Anthrax lethal toxin paralyzes actin-based motility by blocking Hsp27 phosphorylation

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Inhalation of anthrax causes fatal bacteremia, indicating a meager host immune response. We previously showed that anthrax lethal toxin (LT) paralyzes neutrophils, a major component of innate immunity. Here, we have found that LT also inhibits actin-based motility of the intracellular pathogen *Listeria monocytogenes*. LT inhibition of actin assembly is mediated by blockade of Hsp27 phosphorylation, and can be reproduced by treating cells with the p38 mitogen-activated protein (MAP) kinase inhibitor SB203580. Nonphosphorylated Hsp27 inhibits *Listeria* actin-based motility in cell extracts, and binds to and sequesters purified actin monomers. Phosphorylation of Hsp27 reverses these effects. RNA interference knock-down of Hsp27 blocks LT inhibition of *Listeria* actin-based motility. Rescue with wild-type Hsp27 accelerates *Listeria* speed in knockdown cells, whereas introduction of Hsp27 mutants incapable of phosphorylation or dephosphorylation causes slowing down. We propose that Hsp27 facilitates actin-based motility through a phosphorylation cycle that shuttles actin monomers to regions of new actin filament assembly. Our findings provide a previously unappreciated mechanism for LT virulence, and emphasize a central role for p38 MAP kinase-mediated phosphorylation of Hsp27 in actin-based motility and innate immunity.

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Introduction

Bacillus anthracis endospores are highly suited for a bioterrorist attack. The spores can be readily stored for decades, and are highly resistant to adverse conditions. Their diameter of 1–2 µm is the optimal size for inhalation into pulmonary alveoli, and upon entering the mediastinal lymph nodes, the spores germinate, and bacteria quickly multiply causing overwhelming and lethal bacteremia (Dixon *et al*, 1999). The anthrax exotoxin lethal factor (LF), edema factor (EF), and protective antigen (PA) are primarily responsible for virulence. PA serves to usher EF and LF into the host cell cytoplasm. As its name implies, anthrax LF combined with PA called lethal toxin (LT) is thought to be the major virulence factor responsible for impaired immunity, septic shock, and death in inhalation anthrax; however, the molecular mechanisms underlying these effects remain to be determined (Moayeri and Leppla, 2004). LF is known to be a zinc metalloprotease that specifically cleaves the amino-terminal 7 aa of mitogen-activated protein kinase kinases (MAPKKs or MEKs), blocking their ability to phosphorylate downstream substrates. LF cleaves and inactivates MEK 1–4, 6, and 7. The downstream effects of MEK inhibition are incompletely understood and anthrax investigators continue to explore how inhibition of MEKs affects LT's profound toxicity in mice (Moayeri and Leppla, 2004).

We have recently shown that LT at low concentrations (50 ng/ml) markedly impairs neutrophil chemotaxis, and that this chemotactic defect is associated with a marked reduction in neutrophil actin assembly (During *et al*, 2005). Understanding how LT paralyzes neutrophils promises to provide new therapeutic targets for treating acute anthrax infection and new methods for controlling acute inflammation. Therefore, we have explored the pathways underlying LT inhibition of actin-based motility, and now provide *in vivo* and *in vitro* evidence that blockade of Hsp27 phosphorylation is primarily responsible for the impairment of actin assembly. Furthermore, we have proven that Hsp27 primarily impairs actin assembly by sequestering actin monomers, and does not cap the barbed ends of actin filaments, as reported previously (Benndorf *et al*, 1994). We propose that the phosphorylation–dephosphorylation cycling of Hsp27 shuttles actin monomers to regions of new actin filament assembly. Thus, in addition to uncovering a hitherto unsuspected pathway by which anthrax LT can impair innate immunity, these studies point to a potential role for the p38 MAP kinase pathway and Hsp27 in regulating actin-based motility in nonmuscle cells.

Results

Proteomic and Western blot analysis of LT-treated HeLa cells and human neutrophils

To identify the protein target or targets of LT, we treated both human neutrophils and HeLa cells with LT, and subjected cell

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Figure 1 Western blot analysis showing the effects of LT on the phosphorylation of Hsp27 in HeLa cells (A–C) and human neutrophils (D–E). (A) HeLa cell extract subjected to SDS–PAGE followed by western blot analysis using antibodies that recognized total and P82 phosphorylated Hsp27. Cells were treated with 1000 ng/ml LT for 12 h. A total of 50 µg protein extract was loaded in each lane. (B) HeLa cell extract subjected to two-dimensional gel electrophoresis before and after treatment with LT (1000 ng/ml × 12 h) and followed by western blot analysis as described in (A). Letters identify the various phosphorylated isoforms of Hsp27, d being the most phosphorylated form (most acidic isoelectric point) and a being the least phosphorylated form. Note the near complete absence of the d isoform in LT-treated cells. A total of 600 µg of protein was loaded for each two-dimensional gel. Arrow points to missing polypeptide seen in Supplementary Figure S1 in LT-treated cells. (C) Effects of concentration and incubation time on LT inhibition of Hsp27 phosphorylation. Western blots were performed as in (A). For the different concentrations of LT, the incubation time was 12 h. For the time course, cells were exposed to 1000 ng/ml of LT. A total of 50 µg protein extract was loaded per lane. (D) Neutrophil extract subjected to SDS–PAGE. A polyclonal anti-total Hsp27 antibody was used. Anti-p82 Hsp27 failed to crossreact with Hsp27 in human neutrophil extracts subjected to SDS denaturation. (E) Neutrophil extract subjected to two-dimensional gel electrophoresis exactly as described in (B). Note the marked reduction in the most phosphorylated (d) isoform of Hsp27 in LT-treated cells. Arrow points to the main phosphorylation isoform missing in LT-treated cells.

