Chapter 19
Heat Shock Alters Keratocyte Movement and Morphology: Exploring a Role for HSP27 (HSPB1)

Bindi M. Doshi, Lawrence E. Hightower, and Juliet Lee

Abstract HSP27 is essential for mammalian cell movement. To further explore the effects of heat shock and the mechanistic role of HSP27, we have initiated a study using a well-established model of rapidly moving cells, the fish keratocyte. Here we report that heat shock causes a decrease in cell speed. Since changes in cell morphology can drastically affect cell movement, we also monitored changes in cell morphology. Heat shock caused a decrease in the number of polar cells and an increase in those with one stuck adherent edge, indicating the occurrence of both cytoskeletal re-organization and increased adhesion to substrata. Analyses of HSP27 levels using Western blots showed they were relatively high in keratocytes prior to heat shock and remained high afterward. In contrast, Western blot analysis of HSP70 showed that it was induced strongly by heat shock, indicating that fish keratocytes mounted a robust heat shock response. Surprisingly, given the propensity of HSP27 to localize in nuclear/perinuclear regions following heat shock, the location of HSP27 in fish keratocytes was unchanged as shown by indirect immunostaining with anti-HSP27 antibodies. Fluorescence intensities of immunostained images of cells before and after heat shock were quantified using Image J software. The results of this analysis showed that fluorescence intensity decreased following heat shock, suggesting changes in HSP27 that affected antibody recognition. Possible roles for HSP27 in regulating actin filament dynamics, cell speed and morphology are discussed.

Keywords HSP27 • HSPB1 • Cell motility • Keratocytes • Heat shock • Cell morphology • Actin

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R.M. Tanguay, L.E. Hightower (eds.), *The Big Book on Small Heat Shock Proteins*, Heat Shock Proteins 8, DOI 10.1007/978-3-319-16077-1_19

19.1 Introduction

When we began our studies of human HSP27 (HSPB1), the most convincing evidence linking this protein to actin filament dynamics was its inhibition of actin polymerization in vitro (Miron et al. 1991; Benndorf et al. 1994). Our goal was to demonstrate this link in cells by studying the role of HSP27 in cell motility. We used a human colon cancer cell line and nontumorogenic colonocytes to show that HSP27 is critical for wound healing (Doshi et al. 2009). HSP27 levels were reduced using siRNA, resulting in loss of the cells' ability to close wounds in a cell culture-based assay. Immunoprecipitation pull-down assays were used to show that HSP27 and actin are in the same complex both before and after heat shock. Indirect immunostaining of cells was also done to evaluate co-localization of these two proteins. They showed co-localization before heat shock, little association by 3 h post heat shock, and increased association by 24 h after heat shock. Cells were still capable of movement when cytoprotection was established 3 h after heat shock.

Having established that HSP27 plays a vital role in regulating wound healing, we wished to study a motile cell type in which the effects of heat shock on cell speed and morphology can be measured readily. We chose fish epithelial keratocytes, because they are a well-studied model for fast moving cell types that the Lee laboratory has investigated in detail. In addition, we also have experience in analyses of the induction of HSP27 by heat in a fish hepatoma cell line (PLHC-1). HSPs 70, 30 and 27 have been characterized in these cells derived from the subtropical desert minnow species, *Peociliopsis lucida* (Norris et al. 1997). Using radioisotopic labeling, the heat shock temperature and the HSP27 induction time frame were determined. These results provided a starting point for determination of the heat shock response of the keratocytes derived from the tropical and subtropical species of Black Molly used for the experiments described herein.

The question remains why is HSP27 found with actin? It may regulate actin filament dynamics, and/or it may function as an actin binding protein providing structural support for the cytoskeleton. We sought to further investigate the function of HSP27 and actin interaction in keratocytes.

Keratocytes were obtained from Black Molly, *Molliniesia sphenops*, scales. Fish keratocytes are known for their fan-shaped lamella and tightly coordinated movement. As the leading lamella extends at the front, it pulls the cell body forward, and when the rear of the cell detaches, the entire cell can advance. Keratocyte movement is regulated by stretch-activated calcium channels (SACs) that promote rapid movement by allowing cells to sense and respond to increases in cytoskeletal tension (Lee et al. 1999). For example, when the cell rear is unable to detach, while the front is extending, cytoskeletal tension builds. When tension reaches a critical threshold level, SACs are activated, resulting in an increase in intracellular calcium and consequently release of the cell rear. After retraction at the rear of the cell, cytoskeletal tension drops and the cell is able to continue moving. Thus protrusion and retraction are tightly coordinated between the front and rear of the cell. Actin polymerization occurs at the front of the cell where various proteins are involved in regulating

filament dynamics. Their functions can include stabilizing filaments, capping or uncapping filaments, and severing filaments. It is not known exactly how actin filaments are regulated by HSP27 or how heat shock affects filament dynamics. It is possible that HSP27 may regulate filament dynamics, as suggested by previous findings of HSP27 association with actin (Clarke et al. 2013; Doshi et al. 2009; Graceffa 2011; Miron et al. 1991; Mounier and Arrigo 2002).

