Heat shock protein 27 kDa expression and phosphorylation regulates endothelial cell migration

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ABSTRACT **The effects of enhanced HSP27 expression or expression of a nonphosphorylatable form of HSP27 on the migration of bovine arterial endothelial cells was assessed. Expression of the wild-type protein enhanced migration by twofold compared to control transfectants, whereas expression of the mutant protein retarded migration by 40%. Since homologs of the small heat shock protein inhibit F-actin polymerization *in vitro* and may alter basolateral F-actin content *in vivo*, it was postulated that the 27 kDa heat shock protein affects microfilament extension essential for cell motility. Expression of the wild-type protein promoted the generation of long cellular extensions, whereas expression of the dominant negative mutant protein resulted in a marked reduction of lamellipodia and generated aberrant microfilament morphology at the wound edge. Immunofluorescence combined with phalloidin staining demonstrated the colocalization of the HSP27 gene products with lamellipodial microfilament structures. These data suggest that the 27 kDa heat shock protein regulates migration by affecting the generation lamellipodia microfilaments.—Piotrowicz, R. S., Hickey, E., Levin, E. G. Heat shock protein 27 kDa expression and phosphorylation regulates endothelial cell migration. *FASEB J.* 12, 1481–1490 (1998)**

Key Words: lamellipodia · F-actin · vascular endothelial growth factor · HSP27 · wounding

THE 27 kDa HEAT SHOCK PROTEIN (HSP27)[2] is constitutively expressed in vascular endothelial cells and can be enhanced by exposure of the cells to estrogen (1). Enhanced endothelial cell expression generates phenotypic changes that include enhanced growth (1), an earlier onset of culture senescence (1), and the enhanced secretion of basic fibroblast growth factor (FGF2) in response to estrogen (2). In other cell types, HSP27 has been linked to additional cell processes including regulation of apoptosis (3) and enhanced resistance to anti-cancer drugs (4, 5). The number of different effects attributed to HSP27 may reflect the fact that HSP27 activity is central to several cellular processes. Two distinct activities have been demonstrated for HSP27 *in vitro*: that of a molecular chaperone, facilitating the refolding of partially denatured proteins into active conformations (6–9), and that of a F-actin modulating protein, inhibiting F-actin polymerization (10–12).

HSP27 homologs have been characterized as barbed-end microfilament capping proteins that are inhibited by phosphorylation (10, 11, 13). Placement of wild-type human HSP27 into fibroblasts results in the stabilization of cortical actin filaments that normally depolymerize in response to stress and cytochalasin D treatment (12). Inhibition of kinase activity that results in HSP27 phosphorylation (13) or expression of a nonphosphorylatable mutant HSP27 in the fibroblasts inhibits the filament stabilization (14) and inhibits processes dependent on a dynamic microfilament cytoskeleton (e.g., membrane ruffling and pinocytosis) (14). Thus, the F-actin modulating activity of HSP27 may also be inhibited *in vivo* by HSP27 phosphorylation. HSP27 is a substrate for a unique branch of the mitogen-activated protein kinase (MAPK) kinase cascade that is activated by either cytokine treatment or cellular stress (7, 15–17). Activation of the kinase responsible for HSP27 phosphorylation, MAPK-activated protein kinase 2 (MAPKAPK2) (7, 15–17), would thus release one constraint on F-actin polymerization.

The F-actin modulating activity of HSP27 may be localized to the basolateral membrane of endothelial cells. Recently, we have shown that a portion of endothelial cell HSP27 fractionates with plasma membrane components (18). This pool, and not cytosolic HSP27, is the principal fraction of HSP27 that is phosphorylated by kinase activity induced by phorbol ester treatment (18). A portion of the membrane-

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[2] Abbreviations: HSP27, 27 kDa heat shock protein; FGF2; basic-fibroblast growth factor; BAECs, bovine arterial endothelial cells; DMEM; Dulbecco's modified Eagle's medium; MEM; minimum essential medium; DPBS, Dulbecco's modified phosphate-buffered saline; VEGF, vascular endothelial cell growth factor; PIP2, phosphatidylinositol 4,5-bisphosphate; FITC, fluorescein isothiocyanate; MAPK, mitogen-activated protein kinase; wt, wild-type; mu, mutant; vc, vector control; MAPKAPK2, MAPK-activated protein kinase 2.

associated HSP27 can be purified by streptavidin agarose precipitation of extracts prepared from cells biotinylated on the basal, but not the apical, surface (18). Coprecipitated along with the basolateral membrane-associated HSP27 is a portion of cellular F-actin, suggesting a link between the two proteins at the membrane. Phorbol ester activation of HSP27-expressing endothelial cells increases the amount of F-actin copurified with the membrane-associated HSP27, a result congruous with the demonstration that PMA induces the rapid generation of cortical F-actin concurrent with the induction of membrane ruffling in another cell type, 3T3 cells (19). In contrast, a decreased amount of F-actin is isolated from activated cells expressing a nonphosphorylatable mutant HSP27. These data suggest that enhanced expression of HSP27 at the basolateral membrane regulates actin polymerization at the membrane.

A consequence of the *in vivo* actin modulating activity of HSP27 is presented in this report. Expression in bovine arterial endothelial cells (BAECs) of either the human wild-type (wt) HSP27 or a mutant (mu) gene product (expressing glycine residues at the known sites of serine phosphorylation) induces a change in the basal rate of cell migration as assessed in a wound repopulation assay. wtHSP27 BAECs migrated at a rate double that of BAECs transfected with the empty vector, whereas BAECs expressing the nonphosphorylatable muHSP27 migrated at half the rate of the control cells. Since the generation of lamellipodial microfilaments is a crucial step in the migration of cells (20), this report addresses the likely hypothesis that expression of wtHSP27 and muHSP27 affect lamellipodial extension, and therefore cell migration. Wound cultures were stained with fluorescent-phalloidin and/or anti-HSP27 antibodies, and the number and morphology of lamellipodia of each transfectant type were assessed. Clear differences were observed: wtHSP27 BAECs exhibited numerous thin lamellipodia, the muHSP27 BAECs exhibited short projections with aberrant F-actin structures, and control transfectants exhibited plate-like lamellipodia previously described for migrating endothelial cells in this assay (21). Immunofluorescent microscopy localized HSP27 to the leading edge of lamellipodia, a site where F-actin structures are highly dynamic (20, 22–25). These data strongly suggest that the HSP27 gene products directly affect lamellipodial microfilament polymerization, which in turn affects lamellipodia formation and morphology, and thus cell motility.