extracts to two-dimensional gel electrophoresis. The resulting polypeptide profiles were compared to cells treated with buffer alone. In addition to neutrophils, we chose HeLa cells, because we have found that LT also blocks actin-based motility in this cell type (see below). In HeLa cells, a polypeptide with an isoelectric point of 5.3 and molecular weight of 27 kDa was found to be markedly reduced in LT-treated cells (four of four separate experiments, Supplementary Figure S1). The polypeptide was identified by MALDI-TOF to be the small heat-shock protein Hsp27, a phosphoprotein known to serve as a protein chaperone, and to have actin-regulatory functions (Landry *et al*, 1989; Lavoie *et al*, 1993). Quantitative western blots revealed that Hsp27 represented 0.4% of the total protein in HeLa cell extracts and 0.07% of the total protein in human neutrophil extracts (see Materials and methods). Western blots of HeLa extracts subjected to one-dimensional SDS–PAGE probed with an antibody that recognized total Hsp27 revealed similar concentrations of total Hsp27 in LT-treated and control cells, indicating that Hsp27 was not degraded by toxin treatment. However, analysis of the same sample using a specific anti-phospho-Ser 82 Hsp27 antibody revealed a crossreactive protein in control but not in LT-treated cell extracts (Figure 1A).

To further verify our one-dimensional electrophoresis findings, western blots of two-dimensional gels were analyzed (Figure 1B). Anti-total Hsp27 antibody revealed four isoforms (Figure 1B, spots a–d). In LT-treated cells, the relative content

of the most acidic isoform (most highly phosphorylated) was reduced by >4-fold (spot d) as compared to control samples (Supplementary Table S1), whereas the most alkaline isoform (least phosphorylated) was increased by >3.5 times (spot a). As observed in one-dimensional analysis, the sum of the intensities was nearly identical. Analysis of the same samples using the antiphospho-Ser 82 Hsp27 antibody detected three isoforms (Supplementary Figure S2) and LT reduced the most acidic isoform by >6-fold (spot d) and the third most acidic isoform by >2-fold (spot b). Similar results were obtained in LT-treated neutrophils (Figure 1D and E and Supplementary Table S1). We also assessed the concentration and time dependence of LT-mediated blockade of Hsp27 phosphorylation in HeLa cells (Figure 1C). Minimal differences in inhibition were observed within a concentration range of 50–1000 ng/ml of LT. Reduction in Hsp27 phosphorylation was time dependent, near complete blockade being observed at 12 h.

Effects of LT on *Listeria monocytogenes* actin-based motility

We previously reported that LT blocked neutrophil chemotaxis, and reduced the peak rise in actin filament content by 50% (During *et al*, 2005). To further explore the effects of LT on nonmuscle cell actin assembly, we infected HeLa cells with *L. monocytogenes*. This intracellular bacterium is able to induce the directional assembly of host cell actin to form rocket tails (Dabiri *et al*, 1990). Actin polymerization

propels the bacterium through the cytoplasm, and the velocity of movement directly correlates with the rate of actin assembly (Sanger *et al*, 1992; Theriot *et al*, 1992). Thus, *Listeria* can be used as a probe to assess *in vivo* actin assembly. Treatment with 50 ng/ml of LT reduced mean velocities by over 50% ($P < 0.0001$) (Figure 2B, far left graph, Supplementary Figure S3 and quick-time video, supplement). No further slowing down of velocity was observed by raising the concentration to 500 or 1000 ng/ml

(Figure 2B, middle graph). The slowing down of velocity was accompanied by a significant shortening of *Listeria* actin tail lengths (Figure 2A and Supplementary Figure S4). Identical experiments examining *Shigella* actin-based motility revealed that LT treatment had no significant effect on bacterial velocity, indicating these effects were not due to a nonspecific toxicity (Figure 2C), and that *Shigella* induces actin assembly by a pathway that is different from *Listeria*.

Investigation of the potential role of p38 MAP kinase in regulating actin assembly in HeLa cells and human neutrophils

Impairment of Hsp27 phosphorylation suggested that LT inhibition of actin-based motility may be mediated by the MEK 3/6, p38 MAP kinase pathway, the major signal-transduction pathway leading to Hsp27 phosphorylation. In support of this possibility, western blot analysis confirmed that LT blocked phosphorylation of p38 MAP kinase in both HeLa cells and human neutrophils (Figure 2E, upper panels). Maximum inhibition of p38 MAP kinase phosphorylation was observed at a final concentration of 50 ng/ml and within 6 h of initiation of LT treatment (Figure 1E, lower panel). To further investigate the role of p38 MAP kinase, HeLa cells were treated with the p38 MAP kinase inhibitor SB203580 (100 μ M). Within 30 min, *Listeria* actin-based motility was slowed down to the same extent as LT-treated cells (Figure 2B, left graph). However, this inhibitor had no effect on *Shigella* motility (Figure 2C). These effects were accompanied by blockade of Hsp27 phosphorylation (Supplementary Figure S5). Maximal reductions in *Listeria* velocity were observed at a concentration of $\geq 10 \mu$ M, and minimal reversal of inhibition was observed when SB203580 was removed from the media (Figure 2B, right graph). Treatment of human neutrophils with SB203580 (100 μ M) also reduced chemoattractant-associated actin assembly to the same extent as LT treatment (Figure 2D) (During *et al*, 2005).

