HSP27 phosphorylation and interaction with actin-myosin in smooth muscle contraction

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Bitar, Khalil N. HSP27 phosphorylation and interaction with actin-myosin in smooth muscle contraction. Am J Physiol Gastrointest Liver Physiol 282: G894–G903, 2002.—We have investigated the role of heat shock protein 27 (HSP27) phosphorylation and the association of HSP27 with contractile proteins actin, myosin, and tropomyosin. Smooth muscle cells were labeled with [32P]orthophosphate. C2-ceramide (0.1 μM), an activator of protein kinase C (PKC), induced a sustained increase in HSP27 phosphorylation that was inhibited by calphostin C. C2-ceramide-induced (0.1 μM) sustained colonic smooth muscle cell contraction was accompanied by significant increases in the association of HSP27 with tropomyosin and in the association of HSP27 with actin. The significant increases occurred at 30 s after stimulation and were sustained at 4 min. Contraction was also associated with strong colocalization with tropomyosin and with actin as observed after immunofluorescent labeling of tropomyosin, actin, and HSP27 followed by confocal microscopy. Transfection of smooth muscle cells with HSP27 phosphorylation mutants indicated that phosphorylation of HSP27 could affect myosin association with actin. In conclusion 1) HSP27 phosphorylation appears to be necessary for reorganization of HSP27 inside the cell and seems to be directly correlated with the PKC signal transduction pathway, and 2) agonist-induced phosphorylation of HSP27 modulates actin-myosin interaction through thin-filament regulation of tropomyosin.

HEAT SHOCK PROTEIN 27 (HSP27) is a member of the mammalian small HSP family. HSP27 is expressed in a variety of tissues in the presence or absence of stress (12). HSP27 is relatively abundant in all types of cells (36), and it colocalizes with actin filaments in cardiac (24), skeletal (35), and smooth muscle (16). Evidence has shown that HSP27 is important in many cell functions, such as cell survival during stress, apoptosis mediated by Fas/APO1 receptor, and microfilament organization in response to growth factor or stress, as well as smooth muscle contraction (4, 5, 21). HSP27 has been shown to exhibit chaperone activity in vitro (17) and modulate actin filament microdynamics (2, 23). HSP27 becomes phosphorylated in response to heat shock and in response to different stimuli such as cytokines, growth factors, and peptide hormones (21). Landry et al. (21) have mapped the phosphorylation sites in human HSP27 and showed that mitogen-activated protein (MAP) kinase-activated protein kinase-2 (MAPKAP2 kinase) phosphorylates human HSP27 protein on Ser15, Ser78, and Ser82. Ser82 appears to be the major site of in vivo phosphorylation, followed by Ser78 and Ser15, the minor sites (21, 33). HSP27 exists as both large oligomers hypothesized to have chaperone-like activity and as smaller oligomers that bind to and cap the barbed end of microfilaments and stabilize them (35). Phosphorylation is accompanied by a decrease in the size of HSP27 oligomers. Phosphorylation of HSP27 changes the actin cytoskeleton and actin-dependent events.

Because HSP27 may be of importance in smooth muscle contraction, studies of its structure-function relationship together with its interaction with other thin- and thick-filament proteins, namely actin, myosin, and tropomyosin, will greatly complement our knowledge of the contractile machinery in smooth muscle cells.

Tropomyosins are widely distributed in virtually all eukaryotic cells. They function as a crucial part of the contractile apparatus and of thin-filament assemblies of both muscle and nonmuscle cells. Tropomyosin assemblies into an α-helical coiled-coil dimer, with each molecule interacting with six or seven monomers of actin (13). Tropomyosin also binds to itself and helps wrap around the actin molecule to stabilize thin-filament assembly (31).

Although the basic molecular architecture is the same, the functions of tropomyosin vary in different tissues and cell types, as well as in their location and roles within a single cell type (31). In striated muscle, whether skeletal or cardiac, tropomyosin together with the troponin complex provides the calcium switch for the turning on and off of the actin/myosin/ATPase activity associated with contraction/relaxation. In smooth muscle, the role of tropomyosin is less well understood. In smooth muscle, calcium regulation of the phosphorylation and dephosphorylation of myosin light chains is well established, but the control by...
calcium at the level of the thin filaments is not well clarified. In smooth muscle, tropomyosin plays a central role in such processes, but the knowledge of the structural features and interaction properties with other thin-filament proteins is crucial to our understanding of the system.

