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Heat Shock Response and Protein Degradation: Regulation of HSF2 by the Ubiquitin-Proteasome Pathway

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Mammalian cells coexpress a family of heat shock factors (HSFs) whose activities are regulated by diverse stress conditions to coordinate the inducible expression of heat shock genes. Distinct from HSF1, which is expressed ubiquitously and activated by heat shock and other stresses that result in the appearance of nonnative proteins, the stress signal for HSF2 has not been identified. HSF2 activity has been associated with development and differentiation, and the activation properties of HSF2 have been characterized in hemin-treated human K562 erythroleukemia cells. Here, we demonstrate that a stress signal for HSF2 activation occurs when the ubiquitin-proteasome pathway is inhibited. HSF2 DNA-binding activity is induced upon exposure of mammalian cells to the proteasome inhibitors hemin, MG132, and lactacystin, and in the mouse ts85 cell line, which carries a temperature sensitivity mutation in the ubiquitin-activating enzyme (E1) upon shift to the nonpermissive temperature. HSF2 is labile, and its activation requires both continued protein synthesis and reduced degradation. The downstream effect of HSF2 activation by proteasome inhibitors is the induction of the same set of heat shock genes that are induced during heat shock by HSF1, thus revealing that HSF2 affords the cell with a novel heat shock gene-regulatory mechanism to respond to changes in the protein-degradative machinery.

The cellular response to stresses such as heat shock is tightly controlled at the level of transcription, and in larger eukaryotes it is mediated by a family of heat shock transcription factors (HSFs) corresponding to HSF1 through HSF4 (37, 38, 65), which recognize and bind to heat shock elements (HSEs) present in the promoter regions of heat shock genes (11). The expression of multiple HSF family members in larger eukaryotes endows the cell with a mechanism to sense and respond to diverse forms of stress. HSF1 and HSF3 are activated following exposure to traditional forms of environmental and physiological stress such as heat shock and chemical stress (37, 38, 41, 65). In avian cells expressing HSF1 but in which the HSF3 gene is deleted, the heat shock response is strongly diminished, which reveals a new level of regulatory interaction among members of the HSF family (57). The suggestion that HSFs may exhibit complex interactions with other transcription factors is further demonstrated by the observation that HSF3 expressed in avian cells can be activated in the absence of stress by direct protein-protein interaction with the DNA binding domain of the c-Myb proto-oncogene (27).

Another member of the HSF family, HSF2, is 40% related in sequence to HSF1 and HSF3, with the regions of highest sequence conservation corresponding to the DNA-binding and heptad repeat regions. However, unlike HSF1 and HSF3, HSF2 is not activated in response to heat shock and most other forms of cellular stress (37, 38, 65). HSF2 has been described as having properties of a development- and differentiation-associated transcription factor, in part due to observations of HSF2 activation during murine embryogenesis and spermatogenesis (36, 45, 48). The regulatory and biochemical properties of HSF2 have been characterized during hemin-induced differentiation of K562 human erythroleukemia cells; under these conditions, HSF2 is activated from an inert dimer to a DNA binding, transcriptionally active trimer (55, 56, 58). Despite the distinctions in activation signals for HSF1 and HSF2, we have observed that a similar profile of heat shock genes is transcriptionally induced when either is activated (55, 56). It has however, been unclear whether HSF1 and HSF2 display redundancy in target gene expression or whether there are differences in the patterns of genes expressed. Random oligonucleotide selection experiments using recombinant HSF1 and HSF2 have shown that both factors bind to the same 5′-NGAAN-3′ motif of the HSE, although they bind preferentially to slightly different configurations of the HSE sequence (29). These experiments, in conjunction with in vivo and in vitro analyses of HSE promoter occupancy, also revealed that HSF2, unlike HSF1, does not bind to DNA in a cooperative manner (29, 30, 55, 56). Such studies have raised the possibility that HSF2 may have target genes distinct from those of HSF1, as well as differing specificities for common target genes. These speculations have been corroborated by various recent observations. Analyses of the transcriptional properties of human HSF1 and HSF2 in yeast have identified differences in which target stress genes are induced preferentially (35). Furthermore, examination of transcripts differentially expressed under conditions of HSF1 and HSF2 activation in K562 cells facilitated identification of the thioredoxin gene as an HSF2-specific target, although the presence of HSEs in the thioredoxin gene promoter has yet to be confirmed (33).