To begin characterizing the heat shock response in keratocytes, changes in speed and morphology were observed before and after heat shock using phase contrast microscopy. Indirect immunostaining was used to determine HSP27 localization before and after heat shock. Western blot analysis was used to measure changes in HSP70 and HSP27 protein expression levels following heat shock.

19.2 Results

19.2.1 Heat Shock Decreased Cell Speed

To establish the effect of heat shock on cell movement, keratocytes were imaged in a heated chamber using differential interference (DIC) microscopy before, during, and after a 1-h heat shock at temperatures ranging from 37 to 41 °C. The observations after heat shock were made 1 h and 24 h after the termination of the heat shock. The speed of individual cells was obtained from time-lapse movies using the MTrackJ tool in Image J software. The back edge of each cell in the field of view was tracked throughout the image series to obtain the x, y coordinates of the cell position over time, which were used to calculate speed. We found that cell speed decreased in cells receiving heat shock at either 37 °C or 39 °C (Fig. 19.1). At 37 °C, average cell speed decreased significantly after a 1-h recovery period following heat shock, and decreased further by 24 h post heat shock. At 39 °C, average cell speed was also significantly decreased by 1 h and 24 h after heat shock. Heat shock temperatures of 40 and 41 °C for 1 h resulted in cell death (data not shown).

Changes in cell morphology can drastically influence cell speed. For example, keratocytes move most rapidly when they exhibit a fan shape that is indicative of a high degree of polarity. However, cell speed is reduced if polarity is lost such as when one edge becomes stuck, or the cell develops two lamellae. Many keratocytes showed a change in cell morphology as a result of heat shock (Fig. 19.2). To determine how the decrease in cell speed is related to changes in cell morphology, heat shocked cells were sorted into categories; polar, bipolar, one stuck edge, apolar, and rounded cells (Fig. 19.2a). Apolar cells were identified as cells having a lamella surrounding the cell body whereas rounded cells entirely lacked a lamella. The number of polar cells decreased after either a 37 or 39 °C heat shock. After a 37 °C heat shock for 1 h, keratocytes showed a doubling in the percentage of bipolar cells, no change in the percentage of cells with one stuck edge, apolar and rounded cells, consistent with substantial cytoskeletal rearrangement (Fig. 19.2b). After a 39 °C

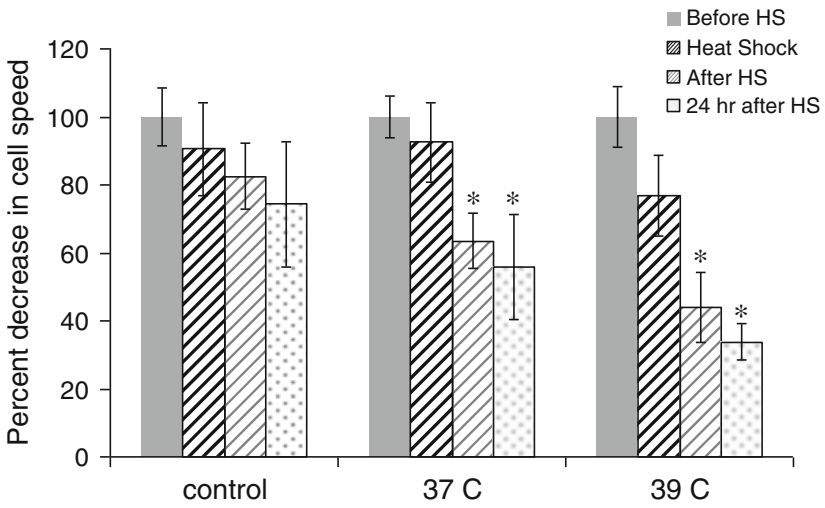

Fig. 19.1 Cell speed decreases in response to heat shock. Keratocyte average cell speed was measured before heat shock, during heat shock, 1 h after heat shock and 24 h after heat shock. Cells received a 1-h heat treatment at either 37 or 39 °C. Cells were maintained at room temperature at all other times. Control cells were monitored under the same conditions with the exclusion of a heat treatment. Individual cells were monitored and the results were averaged: Before HS (before heat shock) n = 19 for the 37 °C experiment and n = 34 for the 39 °C experiment; Heat Shock (during heat shock) n = 12 for 37 °C and n = 17 for 39 °C; After HS (1 h after heat shock) n = 12 at 37 °C and n = 11 at 39 °C; 24 h after HS (24 h after heat shock) n = 15 at 37 °C and n = 9 at 39 °C. Error bars denote standard error of the means. *Statistically significant at $P < 0.05$ (Student *t*-Test)

heat shock, the keratocyte population showed a greater percentage change in the same categories of cell morphology including a doubling of the number of cells with one stuck edge, indicating the occurrence of both cytoskeletal re-organization and increased adhesion strength (Fig. 19.2c).

