The role of Hsp27 and actin in the regulation of movement in human cancer cells responding to heat shock

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Received: 9 October 2008 / Revised: 12 December 2008 / Accepted: 30 December 2008 / Published online: 18 February 2009
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Abstract Human heat shock 27-kDa protein 1 (HSPB1)/heat shock protein (Hsp) 27 is a small heat shock protein which is thought to have several roles within the cell. One of these roles includes regulating actin filament dynamics in cell movement, since Hsp27 has previously been found to inhibit actin polymerization *in vitro*. In this study, the role of Hsp27 in regulating actin filament dynamics is further investigated. Hsp27 protein levels were reduced using siRNA in SW480 cells, a human colon cancer cell line. An *in vitro* wound closure assay showed that cells with knocked down Hsp27 levels were unable to close wounds, indicating that this protein is involved in regulating cell motility. Immunoprecipitation pull down assays were done, to observe if and when Hsp27 and actin are in the same complex within the cell, before and after heat shock. At all time points tested, Hsp27 and actin were present in the same cell lysate fraction. Lastly, indirect immunostaining was done before and after heat shock to evaluate Hsp27 and actin interaction in cells. Hsp27 and actin showed colocalization before heat shock, little association 3 h after heat shock, and increased association 24 h after heat shock. Cytoprotection was observed as early as 3 h after heat shock, yet cells were still able to move. These results show that Hsp27 and actin are in the same complex in cells and that Hsp27 is important for cell motility.

Keywords Hsp27 · Actin · Cell motility · Heat shock · Cancer

Electronic supplementary material The online version of this article (doi:10.1007/s12192-008-0098-1) contains supplementary material, which is available to authorized users.

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Introduction

Heat shock proteins (Hsps) are evolutionarily highly conserved proteins present in both prokaryotic and eukaryotic species (Schlesinger 1990). They are ubiquitous and have different roles depending on their molecular mass and structure. Hsps below 30,000 Da are considered small Hsps (sHsps) and are part of the alpha crystallin family, which share a conserved 80–100 amino acid sequence (Merck et al. 1993; Kim et al. 1998). Small Hsps are activated in times of cell stress but have physiological roles under nonstressed conditions as well. During times of stress, small Hsps are thought to hold and protect protein folding intermediates, until chaperones can facilitate folding into their proper conformations, thus sHsps also prevent protein aggregation within the cell (Jakob et al. 1993).

Human HSPB1/Hsp27 is a small Hsp of 27 kDa molecular mass (Kampinga et al. 2009). It can exist in different multimeric states, ranging from monomers and dimers to oligomers, depending on its phosphorylation state (Arrigo et al. 1988; Kato et al. 1994). Hsp27 has three conserved phosphorylation sites, ser 15, ser 78, and ser 82 (Gaestel et al. 1991; Landry et al. 1992). Phosphorylation occurs through the mitogen-activated protein kinase/p38 pathway (Stokoe et al. 1992; Rouse et al. 1994). *In vitro*, when Hsp27 is unphosphorylated, it exists in a monomeric state, and it has been shown to prohibit polymerization similar to previously studied actin filament capping proteins (Miron et al. 1991; Benndorf et al. 1994). The *in vivo* existence of monomeric Hsp27 has not been demonstrated. Nonphosphorylated forms of Hsp27 are present as large oligomeric complexes. As an oligomer, Hsp27 acts as a chaperone and prevents protein aggregation, by holding partially folded proteins in place (Rogalla et al. 1999). The role of Hsp27 is altered in response to cell stress such as

heat shock due to changes in phosphorylation and conformation (Theriault et al. 2004). During an unstressed state, Hsp27 is located in the cell cytoplasm. In a stressed state, Hsp27 is present in cells as an oligomer and binds to actin in vitro. However, after heat shock, nuclear/perinuclear translocation occurs which is a marker of the heat shock response (Arrigo et al. 1988).

Actin is a cytoskeletal protein present within all eukaryotic cell types. It has a major role in cell movement and is regulated by many other proteins. The regulation of actin filament turnover is essential for cell movement (Pollard and Borisy 2003). Hsp27 is known to interact with actin in vitro; however, its role in this interaction within the cell is unclear. The goal of this study is to determine if Hsp27 is involved in cell movement and whether it interacts with actin in vivo (in this paper, in vivo is used to denote in living cells).

To answer these questions, we first established that a heat shock response occurs in the SW480 cell line. We heat shocked SW480 cells stably expressing Hsp27 (SW480-/Hsp27) and observed fluorescent localization before and after heat treatment. Cytoplasmic to nuclear/perinuclear translocation, a marker of heat shock, was observed. To detect when these cells are cytoprotected, a standard cytoprotection assay was done. Cells were cytoprotected as soon as 3 h after receiving a heat shock. SW480 cells were used in wound assays to observe wound closure, because these cells grow to a confluent sheet, are easily wounded, and readily migrate to close the wound. We observed the effects of knocking down Hsp27 protein levels in SW480 cells, on the rate of wound closure, before and after heat shock. We also observed Hsp27 localization with actin before and after heat shock, using immunofluorescence. Immunoprecipitation (IP) was done to determine if Hsp27 and actin form a complex within the cell.