HSF1 activation occurs as a general response to conditions such as heat shock, oxidative stress, and exposure to amino acid analogs, which lead to the appearance of nonnative proteins (37, 38, 48a, 52a, 65). Because heat shock also causes an inhibition of protein synthesis and in doing so prevents the appearance of potentially misfolded nascent polypeptides, it has been considered that the role of HSF1 is to respond to the appearance of potentially damaging proteins by enhancing the expression of heat shock proteins. The fate of nonnative proteins in the environment of the stressed cell is therefore de-

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pendent upon chaperone activity; chaperones may maintain intermediate folded states, refold the proteins to the native state, or target them for degradation (19, 39). In contrast to our state of understanding of HSF1, HSF2 has remained a puzzle. The only well-established regulator of HSF2 activity is hemin, which, although effective for K562 erythroleukemia cells, was ineffective as an inducer of other vertebrate cells (58). Hemin is an iron-containing protein with potential for oxidative damage; however, it is unlikely that its HSF-activating properties involve oxidative stress, as we and others have shown that conditions known to induce oxidative stress activate HSF1 and not HSF2 (26, 28, 34, 47). Hemin also has the distinctive characteristic of affecting the function of the ubiquitin-proteasome pathway in eukaryotes (9, 20, 63). In this study, we show that down-regulation of the ubiquitin-proteasome pathway by inhibitors such as hemin, MG132, or lactacystin activates HSF2 DNA-binding activity in a cell type-independent mechanism. Consistent with this, HSF2 is a labile protein which accumulates upon arrest of proteasome activity. Thus, HSF2 is regulated by signaling mechanisms distinct from those for HSF1 activation.

MATERIALS AND METHODS

Preparation of cell extracts and gel mobility shift assays. The human tissue culture cell lines K562 (grown in RPMI 1640 supplemented with 10% fetal calf serum), HeLaS3 (grown in Joklik's medium with 5% calf serum), and HepG2 (grown in Eagle's minimal essential medium with 10% fetal calf serum, sodium pyruvate, and nonessential amino acids), and mouse embryo fibroblasts (MEF; a gift of I. J. Benjamin, Southwestern Medical School) (grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum, nonessential amino acids, and 0.5 μM β-mercaptoethanol), were treated with 20 μM bovine hemin (Aldrich), 10 μM cycloheximide (Sigma), 10 μM MG132 (Peptides International), or 10 μM lactacystin (E. J. Corey, Harvard University) as indicated. Cells were alternatively heat shocked at 42°C and allowed to recover for the lengths of time indicated. The ts85 cells (a gift of M. Rechsteiner, University of Utah School of Medicine) were maintained at 30°C (10% CO_2) in McCoy's modified 5A medium plus 10% fetal calf serum or were shifted to 39.5°C for the lengths of time indicated. The cells were harvested for the preparation of whole-cell extracts and analyzed for HSF DNA-binding activity in the gel mobility shift assay by using labeled HSE-containing oligonucleotides, and specific antibodies to HSF2 or HSF1 as described previously (40, 47), to establish the composition of the HSE-binding activities obtained.

Immunological analyses. For immunoblot analyses, cell extracts (10-μg protein samples) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8 or 10% polyacrylamide) and transferred to nitrocellulose, and HSF2 protein was detected by using polyclonal sera raised against murine HSF2 (1:10,000 dilution of serum), as described previously (47). Other antibodies used were the murine HSF1-specific polyclonal sera (47) (1:2,000 dilution of serum), the Hsp70-specific monoclonal antibody 4G4 (40a) (1:10,000 dilution of ascites fluid) or 3A3 (2) (1:20,000 dilution of ascites fluid), and polyclonal sera raised against Hdj-1 (16a) (1:2,500 dilution of serum) and ubiquitin (a gift of A. Ciechanover, Technion–Israel Institute of Technology) (1:5,000 dilution of serum). Immunoreactivity was detected by ECL (Amersham).

Cell extracts (100 to 150 μg of protein) were alternatively used for immunoprecipitation analyses, incubated either with 0.4 μg of ascites protein containing monoclonal anti-HSF2 antibody (3E2) (6a) and 20 μg of rabbit anti-rat linker antibody (Jackson Laboratories) or with linker antibody alone for 2 h. Following the addition of protein A-Sepharose beads (Pharmacia), the samples were incubated for an additional 1 to 2 h at 4°C. The beads were washed with radioimmunoprecipitation assay buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS) and boiled in 2× Laemmli buffer, and the eluates were resolved by SDS–8% PAGE.