MATERIALS AND METHODS

Materials

All cell culture reagents were obtained from BioWhittaker, Inc. (Walkersville, Md.) except for fetal calf serum, which was purchased from Intergen, Inc. (Purchase, N.Y.). The anti-HSP27 (G3.1) monoclonal was purchased StressGen, Inc. The secondary antibody used for this study was fluorescein isothiocyanate (FITC) -donkey anti-mouse IgG Fab′$_2$ from Jackson Laboratories (West Grove, Pa.). For transfection and selection, the cationic lipid Lipofectamine and antibiotic G418 were obtained from GIBCO/BRL, Inc. (Gaithersburg, Md.). FITC and rhodamine-conjugated phalloidin was obtained from Molecular Probes, Inc. (Eugene, Oreg.). Vitronectin was a gift from Dr. B. Felding-Habermann of The Scripps Research Institute.

Generation and culture of BAEC transfectants expressing HSP27

Stable clonal cells lines of BAECs were prepared with modifications of what has been previously described (1). Briefly, low passage BAECs, isolated from pulmonary arteries, were plated at a density of $4 \times 10^4/cm^2$ in 96- and 6-well plates. The cells were transfected with plasmids containing a genomic clone of human HSP27 (26), a mutagenized HSP27 gene in which the codons for Ser^{15,78,82} were altered to encode glycine residues (14), or vector plasmid without insert (pBluescript, pBS) using the cationic lipid Lipofectamine. A plasmid conferring neomycin resistance, pCDM8$_{neo}$, was cotransfected at a one-tenth molar ratio for the purpose of antibiotic selection. Transfected populations were grown for 72 h at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM HEPES and 4.0 g/l glucose supplemented with 1 mM sodium pyruvate, 1 mM nonessential amino acids, 1 mM penicillin/streptomycin, and 10% fetal calf sera. The cells grown in the 6-well plates were screened for transfection efficiency via immunoblot analysis and immunofluorescence microscopy using the anti-HSP27 monoclonal antibody G3.1. Selection media, culture media containing 700 μg/ml G418, was then added to the 96-well plates. Media was changed every 3 days as nontransfected cells died. After 2 wk of culture in selection media, the 96-well plates were viewed via phase-contrast microscopy and the wells containing single colonies were noted. These clones were subcultured in selection media. Following this protocol, a single transfection performed in three 96-well plates generated 15–25 clones. Clones developed from several different transfections expressing the same levels of wt or muHSP27 (1–2 ng/μg Triton X-100 lysate) were used for this study. This constitutes a two- to threefold increase in the total level of HSP27 in these cells. The HSP27 BAEC clones generated do not result in alteration of subcellular location, expression, or phosphorylation of the endogenous BAEC homolog HSP25 (18). Thus, the exogenous gene products add to the pool of cellular HSP25.

Wounding assay

The transfected BAECs were cultured in 9 cm^2 wells to confluence. Five days after confluence, a linear wound was made in the middle of the well using the narrow end (3.5 mm) of a cell scraper. The wells were washed twice and then cultured in serum-free assay media (phenol red-free minimum essential medium supplemented with 1 mM sodium pyruvate and 0.3% lactalbumin hydrolysate) overnight. Phase-contrast photomicroscopy was performed using TMAX ASA 400 (Kodak) black and white film at four distinct sites along each wound, with the center of the wound in the middle of each frame. At 10× magnification, the initial wound width fills the frame and the number of cells in each frame represents the number of cells that migrated into the wound. Contact prints were made and the number of cells in each frame counted. In some experiments, 5 μg/ml of the blocking FGF2 mono-

clonal antibody FB8 (Sigma, St. Louis, Mo.) or control IgG was included in the assay media. In other experiments, 10 ng/ml of recombinant 18 kDa FGF2 (Sigma) was added to the migration assay. Replicative counts of the same frame generated standard deviations in the range of 2%. Previous studies using ^{3}H-methyl thymidine incorporation as a measure of DNA synthesis have demonstrated that cell proliferation does not occur in the time course of the experiments presented in this report, and therefore wound repopulation is solely due to cell migration (27).

Immunofluorescence microscopy and fluorescent phalloidin staining

Glass coverslips were coated with 5 μg/ml vitronectin in Dulbecco's modified phosphate-buffered saline (DPBS) overnight at 4°C. Wounds were made in confluent monolayers that were 3–5 days postconfluent, as described above. The cells were fixed in freshly prepared 4% paraformaldehyde in DPBS for 10 min. After washing with DPBS three times, the cells were permeabilized with 0.2% Triton X-100 in 10 mM imidazole, pH 7.15, 40 mM KCl, and 10 mM EGTA for 15 min. The imidazole lysis buffer stabilizes microfilaments, retarding depolymerization when cells are lysed at room temperature (28, 29). For phalloidin staining, 6 U/ml of FITC phalloidin was dissolved in the imidazole buffer containing 0.5% bovine serum albumin (binding buffer) and placed onto the coverslips for 30 min. For antibody staining, 10 μg/ml of antibody in binding buffer was placed onto the coverslips and incubated for 1 h. The cells were washed three times in binding buffer and then incubated with a 1/50 dilution of FITC-donkey anti-mouse IgG Fab′$_2$ with or without 3 U/ml of rhodamine-phalloidin. After an hour incubation, the coverslips were washed three times in binding buffer and then mounted onto glass slides with Vectashield (Vector Laboratories, Burlingame, Calif.) mounting medium and sealed with nail polish. Cells were viewed on an Olympus BH2-RFCA epifluorescence microscope and photographed with an Olympus 35-AD4 camera attached to a PMTOADS-Olympus automicrographic system. The stained samples were photographed with 400 ASA Ektachrome or 400 ASA TMAX black on white film pushed to 1600 ASA.