Materials and methods

Cell culture and treatment

SW480 human colon cancer cells and CRL1807 human nontumorigenic colonocytes were purchased from the American Type Culture Collection (Manassas, VA, USA). CRL1807 cells were SV40 transformed for immortalization. Both cell types were cultivated in McCoy's Modified 5A media, supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 50 U/ml streptomycin, and 50 U/ml penicillin. Cells were maintained in a 5% CO₂ 37°C incubator. Heat shock was performed by placing the cells in 5% CO₂ 42°C incubator for 1 h, followed by a 24-h recovery period at 37°C.

GFP-/Hsp27 construct

Hsp27 cDNA (NCBI Ref: U85501) was isolated and purified from the desert fish *Poeciliopsis lucida* by Dr. Carol Norris. The three phosphorylation sites noted previously in human Hsp27 are conserved in *P. lucida* DNA sequence. The Hsp27 cDNA was amplified by polymerase chain reaction and restriction enzyme sites BamH1 and Kpn1 were added. The DNA insert encoding Hsp27 was then ligated into permuted enhanced green fluorescent protein (pEGFP; Clontech, Palo Alto, CA, USA) at BAMH1 and KPN1 restriction sites at the C terminus of the multiple cloning site on the pEGFP backbone. In the resulting protein, EGFP was fused to the N terminus of Hsp27 (GFP-/Hsp27). GFP expression was driven constitutively by a cytomegalovirus promoter. The GFP-/Hsp27 product was verified by DNA sequencing of the construct.

GFP-fHsp27 transfection

SW480 cells were stably transfected with the GFP-fHsp27 construct using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the protocol provided. Cells were then placed in complete McCoy's medium with 0.68 mg/ml G418 selection antibiotic. Cells with endogenous Hsp27 expression are termed wild type (WT), while SW480 cells expressing GFP-/Hsp27 are referred to as SW480-/Hsp27 cells.

Knockdown of human Hsp27

Oligomers for knockdown of human HSP27 were purchased from Dharmacon (Chicago, IL, USA) (Table 1). SW480 cells plated in 12-well cell culture dishes were transfected using Dharmafect (Dharmacon, Chicago, IL, USA) according to the manufacturer's protocol using 0.05 nM of the RNAi oligomers. Cells were incubated at 37°C and used for wound assays 44 h later.

Fluorescence quantification of GFP-/Hsp27

SW480-/Hsp27 cells were observed at ×400 total magnification. Ten different fluorescent fields of view were imaged before heat shock, ten fields of view were imaged 3 h after a 1-h heat shock, and ten fields were imaged 24 h after heat shock. Images were analyzed using MetaMorph software. The "linescan" tool was used to measure the fluorescence intensity versus distance along a line drawn through single cells. The data were plotted and graphed into Excel. A graph showing fluorescence intensity in the shape of a bell curve is considered to have brighter nuclear GFP localization, while a graph in the shape of an inverse bell curve has

Table 1 SiRNA sequences of Hsp27

Gene	Duplex #	Sense	Antisense
HSPBI	1	CCGAUGAGACUG	UUGGCGGCAGU
		CCGCCAAUU	CUCAUCGGUU
	3	GGCAGGACGAGC	UAGCCAUGCUCG
		AUGGCUAUU	UCCUGCCUU
	4	CCGGAGGAGUGG	ACUGCGACCACU
		UCGCAGUUU	CCUCCGGUU
	5	CAAGUUUCCUCCU	ACAGGGAGGAGG
		CCCUGUUU	AAACUUGUU
		UAAGGCUAGAAGA	UUAUUCGUAUC
		GAUACUU	UUCUCUAUG
Nonspecific control II	1		

SiRNA oligomers were purchased from Dharmacon. The sequences for Hsp27 as well as the nonspecific oligomers are listed

bright GFP fluorescence in the cytoplasm. The locations of GFP-*f*Hsp27 in all cells in the fields of view were quantified in this manner.

Cytoprotection assay

SW480-*f*Hsp27 cells were used to detect cytoprotection after heat shock and heat challenge according to (Noonan et al. 2008). Briefly, four conditions were tested. In the first condition, cells were maintained at 37°C until passage. In the second condition, cells were placed at 42°C for 1 h (mild heat shock) and passaged 24 h after heat treatment. In the third condition, cells were placed at 47°C for 1 h (lethal heat shock) and passaged 24 h after heat treatment. In the fourth condition, cells were exposed to mild 42°C heat shock for 1 h, followed by a lethal 47°C heat challenge. Cells were challenged at 3 and 24 h after receiving a mild heat shock. After the heat challenge, cells were placed in 37°C for 24 h and then passaged. In all cases, cells were given 24 h after passaging to allow them to reattach to culture dishes. At this point, cells were counted using a hemocytometer for cell survival quantification. The number of cells able to reattach to the cell culture dish indicated cell viability.