We have investigated how HSP27 modulates agonist-induced smooth muscle contraction and the role of its phosphorylation. Our findings indicate that 1) phosphorylation of HSP27 in response to C2-ceramide (0.1 μM) is PKC-mediated; 2) contraction is associated with a sustained increase in the association of HSP27 with actin and with tropomyosin; 3) HSP27 strongly colocalizes with the thin filaments actin and tropomyosin in transverse bands during contraction; and 4) phosphorylated HSP27 is necessary for actin-myosin association. Data suggest a role for HSP27 in modulation of smooth muscle contraction through its phosphorylation, possibly by interacting with the thin filaments.

MATERIALS AND METHODS

Materials

The following reagents were purchased. Monoclonal and polyclonal rabbit anti-tropomyosin antibody and monoclonal mouse anti-myosin heavy-chain antibodies were from Sigma (St. Louis, MO). Monoclonal mouse anti-smooth muscle cell actin antibody was from Chemicon (Temecula, CA). Oregon green 412 conjugated phalloidin and Prolong antifade mounting reagents were from Molecular Probes (Eugene, OR). Cy-3-conjugated AffiniPure F(ab’)2 goat anti-mouse IgG, and Cy-4-conjugated AffiniPure F(ab’)2 goat anti-mouse IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Monoclonal mouse anti-HSP27 antibody (2B4-123) was previously described (4).

C2-ceramide (0.1 μM) was from Matreya (State College, PA), and collagenase type II was purchased from Worthington Biochemical (Freehold, NJ). Protein G-Sepharose was from Pharmacia Biotech (Uppsala, Sweden). Polyvinylidene fluoride (PVDF) membranes were from Bio-Rad (Hercules, CA). Qiagen. AffiniPure F(ab’)2 goat anti-mouse IgG, and Cy-3-conjugated AffiniPure F(ab’)2 goat anti-mouse IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Monoclonal mouse anti-HSP27 antibody (2B4-123) was previously described (4).

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Isolation of smooth muscle cells from rabbit rectosigmoid. Smooth muscle cells of rabbit rectosigmoid were isolated as described (4). Briefly, the internal anal sphincter from anesthetized New Zealand White rabbits, consisting of the distalmost 3 mm of the circular muscle layer, ending at the junction of skin and mucosa, was removed by sharp dissection. A 5-cm length of the rectosigmoid orad to the junction was dissected and digested to yield isolated smooth muscle cells. The tissue was incubated for two successive 1-h periods at 31°C in 15 ml of HEPES buffer (pH 7.4). The composition of the buffer was (in mM): 115 NaCl, 5.7 KCl, 2.0 KH2PO4, 24.6 HEPES, 1.9 CaCl2, 0.6 MgCl2, and 5.6 glucose containing 0.1% (wt/vol) collagenase (150 U/ml, Worthington CLS Type II), 0.01 (wt/vol) soybean trypsin inhibitor, and 0.184 (wt/vol) DMEM. At the end of the second enzymatic incubation period, the medium was filtered through 500 μm Nitex mesh. The partially digested tissue left on the filter was washed four times with 10-ml collagenase-free buffer solution. Tissue was then transferred into 15 ml of fresh collagenase-free buffer solution, and cells were gently dispersed. After a hemocytometric cell count, the harvested cells were resuspended in collagenase-free HEPES buffer (pH 7.4). Each rectosigmoid yielded 10–20 × 106 cells.

Metabolic labeling of smooth muscle cells. Smooth muscle cells from rabbit colon were isolated as described, and the last suspension was done in phosphate-free DMEM. [32P]orthophosphate (0.8–1.0 μCi/ml) was added, and the cells were labeled at 37°C for 4 h.

Cell stimulation and immunoprecipitation of phosphorylated HSP27. To test the hypothesis that phosphorylation of HSP27 is an important step in smooth muscle contraction, we studied the effect of the contractile agonist C2-ceramide (0.1 μM). After radiolabeling, isolated colonic smooth muscle cells were stimulated with C2-ceramide (0.1 μM) for 30 s and 4 min, with or without a 20-min preincubation with the PKC inhibitor H9262, tropomyosin (10−4 M). The reaction was stopped by quickly removing the supernatant and placing the cells on ice. The cells were rinsed three times with ice-cold PBS, then lysed in 2% SDS. The lysate was transferred to a 1.5-ml microfuge tube, boiled for 3 min, vortexed, and centrifuged for 5 min at 12,500 g. The supernatant was then transferred to a new microfuge tube and used for the subsequent steps.

