HSP27 Phosphorylation-mediated Resistance against Actin Fragmentation and Cell Death Induced by Oxidative Stress

Jacques Huot, François Houle, Douglas R. Spitz, and Jacques Landry

Centre de Recherche en Cancérologie de l’Université Laval, L’Hôpital-Dieu de Québec, 11 Côte du Palais, Québec, G1R 2J6 Canada [J. H. F., H. J. L.], and Washington University School of Medicine, Section of Cancer Biology, St. Louis, Missouri (D. R. S.)

ABSTRACT

Phosphorylation of heat shock protein 27 (HSP27) has been suggested to play an important role in the regulation of F-actin dynamics in response to growth factors and stress. Because the microfilament network is one of the earliest targets of oxidative stress and because phosphorylation of HSP27 is strongly induced by reactive oxygen metabolites, we have investigated the role of HSP27 phosphorylation in regulating actin dynamics in response to oxidative stress. Experiments were done in Chinese hamster CCL39 cell lines overexpressing various levels of the wild-type or a nonphosphorylatable form of human HSP27 (pm3 HSP27). In control cells, both H2O2 and menadione induced fragmentation of F-actin. In contrast, cell lines expressing pm3 HSP27 were as sensitive as controls to the lethal effect of H2O2. These results suggest that phosphorylation of HSP27 is causally related to the regulation of microfilament dynamics following oxidative stress and may be involved in mediating an adaptive response to oxyradical-generating agents such as carcinogens, anticancer drugs, and other xenobiotics.

INTRODUCTION

ROMs have been implicated as mediators in a vast array of biochemical mechanisms regulating major physiological functions such as cell growth and differentiation (1, 2). They are produced or released from normal oxygen metabolism, from the respiratory burst of activated phagocytic cells, or from oxidizable xenobiotics (reviewed in Refs. 3 and 4) and are considered as the putative mediators of various physiopathological agonists such as tumor necrosis factor-α, transforming growth factor β1, and bradykinin (5–9). In abnormally high concentrations, ROMs are thought to be involved in many pathological disorders including ischemia-reperfusion injury, atherosclerosis, inflammation, and Alzheimer disease (4, 10–13). They are produced by many anticancer agents and are responsible in part for their toxicity (11, 12). Oxyradicals are also potent mutagens and are believed to be involved in tumor promotion by various agents such as UV light (16, 17). To cope with oxidative environments, cells are equipped with highly effective antioxidant enzymes that rapidly quench the effects of oxyradicals (4, 18). Cells also possess oxyradical-sensitive signaling pathways that, via activations of protein kinases, may lead to the transcriptional activation of genes coding for heat shock proteins (HSP) (22—24, 27–29). HSP27, also called stress-sensitive kinase, which is sequentially activated in a cascade of phosphorylation events, is involved in modulating actin microfilament dynamics (30–32). Phosphorylation of HSP27 is involved in polymerization of F-actin in response to oxidative stress. At the highest concentrations of HSP27, stabilization of actin microfilaments is associated with an increased cellular resistance to the lethal effects of oxidative stress. It is suggested that oxidants activate a stress-sensitive MAP kinase signal transduction pathway that triggers HSP27 phosphorylation and may lead to cytoplasmic protection characterized by resistance to oxidative stress-induced actin fragmentation.

MATERIALS AND METHODS

Materials. [γ-32P]ATP (6000 Ci/mmol) was purchased from New England Nuclear. H2O2 (30%) was from Sigma Chemical Co. Chemicals for electrophoresis were purchased from Bio-Rad and ICN Biochemicals. The other chemicals were obtained from various sources. Hu27ab is a rabbit antisemirum that specifically recognizes human HSP27 (35).

Cell Lines. Three groups of stable transfectant cell lines derived from CCL39 cells were used in this study. These cell lines were described previously (22, 34). In the first group, the cell lines are designated Hu27 clones (#6, B2, and B12), and they expressed varying levels of human HSP27 in addition to the marker neo gene. The second group contains cell lines that express the marker neo gene and varying levels of a nonphosphorylatable form of human HSP27 and which are referred to as Hu27 pm3 clones (V, CIII, CVIII, and CXV). The third group comprises control cell lines that expressed only the marker neo gene (clones 3 and 4). Stable transfectants were routinely maintained in standard DMEM (GIBCO) containing 400 ng/ml G-418, 2.2 g/liter NaHCO3, 4.5 g/liter glucose, and 5% FCS (GIBCO). Seven to 10 days before the experiments, the cell lines were transferred into DMEM media without G-418. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2. The transfectant cell lines were used at passage numbers lower than 17.