Wound assay

Wild-type and SW480-*f*Hsp27 cells were plated into 12-well cell culture dishes. Confluent cells were given a 1-h heat shock at 42°C, followed by a 24-h recovery period at 37°C. Wounds were created in a straight line, with a yellow pipette tip approximately 2 mm wide, in wild-type and SW480-*f*Hsp27 cultures, following a 24-h recovery period. Cells were washed three times with 1× phosphate buffered saline (PBS) and replaced in complete McCoy's media. Wounds were imaged immediately after wounding and after 4, 10, and 24 h and when wounds had closed, using a Leica

inverted epifluorescence widefield microscope, with a 20 times objective.

Protein extraction

After wound closure, SW480 cells were washed twice with cold 1× PBS. Cells were incubated at 4°C for 8 min in 75 µl lysis buffer with 0.1% NP-40 [10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM MgCl₂, 0.5 M sucrose, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonylfluoride (PMSF), and leupeptin at 2 µg/ml]. Cells were detached from the dish by scraping and then centrifuged (15,000×g) at 4°C for 10 min. This supernatant was used as the cytosolic fraction. The remaining pellet was washed with 100 µl of the above buffer without NP-40, resuspended in 10 µl buffer [20 mM HEPES-KOH, pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 5% glycerol, 1 mM DTT, 0.5 mM PMSF, and leupeptin at 2 µg/ml], and incubated on ice for 40 min followed by centrifugation (15,000×g) at 4°C for 10 min. This supernatant was used as the nuclear fraction. Bio-Rad Protein Assay (Bio Rad Laboratories, Hercules, CA, USA) was used to determine protein concentrations.

Immunoblotting

Cytosolic or nuclear protein extracts (10 µg) were denatured under reducing conditions, separated on 12.5% sodium dodecyl sulfate polyacrylamide gels, and transferred to nitrocellulose by voltage gradient transfer. Blots were blocked with 5% nonfat dry milk and washed twice with 1.0% PBS and 0.1% Tween buffer. Antibodies used in detection were primary rabbit anti-human Hsp27 (1:3,000), a kind gift from Dr. Robert Tanguay, and anti-GFP (1:1,000), a kind gift from Dr. David Knecht. Complementary horseradish peroxidase-labeled secondary antibodies

(Sigma) were used for protein detection by enhanced chemiluminescence.

Indirect immunostaining

Indirect immunostaining for Hsp27 and actin was performed with CRL1807, an immortalized human colon epithelial cell line. Cells were seeded onto a sterile coverslip at low density before fixation. Secondary goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated anti-body (Jackson ImmunoResearch, West Grove, PA, USA) was used for fluorescence detection. Actin was detected using rhodamine phalloidin (Molecular Probes, Eugene, OR, USA). Images were captured using a $\times 400$ total magnification, on a Nikon-inverted epifluorescence widefield microscope. The following fixation/staining protocols were carried out at room temperature. Cells were fixed using 4% paraformaldehyde for 15 min and permeabilized with 0.25% Triton X-100 for 15 min. Rhodamine phalloidin, diluted at 1:50 dilution in PBS, was added for 15 min, followed by incubation in 2% bovine serum albumin for 30 min. Primary Hsp27 antibody at a 1:500 dilution was left on the coverslip for 1 h. Secondary FITC-conjugated antibody at 1:100 dilution was incubated with the cells for 1 h, which was followed by mounting the coverslips on microscope slides in slow-fade mounting solution (Molecular Probes, Eugene, OR, USA) and sealing them with nail polish.

Immunoprecipitation pull down assay

Immunoprecipitation of GFP-fHsp27 and actin was performed using SW480 cells stably expressing GFP-fHsp27. Cells were grown to 80–90% confluency. Soluble and insoluble extracts were produced, as previously described (Dulyaninova et al. 2007) with the following exception: After 5 min incubation with Triton X-100, the insoluble fraction was isolated by scraping cells into Laemmli buffer without 2-mercaptoethanol, diluted into an approximately equal volume of sample (Laemmli 1970). Protein concentrations were then determined using BioRad Protein Assay. The soluble and insoluble fractions were incubated at 4°C overnight, with rabbit polyclonal GFP antibodies (Abcam, Cambridge, MA, USA). Protein A sepharose beads were added and the samples were rocked at 4°C for 3 h. Immune complexes were collected by centrifugation and washed with PBS. Laemmli buffer (2 $\times$) was added to the samples and boiled. Proteins were separated on a 12.5% sodium dodecyl sulfate polyacrylamide gel. GFP-fHsp27 and actin were detected using immunoblotting, as described above. Actin was detected using 1:1,000 dilution mouse monoclonal antibody (Abcam) and GFP-fHsp27 was detected using a 1:1,000 dilution anti-GFP antibody (Rockland Immunochemicals Inc.).