One- and two-dimensional protein analyses of ^{35}S-labeled extracts. K562 cells were pulse-labeled with Tran^{35}S label (ICN), 200 μCi/ml, in Met- and Cys-deficient RPMI or Dulbecco's modified Eagle's medium (ICN) for 15 min following treatment with MG132 for 0, 2, or 6 h and were used for immunoprecipitation assays to detect HSF2. The immunoprecipitates were resolved on an SDS–8% PAGE gel and analyzed by fluorography.

For analyses of chaperone expression, K562 cells were pulse-labeled with Tran^{35}S label (ICN), 50 μCi/ml, in Met- and Cys-deficient RPMI medium for 30 min following their respective treatments with proteasome inhibitors or heat shock. However, the HS sample was additionally allowed to recover for 30 min at 37°C prior to the ^{35}S labeling. Cell extracts were prepared as described above, and 10 μg of each sample was resolved by SDS–10% PAGE. The samples were transferred onto nitrocellulose and visualized on a PhosphorImager (Molecular Dynamics). One hundred micrograms of ^{35}S-labeled extracts were subjected to two-dimensional protein analysis using ampholines (pH 3 to 10) for isoelectric focusing and SDS–10% PAGE for the second dimension (42). The proteins were then visualized on the PhosphorImager.

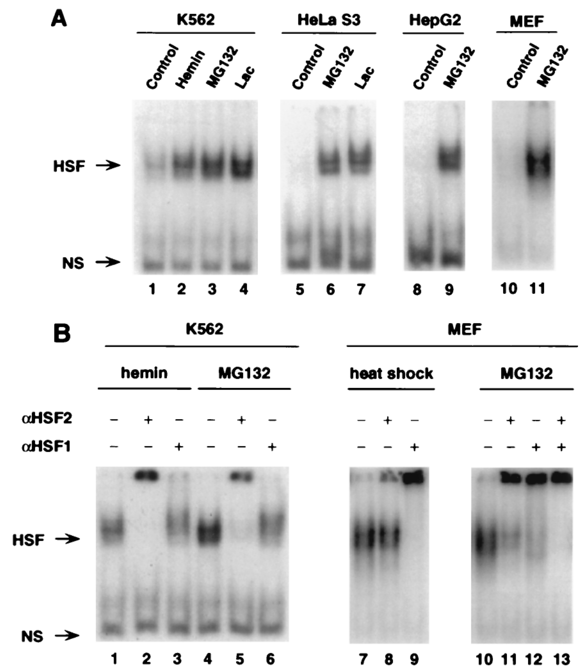

FIG. 1. Activation of HSF2 by treatment of cells with specific proteasome inhibitors. (A) Gel mobility shift assays to analyze formation of HSF-HSE complexes using whole-cell extracts prepared from untreated K562, HeLaS3, and HepG2 cells and MEF (lanes 1, 5, 8, and 10, respectively), from K562 cells treated with hemin for 12 h (lane 2) or with MG132 or lactacystin (Lac) for 2 h (lanes 3 and 4, respectively), from HeLaS3 cells treated with MG132 or lactacystin for 6 h (lanes 6 and 7, respectively), from HepG2 cells treated with MG132 for 2 h (lane 9), and from MEF treated with MG132 for 2 h (lane 11). (B) Identification of the DNA-binding activity as primarily HSF2 by antibody supershift assays. Extracts from hemin- and MG132-treated K562 cells (lanes 1 to 3 and 4 to 6, respectively) were incubated either with or without a 1:50 dilution of specific HSF2 or HSF1 antisera, as indicated, prior to the gel mobility shift assay. Similarly, extracts from MEF heat shocked at 42°C for 1 h (lanes 7 to 9) or treated with MG132 for 6 h (lanes 10 to 13) were incubated in the presence or absence of either the antiserum specific to HSF1, the antiserum specific to HSF2, or both. The HSF DNA-binding activities are indicated by arrows. NS, nonspecific binding.

