHSP₂–HSP70–GFP was performed on BAECs. Eight well-separated drug-surviving single colonies (CHSP1 to 8) that had constitutively high GFP expression without heating were selected and expanded in culture. Western blotting with anti-HSP70 (Noventa, Ann Arbor, MI, SPA-810D) was used to further select clonal cell lines which had high constitutive HSP70–GFP expression but the same low level of endogenous HSP70 as the wild-type BAEC.

Two clonal cell lines (CHSP2 and CHSP8) with the constitutive HSP70–GFP fusion protein expression and one wild-type BAEC specimen were used for luciferase heat inactivation experiments. Three cell lines were each grown in culture in T175 flasks. Upon confluence all samples were transfected with pRL-TK luciferase control reporter vector (Promega, Madison, WI) using Fugene 6 (Roche, Basel, Switzerland). Then, 24 h after transfection, cells in each flask were distributed into 12 T25 phenolic flasks (three samples for each of the four heating durations considered) each containing ~1.0 × 10⁶ cells. Phenolic flasks were employed to prevent contamination from leakage during the heating process. Following 24 h after cell seeding, all samples were rinsed with PBS to prevent cell damage caused by the degradation of L-glutamate at high temperatures. Flasks were filled with heating medium (Eagle’s MEM without L-glutamate) and then submerged in a water bath at a temperature of 42°C for varying heating durations of 0, 5, 10, and 15 min. The method of thermal stimulation and calibration has been previously described (Rylander et al., 2005). Immediately following heating, cells were lysed with Passive Buffer (Promega). The lysate was frozen at −20°C before measurement of luciferase activity. The luciferase activity was measured after the addition of substrates by Dual Luciferase Reporter Assay kit (Promega) with CENTRO LB 960 luminometer (Berthold Technologies, Oak Ridge, TN) according to the manufacturer’s protocol. The luciferase activity before heat shock was taken as 100%, and results from luciferase measurements during inactivation were normalized with that of their corresponding initial samples.

The Perfusion Heating Stage With Cell Culture Ability

A heating stage based on the ΔT culture dish system from Biopetchs (Butler, PA) was modified to achieve uniform spatial temperature distribution throughout the special culture dish over the range of 35–45°C. The heating stage has three components: a thermal controller, an adapter for the Olympus CK40 inverted fluorescence microscope, and a culture dish with an electrically resistive indium tin-oxide coating. The controller regulates an electrical current through the resistance heater in response to a feedback signal from a thermistor monitoring the bottom surface temperature at a point midway between the center and the edge of the dish. To reduce the thermal gradient across the stage, the bottom of the stage was sealed with a piece of transparent film to prevent free convection between the dish and air. A perfusion system was added to the heating stage to further improve the temperature uniformity and to provide cells with fresh medium during long experiments. Perfusion was achieved with a Lambda micro-perfusion peristaltic pump (Biopetchs, #0420131616) and fresh medium, which was vented with 5% CO₂ and connected from a heat supply flask to the stage via an insulated 1/16” tube wrapped with rope heaters (OMEGA Engineering, Stamford, CT, #FG-030). The perfusion system was calibrated for flow rates from 9 to 74 mL/h.

The two-dimensional heating pattern on the stage was measured with a thermal camera (Indigo Systems, Santa Barbara, CA, Phoenix-Mid Insb 320X256FPA). The qualitative temperature distribution was approximately symmetric about the stage center and decreased toward the perimeter. The temperature distribution on the stage was also calibrated via readings from four thermocouples, three of which were affixed radially from the center to the perimeter and one at the perfusion outlet port. The thermocouples were read and recorded with a computer interface device (Analog Devices, Norwood, MA, 6BP16-2) and LabView software. The calibration protocol commenced from an initial state at 25°C, followed by perfusion at 42°C for 2 h and then a step change to 37°C perfusion to the termination. Data for the four thermocouples and the stage thermistor are presented in Figure 1A. The calibration data were applied to derive a linear correlation between the dish center, Tc, and perfusion outlet, Tp, temperatures (R² = 0.9963, Tp = 0.8526 × TC + 2.5659). As illustrated by the data in Figure 1B, the specimen temperature could be assessed in the absence of a probe inserted directly into the cell suspension. This calibration allowed the perfusion outlet temperature to be used as an indicator of the cell state within the growth medium on the heating stage, dispensing with the need for a control thermocouple to be inserted directly into the cell culture environment.