Results

GFP-fHsp27 translocates to the perinuclear region after heat shock

Previous studies have shown that one characteristic of the heat shock response is the translocation of Hsp27 from the cytoplasm to either the nuclear or perinuclear region depending on the cell type (Arrigo et al. 1988; Lavoie et al. 1995). Because we used fish Hsp27 cDNA to make the GFP-fHsp27 construct, we sought to verify that this fusion protein could act as a marker for the heat shock response in SW480 cells. The localization of GFP-fHsp27 fluorescence was observed in SW480 cells expressing GFP-fHsp27 (SW480-fHsp27) before and after a 42°C heat shock of 1 h duration. Ten different images of nonheat shocked cells and heat shocked cells were analyzed, using the Line Scan tool in Metamorph. The fluorescence intensity across each cell, in every field of view, was quantified with the Line Scan tool and categorized depending on its fluorescence intensity profile. Cells with profiles in the shape of an inverse bell curve were categorized as having GFP-fHsp27 in the cytoplasm only. Cells with a bell-shaped profile were categorized as possessing perinuclear localization of GFP-fHsp27, and cells with a relatively flat fluorescence intensity profile were categorized as having GFP-fHsp27 in the perinuclear region and cytoplasm. Prior to heat shock, GFP-fHsp27 was localized in the cytoplasm in 72% of the cell population, while only 2.8% of cells showed fluorescence in the perinuclear region (Fig. 1a, d). In 25.2% of cells, fluorescence was detected in the cytoplasm and perinuclear areas. In cells that received a 1-h heat shock, followed by a 3-h recovery period at 37°C, 52% displayed GFP-fHsp27 localization in the perinuclear region, and only 22% of cells showed cytoplasmic localization of GFP-fHsp27 only (Fig. 1b, d). Twenty-four hours after heat shock, 63% of the cells showed GFP-fHsp27 fluorescence in the cytoplasm, but not in the perinuclear region (Fig. 1c, d). Thus, SW480-fHsp27 cells show the characteristic response to heat shock, as has been observed previously in other cell types. This result supports the use of the GFP-fHsp27 construct as a marker for Hsp27 activity in SW480 cells.

SW480-fHsp27 cells are cytoprotected by 3 h after heat shock

Cytoprotection was verified in SW480-fHsp27 cells by challenging the cells with lethal heat stress at different times after heat shock. Cells were tested at 37°C, 42°C (conditioning heat stress), and 47°C (lethal heat stress). Cells were subjected to lethal heat stress at 3 and 24 h after preconditioning heat shock (Fig. 2). Heat shocked cells

Fig. 1 Translocation of GFP-fHsp27 due to heat shock treatment. SW480 cells stably transfected with GFP-fHsp27 were observed at $\times 20$ magnification (**a**) before heat shock. Prior to heat shock, cells showed mainly cytosolic localization of GFP-fHsp27. Three-hour recovery period at 37°C following heat shock at 42°C (**b**) shows a majority of the cells with GFP-fHsp27 in the perinuclear region. Twenty-four hours after recovery after a 1-h heat shock (**c**), cells show near equal

distribution in cytosol and perinuclear regions. Line graph tool was used on Metamorph to determine fluorescence intensity throughout the cell (**d**). Ten different fields of view were observed. Two hundred twenty-eight total cells were counted. Seventy-eight total cells were analyzed before heat shock, 71 total cells were analyzed 3 h after heat shock, and 79 total cells were analyzed after 24 h after heat shock. Two independent experiments were done. Scale bar = $30\ \mu\text{m}$

that were cytoprotected, but not exposed to lethal heat shock, showed a 94.7% cell survival rate, whereas only 4% of cells exposed to a lethal heat dose survived. However, 37.9% of cells survived a lethal heat challenge, 3 h after preconditioning heat shock (Fig. 2a). A similar result was obtained from the cytoprotection assay performed 24 h after heat shock. A 76.9% cell survival rate was obtained following a preconditioning heat shock at 42°C , but only 2.8% of cells survived a lethal heat challenge. In contrast, when cells were given a preconditioning heat shock, prior to a lethal heat challenge, 37.9% of cells survived (Fig. 2b). These results show that SW480-fHsp27 cells are cytoprotected by 3 h after conditioning heat shock.