RESULTS

HSF2 activation by inhibition of proteasome activity. Incubation of human K562 cells with a proteasome inhibitor—hemin, the peptide aldehyde MG132 (43, 46), or the *Streptomyces* metabolite lactacystin (10)—resulted in the appearance of HSF DNA-binding activity detected by the gel mobility shift assay (Fig. 1A, lanes 1 to 4). To examine whether this corresponded to either of the predominant HSE-binding activities expressed in mammalian cells, HSF1 or HSF2, we used specific polyclonal antisera for antibody supershift assays (47, 56). As shown in Fig. 1B (lanes 1 to 6), the DNA-binding activity induced by the proteasome inhibitors hemin and MG132 corresponds primarily to HSF2, as detected by the appearance of slower-migrating HSF2 antibody-containing ternary complexes in native-gel electrophoresis. However, unlike the cell type-specific effects of hemin, which induces HSF2 DNA-binding activity only in K562 cells, the HSF2-activating effects of MG132 or lactacystin were observed in a large number of vertebrate (primate, canine, and rodent) cell lines, revealing

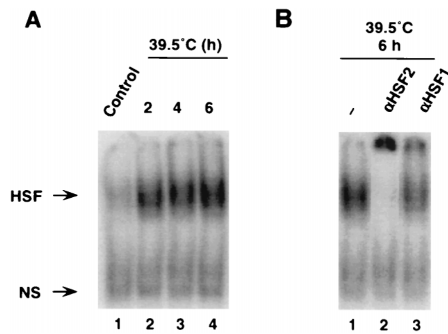

FIG. 2. Inhibition of efficient ubiquitination activates HSF2. (A) Gel shift analysis of ts85 cells maintained at control (30°C) (lane 1) and nonpermissive (39.5°C) (lanes 2 to 4) temperatures for the lengths of time shown. (B) Antibody supershift analyses of the sample at 39.5°C for 6 h were performed by incubation of extracts in the absence (lane 1) or presence of antiserum specific for HSF2 (lane 2) or HSF1 (lane 3).

that inhibition of the ubiquitin-proteasome pathway is a common activator of HSF2 (Fig. 1A, lanes 5 to 11, and data not shown).

Inhibition of the ubiquitin-proteasome pathway resulted in activation of HSF2 DNA-binding activity; however, we also noticed that variable amounts of HSF DNA-binding activity remained after the addition of anti-HSF2 antibodies (Fig. 1B, lanes 5 and 11). One interpretation of this observation is that other HSF DNA-binding activities, presumably HSF1, are also activated in a cell type-dependent manner. One example is in MEF, where MG132 treatment led to the complete coactivation of both HSF2 and HSF1 (Fig. 1B, lanes 11 to 13), whereas only HSF1 was activated upon heat shock (Fig. 1B, lanes 7 to 9).

HSF2 is activated in a cell line expressing a conditional mutation in the ubiquitination pathway. One interpretation of these results is that some property of HSF2 is regulated by the activity of the proteasome. Therefore, as a complement to the use of proteasome inhibitors, we examined the properties of HSF2 in the mouse cell line ts85, which carries a temperature sensitivity mutation in the ubiquitin-activating enzyme E1 that results in reduced levels of ubiquitination at the restrictive temperature (12). Under conditions of normal cell growth (30°C), HSF DNA-binding activity was not detected; however, at the nonpermissive temperature (39.5°C), HSF2 DNA-binding activity was induced (Fig. 2A). As HSF2 DNA-binding activity was not induced at 39.5°C in the parental cell line (data not shown), we conclude that deregulation of proteolytic activity by inhibition at a specific step in the ubiquitination pathway leads to activation of HSF2 DNA-binding activity with negligible effects on HSF1 activity (Fig. 2B). Taken together, the results presented in Fig. 1 and 2, obtained by using either chemical inhibitors of the ubiquitin-proteasome pathway or an E1 enzyme conditional mutant, reveal that the activity of HSF2 is closely linked to changes in the activity of the ubiquitin-proteasome pathway. Thus, conditions and reagents which inhibit the activity of the proteasome pathway serve to induce HSF2 activity in a cell type-independent manner.