Assays for Antioxidative Enzyme Activities. Monolayers of cells grown to 60–80% confluency were scraped into cold saline using a rubber policeman.
Fig. 1. Phosphorylation-dependent protective function of HSP27 against actin fragmentation induced by different concentrations of H$_2$O$_2$. Control neo cells, clone #3 (A, D, and G), Hu27 clone #6 (B, E, and H), and Hu27 pm3 clone V (C, F, and I) were plated on fibronectin-coated glass slides, allowed to attach for 16 h, and then were left untreated (A-C) or were treated for 1 h with 0.8 mM (D-F) or 1.2 mM (G-I) H$_2$O$_2$. Immediately after the treatment, the cells were fixed and stained for F-actin with FITC-conjugated phalloidin. The microfilaments were then visualized by confocal microscopy. Representative fields are shown. Bar, 25 µm.
The effects of oxidative stress on the integrity of the actin microfilament network was investigated in families of CCL39 cell lines that stably express varying levels of either wild-type HSP27 or pm3 HSP27, in addition to a constant amount of 2 ng/μg of endogenous hamster HSP27. These cell lines were described previously (34, 40). Cell lines Hu27, clones B2, #6, B12, and B1 express 3.3, 3.4, 4.8, and 6.0 ng/μg, respectively, of protein of wild-type human HSP27, whereas cell lines Hu27 pm3 clones V, CIII, CXV, and CVIII express 3.7, 4.0, 5.4, and 5.6 ng/μg of protein of pm3 HSP27. These levels represent physiologically relevant levels of HSP27 because CCL39 cells can express up to 10 ng of HSP27 per μg of protein following heat shock gene induction by heat treatments. The clonal isolate #3 expresses only the selectable marker neo gene and was used as control.

 Cultures were exposed for 1 h to 0.8 or 1.2 mM H2O2. As described previously (34, 40), cells were plated on fibronectin-coated glass slides, treated, and then fixed and permeabilized with saponin 0.1% in sodium phosphate buffer (130 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, and 10 mM NaH2PO4, pH 7.5). F-actin was detected using FITC-conjugated phalloidin. Cells were then stained for indirect immunofluorescence confocal microscopy. In control neo cells, H2O2 induced the well-known manifestations of the oxidative toxic manifestations of H2O2, i.e., shortening and fragmentation of actin fibers and formation of F-acm patches concentrated around the cell nucleus. The intensity of these effects was dose-dependent, showing a marked worsening from 0.8 to 1.2 mM H2O2 (Fig. 1, A, D, and G). A protection against actin fragmentation and perinuclear patch formation (Fig. 1, C, F, and I; Fig. 2, G-L). As in the control neo cells, the intensity of the toxic manifestations of H2O2 was dose dependent (Fig. 1, C, F, and I). These observations strongly suggest that expression of wild-type HSP27 can protect against actin fragmentation by H2O2, and that phosphorylation of HSP27 is required to confer this protective effect.

 The protection conferred by HSP27 against actin fragmentation by H2O2 could not be attributed to alterations in the metabolism of H2O2, as in the various cell lines studied. As reported in Table 1, the control clones #3 and #4 and the clonal isolates Hu27 and Hu27 pm3 exhibited comparable levels of glutathione peroxidase and catalase, the two major enzymes involved in the biotransformation of H2O2. The mean levels of glutathione peroxidase and catalase activity of the resistant HU27 clones were not significantly different from the sensitive Hu27 pm3 clones. The catalase activity of clone Hu27 B1 appeared elevated relative to the control cells. However, in this case, it is unlikely that the protection observed against H2O2 could result from increased catalase activity because Hu27 pm3 cells, clone CXV, also exhibited an elevated amount of catalase activity and were, nevertheless, very sensitive to actin fragmentation by H2O2 (Fig. 2L).

 HSP27 kinase activity is highly sensitive to intracellular H2O2, and its activation by this oxidant can be taken as a reliable reflection of functionally available intracellular H2O2 concentrations (22). To more directly ascertain that the HSP27-mediated protection could not be attributed to differences in the metabolism of H2O2, we thus determined the responsiveness of HSP27 kinase to H2O2. Results presented in Fig. 3 show that the concentration-dependent activation of HSP27 kinase by H2O2 was identical in the representative cell lines studied. The EC50 for HSP27 kinase activation by H2O2 were 145, 125, and 120 μM in control neo cells line clone #3, Hu27 clone 6, and Hu27 pm3 clone V, respectively. This indicates that the available concentrations of H2O2 were similar in these cell lines and thus supports the conclusion that the resistance of Hu27 cells cannot be attributed to an increased metabolic breakdown of H2O2.