Heat shock decreases wound closure rates in WT and SW480-fHsp27 cells

Cell lysates were collected roughly 48 h into the wound closure to detect levels of Hsp27 present. Cells expressing GFP-fHsp27 did not have altered levels of human Hsp27 as determined by Western blot analysis (Fig. 3a). GFP-fHsp27 was detected at about 54 kDa on immunoblots using a GFP antibody, and the fusion protein was detected primarily in the cytoplasm both before and after heat shock. The distribution between cytosol and nuclear fractions was similar for GFP-fHsp27 and human Hsp27. We found that without heat shock, endogenous Hsp27 in WT cells, and GFP-fHsp27 in SW480-fHsp27 cells was expressed at

Fig. 2 Cytoprotection occurs by 3 h after heat shock in SW480-fHsp27 cells. SW480-fHsp27 cells were counted after receiving various heat treatments and being replated. Cells were maintained at 37°C, heat shocked at 42°C, challenged at 47°C, or preconditioned at 42°C and then received a 47°C challenge. **a** Cells receiving a heat challenge 3 h after preconditioning. **b** Cells receiving a heat challenge 24 h after preconditioning. Each temperature was tested in triplicate and counted using a hemocytometer. Statistically significant according to Student's *t* test: **P*<0.02

similar levels in cytosolic fractions. However, the levels of both forms of Hsp27 in the cytosol were reduced slightly, 24 h after heat shock. In order to evaluate possible effects of GFP-fHsp27 expression on cell movement, wound closure in SW480-fHsp27 cells was compared to WT cells (Fig. 3b). These results show that expression of GFP-fHsp27 does not affect movement of SW480-fHsp27 cells. This result, combined with perinuclear translocation shown above, indicates that GFP-fHsp27 is a useful marker for studying the role of Hsp27 in movement.

Hsp27 expression is required for wound closure in SW480 colon cancer cells

To further investigate the role of Hsp27 in cell movement and in the heat shock response, its expression was knocked down using RNAi Smartpool containing five Hsp27 oligomers (Table 1). To confirm that the expression of

Hsp27 was knocked down, SW480-fHsp27 cells were lysed and probed for Hsp27 expression levels using immunoblotting (Fig. 4a). The levels of Hsp27 in the cytosolic and nuclear fractions from cells transfected with Hsp27 RNAi were undetectable, compared to Hsp27 levels in nonspecific RNAi transfected cells, and WT cells (Fig. 4a).

Wound closure assays were done with SW480 knock down (SW480-kd) cells and compared with WT and mock-transfected (transfected with nonspecific siRNA oligomers) cells (Fig. 4b, d). The rate of wound closure in SW480-kd cells was dramatically reduced, such that only 20% of the wound had closed by 40 h, whereas in WT and mock-transfected cells, wound closure was completed by this time. To determine the effects of heat shock on wound closure rate, SW480-kd cells were heat shocked and allowed to recover at 37°C for 24 h, before wounding (Fig. 4c, e). Wild-type and mock-transfected cells were able to close their wounds, while wound closure in SW480-kd cells had progressed by only 20% after 40 h. The marked decrease in the rate of wound closure in SW480-kd cells clearly demonstrates that Hsp27 plays a critical role in cell movement. The following caveats have been considered: (1) Using a different siRNA pool (Rousseau et al. 2006) shows that siRNA knock down of Hsp27 impaired human cell migration. This result coupled with ours argues against off target effects by siRNA or nonspecific toxicity. (2) It has been shown that Hsp27 knock down inhibits cell spreading (Hirano et al. 2004). Therefore, since spreading is essential for movement, it is likely that this contributes to the reduced rate of wound closure in addition to the movement between cells. An additional experiment (not shown) in which the cell density of siRNA treated cultures at the time of wounding was the same as control cultures showed the same inhibitory effect on wound closure rates. (3) The possibility that siRNA treated cells cannot close the wound because they are nonviable is ruled out by the fact these cells are proliferative with approximately the same doubling time as control cells. (4) In some siRNA protocols, chemical inhibitors of DNA replication are used to remove the effects of cell proliferation on wound closure in vitro. Heat shock transiently inhibits cell proliferation. In Figs. 3 and 4, we showed that wound closure in heat shocked and nonheat shocked cell cultures had similar kinetics; therefore, cell proliferation did not contribute significantly to wound closure in our study.

GFP-fHsp27 and actin form a complex in vivo

In order to investigate whether GFP-fHsp27 and actin form a complex in vivo, an immunoprecipitation pull down assay was performed. GFP-fHsp27 was used as bait, and actin was prey in this assay. SW480-fHsp27 cells were lysed and incubated with GFP antibody and protein A sepharose

Fig. 3 Effect of GFP-fHsp27 expression on cell movement. SW480-fHsp27 and WT cell cultures were wounded and monitored at different time points after a 24-h recovery period from a 1-h heat shock at 42°C (a). Human endogenous Hsp27 expression levels, sampled at the end of wound closure at 48 h, were analyzed using Western blot. Mock-transfected cytoplasmic fraction, mock-transfected nuclear fraction, SW480-fHsp27 cytoplasmic fraction, and SW480-fHsp27 nuclear fraction are shown in lanes 1–4, respectively. Heat shocked WT cytoplasmic and nuclear fractions are shown in lanes 5 and 6,