Hsp27 localization in polarized human neutrophils

The same anti-Hsp27 antibodies used for western blot analysis were also used to localize total and p82 Hsp27 in neutrophils. Anti-total Hsp27 antibody localized to the lamellipodia and uropods of polarized neutrophils, as well as to the body of each cell, 63% (62/98) of cells showing distinct localization to the peripheral lamellipodia (Figure 3, control, top panels). The anti-p82 Hsp27 antibody staining pattern was distinctly different. A much lower percentage of cells showed staining in the peripheral lamellipodia or uropods (31%, 22/70), and the fainter punctate staining primarily concentrated in central regions of each cell (Figure 3, control, lower panels). Similar differences have been observed in

Figure 3 Immunolocalization of Hsp27 human neutrophils before and after LT treatment: phase and immunofluorescence micrographs of neutrophils exposed to 1 μ M FMLP, for 5 min and stained with anti-Hsp27 antibodies that recognized total Hsp27 and p82 Hsp27. LT-treated cells were incubated with 50 ng/ml of LT for 2 h. Arrows in the control images point to the direction of neutrophil polarity. Arrowheads in the LT-treated cells point to the most rounded cells. Note the marked reduction in p82 phospho-Hsp27 content in these cells (bottom right panel). See text. Scale bars = 10 μ m.

Figure 2 Effects of LT and the p38 inhibitor SB203580 on *in vivo* actin assembly. (A) Fluorescent micrographs of left: control HeLa cells infected with *Listeria* for 4 h stained with Alexa-488 phalloidin (left panel). Note the long actin filament tails. Right: LT-treated HeLa cells were first infected with *Listeria* for 1 h followed by 2 h incubation with 50 ng/ml LT (right panel). Cells were fixed and stained 4 h after initiation of infection. Note the shorter actin tails. Arrows mark the beginning and end of each tail. Scale bar = 10 μ m. (B) Left: bar graph comparing the mean velocities of intracellular *Listeria* in control (white bar), LT-treated (50 ng/ml, dark gray bar) and SB203580-treated (100 μ M, light gray bar) HeLa cells. Brackets show the s.e.m. of $n = 66$ –322 determinations. To allow comparisons on different days, mean velocities were divided by the mean control velocity for each experiment. The actual mean velocities were $0.066 \pm 0.04 \mu$ m/s control versus $0.028 \pm 0.02 \mu$ m/s for LT-treated cells and 0.096 ± 0.011 control versus 0.047 ± 0.002 for SB203580-treated cells, $P < 0.0001$. Cells were treated with LT as describe in (A) or with SB203580 for 15–30 min, and measurements made 4 h after initiation of infection. Middle: bar graph comparing the effects of increasing concentrations of LT on the velocity of *Listeria* actin-based motility. Same experimental conditions as above. Right: bar graph comparing the effects of increasing concentrations of SB203580 on *Listeria* actin-based motility. Black bar, velocity 30 min after washing out (WO) SB203580. Experimental conditions as described above. (C) Effects of LT (50 ng/ml) and SB203580 (100 μ M) on *Shigella* actin-based motility. Cells were treated with LT and SB203580 as described in (B). Measurements were made 2 h after initiation of infection. (D) Graph comparing the rate and extent of f-met-leu-phe-stimulated actin assembly in control (open circles) and SB203580-treated (100 μ M) (closed circles) neutrophils. Cells were stimulated with 1 μ M FMLP at time 0, fixed with formalin at the times depicted, permeabilized with Triton and stained with Alexa 488, followed by FACS analysis of 5000–10 000 cells at each time point as described previously (During *et al*. 2005). (E) LT effects on p38 phosphorylation in HeLa cells. Upper left panel: HeLa cell extract subjected to western blot analysis before and after treatment with LT (1000 ng/ml $\times$ 12 h). Anti-total and phospho-p38 kinase antibodies were used. Protein load is 50 μ g for all three panels. Lower left panel: time course and concentration dependence of LT-mediated reduction in p38 MAP kinase phosphorylation. For time-course experiments, HeLa cells were treated with 1000 ng/ml of LT. For concentration experiments, HeLa cells were incubated with LT for 12 h. Upper right panel: neutrophil extract subjected to one-dimensional SDS-PAGE and western blot analysis as in HeLa cells.

smooth muscles cells, suggesting that phosphorylation of Hsp27 is likely to regulate actin-based motility in many cell types (Pichon *et al*, 2004). When cells were treated with LT (50 ng/ml), as observed previously (During *et al*, 2005), cells were rounded and less polarized. The mean two-dimensional area of cells treated with LT ($91 \pm 3 \mu\text{m}^2$, $n = 201$) was significantly smaller ($P < 0.0001$) than control cells ($114 \pm 3 \mu\text{m}^2$, $n = 194$). As observed for control cells, total Hsp27 was found throughout the cells, including the most peripheral cytoplasm (Figure 3, LT treated, upper panels). The content of phosphorylated Hsp27, as assessed by fluorescence intensity, was reduced by LT treatment. The average relative intensity of anti-p82 Hsp27 antibody-stained cells was significantly lower in LT-treated neutrophils as compared to controls (LT 833 ± 37 relative intensity units, $n = 92$ versus control 1130 ± 35 s.e.m., $n = 96$, $P = 0.0008$). As seen in control cells, phosphoprotein remained localized in the central regions of the cell (Figure 3, LT treated, lower panels). Immunofluorescence was also performed on *Listeria*-infected HeLa cells. However, because of the high concentrations of Hsp27 throughout cytoplasm of HeLa cells, excessive background fluorescence made clear localization of Hsp27 problematic. However, we had the impression that anti-total Hsp27 antibody concentrated in the *Listeria* actin filament tails (Supplementary Figure S6, upper panels), whereas anti-p82 Hsp27 antibody did not (Supplementary Figure S6, bottom panels).

Effects of changes in Hsp27 cellular content on *Listeria* actin-based motility

As final *in vivo* proof that Hsp27 was a primary mediator of LT-induced impairment of actin-based motility, we utilized RNA interference (RNAi) to lower Hsp27 levels by over 50% in HeLa cells using three different RNAi constructs (Figure 4A). Western blot analysis revealed no significant change in the ratio of p82 Hsp27 to total Hsp27 in knockdown cells (Supplementary Table S2). *Listeria* intracellular velocities in knockdown cells were reduced by $\geq 50\%$ by each of the constructs, as compared to infected cells treated with random control RNAi (Figure 4A). LT (50 ng/ml) treatment of control cells subjected to random RNAi also reduced the mean *Listeria* velocity by $\geq 50\%$. However, LT treatment of Hsp27 knockdown cells had no significant effect on *Listeria* actin-based motility as compared to knockdown cells treated with buffer (Figure 4C).