Protein content was quantified using the Bio-Rad assay reagent; an equal amount of protein was used for each sample. Each sample was brought to an equal volume and then diluted 20 times with radioimmunoprecipitation assay (RIPA) buffer of the following composition: 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate Na, 0.50 mM Tris·HCl, and 50 mM NaF.

Immunoprecipitation was then performed at 4°C, and a monoclonal antibody to HSP27 (2B4-123) was added at a 1:50 (wt/vol) dilution and allowed to mix overnight. The next day, protein G-Sepharose (2 μl/100 μl lysate) was added, and the samples were mixed for 2 h. Samples were centrifuged at 12,500 g for 5 min, and the immunocomplexed beads were transferred to 1.5 ml Microfuge tubes after removal of the supernatant. The beads were rinsed 5 times with 1 ml of RIPA buffer containing 0.1% SDS but no NaF. The immunoprecipitates were then prepared for 1-D or 2-D SDS-PAGE.

SDS-PAGE and electrophoretic transfer. For 1-D SDS-PAGE, the samples were mixed in an equal volume of ×2 sample buffer consisting of 50 mM Tris, 10% (vol/vol) glycercol, 2% (wt/vol) SDS, and 0.1% (wt/vol) bromophenol blue, pH 6.8. Proteins were separated by 12.5% or 15% SDS-PAGE and transferred onto nitrocellulose membranes or PVDF. Proteins were identified by chemiluminescence. Autoradiography was performed on blots or dried gels using a Phosphor-Imager.

Immunoprecipitation and immunoblotting. Smooth muscle cells (4–5 × 10^6) were either untreated or treated with C2-ceramide (0.1 μM) for up to 4 min. After treatment, cells were washed with buffer A consisting of (in mM): 150 NaCl, 16 Na2HPO4, 4 NaH2PO4, pH 7.4 (PBS), containing 1 mM Na2VO4. The cells were then disrupted by sonication in buffer B consisting of (in mM): 1 Na2VO4, 1 Na2, 2 PMSF, 5 EDTA, 1 Na3MoO4, 1 DTT, 20 NaH2PO4, 20 Na2HPO4, 20 Na3PO4·10 H2O, plus 50 μl/ml DNase/RNase, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml antipain (pH 7.4), and centrifuged for 15 min at 14,000 g. Protein G-Sepharose was washed twice with buffer B to make a 50% suspension. Lysate containing 200 μg protein in a total of 500 μl of buffer B was preclarified with 50 μl of
protein G-Sepharose bead slurry by rocking at 4°C for 30 min. The mixture was spun at 14,000 g for 5 min at 4°C, and 1–2 μg of anti-HSP27 monoclonal antibody was added to the resultant supernatant. The mixture was incubated at 4°C for 1 h, followed by the addition of 50 μl of protein G-Sepharose bead slurry. The mixture was further incubated at 4°C for 1 h and spun at 14,000 g for 5 min, and the supernatant was aspirated off. The pellet was washed three times with buffer A, resuspended in 25 μl of 2× sample buffer, and boiled for 5 min. Immunoprecipitates were subjected to SDS-PAGE, and proteins were electrophoretically transferred to PVDF membranes. Immunoblotting was performed using a monoclonal anti-actin antibody (1:1,000), a monoclonal anti-tropomyosin antibody, or a monoclonal anti-myosin heavy-chain antibody (1:1,000) for 1 h at 24°C. Membranes were then reacted with peroxidase-conjugated goat anti-mouse IgG (1:2,500) antibody for 1 h at 24°C. Enzymes on the membrane were visualized with luminescent substrates.

Coverslip preparation. Glass coverslips were washed in 70% ethanol for 30 min with gentle agitation. They were allowed to air dry, placed in six-well plates, and irradiated with ultraviolet light for 30 min, then coated with 0.5% poly-l-lysine and allowed to air dry. At the end of the second enzymatic digestion, the digested tissue was washed, titrated, and filtered as described before. Dispersed cells were transferred to a six-well tissue culture plate and allowed to settle for 24 h on the poly-l-lysine-coated coverslips in a humidified 5% CO2 environment.

Cell fixation. Cells were fixed with 3.5% formaldehyde in PBS for 10 min. Fixative was removed, and cells were washed twice with 100-mM glycine buffer, pH 7.4, for 5 min followed by one wash with PBS. The cells were then permeabilized by adding 3 ml of the permeabilization solution with 1% Triton X-100 to each coverslip for 10 min. After permeabilization, the cells were rinsed three times with 3-mi aliquots of PBS.

