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Basolateral Membrane-associated 27-kDa Heat Shock Protein and Microfilament Polymerization

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The in vivo activity of the 27-kDa heat shock protein, a barbed-end microfilament capping protein, may be localized to the plasma membrane. To investigate this putative association, bovine endothelial cells expressing the human wild type or a mutant nonphosphorylatable 27-kDa heat shock protein were subjected to subcellular fractionation and immunoblot analysis. The 25-kDa endogenous bovine homolog and both exogenous gene products partitioned with cytosolic or plasma membrane components, indicating that phosphorylation is not required for membrane association. Phorbol ester treatment resulted in phosphorylation of only membrane-associated 25-kDa and wild type 27-kDa heat shock protein and did not induce redistribution. In a second fractionation protocol, streptavidin-agarose precipitation of extracts prepared from cells biotinylated at either the apical or basal surface localized membrane 25- and 27-kDa heat shock proteins exclusively to the basolateral surface. Stimulation of transfected expressing the wild type 27-kDa heat shock protein resulted in its phosphorylation and a doubling in the amount of membrane-associated F-actin precipitated, whereas the mutant protein decreased the amount of F-actin precipitated. These data suggest that membrane-associated 25- and 27-kDa heat shock proteins inhibit the generation of basolateral microfilaments and that phosphorylation releases this inhibition.

Endothelial cell F-actin exists as a dynamic and responsive microfilament cytoskeleton tightly regulated by a number of spatial and temporally regulated microfilament modulating proteins (1–4). One such protein, a barbed-end filament capping protein that is inhibited by its phosphorylation, is the 27-kDa heat shock protein (HSP27) (5–7). The in vivo activity of HSP27 has been inferred from the effects of overexpression of the protein in cultured fibroblasts (8, 9). Expression of human HSP27 in these cells stabilized cortical F-actin microfilaments that normally disaggregate in response to heat shock, acute cytochalasin D treatment, or oxidative stress (10, 11). In unstressed cells, overexpression of HSP27 leads to increased pinocytotic activity and membrane ruffling (9), processes dependent on a dynamic membrane-associated microfilament cytoskeleton (12, 13). Whether the in vivo changes that result from the enhanced expression of HSP27 are due to the microfilament capping activity demonstrated for HSP27 in vitro has yet to be firmly established, however.

HSP27 is phosphorylated by kinase activity induced by a variety of stress, cytokine and mitogenic stimuli (11, 14–17). The control of HSP27 in vitro activity has been linked to its phosphorylation state, a conclusion based on results demonstrating that a non-phosphorylatable mutant (mu) HSP27, expressed in cultured cells, failed to promote the same effects promoted by the wild type (wt) HSP27 (8, 9). Evidence has been presented, however, that contradicts the reliance of HSP27 activity on its phosphorylation. For example, Knauf et al. (18) have found that overexpression of muHSP27 confers the same thermo-resistant traits as the wild type protein and that the in vitro ability of muHSP27 to act as a molecular chaperone is also not affected by the lack of phosphorylation.

To investigate a putative role of HSP27 in controlling the endothelial microfilament cytoskeleton, we have generated stable transfectants of bovine arterial endothelial cells (BAECs) expressing the human HSP27 gene (wtHSP27) (19) or a mutated gene product (muHSP27) lacking the three known phosphorylation sites (9). The transfected BAECs constitutively express the exogenous gene products in addition to the bovine HSP27 homolog, HSP25 (20, 21). Bovine HSP25 and human wtHSP27, but not muHSP27, are phosphorylated to similar levels by kinase activity induced in response to chemical (phorbol 12-myristate 13-acetate, PMA) or mechanical (laminar flow) stimuli (21). The transfected clones express 20–40 ng of exogenous HSP27/20 μg of total transfectant protein, which has no effect on the expression of the endogenous HSP25 (21). In addition, expression of either exogenous gene product does not effect the basal or stimulated level of HSP25 phosphorylation (21). Thus, at the level of expression generated in the BAEC clones, the exogenous gene products simply add to the cellular pool of HSP25. This addition can induce phenotypic alterations. For example, expression of wtHSP27 induces accelerated growth and culture senescence of the BAECs (20).