We next sought to rescue knockdown cells with wild-type (WT) Hsp27, pseudo-nonphosphorylated Hsp27, created by substituting alanines for serine at amino acids 15 and 90 of hamster Hsp27 (called Hsp27AA), and pseudophosphorylated Hsp27, generated by substituting glutamate at these same locations (called Hsp27EE). As shown in Figure 4B, transfection with these hamster Hsp27 cDNA constructs resulted in protein expression in HeLa cells (Figure 4B, upper panels). WT Hsp27 transfection only minimally increased *Listeria* velocity as compared to cells transfected with the empty vector (EV) (Figure 4B, lower panel, compare EV to left WT bar). The Hsp27 content following transfection is variable; therefore, to better control this variable cells were also rescued by microinjecting purified recombinant WT Hsp27 into *Listeria*-infected knockdown cells ($8 \mu\text{M}$ needle concentration, estimated intracellular concentration $0.8 \mu\text{M}$). Introduction of WT Hsp27 significantly increased bacterial velocity (0.62 ± 0.03 relative velocity, s.e.m., $n = 126$) as

Figure 4 Effects of RNAi knockdown of Hsp27 and addition of Hsp27 WT and Hsp27AA and Hsp27EE on *Listeria* actin-based motility. (A) Western blot analysis of Hsp27 in HeLa cell extracts from untreated cells (control), cells treated with RNAi randomer (A) or specific antisense RNA constructs (#1–3), and bar graph comparing the velocities of *Listeria* in control and RNAi knockdown cells, 3–4 h after initiation of infection (brackets depict the s.e.m. of $n = 56$ –125). Methods were identical to Figures 1 and 2. (B) Western blot analysis of Hsp27 in HeLa cell extracts from untreated, RNAi knockdown cells (RNAi #3, labeled ko), RNAi knockdown cells transfected with EV, WT hamster Hsp27, Hsp27AA (AA) or Hsp27EE (EE). Specific antibodies directed against human Hsp27 and hamster Hsp27 were used to detect knockdown and rescue, respectively. Below is a bar graph quantifying the velocity of *Listeria* actin-based motility in control and Hsp27 knockdown cells rescued with the various constructs. For WT, AA and EE, two bars are shown. The left-hand bar shows the average velocity of cells rescued by transfection, and the right hand bar shows the average velocity of cells rescued by the microinjection of recombinant proteins (needle concentration $8 \mu\text{M}$ for all constructs, estimated cytoplasmic concentration $0.8 \mu\text{M}$). Brackets represent the s.e.m. of $n = 20$ –161. (C) Bar graph quantifying the velocity of *Listeria* actin-based motility in control and Hsp27 knockdown HeLa cells (RNAi #1) in the presence and absence of 50 ng/ml LT. Note that the addition of LT to Hsp27 knockdown cells did not add to the reduction in *Listeria* velocity associated with Hsp27 knockdown alone. (D) Effects of addition of increasing concentrations of either recombinant pseudophosphorylated Hsp27EE (open circles) or pseudo-unphosphorylated Hsp27AA (closed circles) to brain extract (10 mg/ml) containing *Listeria*. Following a 90-min incubation, the lengths of the *Listeria* actin tails were measured (see Supplementary Figure S7). The 0 point represents the mean lengths of *Listeria* tails in the absence of added exogenous Hsp27. Brackets represent the s.e.m. of $n = 40$ –50 determinations per concentration.

compared to EV (0.40 ± 0.02 , $n = 161$, $P = 0.03$), but did not increase mean velocity to control values (1.0 ± 0.07 , $n = 56$) (Figure 4B, compare EV to right WT bar). Identical transfection and microinjection experiments with Hsp27AA and Hsp27EE resulted in significant reductions in *Listeria* velocity as compared to cells transfected with EV ($P < 0.001$) (Figure 4B).

Brain extracts. *Listeria* are capable of inducing actin filament tail assembly in brain extracts (Laurent *et al*, 1999); therefore, we also examined the ability of purified nonphosphorylated (Hsp27AA) and pseudophosphorylated Hsp27 (Hsp27EE) to block *Listeria* actin tail formation. Hsp27AA and WT Hsp27 (data not shown) both caused a concentration-dependent reduction in actin tail length, whereas the Hsp27EE failed to inhibit *Listeria*-induced actin assembly (Figure 4D and Supplementary Figure S7).

Effects of purified recombinant native Hsp27, pseudophosphorylated and pseudo-nonphosphorylated Hsp27 mutants on purified actin assembly

In addition to examining Hsp27 function in cells and extracts, we examined the effects of recombinant Hsp27 proteins on purified pyrenyl actin assembly and disassembly (Kouyama and Mihashi, 1981).

Barbed-end capping assay. Previous investigators have suggested that unphosphorylated Hsp27 inhibits actin assembly by capping the barbed ends or fast-growing ends of actin filaments (Benndorf *et al*, 1994; Mounier and Arrigo, 2002). To explore this possibility, we preincubated increasing concentrations of WT Hsp27 with spectrin-4.1 actin nuclei that contain actin filaments with free barbed ends. As shown in Figure 5A, no significant change in the nucleation rate was observed (open circles). However, when Hsp27 was preincubated with the monomeric actin solution rather than with the spectrin-4.1 nuclei, concentration-dependent slowing down of actin assembly was observed (Figure 5A, closed circles). Extrapolation of the lower curve indicated that a concentration of 120 $\mu\text{g/ml}$ would be expected to completely block the assembly of our actin solution. Calculating the stoichiometry of this inhibition requires a knowledge of the native molecular weight of unphosphorylated Hsp27. We and others have found the unphosphorylated WT Hsp27 has stokes radius consistent with a globular 216 kDa protein or a homo-octomer, whereas phosphorylated Hsp27 or Hsp27 EE is a homodimer (Lambert *et al*, 1999; Theriault *et al*, 2004). Thus, 0.55 μM of WT Hsp27 is able to block the assembly of 0.92 μM actin (1 μM minus the critical concentration of 0.08 μM) consistent with one Hsp27 molecule sequestering 1–2 actin monomers with an estimated K_D of 0.25 μM .