The dish temperature could be raised from room temperature to 42°C in as little as 2.5 min and controlled thereafter between 42 and 42.5°C. A perfusion outlet temperature of 38.0–38.3°C corresponded to a dish temperature of 42°C, and 33.0–33.3°C corresponded to 37°C. For long-term 37°C incubation, the dish temperature was regulated between 36.5 and 37°C.

Co-Localization of the Fusion Protein With Endogenous HSP70

Stable transfected BAECs were heated on the microscope perfusion heating stage at 42°C for 90 min, followed directly by 12 h at 37°C. Serial fluorescence images allowed localization of GFP by green fluorescence to be differentiated from endogenous HSP70 immunostained to emit red fluorescence. Fluorescent images were captured by a color CCD camera (Diagnostic Instruments, Sterling Heights, MI, DOI 10.1002/bit).
The primary antibody used in the immunostaining, goat anti-HSP70 (Santa Cruz Biotechnology, sc-1060), reacts only with endogenous HSP70. Western blot analysis showed that this antibody does not recognize the HSP70–GFP fusion protein. A possible explanation is that the recognizing site of this antibody is a very short epitope located at the C terminal of HSP70. In the fusion protein, GFP was adhered after HSP70 at its C terminal by taking off the stop codon of HSP70 gene, which resulted in the disabled antibody for the fusion protein. Texas Red conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, 705-075-147) was used as a secondary antibody. After heat shock, immunostaining was performed immediately in the same dish in which fusion protein expression was imaged in GFP. Cells were fixed with 4% paraformaldehyde and permeabilized with 3% Triton X-100. Non-specific reactivity of the donkey anti-goat IgG preparation was absorbed with 3% normal donkey serum in PBS as a blocking buffer. Since cells were cultured in a medium containing FBS, non-specific absorption of bovine IgG on cell surfaces might also lead to cross reactivity with the anti-goat IgG antibody. Hence, 20 μg/mL non-conjugated Fab fragment of rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, 305-007-0030) was used to block these potential sites.

**Continuous Observation of HSP70–GFP Expression on the Heating Stage**

Clonal cell lines with heat inducible HSP70–GFP expression were removed from 6-well plates and replated in the ΔT culture dishes one day before the heating experiment to achieve ~90% confluence at the time the experiment was initiated. The experiments were performed under sterile conditions. The perfusion coverlid and silicon tubing of the perfusion inlet was sterilized with 70% ethanol and air dried in a biosafety hood. The perfusion medium, without phenol red (to remove its contribution to background fluorescence) was equilibrated in a 1 L sterile glass flask in a 37°C cell culture incubator overnight saturated with 5% CO₂. The following morning, normal growth medium in the ΔT culture dish with the perfusion lid was replaced with perfusion medium. Phase contrast and fluorescence images of the cells in the ΔT culture dish were taken with a SPOT RT camera before initiation of heating. After initial images were captured, the stage position was held constant for all following images during heating and recovery. To initiate heating the dish temperature was increased from room temperature to 42°C in 2.5 min, and at termination it was cooled from 42 to 37°C in 3 min. Cells were heated at 42°C on the perfusion stage for 30 min to 2 h and recovered at 37°C for intervals up to 20 h. Perfusion was stopped for 11 s when an image was recorded. Identical camera exposure parameters were used for every image in an experiment. Phase and fluorescence images were captured every 20–30 min during heating, every 30–60 min during early post-heating or every 2–4 h in the late recovery time. All images were saved as uncompressed TIFF files in order to retain as much information as possible.