 The redox cycling agent menadione is another oxidant that produces marked alterations in the actin microfilament network (41). In addition, menadione is a strong activator of p45—54 HSP27 (22). The possibility that HSP27 phosphorylation could confer resistance against actin fragmentation by menadione was thus investigated. Representative stable transfectant cell lines Hu27, clone #6 and Hu27 pm3, clone V were thus treated with 100 μM menadione, a concentration that produced one-half maximal protection against actin fragmentation similar to H2O2 in Hu27 pm3 cells, whereas Hu27 cells clone #6 were resistant to this effect. This indicates that the expression of wild-type HSP27 protects the cells against actin disruption by menadione and that phosphorylation of HSP27 is required to confer this protective effect.

 Results and Discussion

 Immunofluorescence Microscopy. F-actin immunofluorescence was done as described previously (34, 40). Cells were plated on fibronectin-coated glass slides, treated, and then fixed and permeabilized with saponin 0.1% in sodium phosphate buffer (130 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, and 10 mM NaH2PO4, pH 7.5). F-actin was detected using FITC-conjugated phalloidin (Ref. 33: 3 μg/ml) diluted 1:50 in sodium phosphate buffer. The cells were analyzed as reported previously by confocal microscopy with a Bio-Rad MRC-600 imaging system mounted on a Nikon Diaphot-TMD-equipped with a ×60 objective lens with a 1.4 numerical aperture (34). Representative fields are shown in Figs. 1, 2, and 4.

 Results

 Overexpression of Wild-Type HSP27 but not pm3 HSP27 Stabilizes Actin Filaments against Disruption by Oxidative Stress. The effects of oxidative stress on the integrity of the actin microfilament network was investigated in families of CCL39 cell lines that stably express varying levels of either wild-type HSP27 or pm3 HSP27, in addition to a constant amount of 2 ng/μg of endogenous hamster HSP27. These cell lines were described previously (22, 34). Cell lines Hu27, clones B2, #6, B12, and B1 express 3.3, 3.4, 4.8, and 6.0 ng/μg, respectively, of protein of wild-type human HSP27, whereas cell lines Hu27 pm3 clones V, CIII, CXV, and CVIII express 3.7, 4.0, 5.4, and 5.6 ng/μg of protein of pm3 HSP27. These levels represent physiologically relevant levels of HSP27 because CCL39 cells can express up to 10 ng of HSP27 per μg of protein following heat shock gene induction by heat treatments. The clonal isolate #3 expresses only the selectable marker neo gene and was used as control.

 Cultures were exposed for 1 h to 0.8 or 1.2 mM H2O2 stained for F-actin using FITC-conjugated phalloidin and then were examined by indirect immunofluorescence confocal microscopy. In control neo cells, H2O2 induced the well-known manifestations of the oxidative injury at the level of F-actin, i.e., shortening and fragmentation of fibers and formation of F-actin patches concentrated around the cell nucleus. The intensity of these effects was dose-dependent, showing a marked worsening from 0.8 to 1.2 mM H2O2 (Fig. 1, A, D, and G). A protection against actin fragmentation and perinuclear patch formation by H2O2 was observed clearly in the four Hu27 clonal isolates (Fig. 1, B, E, and H; Fig. 2, A-F). Hu27 cells, clone 6, were even less sensitive to 1.2 mM H2O2 than control cells to 0.8 mM H2O2 (Fig. 1, H versus D). In contrast, all of the Hu27 pm3 cell lines were as sensitive as the neo cells line to H2O2, and most of the cells exhibited...
Fig. 2. Phosphorylation-dependent protective function of HSP27 against actin fragmentation induced by H$_2$O$_2$ in various Hu27 and Hu27 pm3 cell lines. Hu27 clones B1 (A and B), B2 (C and D), B12 (E and F), and Hu27 pm3 clones CIII (G and H), CVIII (I and J), CXV (K and L) were plated on fibronectin-coated glass slides, allowed to attach for 16 h, and then were left untreated (A, C, E, G, I and K) or were treated for 1 h with 0.8 mm H$_2$O$_2$ (B, D, F, H, J and L). Immediately after the treatment, the cells were processed for F-actin staining with FITC-conjugated phalloidin and examined by confocal microscopy. Representative fields are shown. Bar, 25 μm.