Actin monomer sequestration assay. To explore the ability of Hsp27 to sequester actin monomers in more detail, we utilized gelsolin–actin nuclei. Gelsolin caps the barbed or fast-growing ends of actin filaments, leaving the pointed or slow-growing ends free. Disassembly assays using gelsolin-capped actin filaments proved that Hsp27 does not cap the pointed ends of actin filaments (Supplementary Figure S8). Therefore, any slowing of the assembly rate under these experimental conditions could only be explained by actin monomer sequestration. Pseudo-nonphosphorylated Hsp27AA (closed circles, solid line) and WT Hsp27 (closed squares, dashed line) markedly inhibited actin assembly (Figure 5B). Inhibition was concentration dependent, total inhibition of assembly being observed at a final concentration of 8–12 $\mu\text{g/ml}$ (40–55 nM) of Hsp27AA or WT Hsp27. The concentration of monomeric actin available for assembly in these experiments was 1.2 μM (total actin concentration

of 2 μM minus 0.8 μM the critical concentration of the free pointed ends of the gelsolin–actin nuclei). Therefore, one homo-octomer of nonphosphorylated Hsp27 was capable of sequestering 22–30 actin monomers and preventing them from being assembled into filaments. The estimated K_D for this complex was 20 nM. Pseudophosphorylated Hsp27EE failed to significantly slow the assembly rate of gelsolin-capped actin filaments (Figure 5B, open circles). These findings indicate that when the barbed ends of actin filaments are capped, unphosphorylated, but not phosphorylated, Hsp27 has a high affinity and capacity for binding and sequestering actin monomers. Consistent with this high-affinity interaction, we have found that Hsp27 co-associates with actin in HeLa cell extracts immunoprecipitated with an anti-actin antibody (Supplementary Figure S9).

To further explore the effects of phosphorylation on Hsp27 actin monomer sequestration, the monomer sequestering activity of WT Hsp27 (5 and 13 $\mu\text{g/ml}$) was studied by incubating with MAPKAP-2 (2.5 $\mu\text{g/ml}$) and then compared to WT Hsp27 incubated in buffer alone. Under the conditions of our experiments, MAPKAP-2 exposure resulted in phosphorylation of 45% of Hsp27 as assessed by western blot analysis (Figure 5E, inset) and eliminated all monomer sequestration activity (Figure 5E).

Critical concentration studies. Under steady-state conditions, actin filament ends constantly exchange with a fixed concentration of monomeric actin called the critical monomer concentration. This pool of unpolymerized actin would be expected to increase in the presence of a monomer sequestering protein. To test this possibility, increasing concentrations of monomeric actin were polymerized in the presence of 50 and 100 $\mu\text{g/ml}$ of WT Hsp27 and compared to actin polymerized in buffer alone. As shown in Figure 5C (left graph), the concentration of unpolymerized actin increased from 0.2 to 0.35 μM in the presence 50 $\mu\text{g/ml}$, and from 0.2 to 0.45 μM in the presence of 100 $\mu\text{g/ml}$ of Hsp27, consistent with actin monomer sequestration by Hsp27. Assuming unphosphorylated Hsp27 is an octomer, the stoichiometry of Hsp27:actin was approximately 1:1. Identical concentrations of Hsp27EE had not detectable effect on the critical concentration (Figure 5C, right graph).

Measurement of the disassembly rate of the barbed filament end. The critical concentration is primarily determined by the off-rate and on-rate of the barbed end (Korn, 1982). Our experiments with spectrin-4.1 nuclei indicated that Hsp27 does not directly affect the on-rate of the barbed end, but these growth experiments did not assess the effects of Hsp27 on the off-rate. Dilution of spectrin-4.1-nucleated actin filaments below the critical concentration of assembly (2 μM was diluted to 100 nM) in solutions containing WT Hsp27 or Hsp27AA (data not shown) resulted in a concentration-dependent slowing of the disassembly rate. The apparent K_i for both proteins was 0.32 $\mu\text{g/ml}$ (1.5 nM assuming a homo-octomer) (Figure 5D). Pseudophosphorylated Hsp27 (Hsp27EE) also slowed down the disassembly rate of the barbed end, but required somewhat higher concentrations as compared to Hsp27AA or WT to produce comparable slowing down (K_i = 0.64 $\mu\text{g/ml}$ or 11 nM, assuming a dimeric conformation) (Supplementary Figure S12).

Discussion

For over two decades, scientists have been trying to determine why inhalation anthrax is among the most fulminant and deadly of human infections. Genomic analysis has revealed that, as compared to its close phylogenetic neighbors *Bacillus cereus*, *B. anthracis* is unique in possessing genes that express lethal and edema toxin (Read *et al*, 2003). However, the contributions of anthrax toxins to the rapid onset of septic shock and death remain poorly understood. LF has been the primary focus for explaining the presumed paralysis of the innate immune system (Fukao, 2004). This zinc metalloprotease possesses specific domains for binding to and cleaving the amino-terminal kinase-binding site of MEKs (Montecucco *et al*, 2004). LF is known to inactivate MEK 1/2 blocking the ERK pathway, and this pathway