HSF2 is a labile protein which accumulates during proteasome inhibition. Activation of HSF2 during down-regulation of the ubiquitin-proteasome degradative system suggests that either HSF2 or a component in the pathway of HSF2 activation is labile. Consequently, the accumulation either of HSF2 or of another protein leads to HSF2 activation. Exposure of MEF to MG132 resulted in increased levels of the α and β isoforms of HSF2 (15, 18), as detected by immunoblot analysis using anti-HSF2 antibodies, and a parallel increase in HSF2 DNA-binding activity (Fig. 3A, top and middle panels). By comparison, the levels of HSF1 protein in MG132-treated MEF were unaffected (Fig. 3A, bottom panel), although the electrophoretic mobility of HSF1 from MG132-treated cells by SDS-PAGE analysis corresponded to the stress-inducible phosphorylated state of the factor. HSF2 levels also increased in MG132- or hemin-treated K562 cells (Fig. 3C and D and Fig. 4A) and in ts85 cells at the restrictive temperature, corresponding to the appearance of HSF2 DNA-binding activity (Fig. 3B, lanes 1 to 3). Comparison of HSF2 levels in five

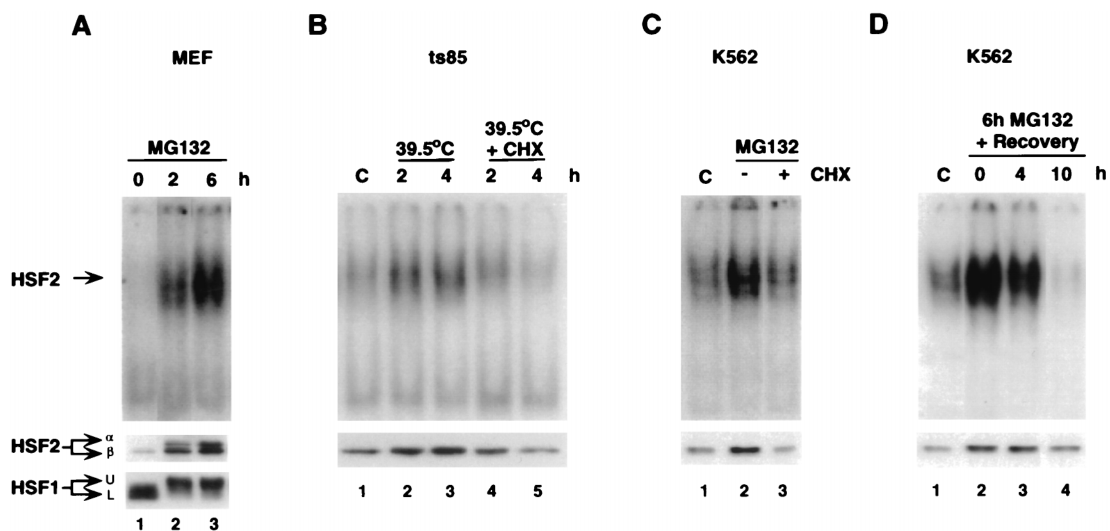

FIG. 3. Coordinate changes in HSF2 DNA-binding activity and protein levels, determined by gel mobility shift (upper panels) and immunoblot (lower panels) assays of whole-cell extracts from MEF treated with MG132 for up to 6 h (A), ts85 cells incubated at the nonpermissive temperature for up to 4 h in the presence (lanes 4 and 5) or absence (lanes 2 and 3) of cycloheximide (CHX) (B), K562 cells left untreated (lane 1) or treated for 2 h with MG132 alone (lane 2) or with MG132 and cycloheximide (lane 3) (C), and K562 cells left untreated (lane 1) or treated with MG132 for 6 h and allowed to recover for 0 (lane 2), 4 (lane 3), and 10 (lane 4) h in inhibitor-free medium (D). C, control.