respectively. Heat shocked SW480-fHsp27 cytoplasmic and nuclear fractions are shown in lanes 7 and 8, respectively (b). Without heat shock, WT cell cultures closed wounds at about 40 h after initial wounding. Following heat shock, WT and SW480-fhsp27 cells closed wounds by 48 h (c). *Graphs of wound widths* were measured from experiments in b and c, respectively (d–e). SEMs are shown for the average of three independent experiments. Each independent experiment was done in six well replicates for each cell type and heat stress condition. Scale bar=50 μm

beads, in order to pull down and collect complexes containing GFP-fHsp27 and actin. To determine whether changes in GFP-fHsp27–actin interaction occur due to heat shock, cell lysates were collected before heat shock and at 1, 3, and 24 h afterward. Lysates were probed for GFP-

fHsp27 and actin using immunoblotting. Western blots showed that GFP-fHsp27 and actin were part of the same complex in untreated and heat shocked cells, thus confirming that these two proteins are present in the same complex in vivo (Fig. 5).

Fig. 4 Hsp27 knockdown resulted in failure of wound closure. Wound closure in SW480-kd, mock-transfected cells, and WT cells were observed at several time points after 24-h recovery from heat shock, as previously described. Lysates were prepared after wound closure at 30 h. Western blot analysis of Hsp27 levels in SW480-kd, mock transfected, and WT cells are seen in **a**. Lanes 1 through 6 that are SW480 cells were not heat shocked. Mock-transfected cytoplasmic fraction, mock-transfected nuclear fraction, transfected cytoplasmic fraction treated with nonspecific RNAi oligomers, and transfected nonspecific RNAi oligomer nuclear fraction, siHSP27 cytoplasmic fraction, and siHsp27 nuclear fraction are shown in lanes 1–6, respectively. Lanes 7 through 12 show mock-transfected cytoplasmic fraction, mock-transfected nuclear fraction, transfected cytoplasmic fraction treated with nonspecific RNAi oligomers, and transfected nonspecific RNAi oligomer nuclear fraction, siHSP27 cytoplasmic fraction, and siHsp27 nuclear fraction, respectively, with heat shock

treatment. **b** Time points of SW480-kd, mock transfected, and WT cells closing wounds over time with no heat shock applied. **c** Wound closure in the same cell types as in (**b**) over time with heat shock applied. **d, e** Graphs of wound closure rates in cells from experiments performed in **b** and **c**, respectively. Wound widths were measured using Image J. SEMs are shown for the average of three independent experiments. In heat shocked and nonheat shocked experiments, cells with reduced Hsp27 levels were unable to close their wounds by 40 h. SEMs are shown for the average of three independent experiments. Note that wound closure rate for WT and mock-transfected cells appears the same in both untreated and heat shocked cells (compare **d** with **e**) and appears to be inconsistent with the data shown in Figs. 3d, e. This is due to the smaller initial wound widths in Fig. 4 and greater variability in wound healing rates that together may mask a relatively small reduction in wound healing rate resulting from heat shock. Scale bar=50 μ m

Fig. 5 GFP-Hsp27 interacted with actin biochemically. Two different 10% gels were run to detect GFP-Hsp27 and actin. GFP-Hsp27 was used as bait in the IP pull down assay that employed anti-GFP antibody and protein A beads (**a**). Lanes 1–2 show unstressed soluble and insoluble cell lysate fractions, lanes 3–4 show soluble and insoluble fractions 1 h after 42°C heat stress, lanes 5–6 show soluble and insoluble cell lysate fractions 3 h after heat stress, and lanes 7–8 soluble and insoluble cell lysate fractions 24 h after heat shock, respectively. Lane 9 is the negative control (protein A beads and lysate only). Actin levels were probed in the cell lysates from the pull down assay (**b**). Lanes were loaded in the same fashion as **a**. Three independent experiments were run

Before heat shock, GFP-Hsp27 levels were higher in the soluble fraction than the insoluble fraction (Fig. 5a). One hour after heat shock, levels of Hsp27 were similar in both the soluble and insoluble fractions. This was also true for cellular fractions collected 3 h after heat shock; however, the amount of Hsp27, in lanes 5 and 6, were reduced in both fractions. At 24 h after heat shock, Hsp27 in the cytosolic fraction was decreased, compared with cells, before heat shock. However, contrary to the result obtained before heat shock, the amount of Hsp27 was greater in the insoluble fraction than in the cytosolic fraction after 24 h. Interestingly, we detected an increase in the amount of actin in the insoluble fraction at all time points after heat shock, compared with preheat shock values consistent with stress fiber formation. These data show that Hsp27 is in the same complex as actin and that heat shock increases the ratio of Hsp27 relative to actin.