Expression of exogenous HSP27 in fibroblasts results in increased cortical actin structure and an enhancement of activities dependent on the membrane-associated microfilament cytoskeleton such as pinocytosis and membrane ruffling (9–11). These data suggest that a component of cellular HSP27 activity is focused to the membrane. To investigate this possibility, we performed subcellular fractionation on BAECs and transfected
BAECs expressing human wtHSP27 and muHSP27. In this report, we demonstrate that a significant portion of cellular HSP25/27 fractionates with plasma membrane components, that phosphorylation is not required for this localization, and that this subpopulation of HSP25/27 is a substrate for kinase activity induced by brief treatment of the cells with phorbol ester. Using a second fractionation protocol, we further demonstrate that a portion of the membrane-associated HSP25/27 localizes to the basolateral membrane where it is substrate for HSP27 kinase activity. Concurrent with phorbol ester-induced HSP25/27 phosphorylation is the generation of additional membrane-associated F-actin. Expression of muHSP27 (which cannot be phosphorylated) inhibits the generation of additional membrane-associated F-actin. These data are consistent with the demonstrated in vitro functioning of HSP27 (i.e., that non-phosphorylated HSP27 inhibits actin polymerization and the phosphorylation abrogates this activity) (7). These data suggest that membrane-associated HSP25/27 may play a role in the regulation basolateral membrane-associated microfilament dynamics.

**EXPERIMENTAL PROCEDURES**

**Culture of Bovine Arterial Endothelial Cells**—Low passage bovine pulmonary arterial endothelial cells were cultured under 5% CO2 in Dulbecco’s modified Eagle’s media (BioWhittaker, Inc.) containing 25 mM HEPES and supplemented with 10% fetal calf serum (Intergen) and 1 mM each of sodium pyruvate, penicillin, streptomycin, and nonessential amino acids. Cells were plated at a density of 0.7–2 × 10^4 cells/cm2 and passed when confluent (approximately 1 × 10^6/cm2).

**Generation and Culture of BAEC Transfectants Expressing HSP27**—All culture reagents, except where otherwise noted were obtained from BioWhittaker, Inc. Stable clonal lines of BAECs were prepared with modifications of what has been previously described (20). Briefly, low passage BAECs, isolated from pulmonary arteries, were transfected with a plasmid containing a genomic clone of human HSP27 (8), a plasmid containing that clone subjected to site-directed mutagenesis in which the codons for the principal sites of HSP27 phosphorylation (Ser18, Ser20, and Ser85) were altered to encode glycine residues (9) or vector plasmid without insert (pBluescript, pBS) using the cationic lipid LipofectAMINE (Life Technologies, Inc.). The cells were plated at a density of 100,000–300,000 cells/cm2 in 96- and 6-well plates. A plasmid expressing neomycin resistance, pCDM8neo, was co-transfected at a one-tenth molar ratio for the purpose of antibiotic selection. Transfected populations containing 100 μg/ml G418 were grown for 72 h at 37 °C under 5% CO2 in Dulbecco’s modified Eagle’s media containing 25 mM HEPES and supplemented with 10% fetal calf serum (Intergen) and 1 mM each of sodium pyruvate, penicillin, streptomycin, and nonessential amino acids. Cells were plated at a density of 0.7–2 × 10^4 cells/cm2 and passaged when confluent (approximately 1 × 10^6/cm2).

**Surface Biotinylation and Streptavidin-Agarose Precipitation**—Apical and basolateral biotinylation of cells was performed essentially as described (23). For apical biotinylation, transfected clones were cultured in tissue culture wells. For basolateral biotinylation, transfected cells were cultured in 6.8-cm2 tissue culture-culture Transwell culture inserts with 3-micron pores. Cells were cultured 1–2 weeks past the point of confluence. For each experiment, fresh succinimidyl-6-(biotinamido)-hexanoate (Pierce) was dissolved in chilled 50 mM NaHCO3, pH 8.2. 15 mg NaCl to yield a concentration of 55 μg/ml. The cells were washed 3 times in prechilled biotinylation buffer; the biotin solution was added and incubated with the cells at 4 °C for 30 min. For the basolateral biotinylation, the biotin solution was stirred in the lower chambers of the Transwell plate with microstir bars. After biotinylation, the cell monolayers were washed 3 times with Dulbecco’s phosphate-buffered saline and lysed in 0.5% Triton X-100 in 10 mM imidazole, pH 7.15, 40 mM KCl, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, 2 mM sodium vanadate, and 0.5 mM sodium fluoride, a buffer that stabilizes and preserves F-actin (3). Cells were lysed in a volume of 200 μl Pretranswell insert. Lysis and all subsequent steps were performed at room temperature without refrigeration. Lysates were briefly centrifuged at 500 × g for 2 min, and a sample was saved for protein determination and immunoblot analysis. The remaining portion of the lysates was incubated with streptavidin-conjugated agarose (SA, Pierce) equilibrated to the lysis buffer at a ratio of 50 μl of prepared SA-agarose beads per 200 μl of lystate. After incubating for 30 min at room temperature while gently mixing, unbound material was removed and three consecutive washes in 100 μl of the same buffer.