Fluorescence images were analyzed with Image-Pro Plus software (Media Cybernetics, Bethesda, MD) to automatically trace the cell boundary, with manual override as necessary. Each isolated cell or multi-cell colony was defined by the operator as an area of interest (AOI). By defining multiple AOIs including a common set of cells in a series of fluorescent images and using the same fluorescence intensity threshold for excluding background fluorescence, images were quantified automatically to determine the relative fluorescence activity (RFA), which was defined as the measure of the HSP70–GFP expression. A green fluorescence intensity histogram was calculated for the same AOIs in each image. RFA was quantified as the area integral of normalized fluorescence intensity above the threshold to represent the amount of fusion protein expression. Images were normalized in fluorescence intensity to the control, based on the same AOIs in the preheating image.
Results

Comparison of HSP70–GFP Fusion Protein and Endogenous HSP70 Expression

Clonal cell lines were heat shocked for 2 h, followed by 12–16 h recovery. Figure 2 shows the Western blot for heat shocked whole cell lysates developed either with anti-HSP70 (A) or anti-GFP (B). Both antibodies recognized HSP70–GFP fusion protein bands at 98 kDa. Cell lines displaying bands for both the endogenous HSP70 and the HSP70–GFP fusion were used for further experiments. The co-migration of the fusion protein bands detected with the GFP antibody in Figure 2B indicated that, following heating, no significant protein degradation had occurred in the selected cell lines.

Figure 3A shows a representative image of cells expressing endogenous HSP70 immunostained in red. In Figure 3B, transfected cells in the same field are identified by their GFP-emitting green fluorescence. The green fluorescence locations mapped exactly onto the red fluorescence, consistent with our contention that HSP70–GFP and endogenous HSP70 co-localize and that fusion protein expression is a legitimate reporter for endogenous HSP70 activity. It is common that a small fraction of stable transfected cells (even monoclonal cells) lose copies of inserted genes over a period of culture time. That may be the reason that a couple of cells in Figure 3B did not have the HSP70–GFP expression but had endogenous HSP70 expression.

In order to confirm that HSP70–GFP fusion protein has the similar chaperon function as HSP70 itself, effects of HSP70–GFP on heat inactivation of luciferase were examined. Two stable transfected cell lines, CHSP2 and CHSP8 in Figure 4A, were selected for the heat inactivation experiments because they had high levels of constitutive HSP70–GFP expression but the same low level of endogenous HSP70 as the wild-type (non-transfected) BAEC. Figure 4B shows that luciferase activities in both CHSP2 and CHSP8 are significantly higher ($P < 0.01$) than that in the wild-type BAEC during the 15 min 42°C heating periods. Therefore, the expression of HSP70–GFP attenuates luciferase denaturation during the heat inactivation process.

HSP70 Expression Kinetics Determined From Continuous Fusion Protein Measurement

Images from an exemplar heating stage experiment are presented in Figure 5. The cells were heat shocked at 42°C for 100 min, followed by 16 h recovery at 37°C, during which images were captured every hour. The phase and fluorescence images in Figure 5A and B are preheating controls. Some of the cells in Figure 5B show low level background fluorescence prior to heat shock. This cloned cell line was chosen purposely so that the low background fluorescence enabled location of the focus plane in the optical pathway. Thus, the same focus plane was used for every image throughout the experiment. As shown in Figure 5C–F, HSP70–GFP fusion protein expression increased progressively after heat shock and 3 h recovery, dropped slightly at 11 h, and then increased back at 12 h recovery.

The level of HSP70–GFP expression in this set of images was quantified by fluorescence image analysis and plotted in Figure 6. HSP70 concentration is elevated immediately at the conclusion of heating and continues to an initial first peak at 3 h, followed by a drop from 8 to 11 h, a second peak at 12 h, and a final decrease in fluorescence, presumably due to HSP70–GFP degradation. The similar HSP70–GFP expression kinetics from the cells heated for 30 min on the stage was also observed and included in Figure 6.

Relocalization of HSP70

The images in Figure 5 and others not shown demonstrated an intracellular relocalization of HSP70 over the period of the experiment. For the first several hours of heat shock...
recovery, HSP70 is distributed evenly in the cytoplasm. Later it becomes concentrated around the nucleus in some cells. After 12 h of heat shock we observed relocalization of HSP70–GFP from the cytoplasm into the nucleus (as indicated by arrows in two cells of Fig. 5F). The translocation of HSP70 back to the nucleus in some cells during recovery after heat shock was observed repeatedly, as demonstrated clearly in Figure 7. The cell nucleus can be seen in the phase image (Fig. 7A). Comparison of phase and fluorescence images in Figure 7A and B shows that HSP70–GFP is concentrated in some cell nuclei.