MCF-7 growth assay

To test whether the recombinant FGF2 included in the migration assays was functional, the incorporation of tritiated (methyl) thymidine into growing MCF-7 cells was determined in serum-free conditions in the presence and absence of the FGF2. Cells were plated in wells of 22 mm diameter at a density of 2.5×10^4/well and subcultured for 48 h. The media was changed to the same assay media described above for the migration studies except that some wells received 10 ng/ml recombinant FGF2. After culture for 24 h, 100 μCi/ml of ^{3}H-methyl thymidine (Amersham, Buckinghamshire, U.K.) was added and the wells cultured for an additional 4 h. The amount of radioactivity incorporated into the cells was then determined essentially as described (2).

RESULTS

Linear wounds were made in confluent cultures of BAEC transfectants to determine whether enhanced expression of HSP27 affects the migration of the endothelial cells and whether HSP27 phosphorylation regulates endothelial cell motility. After culture for 20 h, multiple photomicrographs of each wound were taken and the number of cells repopulating the denuded area counted. The width of the micrograph represents the width of the initial wound; therefore, cells present in the micrograph are cells that have migrated into the wound. That wound repopulation solely represents migration and not proliferation was determined by 5-bromo-deoxyuridine incorporation studies performed during migration. Immunohistochemical analysis of the labeled wounded cultures showed no thymidine analog present in any of the cells whereas subconfluent growing cultures stained positive (data not shown). Thus, the repopulation of the wounded area was a result of cell motility and not cell division. In the experiment depicted in **Fig. 1*A***, a greater number of wtHSP27 cells repopulated the wound than control cells, whereas expression of muHSP27 reduced the number of cells entering the wound area. In this experiment, an average of 160, 68, and 116 wt, mu and vector control cells migrated per 24 mm of wound, respectively (Fig. 1*B*). For each experiment, the number of wt and muHSP27 cells entering the wound were normalized to the number of migrating vector control (vc) cells. The wt/vc and mu/vc ratios were averaged with the ratios obtained from other experiments; the means presented with the calculated SDs in Fig. 1*C*. In 11 experiments, using a panel of several different clones of each transfectant type, twice as many wtHSP27 BAECs migrated into the wound as control cells. The migration of muHSP27 cells was 40% that of the control cells. Thus, the data show that expression of wt or muHSP27 altered the basal rate of BAEC migration and that HSP27 phosphorylation is a positive regulatory event in cell migration.

It has been demonstrated that enhanced expression of HSP27 in BAECs facilitates the release of FGF2 (2), a chemokinetic stimulator in the wound assay. To assess whether differences in the level of endogenous FGF2 could account for the differences in migratory rates observed among the transfectants, a blocking FGF2 antibody (FB8) was included in the wound assay. At a concentration of 5 μg/ml, the anti-FGF2 inhibited BAEC migration by approximately 50% whereas control IgG had little effect on the migration of the BAE cells (**Fig. 2*A***). Each transfectant type was inhibited to nearly the same extent, indicating that differences in culture FGF2 levels did not account for the differences observed in the basal rate of migration. In fact, FGF2 appears to be at saturating levels since the addition of 10 ng/ml exogenous recombinant FGF2 to the wound cultures did not enhance the migration of any of the transfectant types, including muHSP27 BAECs (Fig. 2*B*). In five experiments, the average migration rate of each transfectant type in the presence of exogenous FGF2 was nearly equivalent to the migration in the absence of additional FGF2, indicating that saturating levels of