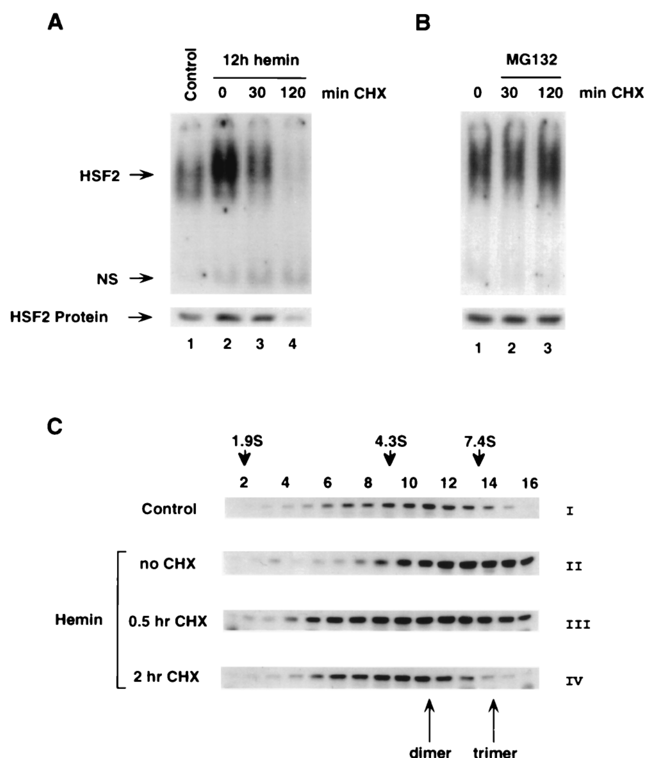

FIG. 4. Inhibition of protein synthesis results in a loss of HSF2 DNA-binding activity and of HSF2 protein levels. (A) Gel mobility shift (upper panel) and immunoblot (lower panel) assays of whole-cell extracts from K562 cells left untreated (lane 1) or induced with hemin for 12 h and treated with cycloheximide (CHX) for 0 (lane 2), 30 (lane 3), or 120 (lane 4) min. (B) The effects of simultaneous inclusion of cycloheximide and MG132, for 0 (lane 1), 30 (lane 2), and 120 (lane 3) min, on hemin-induced cells were also assessed. (C) Glycerol gradient fractionation (55) of K562 cell extracts from control cells (I) and from cells induced with hemin for 12 h (II to IV) and treated with cycloheximide for 0 (II), 30 (III), or 120 (IV) min. Fractions were collected from the top to the bottom of the gradients (fractions 2 to 16). The positions corresponding to dimeric and trimeric HSF2 are shown. The S values from protein standards are indicated (cytochrome *c*, 1.9S; bovine serum albumin, 4.3S; alcohol dehydrogenase, 7.4S).

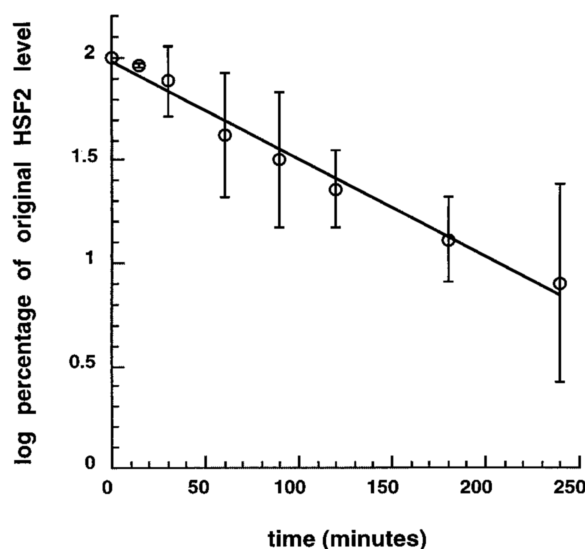

FIG. 5. Estimation of HSF2 half-life. Hemin-treated K562 cells were treated with cycloheximide, and samples were taken at various time points up to 4 h of treatment. Cell extracts were prepared, and 10-μg amounts were used for SDS-PAGE and immunoblot analyses. The HSF2 protein visualized by ECL was quantitated by scanning densitometry. Data compiled from 12 experiments (correlation coefficients, >0.995) are shown. The half-life of HSF2 is calculated to be 60 min from a logarithmic plot of percentages of original HSF2 protein versus time (in minutes).

different mammalian cell lines treated with MG132 revealed increases ranging from 2-fold in K562 to 35-fold in MEF.

Incubation in the presence of cycloheximide, to arrest protein synthesis, abolished both the accumulation and the activation of HSF2 in ts85 cells shifted to the restrictive temperature (Fig. 3B, lanes 4 and 5), as well as in MG132-treated cells (Fig. 3C). Likewise, exposure of hemin-treated cells to cycloheximide resulted in the rapid loss of HSF2 DNA-binding activity (Fig. 4A), which was accompanied by the conversion of HSF2 from the active trimeric state to the inactive dimeric form (Fig. 4C, panels II and III) as determined by glycerol gradient analysis. After 2 h in the presence of both hemin and cycloheximide, all of the HSF2 had been converted to the non-DNA binding state (Fig. 4C, panel IV). Prolonged exposure to cycloheximide additionally resulted in the reduction of the HSF2 level below that observed in control untreated cells (Fig. 3B and C and Fig. 4A). Both the loss of HSF2 DNA-binding activity and the decreased levels of HSF2 protein observed with cycloheximide treatment of hemin-induced K562 cells did not occur in the presence of MG132 (Fig. 4B). The correlation of HSF2 activity with HSF2 protein levels is also observed upon reversal of proteasome inhibition. Incubation of K562 cells in MG132-free medium following a 6-h treatment caused HSF2 DNA-binding activity and HSF2 protein levels to diminish (Fig. 3D).