Triple immunofluorescent labeling for actin, tropomyosin, and HSP27. Triple labeling of actin, tropomyosin, and HSP27 was performed in either resting or contracted smooth muscle cells. Cells were treated with C2-ceramide (0.1 μM), permeabilized, and fixed. Cells were incubated for 1 h with normal goat serum, followed by three 10-min washes in PBS. Cells were then reacted with Oregon green 412 phalloidin (2 units/coverslip) for 20 min and washed with PBS three times for 5 min each time. Cells were incubated for 1 h with first primary antibody, a monoclonal mouse anti-HSP27 antibody (1:50), together with second primary antibody, a polyclonal rabbit anti-tropomyosin antibody (1:100), followed by three 10-min washes in PBS. Subsequently, the cells were incubated with first secondary antibody, 1:500 dilution of AffiniPure F(ab′)2 donkey anti-mouse IgG, together with second secondary antibody, 1:500 dilution Cy-3-conjugated AffiniPure F(ab′)2 goat anti-rabbit IgG, for 1 h, followed by three 10-min washes in PBS. Finally, the cells on the coverslip were mounted on a slide with Prolong antifade mounting reagents. The following controls were also made: 1) Cy-3 only; 2) Cy-5 only; 3) Cy-3 with first primary antibody; 4) Cy-5 with first primary antibody; 5) Cy-3 with second primary antibody, and 6) Cy-5 with second primary antibody.

Excitation parameters for the fluorescent probes were as follows: Oregon green excitation at 492 nm, emission at 520 nm; Cy-3 excitation at 550 nm, emission at 570 nm; Cy-5 excitation at 650 nm, emission at 670 nm. Confocal images were obtained on Noran confocal imaging system. Images were analyzed using Intervision (Silicon Graphics) and Adobe Photoshop.

Results

HSP27 Phosphorylation is Stimulated by C2-ceramide (0.1 μM) and is Inhibited by the PKC Inhibitor Calphostin C

HSP27 was immunoprecipitated from rabbit colonic smooth muscle cells after metabolic labeling with [32P]orthophosphate and stimulation with C2-ceramide (0.1 μM). Proteins were separated in 12.5% SDS-PAGE, the gel was dried, and radioactive bands were visualized by autoradiography. C2-ceramide (0.1 μM) induced phosphorylation of HSP27 as early as 30 s after stimulation (47.15 ± 10% increase above control, n = 6, P < 0.02). The phosphorylation was sustained at 4 min after stimulation (52.5 ± 8.5% increase above control, n = 6, P < 0.01) (Fig. 1). Phosphorylation was also induced by bombesin, a peptide shown to induce sustained contraction of smooth muscle cells from the rabbit colon (16.9 ± 3.9% increase above control at 30 s and 33.6 ± 2.7% at 4 min, n = 3). Preincubation of smooth muscle cells with calphostin C (10−6 M) resulted in inhibition of C2-ceramide-induced (0.1 μM) phosphorylation of HSP27 (76 ± 14% inhibition of phosphorylation, n = 3, P < 0.01).
Sustained Phosphorylation of HSP27 in Colonic Smooth Muscle Cells Independent of Activation of Myosin Light Chain Kinase

We next examined the effect of C2-ceramide (0.1 μM) on the phosphorylation of HSP27 by isoelectric focusing followed by Western blotting using a specific monoclonal antibody (2B4–123). In untreated smooth muscle cells, all four isoforms (phosphorylated, mono-, di-, and triphosphorylated) were found, indicating a basal level of phosphorylation. C2-ceramide (0.1 μM) induced an increase in the di- and triphosphorylated isoforms at 30 s (134.6 ± 23.6 and 144.4 ± 26.3% increase, respectively, P < 0.05) and at 4 min after stimulation (163.9 ± 13.5 and 160.6 ± 8.7% increase, respectively, P < 0.01) (Fig. 2). There was also a concomitant decrease in the amount of unphosphorylated isoform both at 30 s (66.4 ± 5.2% decrease, n = 4, P < 0.002) and at 4 min (60.3 ± 3.7% decrease, n = 4, P < 0.001).