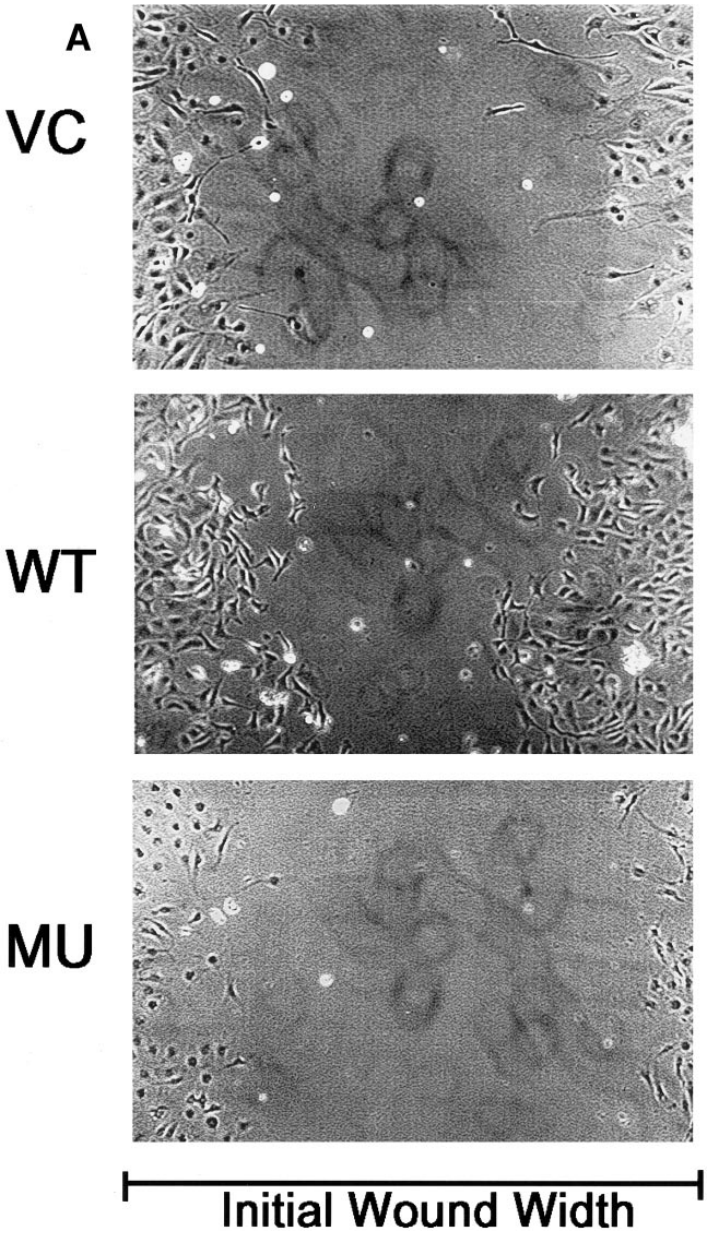
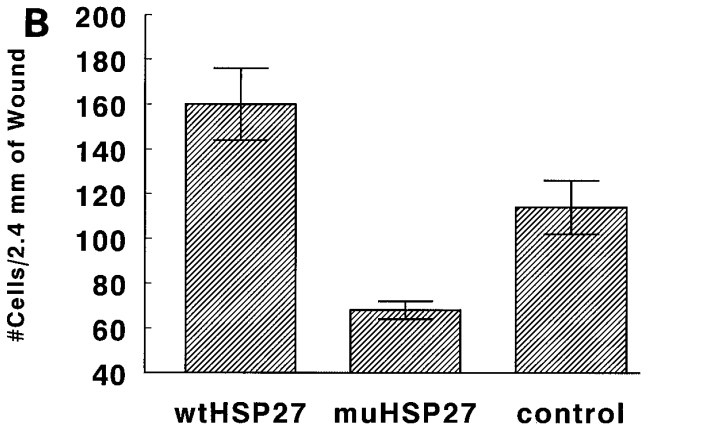
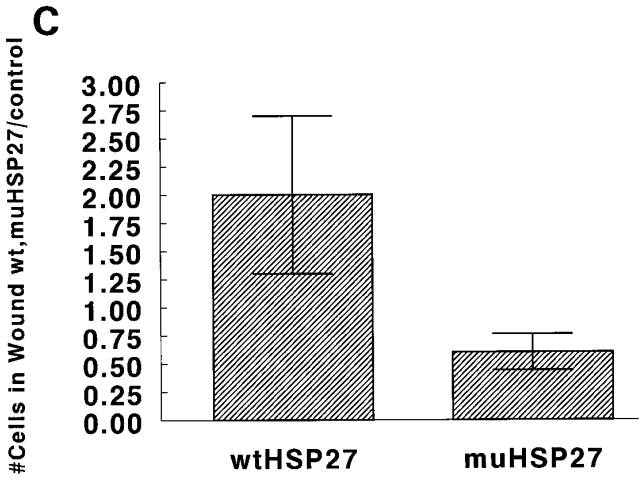


Figure 1. *A)* Phase-contrast micrographs of control (VC), wtHSP27 (WT) and muHSP27 (MU) BAEC cultures 20 h after wounding. The width of the initial wound (3.5 mm) is indicated and represents the length of the micrographs. Magnification is 10×. *B)* The number of cells migrating into the wound was determined from four micrographs of each type of transfectant. The results were averaged and presented with the SD. *C)* The average relative rates of migration of wtHSP27 and muHSP27 BAECs compared to control cells were determined. For each experiment, the average number of wt or muHSP27 cells repopulating a wound were divided by the average number of vector control cell migrating into the wound. The ratios obtained from 11 experiments were averaged and are presented with the SD.





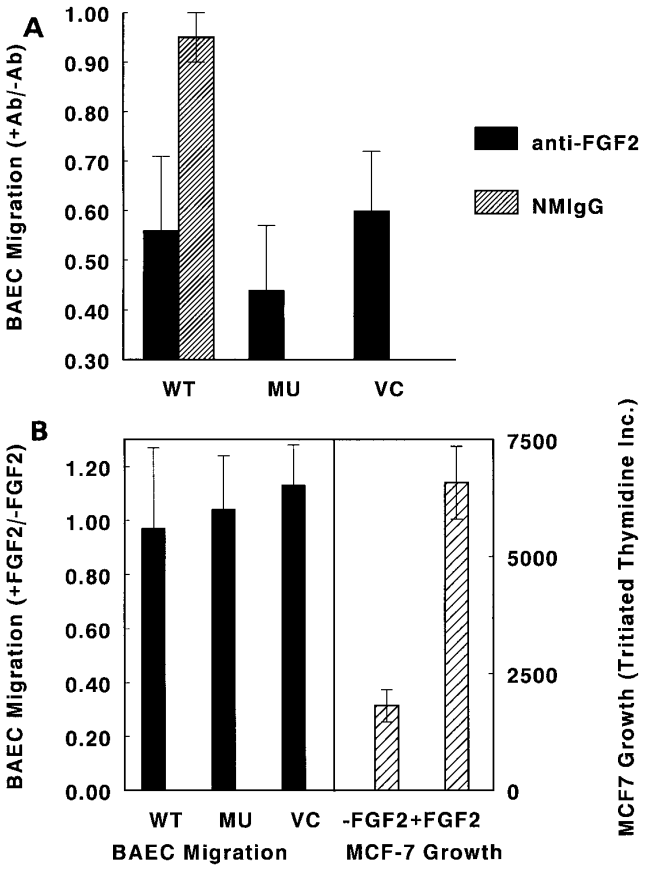


Figure 2. *A)* The effect of including 5 μg/ml of anti-FGF2 in the wound assay on the migration of wt, muHSP27, and vector control BAECs. Data are presented as the ratio of migration obtained in the presence of the antibody over that obtained in the absence of antibody (n=5). *B)* The effect of including 10 ng/ml recombinant 18 kDa FGF2 on the migration of the BAEC transfectants. Data are presented as migration obtained in the presence of FGF2 over that obtained in the absence (n=5). To demonstrate functionality of the FGF2, the growth of the breast adenocarcinoma cell line MCF-7 in the presence and absence of FGF2 was determined by measuring incorporated tritiated thymidine.