19.2.2 HSP27 Localization Is Unaltered After Heat Shock

HSP27 is known for its translocation from the cytoplasmic region to the perinuclear/nuclear region after cells experience stress (Arrigo et al. 1988; Borrelli et al. 2002). As we observed previously (Doshi et al. 2009), SW480 colon cancer cells display HSP27 translocation to the perinuclear region as a result of heat shock. To establish whether HSP27 translocated to the perinuclear region in keratocytes following heat shock, indirect immunostaining was used to visualize HSP27 localization. Indirect immunostaining was done before and after heat shock, using primary HSP27 antibodies (Shelden laboratory) that were detected with a fluorescent secondary antibody. To monitor changes in cytoskeletal organization, fluorescent phalloidin was used to visualize actin (Fig. 19.3a). Immunofluorescence staining was performed on keratocytes prior to heat shock (Before 39 °C HS), immediately after receiving a 1-h heat shock (After 39 °C HS), and either 3 or 24 h after heat shock. Wide-field fluorescence microscopy was used to detect

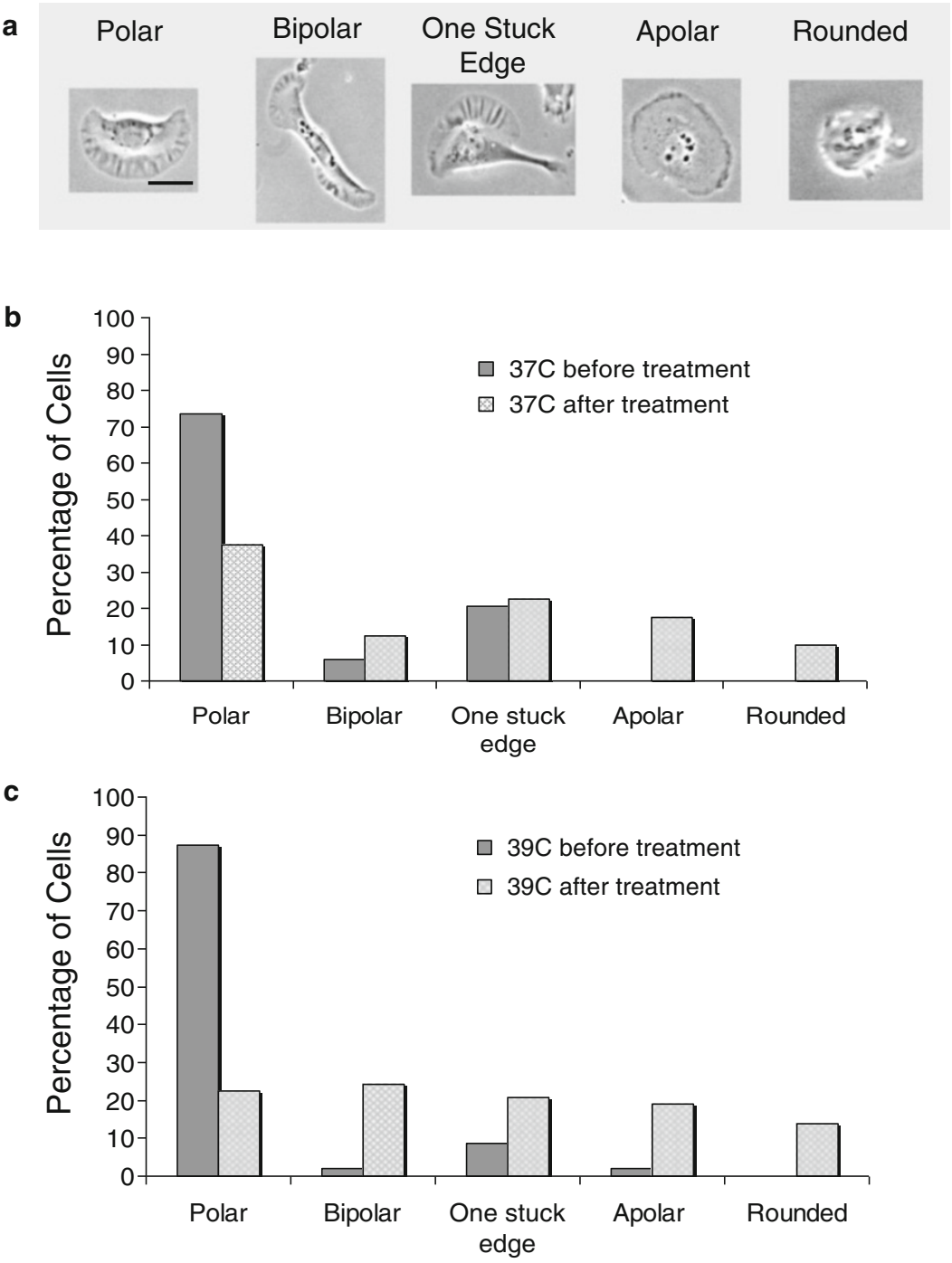

Fig. 19.2 Changes in cell morphology in response to heat shock. Individual keratocytes were classified into one of six different morphological categories (**a**). (**b**) Cell morphology before/after a 37 °C heat shock. Cell morphology was determined before (*dark grey bars*) and after (*light grey bars*) at 37 °C heat shock. Thirty-four cells were assessed before heat shock and 40 cells were assessed after a 39 °C heat shock. (**c**) Cell morphology before/after a 39 °C heat shock. Cell morphology was determined before (*dark grey bars*) and after (*light grey bars*) at 39 °C heat shock. Forty-seven cells were assessed before heat shock and 58 cells were assessed after a 39 °C heat shock