Cellular material was eluted off the SA-agarose by boiling in Laemmli SDS-PAGE sample buffer for 3 min. Samples were reduced by the addition of 5% β-mercaptoethanol and then analyzed for the presence of HSP27 and actin by immunoblot analysis. In some experiments, transfected BAECs were radiophosphorylated (as described above) prior to basal biotinylation. In every experiment, serially diluted Triton X-100 lysates were also subjected to immunoblot and autoradiographic analyses for the purpose of demonstrating linearity of the immunoreactive and autoradiographic signals (see below).

**Immunoblotting**—Cell subfractions, lysates, or streptavidin eluates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on 12% (w/v) polyacrylamide gels (24). Upon completion of electrophoresis, proteins were transferred to nitrocellulose membranes that were then blocked for 1 h at room temperature in 5% nonfat milk in 20 mM Tris, pH 7.4, 145 mM NaCl with 0.05% Tween 20 (TTBS). The membranes were then incubated with dilutions of rabbit antisera raised against murine HSP25 (Stress Gen, Inc.) or a murine monoclonal antibody (G3.1, Stress Gen) specific for human HSP27, an antiserum to a recombinant antibody specific for human nonmuscle β-actin (clone AC-15, Sigma) or against mouse beta-actin (1.0 mg/ml bovine serum albumin). Bound antibody was detected using donkey anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Jackson Laboratories) and the enhanced chemiluminescence reagent (Amersham Corp.). The stained immunoblots were placed against x-ray film (BioMax, Eastman Kodak Co.) to generate chemilumigrams.

**Densitometric Analyses and the Demonstration of Linearity of Chemi-
HSP25, wtHSP27, and muHSP27 Are Present in Cell Fractions Enriched for Plasma Membrane Components—Overexpression of HSP27 in transfected cell lines generates enhanced cortical F-actin structure and alters cellular processes dependent on a dynamic membrane-associated microfilament cytoskeleton (e.g. pinocytosis and membrane ruffling) (9–11), suggesting a functional association with the plasma membrane. To determine if endothelial cell HSP25 localizes to this cell compartment, BAECs were fractionated by sequential hypotonic lysis, homogenization in the presence of Nonidet P-40, and differential centrifugation (22). Using this protocol, BAEC cell fractions enriched for nuclear (N), plasma membrane (P), microsomal (M), and cytosolic components (C) were generated and subjected to immunoblot analysis using rabbit antisera raised against murine HSP25 (Fig. 2, panel a). HSP25 primarily fractionated with either the Nonidet P-40 homogenate containing solubilized plasma membrane components (P) or the hypotonic lysate containing cytosolic proteins (C).

The protocol employed efficiently fractionates the plasma membrane compartment from the cytosolic compartment as demonstrated by the lack of anti-glucose-6-phosphate dehydrogenase (G6PDH) immunoreactive material in the plasma membrane fraction (P) but staining of immunoreactive material in the cytosolic (C) fraction (Fig. 2, panel b). Conversely, cytosolic material was not contaminated with plasma membrane components as indicated by the lack of anti-integrin β3 immunoreactivity in the cytosolic fraction but positive staining of a band with the appropriate apparent molecular mass (100 kDa) in the plasma membrane fraction (Fig. 1, panel C, lane P).

