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

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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 actin. In pm3 HSP27 overexpressing cells, both H2O2 and menadione induced fragmentation of actin, which forms aggregates and patches concentrated around the nucleus. Stable overexpression of wild-type HSP27, but not of pm3 HSP27, conferred resistance against actin fragmentation, suggesting that HSP27 has a phosphorylation-activated protective function against actin disruption by oxidative stress. Cell lines that overexpressed the highest levels of the wild-type form of human HSP27 also showed an increased cell survival following exposure to H2O2. In contrast, cells expressing pm3 HSP27 were as sensitive as the 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 oxiradical-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 β, 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 (14, 15). Oxiradicals 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 oxiradicals (4, 18). Cells also possess oxiradical-sensitive signaling pathways that, via activations of protein kinases, may lead to the transcriptional activation of genes coding for proteins with protective functions (19–21).

We reported recently in Chinese hamster fibroblasts and human HeLa cells that one of the earliest manifestations of oxidative stress is the activation of MAPKAP kinase-2, a mediator of stress-sensitive kinase, which is sequentially activated in a cascade of kinases involving the new MAP kinase p38/Hog-1 (22–26). When activated, MAPKAP kinase-2 phosphorylates the small molecular weight heat shock protein, HSP27 (22–24, 27–29). HSP27, also called HSP25 or HSP28 (30), behaves in vitro as a F-actin cap-binding protein, and its phosphorylation has been proposed as a key determinant in modulating actin microfilament dynamics (30–32). Phosphorylation of HSP27 is involved in polymerization of F-actin in response to growth factors, in membrane ruffling, and pinocytosis (33). In addition, phosphorylation of HSP27, which is induced during the first minutes of exposure to high temperatures, is an essential step in the activation of its thermoprotective function at the level of actin microfilaments (34). The regulation of actin microfilament dynamics by phosphorylation of HSP27 has been attributed to the phosphorylation-induced modifications in the oligomeric structure of HSP27 (32, 34). Given the fact that microfilaments are highly sensitive to ROMs and given the fact that phosphorylation seems to trigger a regulatory function of HSP27 at the level of actin, it was thus suggested that the early and strong phosphorylation of HSP27 by oxidative stress may trigger an adaptive stress response at the level of actin. We report that overexpression of wild-type but not of a nonphosphorylatable form of HSP27 can mediate protection against actin fragmentation by oxidative stress. At the highest concentrations of HSP27, stabilization of microfilaments is associated with an increased cellular resistance to the lethal effect 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 antisemum 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, B1, 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 cells that expressed only the marker neo gene. The second group contains cell lines that express the marker neo gene and 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 cells 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.

and centrifuged at 400 × g; then the supernatant was discarded, and the cell pellets frozen at −80°C. The pellets were thawed, resuspended in 50 mM phosphate buffer (pH 7.8) containing 1.34 mM diethylenetriamine pentaacetic acid and sonicated on ice with 10 × 1-s bursts using a Biosonik sonicator equipped with a microtip set at 30% output. The homogenates were assayed for catalase activity using the method of Beers and Sizer (36) and expressed in mK units/mg protein as described by Aebi (37). Glutathione peroxidase activity was determined by the method of Lawrence and Burk (38) using cumene hydroperoxide as the substrate, and one unit of enzyme activity was defined as the amount of protein that oxidized 1 μmol of NADPH/min. Protein content was determined by the method of Lowry et al. (39).

Cytotoxic Treatments. Monolayers of cells in their exponential phase of growth were treated for 1 h with various concentrations of H$_2$O$_2$ or menadione. Immediately after treatments, the cells were tested for survival or processed for HSP27 kinase assays or for immunofluorescence microscopy.

Cell Survival. After each treatment, the cells were washed twice, trypsinized, and plated at appropriate dilutions in triplicate. Relative survival was calculated from the number of single cells that formed colonies of more than 50 cells within 7–10 days. The survival data were corrected for the plating efficiency of the appropriate controls.

HSP27 Kinase Assay. HSP27 kinase activity was determined as described previously (22, 28). At the end of the treatments, extracts were prepared and incubated for 15 min at 30°C in the presence of [γ-32P]ATP and purified recombinant HSP27. Transfer of 32P on recombinant HSP27 was visualized by autoradiography after separation of the extracts by SDS-PAGE. Phosphorylation was quantified as described previously (28).
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 were 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). 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 actin and examined in fluorescence microscopy. Results presented in Fig. 4 show that menadione induced 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.

Overexpression of Wild-Type HSP27 but not pm3 HSP27 Protects against Clonogenic Cell Death Induced by H2O2. The relationship between the expression of HSP27 and resistance to cell death induced by H2O2 was first determined by comparing the effects of increasing concentrations of H2O2 on the clonogenic survival of both the control neo cells and the Hu27, clone B1, the clone that expresses the highest amount of human HSP27. In this study, the results were expressed as a function of µmoles H2O2/106 cells to minimize the effect of cell density on the evaluation of H2O2 toxicity (42). The results obtained from 10 different experiments indicated that the clonal isolate Hu27 B1 was more resistant than the control cell line #3 to a 1-h exposure to various concentrations of H2O2. At concen-

### Table 1

<table>
<thead>
<tr>
<th>Cell linesa</th>
<th>GPX (milliunits/mg)</th>
<th>Catalase (mK units/mg)</th>
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<tbody>
<tr>
<td>Neo cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#3</td>
<td>2.71</td>
<td>29.3</td>
</tr>
<tr>
<td>#4</td>
<td>2.40</td>
<td>29.0</td>
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<tr>
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<td>2.55 ± 0.21</td>
<td>29.1 ± 0.02</td>
</tr>
<tr>
<td>Hu27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#6</td>
<td>1.09</td>
<td>27.8</td>
</tr>
<tr>
<td>B1</td>
<td>1.99</td>
<td>45.0</td>
</tr>
<tr>
<td>B2</td>
<td>2.99</td>
<td>27.7</td>
</tr>
<tr>
<td>B12</td>
<td>3.19</td>
<td>32.4</td>
</tr>
<tr>
<td>Average</td>
<td>2.31 ± 0.97</td>
<td>33.2 ± 8.10</td>
</tr>
<tr>
<td>Hu27 pm3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>2.21</td>
<td>33.9</td>
</tr>
<tr>
<td>CIII</td>
<td>3.05</td>
<td>28.2</td>
</tr>
<tr>
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<td>3.17</td>
<td>34.5</td>
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<tr>
<td>CXV</td>
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<td>42.5</td>
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<tr>
<td>Average</td>
<td>2.70 ± 0.48</td>
<td>34.8 ± 5.90</td>
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a Specific enzymatic activities were determined on exponentially growing cells in each group of cell lines, as described in "Materials and Methods."

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.

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's 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 kinase (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 activation of HSP27 kinase in CCL39 cells (22). After treatment, the cells were stained for actin and examined in fluorescence microscopy. Results presented in Fig. 4 show that menadione induced 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.
trations higher than 2 μmoles H₂O₂/10⁶ 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 H₂O₂ was further ascertained by studying the effect of a single concentration of H₂O₂ (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 H₂O₂ 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 H₂O₂ (Fig. 5B). Clone CXV was, in fact, more sensitive than the controls to H₂O₂.
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

**DISCUSSION**

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.
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 oxidents, 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 vitro, 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, carcinogens, and other xenobiotics.

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