partially mediates cytokine production by dendritic cells (Agrawal *et al*, 2003) and is a key regulator of cell proliferation (Roux and Blenis, 2004). Although the ERK pathway may help to mediate the innate immune response, the p38 MAPK pathway is likely to play a more central role (Fukao, 2004), and LF is known to block this pathway via inhibition of MEK 3/6. The p38 MAPK pathway also mediates cytokine production in dendritic cells (Agrawal *et al*, 2003) and the discovery of specific inflammatory inhibitors is being pursued by pharmaceutical industry (Schieven, 2005). Investigators of anthrax pathogenesis have also emphasized the role of the p38 pathway in promoting macrophage survival, and LF blockade of this pathway has been shown to induce macrophage apoptosis in susceptible mouse strains (Park *et al*, 2002). In other rodents, macrophages are resistant to LF-induced apoptosis; however, these animals also quickly

succumb to treatment with LT, indicating that other mechanisms are likely to mediate the LT-induced sudden death (Moayeri and Leppla, 2004). We have focused on an additional mechanism for LT-mediated impairment of innate immunity, paralysis of neutrophil chemotaxis. Impairment of neutrophil actin-based motility is not accompanied by an acceleration of apoptosis (During *et al*, 2005), and resistance of neutrophils to LT-induced apoptosis has recently been confirmed (Crawford *et al*, 2006).

Using a proteomics approach, we have identified blockade of Hsp27 phosphorylation as the potential explanation for LT-mediated impairment of actin-based motility in both human neutrophils and the human-derived HeLa cells. We have shown that LT blocks both p38 MAP kinase and Hsp27 phosphorylation in these two cell types (Figures 1 and 2). Furthermore, we have compared the effects of LT to the p38 MAP kinase inhibitor SB203580 and found that this inhibitor mimics LT in its ability to block FMLP- and *Listeria*-induced actin assembly, providing strong evidence that the effects of LT on actin dynamics are primarily mediated by the p38 MAP kinase pathway (Figure 2B and D).

Hsp27 has been extensively studied for over two decades, and a large scientific community has been dedicated to studying heat-shock proteins. These investigators have suggested that in addition to serving as a protein chaperone, Hsp27 is an actin-regulatory protein (Mounier and Arrigo, 2002); however, the mechanisms by which Hsp27 regulates actin assembly are poorly understood. Previous investigators suggested that purified Hsp27 capped the barbed ends of actin filaments. However, the high concentrations of protein required to slow down actin assembly (0.4–1 μ M Hsp27 for 1 μ M monomeric actin) (Benndorf *et al*, 1994) raised concerns about the physiologic relevance of Hsp27 actin-regulatory function. We have used highly purified recombinant Hsp27 proteins to more completely characterize actin-regulatory function, and now show that Hsp27 is not a barbed end capping protein, but is primarily an actin monomer sequestering protein that functions at physiologic concentrations. We estimate that the cytoplasmic concentration of the unphosphorylated Hsp27 octomer is 0.75 μ M in HeLa cells

and 0.13 μ M in neutrophils. The dissociation constant for the Hsp27-monomeric actin complex is estimated to be 20 nM, well below its total cellular concentration. Stoichiometric analysis reveals that the Hsp27 octomer has a high binding capacity, one homo-octomer being capable of sequestering 22–30 actin monomers (Figure 5B). Most exciting, we find that phosphorylation of Hsp27 by MAPKAP-2 kinase eliminates actin monomer sequestering activity, as does generation of mutant pseudophosphorylated protein (Figure 5B and E). Furthermore, we have found that the ability to efficiently sequester actin monomers requires that the barbed ends of actin filaments be capped (compare Figure 5A to B). Our studies reveal that both unphosphorylated and pseudophosphorylated Hsp27 interact with the free barbed end, slowing the off-rate of actin monomers (Figure 5D and Supplementary Figure S10), but have no effect on the on-rate (Figure 5A). This condition would be expected to lower the K_D (off-rate/on-rate) of the barbed end for actin monomers and allow the barbed end to more readily attract actin monomers complexed to actin monomer sequestering proteins. This characteristic explains why previous investigators who studied the growth of actin filaments with free barbed ends demonstrated such weak inhibition of actin assembly by unphosphorylated Hsp27 (Benndorf *et al*, 1994).

How does Hsp27 function in the cell, and how does LT blockade of Hsp27 phosphorylation impair actin-based motility? Our RNAi knockdown and rescue studies provide insight into the *in vivo* function of Hsp27. Reduction in the concentration of this protein slows down actin-based motility, and the addition of LT to knockdown cells fails to cause additional inhibition (Figure 4C). These findings suggest that Hsp27 enhances new actin filament assembly, and that LT inhibition of actin-based motility is primarily mediated by altering Hsp27 *in vivo* function. We also find that, in the unphosphorylated state, Hsp27 concentrates in the lamellipodia of polarized human neutrophils (Figure 3), and may also concentrate in the actin filament tails of *Listeria* (Supplementary Figure S6). Thus, unphosphorylated Hsp27 is concentrated in regions where new actin assembly is occurring in the cell. When Hsp27 is phosphorylated, the protein no longer loca-