To determine whether phosphorylation of HSP27 is necessary for the partitioning of this protein into the membrane fraction, clones of BAECs expressing HSP27 or the non-phosphorylatable mutant HSP27 were also fractionated by this method and analyzed with the monoclonal antibody G3.1 that is specific for the human protein. Both wtHSP27 (Fig. 2, panel d, lanes 1–3) and muHSP27 (Fig. 2, panel d, lanes 4 and 5) partitioned with the plasma membrane fraction indicating that phosphorylation of HSP27 is not required for the association of HSP25/27 with the membrane components. In panel d, a greater percentage of the plasma membrane fractions was subjected to analysis than the hypotonic lysates containing cytosolic components. This accounts for the greater signals obtained for the HSP25/27 in the plasma membrane fractions of the individual clones.

Quantitation of the Membrane-associated HSP25/27—Cell fractionation experiments detailed above were performed, and the relative antigen level in each fraction was determined by immunoblot analysis. The antigenic signals obtained for the membrane-associated or cytosolic HSP25/27 from the fractionated transfectants fell within the linear range of immunodetection (Fig. 1), allowing the comparison of signal intensities. The relative amounts of HSP25/27 in the cytosolic fractions and the plasma membrane fractions of BAECs, wtHSP27-, or mu-HSP27-expressing BAECs were calculated from the densitometric signals obtained from the immunoblot and the percentage of each fraction analyzed. The average percentages of total cellular HSP25, wtHSP27, and mu-HSP27 that partitioned with the membrane fractions were determined to be 27 ± 9 (n = 14), 26 ± 12, and 24 ± 15%, respectively.

Thus a significant portion of the endogenous HSP25 and the exogenous HSP27 gene products fractionate with membrane components. These results are not due to the nonspecific trapping of large oligomeric complexes containing HSP25/27 since glucose-6-phosphate dehydrogenase, which also exists in heter-oligomeric complexes exhibiting molecular mass of up to 250 kDa (25, 26), was not detected in the plasma membrane fractions.

RESULTS

HSP25, wtHSP27, and muHSP27 Are Present in Cell Fractions Enriched for Plasma Membrane Components—Overexpression of HSP27 in transfected cell lines generates enhanced cortical F-actin structure and alters cellular processes dependent on a dynamic membrane-associated microfilament cytoskeleton (e.g. pinocytosis and membrane ruffling) (9–11), suggesting a functional association with the plasma membrane. To determine if endothelial cell HSP25 localizes to this cell compartment, BAECs were fractionated by sequential hypotonic lysis, homogenization in the presence of Nonidet P-40, and differential centrifugation (22). Using this protocol, BAEC cell fractions enriched for nuclear (N), plasma membrane (P), microsomal (M), and cytosolic components (C) were generated and subjected to immunoblot analysis using rabbit antisera raised against murine HSP25 (Fig. 2, panel a). HSP25 primarily fractionated with either the Nonidet P-40 homogenate containing solubilized plasma membrane components (P) or the hypotonic lysate containing cytosolic proteins (C).

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Membrane-associated HSP25 Is Substrate for HSP25/27 Kinase Activity—The microfilament capping activity of HSP25, which inhibits actin polymerization in vitro, is abrogated by HSP25 phosphorylation (7). To investigate whether membrane HSP25 is phosphorylated in response to kinase activity induced by phorbol esters, BAECs were radiolabeled with $^{32}$P, stimulated with PMA, and then fractionated by the above procedure. To evaluate the relative levels of HSP25 phosphorylation in the two cellular compartments, autoradiographic and immunoblot analyses of the cytosolic and plasma membrane fractions obtained from PMA-stimulated, $^{32}$P-labeled BAECs were performed (Fig. 3, panel A). Because two-dimensional isoelectric focusing/SDS-PAGE demonstrated that no other phosphoproteins in endothelial cell lysates co-migrate in the second dimension with phospho-HSP25 (27, 28), one-dimensional SDS-PAGE was deemed adequate for this study. Immunostaining for HSP25 demonstrated equivalent loading of HSP25 in the membrane and cytosolic fractions for both stimulated (+) and non-stimulated (−) cells. PMA stimulation did not result in a change in the relative distribution of cellular HSP25 between the two compartments (Fig. 3, panel A). The percentage of cellular HSP25 that partitioned with the plasma membrane fraction was determined to be 37 ± 9 and 35 ± 10% (n = 5) for non-stimulated and stimulated BAECs, respectively. The autoradiographic intensity of the cytosolic HSP25 did not increase as a result of PMA stimulation (Fig. 3, panel A). In contrast, the autoradiographic intensity of the membrane-associated HSP25 exhibited a 2.7-fold increase upon treatment with PMA.