FGF2 were present in the cultures of each transfectant type. The recombinant FGF2 used for the migration assay stimulated the proliferation of cultured cells (e.g., MCF-7 cells, Fig. 2*B*) and was deemed functional. Thus, the differences in cell motility induced by expression of the exogenously introduced HSP27 were not due to differences in the level of chemokinetic stimulator FGF2 in the culture and reflect cellular differences among the transfectant types.

A cellular consequence of expression of wtHSP27 or muHSP27 in BAECs is the alteration of lamellipodia F-actin. This is demonstrated in photomicrographs of FITC-phalloidin-stained wound cultures (**Fig. 3**). The confluent monolayers of each type demonstrated the typical staining pattern of postconfluent cultures of endothelial cells: faint cortical F-actin and randomly arranged stress fibers (21, 30). Initial wounds left a straight edge of folded cells (at the indicated triangles) that spread within the first few hours of the assay. At 12 h postwounding of vector control cultures, broad lamellipodia with densely stained fibers at the leading edge were evident (see arrows in the lower right panel of Fig. 3). wtHSP27 cells exhibited these structures in addition to long and slender extensions that protruded into the wound (upper right panel of Fig. 3). In addition to membrane-associated F-actin fibers on both sides of the thin cellular extensions, structures at the tip of the lamellipodia, likely representing a meshwork of nascent F-actin fibers (20, 22–24), were also stained (arrows in upper right panel).

In contrast to what was observed for the wtHSP27 and vector control BAECs, few cells expressing muHSP27 demonstrated cellular extensions into the wound area (Fig. 3). When present, however, the extensions were devoid of intense phalloidin staining (e.g., at arrow in the middle panel of Fig. 3). The cells at the wound edge not presenting identifiable protrusions exhibited punctate phalloidin staining within the cell body.

The overall F-actin morphology of the transfectant monolayers at 5 and 15 h postwounding is depicted in **Fig. 4** (magnification 200×). At 5 h, the wound edge of the vector control culture was lined by cells exhibiting broad lamellipodia, with caps of densely stained fibers at the leading edge (indicated by arrows). The wtHSP27 BAECs at this time point presented many cells exhibiting long protrusions into the wound area. In contrast, the muHSP27 BAECs at the wound edge presented only short extensions with patches of phalloidin along the membrane. These cells failed to exhibit the cellular spreading associated with normal lamellipodia formation.

At 15 h postwounding, the wtHSP27 BAECs at the wound edge were elongated (Fig. 4). Many cells exhibited slender extensions that protruded from the spread cells of the monolayer or from cells apart from the monolayer. The cellular extensions protruded perpendicular to the monolayer into the wound space. Control cultures exhibited broad cells at the wound edge that spread to a somewhat lesser extent than the wtHSP27 BAECs. Both wt and control cells were opaque with numerous stress fibers. The monolayers behind the wound edge of both the wtHSP27 and control BAECs had fewer gaps between the cells than at the 5 h stage and presented intense staining of cortical F-actin structures. This result, which was highly consistent, may represent the lateral spreading of the cells to fill small spaces left by the forward extension of the migrating cells. At 15 h postwounding, muHSP27 cultures were clearly different from either the wtHSP27 or control BAEC cultures (Fig. 4). Cells at the wound edge were slightly separated from the monolayer, with most orientated parallel to the wound. These cells presented patches of phalloidin