Concentrations higher than 2 μmoles H$_2$O$_2$/10$^6$ cells, a 60-fold increase in cell survival was consistently found in clone B1 cells relative to the neo clone #3 cells (Fig. 5A).

The relationship between expression of HSP27 and resistance to H$_2$O$_2$ was further ascertained by studying the effect of a single concentration of H$_2$O$_2$ (1.2 mm for 1 h) on the survival of four clonal isolates that express varying amounts of HSP27. The results showed that there was a correlation between survival after H$_2$O$_2$ and the amount of wild-type human HSP27. At 5.8 ng of human HSP27 per μg of protein (Hu27 cells, clone B1), there was a 30-fold increase in cell survival as compared to control neo cells, clone #3. In comparable experiments, Hu27 pm3 cells were as sensitive as the controls to the toxic effect of H$_2$O$_2$ (Fig. 5B). Clone CXV was, in fact, more sensitive than the controls to H$_2$O$_2$.
**DISCUSSION**

The observation that overexpression of wild-type human HSP27 but not the pm3 HSP27 increased resistance to F-actin fragmentation induced by H$_2$O$_2$ and menadione is strong evidence that phosphorylation of HSP27 is required to protect F-actin from oxidative stress and strongly suggests that the pathway leading to HSP27 phosphorylation is an essential component of the adaptive response to oxidants. It is unlikely that the increased resistance of the Hu27 cells to F-actin fragmentation by H$_2$O$_2$ results from clonal effects because the protection was observed in all four Hu27 cell lines tested and in none of the four Hu27 pm3 cells. It is also unlikely that the results obtained could be attributed to an increased metabolism of H$_2$O$_2$ in the resistant Hu27 cells because the levels of catalase and glutathione peroxidase expressed by these cell lines are comparable to those expressed by the more sensitive control neo cells and Hu27 pm3 cells. This is further supported by the observation that the activation of HSP27 kinase in response to increasing concentrations of H$_2$O$_2$ was identical in the resistant versus the sensitive transfected cell lines studied. Activation of HSP27 kinase is highly sensitive to intracellular H$_2$O$_2$ and thus can

![Fig. 3. Activation of HSP27 kinase by various concentrations of H$_2$O$_2$ in representative Hu27 and Hu27 pm3 cell lines. Exponentially growing control neo cells, clone #3 (A), Hu27 clone #6 (B), and Hu27 pm3 clone V (C) were incubated for 30 min in the presence of varying concentrations of H$_2$O$_2$. At the end of the treatments, extracts were prepared and processed for HSP27 kinase activity. Representative autoradiograms are shown. Lane 1, untreated; Lane 2, 0.1 mM; Lane 3, 0.25 mM; Lane 4, 0.5 mM H$_2$O$_2$.](image)

![Fig. 4. Phosphorylation-dependent protective function of HSP27 against actin fragmentation by menadione. Hu27 clone #6 (A) and Hu27 pm3 clone V (B and D) were plated on fibronectin-coated glass slides, allowed to attach for 16 h, and then were left untreated (A and B) or were treated for 1 h with 100 μM menadione (C and D). Immediately after the treatment, the cells were fixed and processed for F-actin staining with FITC-conjugated phalloidin. The microfilaments were then visualized by confocal microscopy. Representative fields are shown. Bar, 20 μm.](image)

Taken together, the survival data suggest that overexpression of HSP27 can increase the cellular resistance to H$_2$O$_2$ and that the phosphorylation of HSP27 is required to trigger its protective function.

![Fig. 5. HSP27 but not pm3 HSP27 protects against cell death induced by H$_2$O$_2$. A. control neo cells line, clone #3 (○) and Hu27 clone B1 (●) were treated for 1 h with varying concentrations of H$_2$O$_2$, and cell survival was determined by colony formation. The points represent values taken from 10 different experiments over a period of 1 year. The concentrations of H$_2$O$_2$ are expressed as μmoles H$_2$O$_2$/10$^6$ cells. B. control neo cells clone #3 and clonal isolates Hu27 (●) and Hu27 pm3 (○) were treated for 1 h with 1.2 mM H$_2$O$_2$, and then were plated at appropriate concentration for cell survival evaluation by colony formation at 37°C. The results are expressed as the relative survival of the various cell lines as a function of human HSP27 or pm3HSP27 in the cells. Levels of human HSP27 were evaluated by densitometric analysis of the Western blots obtained using Hu27ab, an antibody that specifically recognized human HSP27 and pm3HSP27.](image)
be used as a suitable end point to evaluate the relative intracellular concentration of H$_2$O$_2$ (22).