To compare the relative levels of PMA-induced phosphorylation of membrane-associated HSP25 to cytosolic HSP25, the autoradiographic integrated densities (Rad) of cytosolic HSP25 and membrane-associated HSP25 were determined for quiescent and stimulated BAECs. The autoradiographic densitometric values (Rad) obtained for the cytosolic and membrane-associated HSP25 present in fractions obtained from quiescent or PMA-stimulated BAECs were divided by the densitometric values (Ag) obtained for the anti-HSP25 immunostained bands of those fractions. The Rad/Ag ratios were averaged and are presented with the calculated standard deviations.
Transfectants were biotinylated either at the apical (panels A and B) or basal (panels C and D) surface, lysed with 0.5% Triton X-100, and then subjected to streptavidin-agarose precipitation. The starting and eluted material were analyzed via immunoblotting using the anti-HSP27 monoclonal antibody G3.1 (panel A) and a functional association of HSP27 with membrane-associated microfilaments has been suggested (9–11), it is likely that these microfilaments binds the microfilament binding proteins α-actinin and myosin light chain (Fig. 5, panel B). These proteins have filament bundling and contractile activities, respectively, and do not interact with G-actin (29–31). That the SA-precipitated actin represents filamentous and not monomeric actin is also suggested by the fact that endothelial cell F-actin, including cortical structures, exists below tight junctions (1) that are impermeable to apically applied biotin. The fact that apical biotinylation failed to result in the isolation of any actin suggests that surface biotinylation and subsequent streptavidin precipitation does not precipitate monomeric actin and that the actin isolated from the basally biotinylated cells represents F-actin.

PMA stimulation did not result in a significant change in the amount of HSP25/27 precipitated from basally biotinylated cells via SA precipitation (Fig. 6, panel A). For example, densitometric analysis revealed that the ratios of HSP27, muHSP27, and HSP25 precipitated from stimulated cells over that precipitated from quiescent wtHSP27 BAECS, muHSP27 BAECS, and control cells were 1.1, 1.2, and 0.9, respectively.

In contrast to the precipitation of basolateral HSP25/27, PMA stimulation resulted in the isolation of additional actin from basally biotinylated HSP27 BAECS and control BAECS (Fig. 6, panel A). For the wtHSP27 BAECS, PMA stimulation resulted in an average 1.8 ± 0.3-fold (n = 5) increase in the amount of actin precipitated as determined by densitometric analysis of the immunoblot stained with a monoclonal antibody specific for non-muscle β-actin (Fig. 6, panel A). PMA stimulation of control BAECS resulted in a more modest 1.3 ± 0.18-fold (n = 3) increase in the amount of actin precipitated (Fig. 6, panel A). In contrast, expression of muHSP27 at the mem-
Basal surface and then lysed with 0.5% Triton X-100. The lysates were subjected to streptavidin-agarose precipitation. The lysates (lanes 2 and 4) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were subjected to immunoblot analysis using monoclonal antibodies specific for human HSP27 and non-muscle β-actin. Panel B, the streptavidin-agarose eluates obtained from quiescent or PMA-stimulated basally biotinylated wtHSP27 (n = 4), muHSP27 (n = 3), and vector control cells (n = 3) were subjected to immunoblot analysis using the anti-β-actin monoclonal antibody. The ratio of densitometric values obtained for the membrane-associated F-actin obtained for the actin bands of stimulated cells over that obtained for the nonstimulated cells was then calculated.