Fluorescence intensity analysis was performed separately for the cytoplasm and nucleus of three cells in Figure 8 to track fusion protein translocation during the 6–16 h recovery window. The nucleus area was identified semi-manually in the corresponding phase-contrast images using Image-Pro Plus as described in the Materials and Methods Section and mapped to the fluorescence images in Figure 8. The mean fluorescence intensity (MFI) was measured as representative of the density of HSP70 in the cytoplasm and the nucleus, and the summation of fluorescence intensities (SFI) as representative of the total HSP70 expression for the entire cell. Distribution of HSP70 expression within the cell was described by the ratio of MFI in the nucleus to the cytoplasm. The change of the distribution ratio for 6–16 h recovery is summarized in Table I.

The ratios of SFI at 6 h recovery (SFI$_{R6}$) to SFI at 16 h recovery (SFI$_{R16}$) for three cells were all less than one (Table I: 0.30, 0.47, and 0.79), indicating an increase in HSP70 expression during this period. At 6 h HSP70 was evenly distributed throughout each cell, with the majority in the cytoplasm, giving $D_{R6}$ for three cells of ~1.0 (Table I: 1.06, 1.04, and 0.97). At 16 h recovery, fluorescence images showed that HSP70 was more concentrated in and around the nuclei than in the cytoplasm. The quantitative analysis of HSP70 expression in those three cells at 16 h recovery results in $D_{R16}$ around 1.1 (Table I: 1.19, 1.17, and 1.09), confirming a greater density of HSP70 in the nuclei. Comparing averages of $D_{R6}$ (1.02) with $D_{R16}$ (1.15), the $P$-value is less than 0.05 using a Student’s $t$-test showing that HSP70 translocation from the cytoplasm to the nucleus is statistically significant. Interestingly, three cells had a similar percentage of HSP70 distribution change (~12.4%), even though they had significantly different levels of initial HSP70 expression at 6 h recovery and different total HSP70 expression change from 6 to 16 h recovery.

Conclusions and Discussion

In this study, molecular cloning and stable ectopic cellular expression techniques were employed to generate a reporter system to afford an optical means for identifying HSP70 expression kinetics. Demonstration of HSP70–GFP protein integrity by Western blot analysis, chaperon function verification of the fusion protein by heat inactivation of
luciferase along with the observed co-localization of the endogenous and fusion proteins following heat shock confirmed the utility of the HSP70–GFP approach. Thereby long-term continuous observation of HSP70 expression kinetics was achieved with a controlled temperature perfusion stage. An HSP70 expression kinetics curve was obtained in real time for single cell specimens rather than by serial experiments terminated at differential times for different specimens.

The continuous expression kinetics of HSP70–GFP had the same bimodal pattern over time as that shown previously (Wang et al., 2003); a first peak at 3 h recovery was followed by a second peak at 12 h. Thus, the present data are consistent with existing expression kinetics data for endogenous HSP70. However, the absolute expression level
of HSP70–GFP is not comparable with endogenous HSP70. For example, the maximum endogenous HSP70 expression in BAECs after heat shock and recovery can reach ten times greater than the control (Wang et al., 2003), while the maximum HSP70–GFP fusion expression reported in this article is increased only threefold. Furthermore, the absolute amount of HSP70–GFP expression most likely differs from one stable transfected cell line to another. This effect is anticipated because stable transfection methods such as used in this study cannot control the copy numbers of the integrated DNA nor its insertion locations within the cellular genome. More advanced molecular techniques, such as gene disruptions/insertions achieved by homologous recombination, would be required to construct a transgenic model or a stable transfected cell line such that each copy of HSP70 within the genome is replaced with a HSP70–GFP fusion reporter.