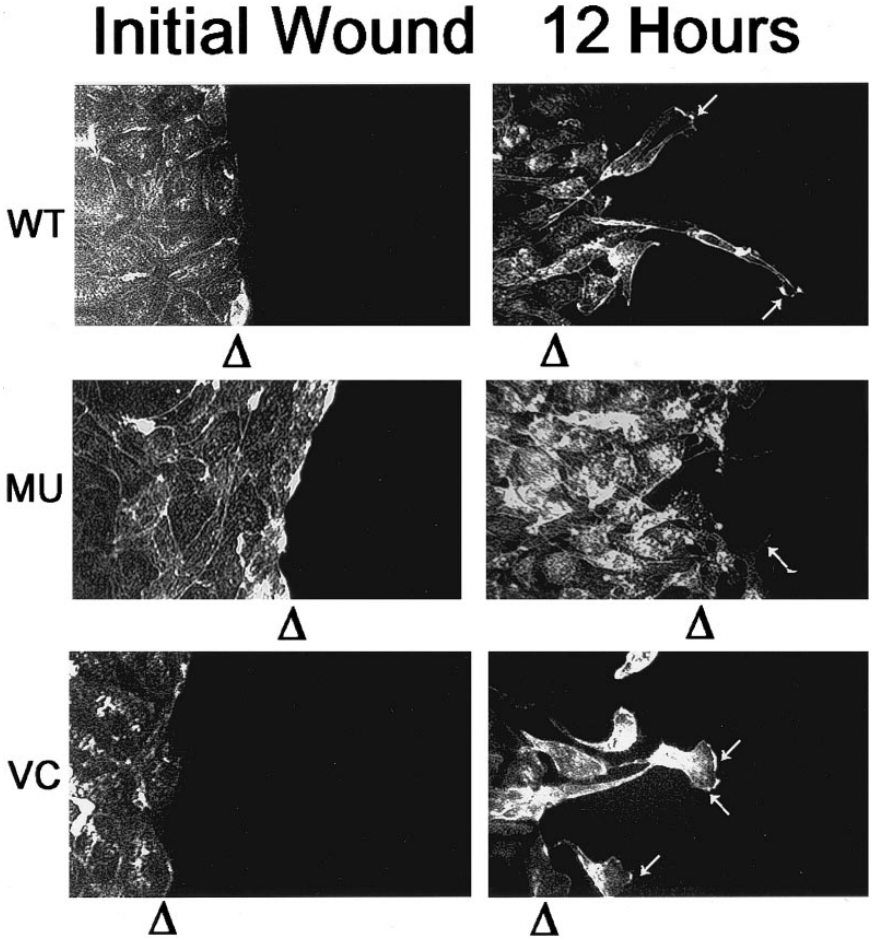


Figure 3. Cultures of wtHSP27 (WT), muHSP27 (MU), and vector control (VC) BAECs were wounded and stained immediately (initial wound) or 12 h after wounding with FITC-phalloidin. Triangles represent the approximate edge of the initial wound. Arrows in the micrographs of the wtHSP27 and vector control BAECs at the 12 h time point demonstrate phalloidin staining at the leading edge of the cell extensions and lamellipodia. The arrow in the micrograph depicting muHSP27 BAECs at the 12 h time point demonstrate cellular extensions that are devoid of F-actin. Magnification is 500×.

staining along the membrane (indicated by arrows). Cells of the monolayer adjacent to the wound failed to present the well-defined F-actin structures present in the wt and vector control BAEC cultures.

Thus, expression of wtHSP27 and muHSP27 in the BAECs alters the morphology of lamellipodial structures. That these effects are due to wtHSP27 or muHSP27 acting directly at the sites of microfilament assembly is suggested by the fact that the exogenous HSP27 localized to sites where lamellipodial F-actin is dynamic (20, 24, 25, 31). The colocalization is demonstrated in **Fig. 5**, in which wtHSP27 BAEC wound cultures were double-stained with an anti-HSP27 monoclonal antibody (G3.1) and phalloidin. Both the antibody and phalloidin stained the leading edge of migrating wtHSP27 BAECs (see arrows). The anti-HSP27 antibody also exhibited diffuse staining of the perinuclear region of the cells. Micrographs in which cells were stained with normal mouse IgG and photographed for the same length of time used to document the G3.1 staining failed to generate images. Thus, the G3.1-stained structures do not represent cross-channel contamination from the phalloidin-stained material. The localization of HSP27 to dynamic F-actin structures on lamellipodia is consistent with an earlier report demonstrating anti-HSP27 staining of lamellipodia of HSP27-transfected fibroblasts (14). MuHSP27 and vector control BAECs were also subjected to double labeling (**Fig. 6**). The muHSP27 cells exhibited diffuse anti-HSP27 staining at the site of truncated projections along with the perinuclear staining. Control cells exhibiting broad lamellipodia presented a leading edge that was stained with both phalloidin and anti-HSP27 (arrow), demonstrating colocalization of the endogenous HSP27 homolog (HSP25) with the membrane-associated F-actin in the lamellipodia.

DISCUSSION

The migration of endothelial cells from a confluent monolayer into an area denuded of cells has been used extensively as a migration assay (21, 30, 32). Wound repopulation is the result of the migration of individual cells and of the monolayer as a unit. For the latter, distinct changes in endothelial microfilament architecture have been described (21). Initially, cortical actin structures of the cells at the wound's edge break down as cellular extensions are generated. By 2 h, fibers running parallel to the wound edge are formed in the lamellipodial extensions,

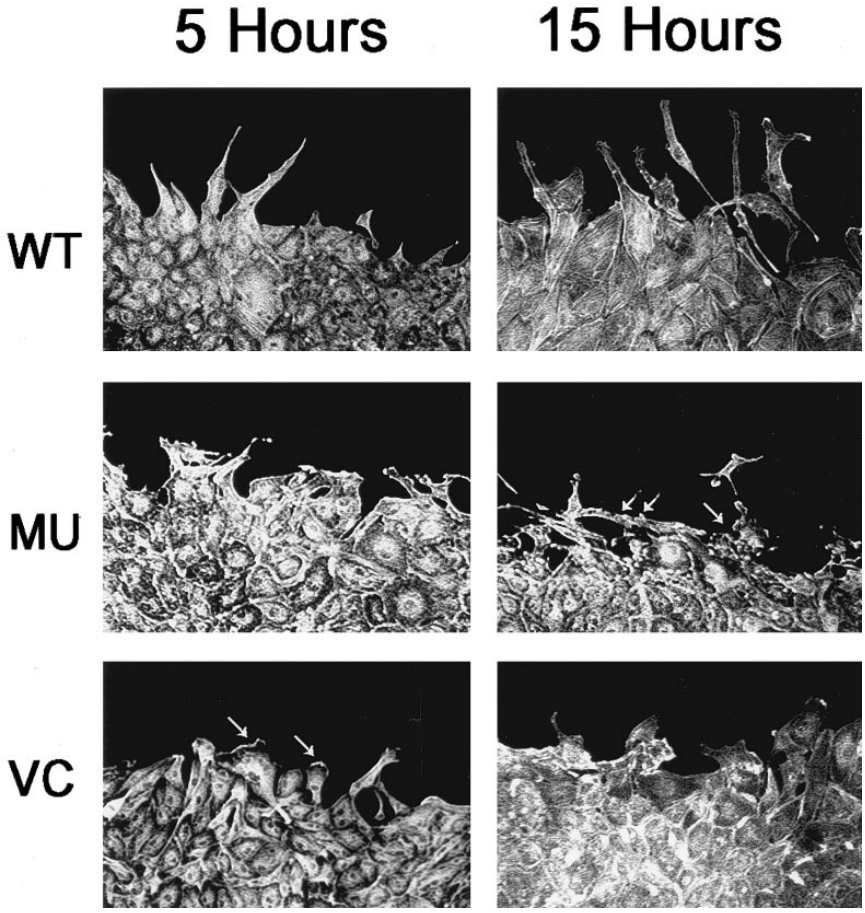


Figure 4. Cultures of wtHSP27 (WT), muHSP27 (MU), and vector control (VC) BAECs were stained at 5 and 15 h after wounding with FITC-phalloidin. Arrows indicate phalloidin staining at the leading edge of the vector control cells at the 5 h time point. In the micrograph depicting muHSP27 BAECs at the 15 h time point, the arrows demonstrate patches of phalloidin-stained material along the edge of the membrane. Magnification is 200×.