The rapidity and the kinetics of the phosphorylation of HSP27 seem to correspond to the contractile response in colonic smooth muscle cells. We next tested whether C2-ceramide-induced phosphorylation of HSP27 was mediated by activation of myosin light chain kinase. Pretreatment of the cells with the myosin light chain kinase inhibitor ML7 (20 μM for 30 min) had no effect on the phosphorylation of HSP27 induced by C2-ceramide (0.1 μM). C2-ceramide (0.1 μM) induced an increase in the diphosphorylated isoforms at 4 min after stimulation (120.7 ± 4.1% increase above control, P < 0.07) and in the triphosphorylated isoform (157.6 ± 10.0% increase above control, P < 0.001). There was a concomitant decrease in the amount of unphosphorylated isoform [52.7 ± 2.4% of control, P < 0.006 (Fig. 3)]. Data thus seem to suggest that the sustained phosphorylation of HSP27 and its role in smooth muscle contraction is independent of activation of MLC kinase but could be mediated by PKC activation in colonic smooth muscle cells.

HSP27 Coimmunoprecipitated with Actin and with Tropomyosin

Actin, myosin, and tropomyosin are the major contractile proteins involved in generation of contraction in smooth muscle. We have previously examined the association of HSP27 with other contractile proteins (16). HSP27 coimmunoprecipitated with actin, myosin,
A decrease in the amount of unphosphorylated isoform (52.7 ± 0.3%) is independent of the activation of myosin light-chain kinase. We have measured changes in contractile proteins. We have investigated the possible interactions between HSP27 and actin and tropomyosin, as revealed by immunoblotting. The observed binding affinity between those proteins suggested the possible interactions between HSP27 and contractile proteins. We have measured changes in immunoprecipitates in response to stimulation by the contractile agonist C2-ceramide (0.1 µM). Immunoprecipitations were carried out using a mouse anti-HSP27 monoclonal antibody (2B4–123). Pretreatment of the cells with the myosin light-chain kinase inhibitor ML7 (20 µM for 30 min) had no affect on the phosphorylation of HSP27 induced by C2-ceramide. C2-ceramide induced an increase in the diphosphorylated isoforms at 4 min after stimulation (120.7 ± 4.1% increase above control, P < 0.07) and in the triphosphorylated isoform (157.6 ± 1.0% increase above control, P < 0.01) and induced a decrease in the amount of unphosphorylated isoform (52.7 ± 2.4% of control, P < 0.01). Data thus seem to suggest that the phosphorylation of HSP27 and its role in smooth muscle contraction is independent of the activation of myosin light-chain kinase. Top, representative blot of 4 independent experiments.

And tropomyosin, as revealed by immunoblotting. The observed binding affinity between those proteins suggested the possible interactions between HSP27 and contractile proteins. We have measured changes in immunoprecipitates in response to stimulation by the contractile agonist C2-ceramide (0.1 µM). Immunoprecipitations were carried out using a mouse anti-HSP27 monoclonal antibody (2B4–123) (16). The immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with either an anti-actin monoclonal antibody or anti-tropomyosin antibody. In the control unstimulated smooth muscle cells, there was a small amount of tropomyosin that coimmunoprecipitated with HSP27. C2-ceramide (0.1 µM) induced a marked increase (54 ± 3%, n = 3, P < 0.05) in the tropomyosin protein that immunoprecipitated with HSP27 at 30 s. The increase in the association of HSP27 with tropomyosin was sustained at 4 min after stimulation (Fig. 4A). Similarly, in the control unstimulated smooth muscle cells, actin coimmunoprecipitated with HSP27. C2-ceramide (0.1 µM) induced a marked increase in the actin protein that immunoprecipitated with HSP27. The increase in the association between HSP27 and actin occurred at 30 s and was sustained at 4 min (51 ± 5%, n = 3, and 60.6 ± 3%, n = 3, respectively, P < 0.05) (Fig. 4B). The increased associations of HSP27 with actin and with tropomyosin suggest that HSP27 might act as a modulator, linking these proteins together to bring about sustained smooth muscle contraction.

**HSP27 Colocalizes with Tropomyosin and Actin During Smooth Muscle Contraction**

We further investigated the cytoskeletal reorganization of actin, tropomyosin, and HSP27 in response to contraction induced by C2-ceramide (0.1 µM). Smooth muscle cells were isolated and allowed to attach to poly-L-lysine-coated coverslips. The cells were stimulated with C2-ceramide (0.1 µM) and immunostained with either a polyclonal anti-tropomyosin antibody or a monoclonal anti-HSP27 antibody (2B4–123) (4). The immunolocalizations were then examined under a confocal microscope. Evaluation was carried out on optical sections of reconstructed 3-D images prepared from a Z series. Immunostaining of actin, tropomyosin, and HSP27 followed by confocal microscopy revealed that in response to C2-ceramide (0.1 µM), tropomyosin and HSP27 strongly colocalized in transverse bands, similar to previously described localization of actin with HSP27 in contracted smooth muscle cells of the colon (16). We further examined the localization of actin together with tropomyosin and HSP27. In a fully contracted cell, actin and tropomyosin reorganized their distribution. The strong colocalization of actin and tropomyosin seems very similar in shape and pattern to the colocalization of tropomyosin and HSP27. When those bands were examined under high-power (×100 oil immersion) confocal microscope lens and reconstructed digitally from the serial Z sections, the bands showed a very characteristic coiled-coil pattern (Fig. 5).