Hsp27 colocalizes with actin before and after heat shock

To observe the location of endogenous Hsp27 within the cell, CRL1807 cells—human nontumorigenic colonocyte cells—were immunostained for Hsp27 and actin (Fig. 6). CRL1807 cells were used for this study because they have a large, flat lamella in which Hsp27 and actin localization can be seen clearly. Prior to heat shock, Hsp27 colocalized with stress fibers and was also diffusely distributed in the cytoplasm (Fig. 6a–c). Three hours after heat shock, Hsp27

was found primarily in the nucleus and its colocalization with the actin cytoskeleton was reduced, even though actin stress fibers were not disrupted (Fig. 6d–f). The cytoplasmic distribution of Hsp27 was diffuse, yet granular in appearance (Fig. 6g), and colocalized with stress fibers (Fig. 6i). In addition, Hsp27 immunostaining revealed “chains” of Hsp27 granules that were not associated with stress fibers (white arrowheads, Fig. 6g) and which were not seen prior to heat shock. There appears to be a qualitative increase in the number and/or thickness of stress fibers (Fig. 6h) as compared to cells prior to heat shock (Fig. 6b). These results demonstrate the close association between Hsp27 and actin *in vivo* and that this is increased following heat shock.

Discussion

We have shown that GFP-Hsp27 expressed in SW480-Hsp27 cells is located in the cytoplasm in unstressed cells and translocates to the perinuclear region upon heat stress. Thus, GFP-Hsp27 can act as a marker for the heat shock response. In addition, we established that this marker does not alter endogenous levels of human Hsp27 in untreated and heat shocked cells, and it does not affect the rate of wound closure, compared with WT cells. When Hsp27 expression was knocked down, wound closure was drastically reduced, in both heat shocked and untreated cells, indicating a pivotal role for Hsp27 in regulating cell motility. Consistent with this, immunoprecipitation pull down assays and immunofluorescence studies showed that Hsp27 associates with actin *in vivo*. Furthermore, heat shock led to an increase in association of Hsp27 with actin. Our data suggest that in addition to its cytoprotective function in heat shocked cells, Hsp27 has two distinct roles in regulating the actin cytoskeleton and, consequently, cell movement, depending on whether the cell has been heat shocked or not.

The role of Hsp27 in the cellular response to heat shock is well documented and has been shown to involve translocation of Hsp27 from the cytoplasm to the nucleus/perinuclear region (Arrigo et al. 1988; Borrelli et al. 2002). Hsp27 migrates either to the nucleus or to the perinuclear region depending on the cell type (Lavoie et al. 1995). In the nucleus, Hsp27 was detected as well-defined punctate structures by immunogold electron microscopy (Arrigo et al. 1988). The function of these structures is not known but they are probably not involved in maintaining cytoskeletal structure. Using live-cell fluorescence imaging, we directly observed the cytosolic–perinuclear translocation of GFP-Hsp27 and had confirmed that this coincides with the onset of the cytoprotected state in SW480-Hsp27 cells (Fig. 2). In contrast to previous studies, we showed that the

Fig. 6 Colocalization of Hsp27 with actin. Endogenous Hsp27 was detected using primary Hsp27 antibodies and secondary FITC antibodies (**a, d, g**) in human colon CRL-1807 cells. Actin was detected using rhodamine phalloidin (**b, e, h**). Images were captured in separate channels and merged using Image J to analyze colocalization between

the two proteins (**c, f, i**). Cells were observed in unstressed conditions (**a–c**), 3 h after heat stress (**d–f**) and 24 h after heat stress (**g–i**). *White arrowheads (g) indicate Hsp27 chains*. All images were taken at $\times 400$ total magnification. *Scale bar*=15 μm

cytoprotected state is attained as early as 3 h postheat shock of SW480 cells, which have high basal levels of Hsp27, instead of 24 h, which is typical of other cell types. However, the levels of newly induced Hsp27 did not increase significantly by 3 h. We also noted that translocation of Hsp27 to the perinuclear region occurs by 3 h postheat shock and may contribute to cytoprotection as well.

A growing number of studies have shown that Hsp27 is involved in regulating actin filament dynamics and, consequently, cell movement. For example, Hsp27 has been found in actin-rich structures such as lamellipodia, filopodia, and membrane ruffles (Lavoie et al. 1995). A related protein, αB -crystallin, localizes to the leading edge of migrating lens epithelial cells (Maddala and Vasantha Rao 2005). Hsp27 has also been shown to increase the rate

of cancer cell motility (Lemieux et al. 1997; Rust et al. 1999). It has been suggested that the onset of cytoprotection coincides with increased stability of the actin cytoskeleton (Guay et al. 1997; Mounier and Arrigo 2002). These findings are supported by our observations that wound closure is slowed (Fig. 3), and the association of Hsp27 with the cytoskeleton is increased, following heat shock (Figs. 5 and 6). Although chaperone activity and stabilization of the cytoskeleton may both be involved in cytoprotection, our data suggest that they do not occur concurrently in SW480/Hsp27 cells, since the translocation of GFP-Hsp27 is apparent, before any increase in association of Hsp27 with the cytoskeleton is detected. Instead, we propose that the response to heat shock occurs in two phases. According to this idea, the first phase would involve the chaperone activity of Hsp27, leading to

cytoprotection by 3 h after heat shock. The second phase of the heat shock response would occur at ~24 h, as evidenced by the increase in number of stress fibers and the ratio of Hsp27 associated with actin.