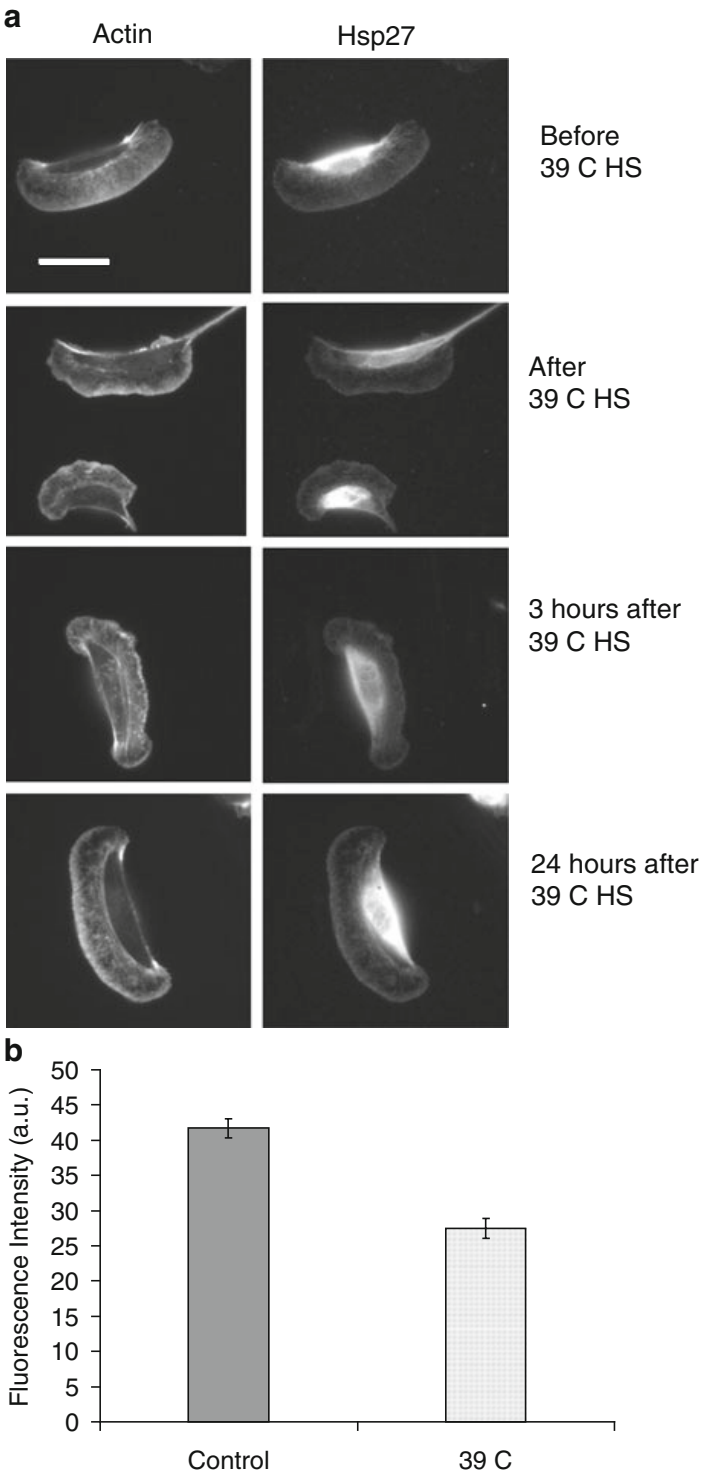

Fig. 19.3 Cellular location of HSP27 before and after heat shock. Keratocytes were fixed and stained for endogenous HSP27 using anti-HSP27 primary antibodies obtained from the Shelden laboratory and actin was detected using rhodamine-labeled phalloidin. Heat was applied at 39 °C for 1 h. (**a**) Images of representative unheated control cells fixed and subjected to indirect immunostaining (Before 39 °C HS, n = 33), cells stained immediately after heat shock (After 39 °C HS, n = 23), cells stained 3 h after heat shock (3 h after 39 °C HS, n = 31) and cells stained 24 h after heat shock (24 h after 39 °C HS, n = 20). Scale bar, 10 uM. (**b**) Fluorescence intensity levels derived from Image J software analysis of HSP27-stained cells shown in previous panel. The bar marked Control shows data obtained from cells without heating and the bar marked 39 °C shows data obtained from cells after a 1-h heat shock. The standard error of the means are shown

localization of HSP27. No change in HSP27 localization was observed between non-heat shocked and heat shocked cells. This result was confirmed using a primary HSP27 antibody from the Tanguay laboratory.

To quantify the amount of HSP27 immunostaining inside keratocytes, fluorescence intensity was measured using Image J Software. Fluorescence intensity of areas in cells stained with anti HSP-27 antibodies decreased after cells received heat shock (Fig. 19.3b).

19.2.3 HSP27 Expression Levels Were Unchanged Following Heat Shock