**HSP27 Phosphorylation Modulates the Thin-Filament Regulation of Smooth Muscle Contraction**

To further understand the regulatory role of HSP27 in contraction, we examined the effects of HSP27 phosphorylation on the interaction of contractile proteins. We studied the gain-of-function and loss-of-function HSP27 phosphorylation mutants in smooth muscle cells. We transfected smooth muscle cells with phosphorylation mutants of HSP27: 3D or 3G HuHSP27. In the 3G HSP27 mutant, all three serine phosphorylation sites (Ser15, Ser78, and Ser82) of the human HSP27 cDNA were replaced with glycine, to mimic nonphosphorylatable serine residues. In the 3D mutant, Ser15, Ser78, and Ser82 were mutated into aspartate to mimic constitutively phosphorylated residues. These mutant constructs were expressed in smooth muscle cells, and the effects of HSP27 phosphorylation on the association of contractile proteins were investigated. We ex-
amined the association of actin with myosin in cells transfected with these two HSP27 mutants. Out of $5 \times 10^6$ cells transfected through G-418 selection, the transfection rate was estimated to be $\sim 40\%$. Transfections were successfully demonstrated by detection of the mutant HuHSP27 protein via immunoblotting of the transfected cell lysates with HuHSP27-specific antibody, which has been shown not to have any cross activity between rabbit and mouse. Lysates extracted from the transfected cells were immunoprecipitated with monoclonal mouse anti-smooth muscle cell actin antibody. The immunoprecipitates were then subjected to immunoblotting using monoclonal mouse anti-myosin heavy-chain antibody. In the cells transfected with 3D HSP27 mutant, the amount of myosin binding to actin increased compared with normal nontransfected cells. C2-ceramide-induced (0.1 $\mu$M) association of actin-myosin greatly decreased in 3G HSP27 mutant transfected cells (48.5 $\pm$ 4.4%, $P < 0.005$) (Fig. 6). Data indicated that HSP27 phosphorylation might modulate the actin-myosin interaction by regulating the thin filaments of smooth muscle. Our data suggest that in response to a contractile agonist, phosphorylation of HSP27 seems to facilitate the interaction of actin with myosin, probably as a result of a stronger association of HSP27 with tropomyosin.

**DISCUSSION**

We have examined the possible role of HSP27 and its phosphorylation during cytoskeletal reorganization in smooth muscle contraction. We have examined the association of HSP27 with tropomyosin and its effects on the interaction of actin with myosin. We propose that HSP27 may affect smooth muscle thin-filament regulation through its phosphorylation and play an active role in the contractile machinery through its association with contractile proteins in smooth muscle.

We have previously reported that preincubation of smooth muscle cells from the rabbit rectosigmoid with a monoclonal antibody to HSP27 inhibits PKC-mediated contraction induced by bombesin (4). We have demonstrated that HSP27 and MAP kinase colocalize in smooth muscle cells in response to contraction induced by bombesin (37). These findings give support to a role for HSP27 in PKC-mediated contraction. Treatment of the cells with C2-ceramide (0.1 $\mu$M) resulted in an increase in phosphorylation of HSP27 detected by autoradiography at 30 s and 4 min. This increase in phosphorylation was inhibited by preincubation of the cells with calphostin C ($10^{-6}$ M). Data suggest that inhibition of PKC leads to inhibition of HSP27 phosphorylation. The following physiological paradigm arises: is HSP27 phosphorylation required for contraction and, if so, does phosphorylation of HSP27 modulate the increase in association of contractile proteins, and thus modulation of contraction?