Figure 5 Effects of purified Hsp27 on purified pyrene-labeled actin assembly. (A) Effects of Hsp27 on spectrin-4.1 nucleation. Spectrin 4.1 nuclei (20 μ g/ml) were incubated with increasing concentrations of WT Hsp27 (open circles) and then added to a final concentration of 1 μ M pyrenyl actin. Minimal impairment of nucleation was observed. However, preincubation of the same concentration of G-actin with WT Hsp27 caused a concentration-dependent inhibition in the rate of actin assembly, after addition of spectrin 4.1 (closed circles). The 0 concentration points represent the rate of actin assembly in the absence of Hsp27 in each of the two experiments (for the actual kinetic curves, see Supplementary Figure S10A and B). (B) Effects of Hsp27 on the pointed end assembly rate of actin (a measure of actin monomer sequestration). The assembly rates of purified pyrenyl actin (2 μ M) nucleated by gelsolin-actin seeds (molar ratio 1:20 gelsolin to actin) were determined for each concentration of Hsp27EE (open circles), Hsp27AA (closed circles) as well as WT Hsp27 (closed squares). A concentration-dependent decrease in actin assembly was seen with Hsp27AA and WT Hsp27 with complete inhibition being observed at final concentrations of 8–12 μ g/ml. The 0 concentration points represent the rates of actin assembly in the absence of Hsp27 in each of the three experiments (for the actual kinetic curves, see Supplementary Figure S11A and B). (C) Effects of Hsp27 on the actin critical concentration. Left graph: the effects of unphosphorylated WT Hsp27. Increasing concentrations of monomeric pyrenyl actin were incubated with buffer alone (open circles), buffer containing 50 μ g/ml (closed circles) and 100 μ g/ml (closed squares) of Hsp27 followed by the addition of F-actin nuclei and a final concentration of 0.1 M KCl and 1 mM $MgCl_2$. The fluorescence intensity at steady state (1.5–2 h) was plotted versus actin concentration. Right graph: the effects of pseudophosphorylated Hsp27EE. The identical conditions described for WT Hsp27 were used. Closed circles represent 50 μ g of Hsp27EE and open circles 100 μ g/ml. (D) Effects of WT Hsp27 on the disassembly rate of actin filaments. A 2 μ M portion of spectrin-4.1 nucleated pyrenyl actin filaments was diluted to 100 nM in buffer containing increasing concentrations of Hsp27 (closed symbols, μ g/ml shown for each curve). Note the marked slowing down of actin assembly as compared to F-actin diluted in buffer alone (open circles). Identical curves were seen with pseudo-nonphosphorylated Hsp27AA (data not shown). (E) Effects of MAPKAP-2 phosphorylation of Hsp27 on actin monomer sequestration. Incubation of G-actin with WT Hsp27 (5 μ g/ml black solid squares and 13 μ g/ml black solid circles) reduced the actin assembly rate as compared to gelsolin nuclei incubated in buffer (open circles). Incubation of the same concentrations of Hsp27 with 2.5 μ g/ml of MAPKAP-2 for 30 min at 25°C eliminated this inhibition (gray circles represent 13 μ g/ml Hsp27 and gray squares 5 μ g/ml Hsp27). The identical conditions used in (B) were employed except that the initial monomeric actin concentration was 1 μ M. Inset shows western blots of Hsp27 protein using anti-total Hsp27 and anti-p82 Hsp27 antibodies after incubation with buffer or MAPKAP-2. Densitometry revealed that 45% of the total Hsp27 was phosphorylated by the kinase under the conditions of our experiment.

Figure 6 Schematic diagrams showing the basic components of the signal-transduction pathways for actin assembly, and showing the phosphorylation cycle for the shuttling of actin monomers by Hsp27 to regions of new actin assembly. (A) The signal-transduction pathways that allow FMLP receptor binding to activate the uncapping of actin filaments, Arp2/3 nucleation and the release of actin monomers by Hsp27. The first two pathways on the left-hand side have been emphasized. All three pathways are initiated by the heterotrimeric G-protein complex linked to the FMLP receptor. The far left pathway involves phosphoinositide kinases that generate PtdIns (4,5) and PtdIns (3,4,5) that inactivate the barbed end capping proteins gelsolin and CapZ, resulting in free barbed ends. The middle pathway is less well understood, but results in the activation of the GTPase Cdc 42, which in turn activates N-WASP, which binds to and activates Arp2/3 to nucleate the formation of new actin filaments. The far right pathway involves the activation of MAP kinase kinase kinases (MKKKs) to phosphorylate MAP kinase kinases including MAP kinase kinases 3 and 6 (MEK 3/6). These proteins then phosphorylate p38 MAP kinase that phosphorylates and activates MAPKAP kinase 2/3 to phosphorylate Hsp27 causing the release of sequestered actin monomers. LT cleaves the amino-terminus of MEK 3/6, preventing downstream phosphorylation of Hsp27. (B) Model of how Hsp27 phosphorylation and dephosphorylation could serve to shuttle actin monomers to sites of new actin filament assembly. LT blocks MEK 3/6 function and prevents Hsp27 phosphorylation. See text for details.

lizes to these regions (Figure 3). Previous work has revealed that FMLP receptor agonists activate the p38 MAPK pathway and increase the phosphorylation of Hsp27 in neutrophils (Zu *et al*, 1996). However, recent reviews of the signal-transduction pathways mediating actin-based cell motility have emphasized phosphoinositides and small G-proteins as the primary mediators of actin-regulatory protein function and have ignored Hsp27 (Cicchetti *et al*, 2002; Parent, 2004). The phosphoinositide and G-protein pathways would be expected to work in concert with the p38 MAPK/Hsp27-activation pathway (Figure 6A). In the resting cell, actin filaments are capped at their barbed ends and Hsp27 would be expected to maximally sequester actin monomers. Agonist stimulation would serve to uncapped actin filaments, activate Arp2/3 nucleation, and phosphorylate Hsp27 freeing sequestered actin monomers. These three events would serve to promote the assembly of new actin filaments.

We propose that, under normal conditions, Hsp27 facilitates actin-based motility through a phosphorylation cycle (Figure 6B). Unphosphorylated Hsp27 with bound actin monomers concentrates at the leading edge of motile cells, where upon chemoattractant stimulation, the p38 MAPK signal-transduction cascade induces Hsp27 phosphorylation, releasing actin monomers for new actin filament assembly. Phosphorylated Hsp27 then moves toward the center of the cell, where it is dephosphorylated, again binds actin monomers, and then shuttles back to the leading edge. LT blocks the Hsp27 phosphorylation cycle, impairing actin assembly and chemotaxis. Our RNAi knockdown experiments are consistent with this model. Reduction of Hsp27 content below a critical level would be expected to impair Hsp27-facilitated actin-based motility, and rescue with constructs that are incapable of phosphorylation or dephosphorylation would prevent Hsp27 cycling between the unphosphorylated and phosphorylated forms. The recent finding that introduction of a phosphorylation-resistant Hsp27 recombinant protein into human neutrophils impairs chemotaxis provides additional support for our proposed mechanism (Jog *et al*, 2007).