To detect changes in HSP70 and HSP27 expression levels before and after heat shock, protein levels were detected using Western blot analysis. HSP70 induction is a common characteristic of a heat shock response by virtually all organisms including fish (Norris et al. 1997). HSP70 protein expression levels were probed on immunoblots of SDS-PAGE gels of keratocyte lysates before and after heat shock (Fig. 19.4a). HSP70 levels increased compared to actin controls by 3 h after a 39 °C heat shock for 1 h, confirming that fish keratocytes could mount a heat shock response under our experimental conditions.

To determine whether HSP27 expression levels are affected by heat shock, immunoblots of cell lysates were made before and after heat shock and visualized using anti-HSP27 antibodies from either the Shelden or the Tanguay laboratory. No significant increases in HSP27 protein levels were detected 24 h after either a 37 or

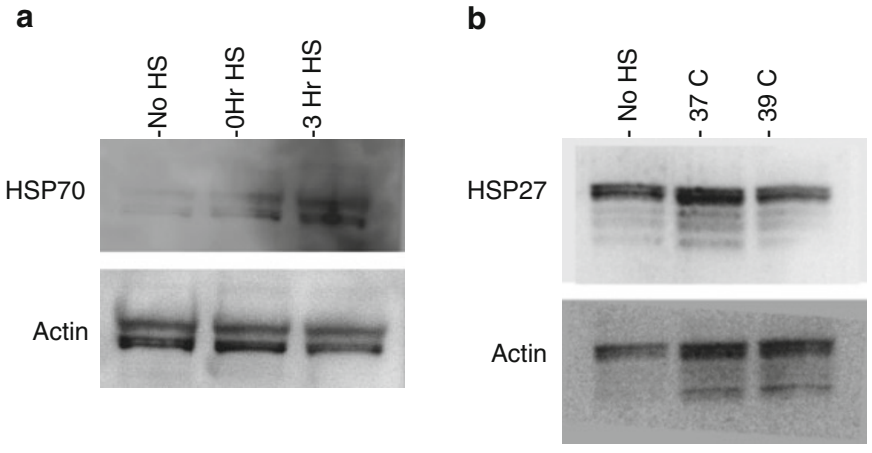

Fig. 19.4 Immunoblot analysis of HSP70 and HSP27 levels in keratocytes before and after heat shock. (**a**) Cell lysates were made from unheated control cells (No HS) and immediately after (0 h HS) or 3 h (3 h HS) after a 39 °C heat shock for 1 h. Proteins were separated by SDS-PAGE and the gels were processed by Western blotting using anti-actin and anti-HSP70 primary antibody. (**b**) Cell lysates were made from unheated control cells (No HS) and from cells 3 h after heating for 1 h at either 37 or 39 °C. The lysates were processed by Western blotting using anti-actin and anti-HSP27 (Shelden antibody)