causing extensive cell spreading. Net actin polymerization is greatest near the distal membrane of the cell and is evident in some cells as densely stained F-actin structures at the leading edge of the lamellipodia. After several hours, stress fibers associated with vinculin-rich plaques form perpendicular to the wound and cell translocation begins. Similarly, individual migrating cells demonstrate lamellipodial spreading that is concurrent with the net generation of actin fibers at the membrane of the leading edge of the cell extension (20, 24).

Forward membrane extension of motile cells is dependent on the net polymerization of F-actin at the leading edge of the protrusive structures (24, 25). The regulation of lamellipodia microfilament extension is complex, with both F and G actin binding proteins playing a role (20, 24, 25, 31). The subsequent organization of the lamellipodial fibers is cell-specific (33, 34) but generally filaments at the leading edge are orientated with the rapidly growing barbed ends orientated toward the leading edge of the lamellipodia (22–25). Lamellipodial F-actin polymerization may be the result of the uncapping and elongation of existing fibers, the growth of preexisting fibers that have been severed and uncapped, or the de novo nucleation of nascent fibers (20, 31, 35). For example, polymorphonucleocytes exposed to chemoattractants generate an increase in F-actin filaments in the absence of filament elongation, suggesting that filament severing and uncapping and/or filament nucleation are the principal means of filament generation in these cells (36).

Evidence has been presented that supports a role for barbed-end capping proteins in the regulation of F-actin generation and, thus, cell migration. For example, expression levels of capping protein have been demonstrated to affect cell motility in *Dictyostelium* mutants. Mutants exhibiting more capping protein migrated faster whereas those expressing less migrated more slowly (37). Expression of moderate levels of the phosphatidylinositol 4,5-bisphosphate (PIP2) and Ca^{2+} binding capping protein CapG in NIH3T3 cells does not grossly affect microfilament morphology or F-actin content, but does increase the rates of wound healing and cell migration (38). *In vitro* studies of the neutrophil capping protein beta-2 demonstrated that an interaction with PIP2, induced in the intact cell by exposure to chemoattractants, causes the release of the capping protein from microfilaments (39). However, these capping proteins can affect the microfilament cytoskeleton of the entire cell (e.g., in *Dictyostelium*) and/or are integrally related to second messenger systems (e.g., CapG and capping protein beta-2). It cannot be concluded,