An advantage of observing HSP70–GFP expression continuously on a heating stage is the ability to acquire HSP70 localization information in real time. In some cells following heat shock and recovery, we observed translocation from the cytoplasm to nucleus as previously reported by others (Laszlo et al., 1993; Snoeckx et al., 1991; Welch and Feramisco, 1984). HSP70 translocation from the nucleus back to the cytoplasm was found in fibroblasts at about 8 h (Welch and Suhan, 1986), and the redistribution occurred more rapidly in transient or permanently heat-resistant cells than their normal counterpart (Ohtsuka and Laszlo, 1992). Since HSP70 must be in the cytoplasm to provide chaperon function to misfolded and unfolded proteins, perhaps the dynamic relocalization of HSP70 is important in a rescue situation such as when cells are under stress. This phenomenon may be one of the reasons why identical absolute levels of HSP70 expression at 6 h recovery did not result in the same effective protection in rat hearts at 24 h recovery after heat shock (Cornelussen et al., 1998). An extended time heating stage experiment (beyond 24 h recovery) could be conducted to establish the time when HSP70 translocates back to the cytoplasm from the nucleus.

We found it interesting that two dividing cells with different HSP70 localization patterns were observed to have totally different fates. The cells might be in different phases of the cell cycle when heat shock was applied. The cell in Figure 9, which died eventually, had undergone S phase as

Table 1. Quantitative analysis of HSP70 relocalization (R6: 6 h recovery, R16: 16 h recovery, SFI: summation of fluorescence intensities, MFI: mean fluorescence intensity).

<table>
<thead>
<tr>
<th>Cell</th>
<th>#1</th>
<th>#2</th>
<th>#3</th>
<th>Average</th>
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<td>SFIR6/SFIR16</td>
<td>0.30</td>
<td>0.47</td>
<td>0.79</td>
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<td>D[R6]: HSP70 distribution at R6 (MFI_nucleus/MFI_cytoplasm)</td>
<td>1.06</td>
<td>1.04</td>
<td>0.97</td>
<td>1.02 ± 0.047</td>
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<tr>
<td>D[R16]: HSP70 distribution at R16 (MFI_nucleus/MFI_cytoplasm)</td>
<td>1.19</td>
<td>1.17</td>
<td>1.09</td>
<td>1.15 ± 0.053</td>
</tr>
<tr>
<td>Percentage of distribution change (</td>
<td>D[R16] - D[R6]</td>
<td>/D[R6])</td>
<td>12.3%</td>
<td>12.5%</td>
</tr>
</tbody>
</table>

Average numbers are presented as mean ± SD.

Figure 9. Phase and fluorescence images of a dividing cell having undergone S phase in with its chromosomes in the process of alignment in the cell midline at the time of a 1 h heat shock. A: Before heating, B: 1 h recovery, C: 6 h recovery. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]
shown in phase contrast images, and its chromosomes were in the process of alignment at the cell midline when it was heat shocked. Another cell (data not shown), which survived and later divided into two daughter cells, might have undergone mitosis and have its chromosomes condensed in the center of the nucleus at 12 h recovery. Translocation of HSP70 from the nucleus back to cytoplasm after recovery was never observed in the cell that died subsequently. No visible relocation of HSP70 was observed for the surviving cell, and HSP70 was continuously in the cytoplasm during the division process. Therefore, the relocalization of the HSP70 after heat shock might be regulated by the cell cycle and may be indicative of the fate of the cell. However, since no cell cycle indicator or DNA dye was used in this study, the hypothesis need to be further investigated. For example, heat shock experiments can be performed on pHSP<sub>p</sub>-HSP70–GFP stable transfected cells synchronized at different phases in the cell cycle. It had been shown that the pattern of HSP70 nuclear localization and redistribution back to the cytoplasm was related to the cells’ survival ability from moderate heat shock at 41.1 °C (Xu et al., 1998). In this article, authors observed that human colon adenocarcinoma cells (NSY42129 and HT29), which showed HSP70 delocalization from the nucleus, survived and proliferated at 41.1 °C; while HCT15 (human colon adenocarcinoma cells) and U87MG (human glioma cells), which did not show HSP70 delocalization, could not survive or proliferate at 41.1 °C. Although the mechanism to determine the death of small population of cells in our experiment may be totally different from theirs, our data confirmed that the pattern of HSP70 nuclear localization and redistribution could be an indicator of the cell fate.

According to our knowledge, these experiments are the first to measure HSP70 expression kinetics quantitatively in living cells in real time. The technique could be applied to other proteins whose expression, when under the control of the heat shock promoter, could be activated by heating. This method might be a particularly useful way to study the expression of proteins which are toxic to cells when constitutively over-expressed.

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