**Fig. 4. Coimmunoprecipitation of the cytoskeletal proteins actin and tropomyosin with HSP27.** Smooth muscle cells ($5 \times 10^6$) were either untreated or treated with C2-ceramide (0.1 $\mu$M) for 30 s and 4 min. The protein was extracted and immunoprecipitated with monoclonal anti-HSP27 antibody as described in MATERIALS AND METHODS. SDS-PAGE and proteins were electrophoretically transferred to polyvinylidene difluoride membranes. Immunoblotting was performed using a monoclonal anti-actin antibody (1:1,000) or a monoclonal anti-tropomyosin antibody (1:1,000) for 1 h at 24°C. The membrane was then reacted with peroxidase-conjugated goat anti-mouse IgG (1:2,500) antibody for 1 h at 24°C. Enzymes on the membrane were visualized with luminescent substrates. A: the amount of tropomyosin immunoprecipitated with HSP27 increases in response to ceramide stimulation. Immunoprecipitates of HSP27 (IP) from 200 $\mu$g of protein lysate of cells either untreated or stimulated with C2-ceramide were subjected to SDS-PAGE and Western blotted with anti-tropomyosin monoclonal antibody (IB). C2-ceramide induced a significant and sustained (30 s and 4 min) increase in the association of HSP27 with tropomyosin. Inset, representative blot of 4 independent experiments. B: the amount of actin immunoprecipitated with HSP27 increases in response to ceramide stimulation. Immunoprecipitates of HSP27 (IP) from 200 $\mu$g of protein lysate of cells either untreated or stimulated with C2-ceramide were subjected to SDS-PAGE and Western blotted with anti-actin monoclonal antibody (IB). C2-ceramide induced a significant and sustained (30 s and 4 min) increase in the association of HSP27 with tropomyosin. Inset, representative blot of 4 independent experiments.
During prolonged contraction of some smooth muscle types, the association between intracellular calcium concentration, LC20 phosphorylation levels, and cross-bridge cycling grades and force has been reported (7, 25). Others have shown that in a calcium-free medium, PKC-dependent translocation of cytosolic MAP kinase to the membrane occurs before contraction, followed by a second redistribution of MAP kinase to the contractile filaments during cell contraction (20). Sustained smooth muscle contraction is associated with activation of MAP kinase. MAP kinase has been implicated in the maintenance of force in gastrointestinal and vascular smooth muscle cells (1, 8, 18, 37). MAP kinase has been shown to activate a model kinase MAPKAP2.

**Fig. 5.** Dual immunofluorescent labeling of tropomyosin and HSP27 in relaxed and contracted rabbit colonic smooth muscle cells. Smooth muscle cells from the circular muscle layer of the rabbit colon were isolated and allowed to settle on poly-L-lysine-coated coverslips. Cells were fixed with 4% paraformaldehyde in PBS followed by permeabilization with 1% Triton X-100 in PBS with PBS washing in between. Cells were then reacted with Oregon green phalloidin (2 units/coverslip) for 20 min and washed with PBS 3 times for 5 min, followed by tropomyosin and HSP27 immunostaining. Cells were incubated in antibody buffer (10% goat serum, 1% BSA in PBS) for 1 h to remove nonspecific binding and then incubated with monoclonal mouse anti-HSP27 antibody and polyclonal rabbit antitropomyosin antibody for 1 h followed by 1 h incubation with Cy-3 conjugated donkey anti-rabbit antibody and Cy-5 conjugated goat anti-mouse secondary antibodies. Immunofluorescences were visualized under ×100 oil lens by Noran confocal microscope. Colocalization images were generated by Intervision from SGI. A: Colocalization of HSP27 and tropomyosin is shown as pink [a combination of Cy-3 (red) and Cy-5 (blue)]. B: Colocalization of tropomyosin and actin is shown as yellow [a combination of Cy-3 (red) and phalloidin (green)]. On examination under confocal microscopy and Z serial sections, the colocalization showed the very characteristic coiled-coil pattern. In the same cell, the colocalization of HSP27 and tropomyosin is very similar and identical in shape and pattern to the localization of tropomyosin and actin.

**Fig. 6.** The association of myosin, tropomyosin, and actin in transfected smooth muscle cells with mutant HSP27. Smooth muscle cells were cultured in DMEM with 10% FBS and 3% penicillin/streptomycin on collagen IV-coated dishes as described under Methods. The cells were transfected with 3D or 3G human HSP27 as described. After 2 days of transfection, the cells were selected with G-418 (3 mg/ml) for 1–2 days. A: transfections were successfully demonstrated by detection of the mutant human HSP27 (HuHSP27) protein via immunoblotting of the transfected cell lysates with Hu-HSP27-specific antibody, which has been shown not to have any cross activity between rabbit and mouse. B: total lysates were immunoprecipitated with smooth muscle actin antibody (1.5 μl antibody/400 μg total protein) and immunoblotted with anti-myosin heavy-chain antibody. C2-ceramide-induced (0.1 μM) association of actin-myosin greatly decreased in 3G HSP27 mutant transfected cells (48.5 ± 4.4%, P < 0.005). Data suggested that HSP27 through its phosphorylation might regulate microfilament reorganization during smooth muscle contraction. Inset, representative blot of 3 independent experiments.
kinase, which has been shown to phosphorylate HSP27 (28, 32, 33).