39 °C heat treatment compared to actin controls (Fig. 19.4b). To assess whether HSP27 induction was occurring at shorter times post heat shock, protein extracts were taken before 1 h of heat shock at both temperatures, immediately after a 1-h heat shock, 3, 5 and 9 h post heat shock (data not shown). None of the lysates showed increased levels of HSP27 relative to actin controls regardless of which primary anti-HSP27 antibody was used. Immunoblots made from lysates of SW480 colon cancer cells were probed for HSP27 to verify that the antibodies we used could recognize keratocyte HSP27. Western blots showed that HSP27 levels increased after heat shock in SW480 cells, confirming that the antibody recognized HSP27 (data not shown). Since the amount of keratocyte HSP27 was high prior to heat shock and unchanged afterward, these results support the idea that keratocytes may express maximum levels of HSP27 at normal culture temperatures of 22 °C.

19.3 Discussion

HSP27 associates with actin in human cells in culture, indicating that it may be involved in actin filament dynamics (Doshi et al. 2009). It is often stated that HSP27 (HSPB1) is an actin filament barbed-end capping protein in vitro (Guay et al. 1997; Miron et al. 1991). This early work used the murine and avian HSP25 and numerous models of in vivo interactions between human HSP27 and actin filaments often have been based on studies of HSP25. More recent studies by Graceffa and colleagues using HSP27 (Graceffa 2011) and employing analytical ultracentrifugation, fluorescence spectroscopy and electron microscopy (EM) support the conclusion that HSP27 is a weak actin filament (F-actin) side-binding protein in vitro. Additional evidence from this study indicates that HSP27 binds F-actin as a monomer. Other investigators studying the effect of HSP27 on actin polymerization have concluded that it is probably not a barbed-end capping protein (During et al. 2007). They considered HSP27 to be an actin monomer sequestering protein. Graceffa stated in his paper that his work did not rule out the possibility that HSP27 is a barbed-end capping protein but rather it added support to the idea that HSP27 is also an F-actin side-binding protein. In this regard, we found the EM study reported by Graceffa quite interesting in that addition of HSP27 to F-actin did not result in dissociation of the actin filaments, which prompted him to conclude that HSP27 is not a strong sequesterer of actin monomers. It is important to note that the fluorescently tagged forms of HSP27 used in the Graceffa studies did not involve modification of the termini of HSP27, and so these are unlikely to interfere with actin binding. This possibility is supported by the recent finding that the attachment of fluorescent proteins to either the N-terminal or C-terminal ends of HSP27 and several other small HSPs alters their oligomeric structures, their chaperone-like activities and their ability to form hetero-oligomers (Datskevich and Gusev 2013).

A recent cell-based study (Clarke et al. 2013) may be more pertinent to our experiments with keratocytes. PC12 cells, a neuroendocrine rat pheochromocytoma line that expresses HSPB1 endogenously, was used. Association between

HSPB1 and F-actin was investigated using pull-down assays in conjunction with immunocytochemistry, confocal microscopy and Western blotting. The authors showed that F-actin is complexed with both phosphorylated and non-phosphorylated HSPB1 and that this association increases upon heat shock. Even though the details of this response are not known, it may account for the effects of heat shock on keratocytes.

The effects of heat shock on keratocyte movement were investigated to determine whether alterations in motility are related to HSP27 expression (Fig. 19.5a). HSP27 protein expression levels remained unchanged after heat shock, indicating that keratocyte HSP27 did not show the characteristic heat shock response common to many other cell lines. Cell speed decreased in response to heat shock and cell morphology was also affected. Keratocyte morphology changed from a polarized fan shape before heat shock, to either bipolar, apolar, rounded or having a stuck edge, after heat shock. Unlike many other cell types, fish keratocyte HSP27 did not translocate to either the nucleus or perinuclear region after heat shock. However, immunofluorescence staining for HSP27 decreased. This, together with the fact that HSP27 expression levels are unchanged by heat shock, indicates that HSP27 was less accessible to the primary antibody due to either a change in conformation or association state. This possibility is supported by evidence that HSP27-actin binding strength increases in response to heat shock. Given the abundance of actin filament networks in keratocytes, it is possible that HSP27 becomes tightly associated with these networks and unavailable for translocation to the nucleus as well as less accessible to the primary antibody.

Heat shock caused a significant decrease in keratocyte cell speed, which gave a strong indication that actin filament dynamics was altered. This may be due to the interaction of HSP27 with actin filaments. For example, if HSP27 acts as a plus end actin capping protein in vivo, it might promote a high rate of movement by maintaining a large pool of actin monomers in an unstressed cell. However, significant uncapping of HSP27 would decrease cell speed due to the depletion of actin monomers, as might be expected in a cell experiencing heat shock. HSP27 may also stabilize actin filaments during stress by binding to their sides. For example, fibroblasts over-expressing HSP27 were able to adhere better to collagen matrix than control or under-expressing HSP27 cells (Hirano et al. 2004). While its exact function in actin filament dynamics is unclear, numerous studies have shown that HSP27 is essential for cell migration. This has been demonstrated by studies where RNAi mediated reduction of HSP27 levels decreased migration regardless of cell type (Doshi et al. 2009; Garcia-Arguinzonis et al. 2010; Golembieski et al. 2008; Nomura et al. 2007).