The results of the survival studies indicated that there was a correlation between resistance to H$_2$O$_2$ and expression of HSP27. The relationship is a strong argument, suggesting that resistance to H$_2$O$_2$ is causally related to the expression of HSP27. These observations confirm previous reports that showed that overexpression of wild-type HSP27 can confer resistance to H$_2$O$_2$, and also to oxyradical-generating anticancer drugs (43–47). The results of the present study show that phosphorylation of HSP27 is required to trigger its protective function during H$_2$O$_2$ exposures because the cells that overexpressed pm3 HSP27 were either equally sensitive or even more sensitive than the control cell lines to H$_2$O$_2$. The protection by wild-type HSP27 against actin fragmentation was observed in all of the clones investigated. However, resistance to cell death was not invariably found in all of these cell lines. Clone #6, for example, was markedly resistant against actin fragmentation by H$_2$O$_2$, whereas it was as sensitive as the controls against cell death by the oxidant. This indicates that actin is not the only critical cellular target that determines clonogenic survival following H$_2$O$_2$ treatment. ROMs can produce, among others, membrane as well as DNA damage, which may also impair cell survival (48, 49).

Several studies have reported that oxidative stress induces profound alterations of the microfilament network, characterized by the fragmentation of F-actin, which, for the short treatment as used here, is not accompanied by a net change in the levels of monomeric G-actin or polymeric F-actin (50, 51). The mechanisms of the cytoskeleton disruption by oxidative stress are unclear and may involve ATP depletion, oxidation of actin SH group, and cross-linking of actin filaments (41, 51–53). The role of HSP27 at the level of actin, as well as the mechanisms of HSP27 phosphorylation-mediated protection against actin fragmentation by oxidants, is also still unclear. In vivo, HSP27 behaves as a F-actin cap-binding protein (31) and has been shown to inhibit actin polymerization in a phosphorylation-dependent manner (32). In vivo, the fast phosphorylation of HSP27 in response to growth factor stimulation was shown to regulate a function of HSP27 at the level of actin filament dynamics enhancing membrane ruffling and pinocytosis (33). Phosphorylation of HSP27 has also been proposed to play a homeostatic function, characterized by stabilization of actin filament in response to heat shock and cytochalasin D, a specific inhibitor of actin polymerization (34, 40). The results of the present study suggest that phosphorylation of HSP27 also has a homeostatic function in preserving the actin microfilament network integrity following oxidative stress. It is possible that the phosphorylation-induced modifications in the oligomeric structure of HSP27 (34) enhance its actin polymerizing activity and thus increase the stability of the microfilaments during oxidative stress. Another possibility is that HSP27 may exert chaperonin functions protecting F-actin against oxidative stress. However, this is unlikely, because, in vitro, the chaperonin function of HSP27 was shown to be phosphorylation independent (54).

The demonstration that phosphorylation of HSP27 is required to protect F-actin from fragmentation induced by oxidants suggests that the pathway leading to HSP27 phosphorylation may be an important component of the adaptive response to oxidative stress. Some of the events along this pathway began to be identified. We showed previously that in vivo phosphorylation of HSP27 is mediated by activation of p45–54 HSP27 kinase, a stress-sensitive kinase, also coined MAPKAP kinase-2 (22, 27–29). MAPKAP kinase-2 appears to be activated in vivo by p38 MAP kinase, the mammalian homologue of HOG-1, a yeast MAP kinase that, after osmotic stress, is essential to glycerol synthesis and repositioning of actin cytoskeleton at the bud site (23, 24, 55–57). As HOG-1, p38 may be involved in a stress-sensitive pathway, the induction of which is required in the adaptive response against external stresses in mammalian cells. From the present study, it is likely that the p38-associated stress-activated pathways may lead directly, through HSP27 phosphorylation, to cytoplasmic protection and, thereby perhaps, complement other stress-sensitive pathways modulated by other MAP kinases (58–60). It is conceivable that the activation of an oxidant-sensitive pathway leading to microfilament stabilization represents an early primary adaptive response that may contribute to the defense mechanisms against oxidative stress in humans. Such adaptive mechanisms may be of importance in modulating the cellular response to oxyradical-generating agents including some anticancer drugs, carcino gens, and other xenobiotics.

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