MAPKAP2 kinase is the enzyme responsible for the phosphorylation of HSP27 in mammalian cells (32). MAPKAP2 kinase is activated by the extracellular regulated kinase (ERK) MAP kinase family (32). In airway smooth muscle cells, activation of muscarinic receptors seems to phosphorylate HSP27 through tyrosine phosphorylation of p38 MAP kinase (30). In airway smooth muscle cells, activation of MAPKAP2 kinase is the physiological substrate for p38 MAP kinase (22). In acinar cells, it has been shown that CCK, carbachol, and bombesin increase both p38 MAP kinase activity and HSP27 phosphorylation (28, 32, 33).

The evidence suggests that HSP27 may serve as a link between signal transduction pathways leading to contraction and the final contractile apparatus. We have shown that HSP27 can immunoprecipitate with tropomyosin (16). We have also shown that HSP27 can immunoprecipitate with other cytoskeletal proteins including actin, myosin, and caldesmon (15). However, such bindings could be indirect. Data indicate that during smooth muscle contraction, there was increased association between HSP27 and tropomyosin, suggesting that HSP27 might interact with tropomyosin to regulate smooth muscle contraction. Our data, using immunofluorescent dual labeling of tropomyosin and HSP27 followed by confocal microscopy, indicated that in the control relaxed state, tropomyosin and HSP27 are distributed along the longitudinal axis of the smooth muscle cell in a fiber-like manner. C2-ceramide (0.1 μM) induced an increase in the di- and triphosphorylated forms of HSP27 as shown in Fig. 2. Pretreatment of the cells with the myosin light-chain kinase inhibitor ML7 (20 μM for 30 min) had no affect on the phosphorylation of HSP27 induced by C2-ceramide. Data thus seem to suggest that the phosphorylation of HSP27 and its role in smooth muscle contraction is independent of the activation of myosin light-chain kinase but could be mediated by activation of PKC. We (29) previously showed that PKC is an important mediator in ERK kinase activation induced by C2-ceramide. More recently, it was shown that PKC is an important mediator in p38 MAP kinase activation of HSP27 synthesis induced by the contractile agonist endothelin-1 (19).

p38 MAP kinase is activated during adverse environmental conditions and could serve as a homeostatic function in the regulation of microfilament dynamics during stress. p38 Has also been shown to be activated during normal agonist stimulation and may constitute an additional actin-signaling pathway (11). Biochemical analyses have revealed that HSP27 is phosphorylated on the same serine residues and by the same protein kinase regardless of the triggering agent or pharmacological treatment (9). Because of the involvement of MAP kinase in smooth muscle proliferation, growth, caldesmon phosphorylation (6), and colocalization with HSP27, this kinase appears to play a central role in signal transduction for both the contractile and proliferative phenotype of smooth muscle. Agents that increase MAP kinase activity are stimulatory for contraction and proliferation, whereas cAMP inhibits both processes. Thus the function of MAP kinase in the contractile phenotype of smooth muscle, whether vascular or gastrointestinal, is unknown. MAP kinase may be an initiating factor leading to contraction, and MAP kinase signal processes may lead to smooth muscle actin filament restructuring or alterations in contraction (27). We have previously shown that the second redistribution of MAP kinase during smooth muscle cell contraction was colocalized with redistribution of HSP27 when stimulated with the contractile agonist bombesin (37). We have also shown that HSP27 colocalized with the small guanosine 5’-O-(3-thiotriphosphate) binding protein RhoA and with PKC-α on the membrane during contraction (3, 34).
with myosin as immunoprecipitation and immunoblotting data revealed (Fig. 6).

We have presented data that indicate a role for HSP27 and its phosphorylation in smooth muscle thin-filament regulation during smooth muscle contraction. We propose a model whereby phosphorylated HSP27 may undergo conformational changes that lead to the binding of HSP27 with both actin and tropomyosin. When HSP27 is unphosphorylated, the tropomyosins bind to the actin molecules and occupy the potential myosin binding sites on actin. On phosphorylation, HSP27 may bind to tropomyosin and pull tropomyosin away from the myosin head-binding site into the F-actin groove. As a result, the actin molecules are more available to myosin heads. Thus HSP27 may function as a facilitator in thin-filament regulation of smooth muscle contraction.

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