The profound effects of LT on Hsp27 phosphorylation and actin assembly emphasize the importance of the p38 MAPK signal-transduction pathway and Hsp27 for actin-based motility. Targeting of this important pathway by LT is likely to explain how *B. anthracis* paralyzes neutrophils, and impairment of this critical component of the innate immune system helps to explain how this pathogen is able to rapidly multiply in the host. Furthermore, we are presently examining how LT blockade of the p38 MAPK pathway affects other actin-mediated functions, including platelet spreading and hemostasis, as well as vascular endothelial junction integrity. Impairment of these functions would further contribute to LT's deadly effects and provide additional explanations for the fulminant clinical course of inhalation anthrax.

Materials and methods

Toxin purification

Toxin components were purified as described previously (Quinn *et al*, 1988; During *et al*, 2005).

Listeria infection

Listeria infection and phalloidin staining was carried out exactly as described in Dabiri *et al* (1990). *Listeria* motility was observed 3–4 h after infection, velocity and tail lengths being measured using the Metamorph program (Universal Imaging, West Chester, PA) as described previously (Zeile *et al*, 1996).

RNAi

Transfection of HeLa cells was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) by following the manufacturer's protocol. Hsp27 siRNA #1 GGCAGCAGCAGCAUGGCUAUU, #2 GCGGAG GAGUGGUCGAGUUU, #3 UGAGACUGCCGCAAGUAA (target sequence) and control UAAGGCUAUGAAGAGAUAC were purchased from Dharmacon. (Chicago, IL). After 48 h, western blots were also performed using anti-Hsp27 antibodies to confirm knockdown. For rescue experiments, hamster Hsp27 cDNA constructs were transfected after 48 h of siRNA treatment using calcium phosphate as described previously (Lambert *et al*, 1999). Microinjection of recombinant Hsp27 proteins was performed using an Eppendorf FemtoJet (Southwick and Purich, 1994).

Neutrophil purification and assessment of actin assembly

These procedures were performed exactly as described recently (During *et al*, 2005). SB23058 was obtained from Calbiochem

(LaJolla, CA) and added to neutrophils or HeLa cells at a final concentration of 1–100 μ M followed by incubation for 15–30 min at 37°C.

Neutrophil and *Listeria* immunofluorescence

Anti-Hsp27 antibodies (Cell Signaling, Beverly, MA) were used to stain total Hsp27 and phosphorylated serine 82 isoforms in both human neutrophils and *Listeria*-infected HeLa cells as described previously (Laine *et al*, 1997).

Two-dimensional electrophoresis and Western blotting

Two-dimensional SDS–PAGE was carried out exactly as described by the manufacturer (Amersham Pharmacia Biotech, Uppsala, Sweden). HeLa cells were treated with 1 μ g/ml LT overnight, and human neutrophils with 500 ng/ml $\times$ 2 h. DryStrip pH 3–11, 18 cm isoelectric focusing strips were rehydrated with 600 μ g of protein for 12 h followed by isoelectric focusing. Electrophoresis in the second dimension was performed using 12.5% acrylamide gels. Gels were silver stained by using Silver Quest (Invitrogen, Carlsbad, CA). Protein spots were excised from the gel and subjected to QSTAR MALDI-TOF. Protein identification was carried out at the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) core facilities. Hsp27 western blots were performed as described previously (Laine *et al*, 1997). For quantitative western blots, purified human Hsp27 was used to generate a standard curve and compared to three dilutions of HeLa cell and neutrophil extract.

Recombinant Hsp27

WT and phosphorylation mutant Hsp27 proteins were generated, expressed and purified as described previously (Theriault *et al*, 2004). For phosphorylation experiments, 5 or 13 μ g/ml of human Hsp27 was incubated for 30 min at 25°C with 2.5 μ g/ml of active recombinant MAPKAP-2 (Upstate Cell Signaling, Lake Placid, NY) in the manufacturer's recommended kinase buffer. Extensive comparisons between human and hamster WT Hsp27 revealed no differences in the ability of these two proteins to bind and regulate actin assembly. Transfected hamster WT protein is phosphorylated *in vivo* by human cells (J Landry, unpublished data).

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Listeria motility in rat brain extracts

Rat brain extracts were prepared and motility assays were performed as described previously (Egile *et al*, 1999). The slides were left at room temperature for 90 min before viewing. Tail lengths were measured using the Metamorph 4.0 program as described above.

Pyrenyl actin kinetic assays

Pyrene-labeled actin was prepared from rabbit skeletal muscle, actin assembly monitored, and critical concentration determined as described previously (Young *et al*, 1990). For assessment of barbed end capping, spectrin-4.1 nuclei (20 μ l of a 0.83 mg/ml stock solution) were incubated with differing concentrations of Hsp27 in a total volume of 285 μ l S2 buffer (10 mM imidazole HCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 0.25 mM ATP, 0.1 mM KCl and 1 mM DTT (pH 7.5)), followed by the addition of G-actin. To assess actin monomer sequestration, elongation from gelsolin–actin seeds (final concentrations 2 nM gelsolin, 40 nM actin) in a final concentration of 2 μ M actin (50% pyrene labeled) was measured as described previously (Young *et al*, 1994). The actin filament disassembly assay was performed exactly as described previously (Southwick, 1995).

Statistical analysis

A nonparametric Wilcoxin, unpaired, two-tailed test was used to compare differences in the velocities and tail lengths of *Listeria*-infected cells.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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