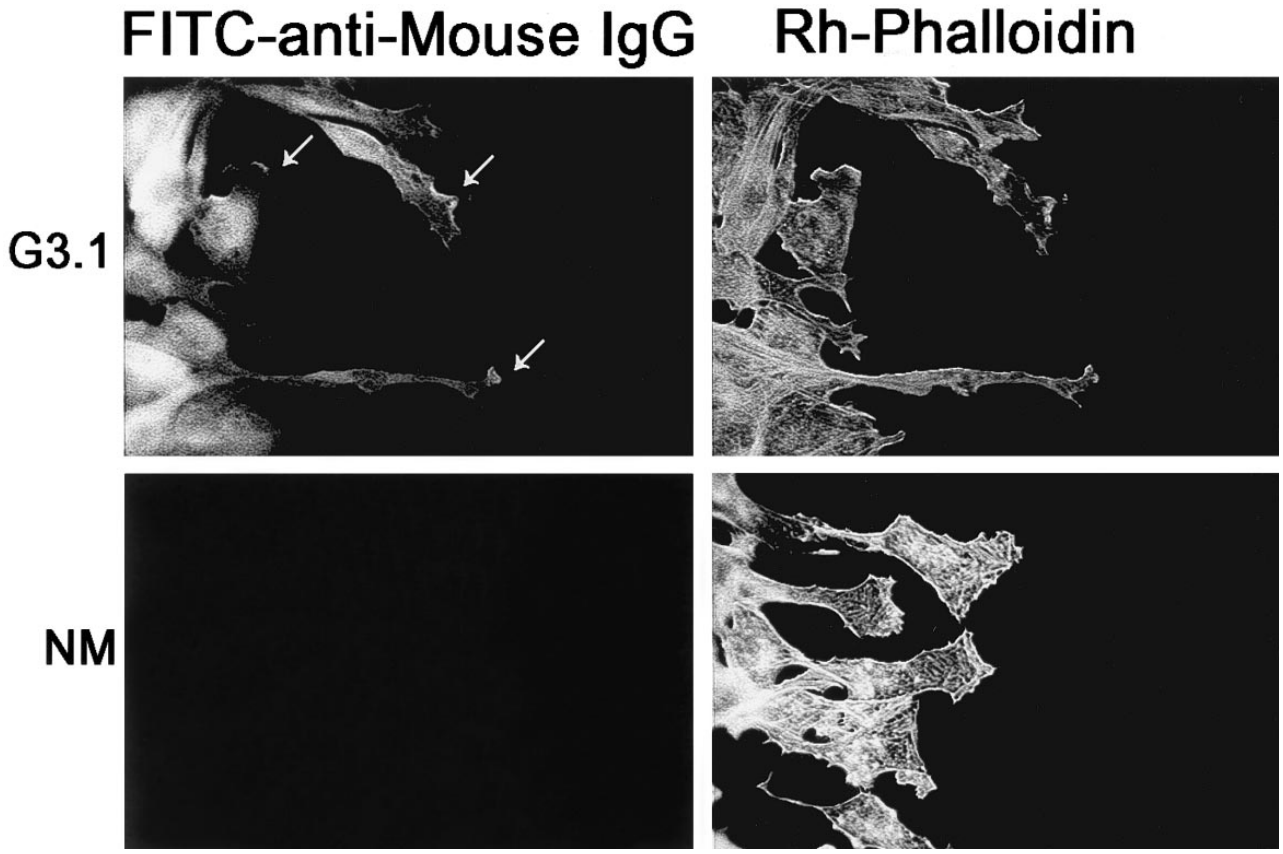


Figure 5. wtHSP27 BAECs were double-stained with rhodamine-conjugated phalloidin (Rh-Phalloidin) and anti-HSP27 (G3.1) or normal mouse IgG (NM). Antibody staining was visualized with FITC-anti-mouse IgG. Arrows indicate anti-HSP27 staining at the tip of the cellular extensions or lamellipodia. The lack of signal obtained with the normal mouse IgG indicates the lack of cross-channel contamination. Magnification is 1000×.

therefore, that their effect on cell migration is due solely to the ability to affect lamellipodial F-actin. Thus, the role of barbed-end capping proteins in cell motility may be of restricted consequence depending on the protein in question, the type of cell, and its physiological state.

HSP27 homologs have been demonstrated to be barbed-end capping proteins (10–12) and thus have

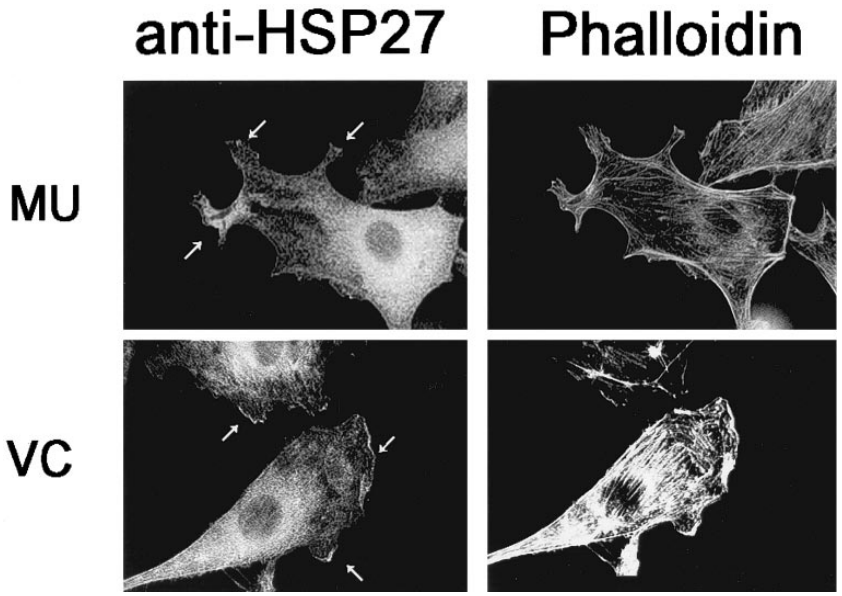


Figure 6. muHSP27 (MU) and vector control (VC) BAECs were subjected to double staining as in Fig. 5. Arrows indicate positive anti-HSP27 staining at truncated cellular extensions of the muHSP27 cells and at the edge of the lamellipodia of the control cells. Magnification is 1000×.

the potential to affect F-actin generation associated with lamella extension and cell motility. Indeed, expression of wtHSP27 and muHSP27 resulted in aberrant lamellipodial microfilament structures. WtHSP27 cells exhibited the propensity to express long projections that extended out perpendicular to the wound, whereas expression of muHSP27 resulted in an inhibition in the generation of microfilament-dependent cellular extensions. Immunofluorescence microscopy localized a pool of HSP27 to the leading edge of the lamellipodia of migrating cells, suggesting that wtHSP27 and muHSP27 have the potential to directly affect microfilament dynamics within the lamellipodia at these sites. The localization of HSP27 to the leading edge of the lamellipodia is consistent with the fact that lamellipodia microfilaments are orientated with the barbed ends at this edge (22–25). Since F-actin generation within the lamellipodia is crucial to cell migration, it is likely that wtHSP27 and muHSP27 expression affect cell migration at this step.

Recently, it was demonstrated that vascular endothelial cell growth factor (VEGF), a potent chemokinetic stimulator of endothelial cells, results in the activation of p38 of the MAPK cascade (40) in human endothelial cells. p38 activates the kinase responsible for HSP27 phosphorylation, MAPKAPK2 (7, 15–17). Specific inhibition of p38 resulted in the abrogation of the VEGF induction of stress fibers, the phosphorylation of HSP27, and cell migration (40). In this report, we demonstrate that it is a downstream substrate of the p38 cascade that affects cell motility: HSP27. If HSP27 activity is regulated by phosphorylation *in vivo* as it is *in vitro* (13, 18), phosphorylation of HSP27 within lamellipodia would release the inhibition of one factor controlling microfilament elongation. Fractionation studies (18) demonstrate that HSP27 phosphorylation does not result in translocation of membrane-associated HSP27. Thus, enhanced HSP27 expression would result in an increase in the pool of phospho-HSP27 and generate a potentiation in lamellipodia F-actin polymerization within the wtHSP27 BAECs. In contrast, the presence of the constitutively active muHSP27 within the lamellipodia would inhibit this shift toward F-actin polymerization. Cells expressing the dominant negative muHSP27 would thus generate the aberrant microfilament structures observed and exhibit decreased cell extension and, consequently, a decreased migration rate. The retardation of cell migration by expression of muHSP27 suggests that the phosphorylation of HSP27 and the uncapping of filaments at the forward edge of the lamellipodia is a critical step in cell motility. FJ

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