The dramatic reduction in wound closure rate seen in Hsp27 knock down cells demonstrates the importance of Hsp27 in regulating cell motility, in both heat shocked and unstressed cells. A surprising aspect of this result is that a heat shock protein, i.e., Hsp27, is fundamental to the movement of unstressed cells. This raises two questions: What is the role of Hsp27 in regulating cell motility, and does this differ depending on whether the cell is responding to heat shock or not? We propose a model that addresses these questions, based on the assumption that the phosphorylated state of Hsp27 determines its function in regulating the actin cytoskeleton (Fig. 7).

In vitro studies have shown that Hsp27, in its unphosphorylated form, binds to the barbed end of actin filaments, and can thus inhibit the polymerization of F-actin (Miron et al. 1991; Benndorf et al. 1994). However, barbed-end capping of F-actin in vivo is required for maintaining a sufficiently large pool of G-actin to fuel the high rates of actin polymerization involved in the formation of protrusive activities, such as the formation of lamellipodia, filopodia, and phagocytic cups (Carlier 1998; Pollard and Borisy 2003). The importance of plus-end capping proteins is further underscored by the finding that a mutated actin capping protein expressed in *Drosophila*, results in embryonic lethality (Hopmann et al. 1996). In relation to our current study, there is evidence that Hsp27 caps the barbed ends of actin filaments in vivo (Pichon et al. 2004). We suggest that in unstressed cells, Hsp27 may serve as a barbed-end actin-capping protein and maintains a high rate of actin turnover, which is necessary for cell motility. The dramatic reduction in wound closure rate we observed in

SW480-kd cells is consistent with the loss of actin capping protein. In support of this idea, a recent study showed that the movement of glioblastoma cells was markedly reduced following Hsp27 knockdown (Nomura et al. 2007). Another indication that Hsp27 caps the barbed ends of F-actin comes from our observation that less Hsp27 is associated with the cytoskeleton in untreated cells compared with heat shocked cells (Figs. 5 and 6). The lower stoichiometry between actin and Hsp27 in unstressed cells is expected for a protein that binds to the ends, not the sides, of actin filaments. Our IP pull down data also point to an association between G-actin and Hsp27 because similar amounts of actin and Hsp27 are found in the cytosolic cell fractions. If so, it is possible that Hsp27 may act as an actin sequesterer, another activity known to be important for maintaining a large pool of G-actin, and which is required for a high rate of actin filament turnover (Pollard and Borisy 2003).

Previous work has shown that Hsp27 becomes phosphorylated, in response to heat shock, and its affinity for the plus end of F-actin is reduced (Benndorf et al. 1994). In addition, the phosphorylation of Hsp27 has been shown to cause disruption of large, multimeric complexes of Hsp27, and the formation of smaller, phosphorylated oligomers (Rogalla et al. 1999) that may bind to the sides of F-actin, thus stabilizing them (Mounier and Arrigo 2002). We suggest that phosphorylation of Hsp27, shortly after heat shock, leads to a transition from its role as a barbed-end capping protein, to one that associates with the sides of actin filaments, ~24 h after heat shock. Our data are consistent with this sequence of events. At 3 h after heat shock, we detected a decrease in the amount of actin-associated Hsp27, and we observed a period of increased lamellar dynamics in SW480-*f*Hsp27 cells responding to heat shock (supplementary movie AfterHS) compared with

Fig. 7 Diagram illustrating how the role of Hsp27 in regulating actin filament dynamics may be altered by phosphorylation, following heat shock

nonheat shocked SW480-/Hsp27 cells (supplementary movie BeforeHS). The uncapping of phosphorylated Hsp27 would increase the number of F-actin barbed ends available for polymerization and so could explain this increase in lamellar dynamics. The reduction in lamellar dynamics seen at 24 h after heat shock could be explained by an increase in F-actin stability. In support of this, we detected an increase in association of Hsp27 with actin (Fig. 5), enhanced stress fiber formation (Fig. 6), and a reduction in rate of wound closure (Fig. 3).

Herein, we show that Hsp27 has an essential role in cell movement in unstressed and heat shocked cells, during in vitro wound closure assays. It is further shown that Hsp27 and actin form complexes in SW480-/Hsp27 cells. Other investigators have proposed and have provided suggestive evidence that the phosphorylation state of Hsp27 may determine its role in regulating actin filament dynamics. Future studies of the effects Hsp27 phosphomimetics in our cell movement assays are planned.

Acknowledgments Many thanks to Dr. Robert Tanguay for providing Hsp27 antibodies, Dr. Charles Giardina for technical assistance for molecular biology assays, and to Dr. Adam Zweifach for Western blot imaging assistance. This work was supported by the National Science Foundation Grants MCB-0114231 and MCB-0724147 to J.L. This work was funded by the University of Connecticut Research Foundation (grant 447393 to L.E.H.).

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