The changes in keratocyte morphology and speed that occurred after heat shock suggest a change in actin filament dynamics. For example, some keratocytes appeared to become more firmly attached to the substratum, as indicated by cells possessing one stuck edge that is unable to retract. In addition, many cells lost polarity, becoming bipolar or apolar. Evidence suggests that the activity of mitogen-activated protein kinase (MAPK) might be involved in these changes, because MAPK phosphorylates HSP27 in response to stress (Stokoe et al. 1992), which is a major determinant of HSP27 function (Fig. 19.5b). Un-phosphorylated HSP27

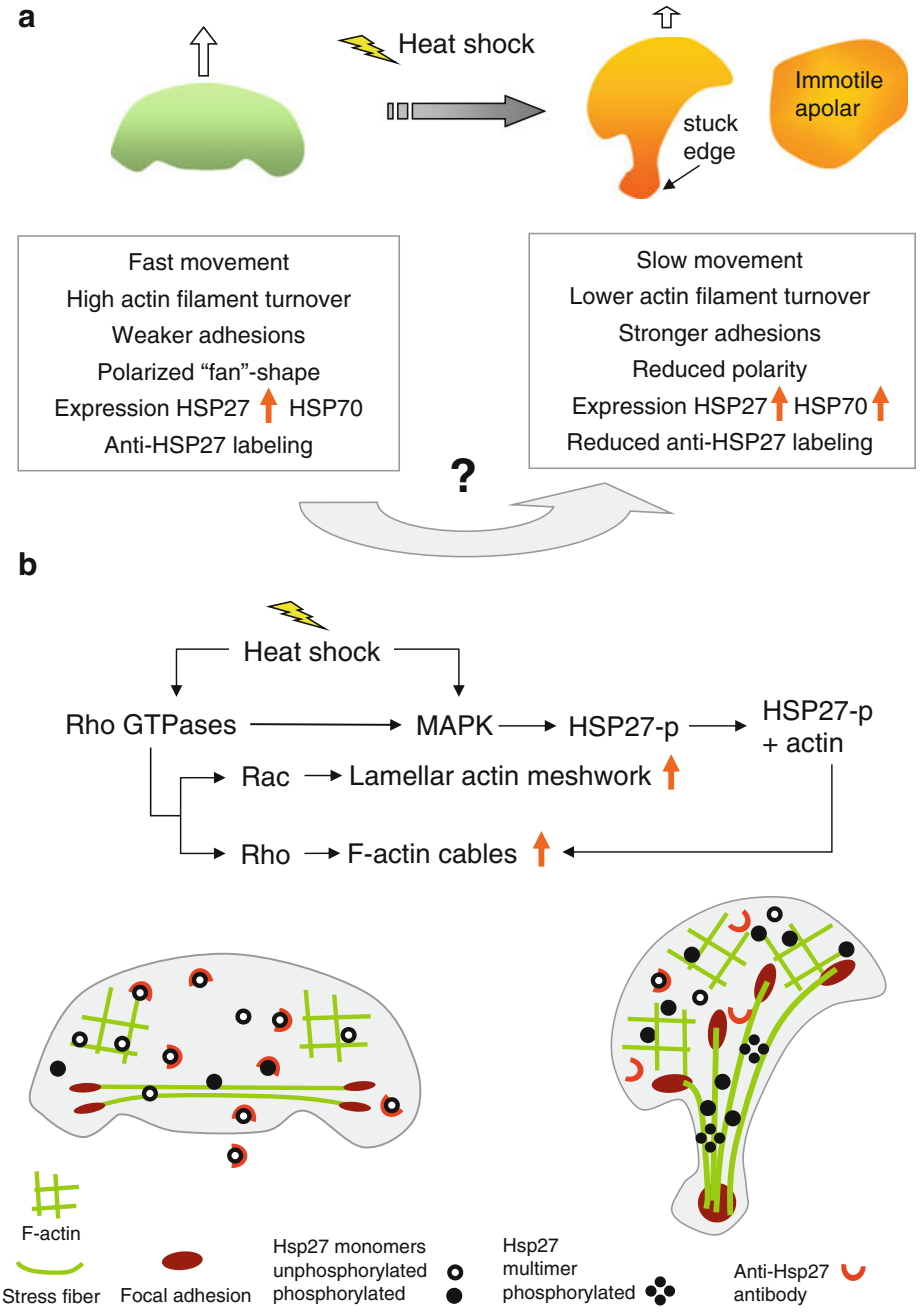

Fig. 19.5 Summary of effects of heat shock on keratocyte morphology and movement. (**a**) Diagram based on a summary of experimental results. (**b**) Diagram based on hypotheses derived from our experiments and current literature