The stabilization of cortical actin filaments in stressful situations, enhanced membrane ruffling, and increased pinocytosis in cells expressing elevated levels of HSP27 suggests that the HSP25/27 activity is spatially regulated. Data presented in this report identified a subpopulation representing nearly 30% of the HSP25/27 antigen isolated to a similar extent as wtHSP27, indicate that association of HSP25/27 at the basolateral membrane is independent of its phosphorylation state. This is in agreement with the results obtained for the entire membrane HSP25/27 pool. Likewise, basolateral HSP25/27 exhibited a greater propensity to serve as substrate for PMA-induced HSP25/27 kinase activity than the remaining HSP25/27, a finding consistent with the partitioning of phospho-HSP25 with the plasma membrane in BAECs fractionated by sequential hypotonic lysis and homogenation.

**DISCUSSION**

The small molecular weight heat shock protein HSP27 has been demonstrated to be an important microfilament modulating protein involved in the control of microfilament dynamics and organization that is regulated by inducible kinase activity. For example, phosphorylation of HSP25/27 retards the inhibitory filament capping activity demonstrable in vitro (5–7). In cultured cells, transfection and expression of the wild type human HSP27 stabilizes cortical filaments that normally disaggregate in response to cytokines, oxidative stress, and hyperthermia, conditions that result in the phosphorylation of HSP27 in vivo (11, 14–17). Enhanced HSP27 expression in these cells conveyed increased thermo-tolerance, whereas expression of a non-phosphorylatable mutant HSP27 did not convey resistance and had no effect on microfilament dynamics or, in some cases, reversed the effect of the wild type protein (9). In non-stressed cultures, expression of the phosphorylatable wild type protein enhanced pinocytic activity and membrane ruffling (9), processes dependent on a dynamic membrane-associating cytoskeleton (12, 13). The reliance on the ability of HSP27 to be phosphorylated to impart biological consequences may not be universal, however. For example, Knauf et al. (18) have shown that a non-phosphorylatable mutant HSP27 conveys the same thermo-tolerance in Swiss 3T3 cells as did the wild type protein. These data indicate that HSP27 phosphorylation, and thus regulation of HSP27 activity, may be spatially and/or temporally regulated.

**FIG. 6.** Streptavidin-agarose precipitation of radio phosphorylated HSP25/27 and membrane-associated F-actin. Panel A, wtHSP27-BAECs, muHSP27-BAECs, and vector control BAECs were radiophosphorylated, allowed to remain quiescent (lanes 1 and 3), or stimulated with PMA (lanes 2 and 4) while growing on Transwell membranes. Biotin was placed in the subliminal chamber and then Triton X-100 extracts were prepared (lanes 1 and 2). The extracts were subjected to SA precipitation and the eluates and extracts (lanes 3 and 4) were subjected to SDS-PAGE and transferred to nitrocellulose which was then exposed to film. After radiography, the blots were immunostained for HSP25 and HSP27.

**FIG. 7.** Autoradiographic analyses of streptavidin-agarose eluates. wtHSP27, muHSP27, and vector control cells were radiophosphorylated with 32P, left quiescent (lanes 1 and 3), or stimulated with PMA (lanes 2 and 4) while growing on Transwell membranes. Biotin was placed in the subliminal chamber and Triton X-100 extracts were prepared (lanes 1 and 2). The extracts were subjected to SA precipitation and the eluates and extracts (lanes 3 and 4) were subjected to SDS-PAGE and transferred to nitrocellulose which was then exposed to film. After radiography, the blots were immunostained for HSP25 and HSP27.

HSP25 and HSP25 phosphorylation increased 1.38- and 2 fold over basal levels (lanes 1 and 3), respectively. In contrast, muHSP27 (lanes 2 and 4) exhibited a 1.70 and 1.4 fold increase, respectively (Fig. 7) which is consistent with the data presented above.
level in the membrane fraction did not change upon PMA stimulation, indicating that phosphorylation did not induce a change in HSP25/27 distribution. Thus, any alteration in the activity of the membrane-associated HSP25/27 caused by PMA stimulation is likely a result of its phosphorylation. Although the kinase present at the membrane responsible for HSP27 phosphorylation has yet to be defined, one kinase that has been shown to phosphorylate HSP27 is the mitogen-activated protein kinase-activated protein kinase 2 (11, 14–17).