cannot initiate new filament growth, and it can inhibit actin polymerization by behaving as an actin plus end capping protein (Benndorf et al. 1994; Miron et al. 1991). However, phosphorylated monomers and non-phosphorylated multimers permit actin polymerization in vitro (Benndorf et al. 1994).

A recent interesting paper (Stöhr and Hüttelmaier 2012) reported that IGF2 mRNA binding protein 1 (IGF2BP1), an oncofetal RNA-binding protein, is an

oncogenic factor that regulates adhesion, migration and ultimately invasiveness of tumor cells through intracellular signaling pathways. Evidence was presented that IGF2BP1 interferes with phosphorylation of HSP27 directed by MAPK-activated protein kinase 5 (MK5). This protein kinase is also known as P38-regulated/activated protein kinase. This occurs indirectly through the facilitation of inhibition of MAPK4 mRNA translation by IGF2BP1. It is proposed that these alterations limit the ability of phosphorylated HSP27 to sequester G-actin, which in turn enhances cell adhesion and increases the speed of tumor cell migration.

Activation of the Rho GTPases has been shown to increase MAP kinase activity in response to stress (Du et al. 2010; Lee et al. 2001) and may also induce changes in keratocyte cell morphology. This is believed to occur through the activation of PAK (p21-activated kinase) which can activate the p38MAP kinase pathway together with Cdc42 and Rac. Increased Rac activity is consistent with our observation of apolar cells with large, spread lamellae, whereas the activation of Rho might explain why some keratocytes appear stuck at one edge, since this GTPase will increase stress fiber and adhesion formation. Increased levels of phosphorylated HSP27 might also contribute to this phenotype by stabilizing actin filaments, and indirectly decreasing adhesion turnover, which could reduce cell speed.

Translocation of HSP27 to the nucleus or to perinuclear regions after a heat shock has become a hallmark of the heat shock response in many different cell types (Arrigo et al. 1988; Ehrnsperger et al. 1999). Often, cytoskeletal networks collapse into the perinuclear region as well (Mounier and Arrigo 2002). We observed that in fish keratocytes the cytoskeletal structure remained stable after heat shock as observed by visualization of actin stress fibers. Under these circumstances, it is possible that cytoplasmic HSP27 becomes fully associated with actin stress fibers to stabilize them during heat shock. Consequently, these HSP27 molecules would not have been available to translocate, and no perinuclear localization of HSP27 due to a collapse of the filament network would have occurred. The decrease in fluorescence intensity of cytoplasmic HSP27 that is observed in keratocytes following heat shock may have been another manifestation of this increased association with the actin cytoskeleton. This is because the antibody binding site(s) on HSP27 may have been shielded by interaction with the actin filament network or conformational changes in HSP27 bound to these filaments may have reduced its affinity for the primary antibodies. Another possibility is that oligomerization of HSP27 following heat shock may have reduced antibody affinity due to conformational changes and shielding of antibody binding sites.

We observed that keratocytes have an elevated level of HSP27 without heat shock, when compared with the minimal levels of HSP27 expression seen in PLHC (*P. lucida* liver cells) without heat shock. Possibly since HSP27 is already upregulated in keratocytes prior to heat shock, HSP27 expression levels may not be able to increase further in response to heat shock. HSP27 present in high amounts in keratocytes and low levels in PLHC cells further exemplifies how regulation of this protein varies among cell types.

Fish have evolved a particularly rapid wound healing response that allows them to quickly repair skin lesions. According to our hypothesis, fish keratocytes would

not require several hours of stress-induced gene expression in order to produce elevated levels of Hsp27 and to achieve maximum changes in cell morphology. This would allow rapid movement of keratocytes into damaged areas of skin to achieve wound closure. Fish liver cells presumably do not require such a rapid response and consequently maintain relatively low basal levels of HSP27. Our analysis of the heat shock response in fish keratocytes indicates that it develops over a period of several hours, during which keratocytes reduce their speed and become more tightly adhered to the substratum. In addition, the dramatic changes in cell morphology are consistent with a change in actin filament dynamics, perhaps involving stabilization of the cytoskeleton. We hypothesize that similar changes may occur during a fish skin wound response after the keratocytes have moved rapidly into the damaged area to initiate wound repair.

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