Although extremely useful in identifying a distinct pool of cellular HSP25/27, the hypotonic buffer used in the fractionation protocol employed does not preserve F-actin structure and therefore is of little use in addressing whether the functional consequences of HSP25/27 phosphorylation involve microfilaments. A second fractionation method in which membrane-associated F-actin was isolated and quantitated was therefore employed. BAEC transfectants were biotinylated at their basal surface, lysed in Triton X-100 in a buffer that stabilizes microfilaments (3), and then were subjected to SA precipitation. In these experiments, 1) HSP25 and HSP27 were detected exclusively in the eluates obtained from basal biotinylated cells, demonstrating an association with basolateral membrane components, 2) muHSP27 was detected in these eluates indicating that membrane association does not require HSP27 phosphorylation, and 3) PMA induced the phosphorylation of the eluted HSP25/27 to over twice that of untreated cells whereas lysate HSP25/27 was less affected. Thus, by two different procedures, it is apparent that membrane association of HSP27 is independent of its phosphorylation state and that this subpopulation of HSP25/27 is substrate for PMA-induced HSP27 kinase activity.

The exact nature of the interaction(s) of HSP25/27 at the basolateral membrane remains to be determined. It is possible that HSP27 associates with the membrane itself, with integral membrane proteins, or through a direct interaction with basolateral microfilaments. The former two possibilities are indicated by the persistence of HSP25/27 with the plasma membrane fragment in buffer conditions in which actin filaments readily depolymerize. An association of HSP25/27 with the membrane would not be novel since other actin filament modulating proteins (e.g. vinculin) also interact with the membrane (32). The possibility that HSP25/27 was fractionated with membrane components due to the interaction with the membrane-associated microfilaments is suggested by the in vitro studies demonstrating that HSP25/27 acts as a filament capping protein (5, 6).

The localization of HSP27 to the basolateral membrane suggests that this subpopulation of HSP27 may have a pivotal role in regulating basolateral membrane-associated microfilaments, particularly when the membrane-associated cytoskeleton is undergoing dynamic changes. In Swiss 3T3 cells, PMA treatment has been shown to stimulate membrane ruffling, a process associated with the enhanced polymerization of cortical actin (33). Likewise, PMA stimulation of wtHSP27 BAECs generated a substantial increase in the amount of F-actin isolated via streptavidin precipitation, whereas muHSP27 expression actually decreased the amount of membrane-associated F-actin precipitated. These changes do not appear to be artifacts of the biotinylation of activated versus non-activated cells since SA precipitated equivalent amounts of biotinylated material and associated proteins. For example, the amount of wt- or muHSP27 isolated from activated or quiescent cells was found to be identical. The increased membrane-associated F-actin in the wtHSP27 BAECs is consistent with the observation that stress, which results in HSP27 phosphorylation, leads to an accumulation of cortical actin in fibroblasts (8, 10, 11). A reduction in the amount of membrane-associated F-actin in muHSP27 expressing cells is congruous with the dominant negative effects of muHSP27 expression on membrane ruffling and pinocytosis (9).

The mechanism by which wtHSP27 or muHSP27 expression affects PMA-induced changes in the basolateral membrane-associated microfilament cytoskeleton is suggested by the in vitro observations of HSP25/27 activity (5–7). The barbed-end capping activity of HSP25/27 inhibits actin polymerization when HSP25/27 is unphosphorylated. Since both wtHSP27 and muHSP27 localize to the membrane, PMA stimulation would result in the phosphorylation of the wild type protein but not the mutant gene product. Thus, cell activation would inhibit wtHSP27 activity, allowing the generation of F-actin. Introduction of muHSP27 would establish a population that is constitutively active and continuously inhibits F-actin generation. In this manner HSP25/27 may contribute to the regulation of membrane-associated F-actin polymerization.

A putative consequence of this regulation is the control of endothelial cell migration. It has been recently demonstrated that the expression of wtHSP27 or muHSP27 enhances or retards BAEC migration, respectively, in a wound assay. Since endothelial cell migration is dependent on the polymerization of a meshwork of lamelipodial actin that is associated with the basolateral membrane (4), HSP25/27 may be regulating migration at the level of lamelipodial extension. This is substantiated by the fact that the F-actin demonstrated to be affected by HSP25/27 and subject to SA precipitation is part of the Triton X-100 soluble pool of cellular F-actin. This pool has been demonstrated in other cell types to be highly dynamic and includes filaments involved in lamelipodial extension and chemotaxis (2, 3).

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