As these results suggested that HSF2 was labile, the half-life of HSF2 was determined by quantitation of HSF2 protein levels in K562 cells at different times following the addition of cycloheximide. Half-lives of 60 min for human HSF2 (Fig. 5) and 70 min for mouse HSF2 were determined. By comparison, HSF1 is a stable protein whose levels did not change during the time course of this experiment (data not shown).

The increased level of HSF2 protein observed upon inhibition of proteasome activity may result either from inhibition of HSF2 degradation or from the increased synthesis of HSF2, or both. By using a pulse-chase metabolic labeling protocol and immunoprecipitation analysis, HSF2 synthesis was examined under conditions of MG132 treatment. Levels of HSF2 synthesis increased twofold for K562 cells and sevenfold for MEF within the time course examined. The immunoprecipitation results for MEF demonstrate increased synthesis of both α and β HSF2 isoforms (Fig. 6A). Upon removal of MG132 from the medium of the tissue culture cells, the rate of HSF2 synthesis decreased markedly (data not shown), which partially explains the return to control levels of HSF2. Pulse-chase analysis of HSF2 protein in MEF (Fig. 6B) revealed loss of both HSF2 isoforms under normal conditions (lane 2). In the presence of MG132, however, HSF2 levels were maintained (Fig. 6B, lane 3). These results provide an explanation for the accumulation of HSF2 protein, which occurs prior to the increased synthesis of HSF2 (Fig. 6A, lane 2).

Inhibition of proteasome activity results in induction of heat shock gene expression. To determine whether activation of HSF2 DNA-binding activity leads to the expression of the known heat shock-regulated genes, we used a quantitative reverse transcription-PCR multiplex assay which uses oligonucleotide primers specific for the genes encoding the cytosolic and nuclear chaperones Hsp90, Hsp70, Hsc70, and Hsp27, the endoplasmic reticulum chaperone Grp78, and the mitochon-

FIG. 6. Elevated synthesis and decreased degradation of HSF2 upon proteasome inhibition. (A) Control MEF (lane 1) and MEF treated with MG132 for 2 (lane 2) and 6 (lane 3) h were pulse-labeled for 15 min, following which cell extracts were prepared for use for immunoprecipitation (upper panel) and immunoblot (lower panel) analyses as described above. The labeled proteins were visualized by fluorography and quantitated by PhosphorImager analysis. The labeled protein band appearing above 208 kDa represents a nonspecific interaction with the antibodies. (B) MEF treated with MG132 for 4 h were pulse-labeled for 15 min and incubated in complete medium with (lane 3) or without (lane 2) MG132 for an additional 4 h. Cell extracts were prepared and used for immunoprecipitation analyses as described above.

drial chaperones Hsp75 and Hsp60. Of these members of the heat shock gene family, the expression of Hsp90, Hsp70, Hsc70, and Hsp27 mRNAs was induced three- to fivefold, that of Hsp60 and Grp78 mRNAs was induced twofold, and Hsp75 expression was unaffected in MG132-treated K562 cells (data not shown). The levels of HSF2 mRNA, in comparison, do not increase with MG132 treatment (data not shown). These results are consistent with those of previous studies showing that the Hsp90 and Hsp70 genes, and not the HSF2 gene (55), were transcriptionally induced upon hemin treatment of K562 cells (56) and that Hsp70 mRNA levels increased upon MG132 treatment of HepG2 cells (67). The inhibition of proteasome activity, therefore, has broad and nonselective effects as a stress inducer and leads to the activation of the same set of genes known to be regulated by HSF1.

The induction of heat shock protein synthesis in MG132- and lactacystin-treated cells was examined following incubation with Tran^{35}S label and analysis by one and two-dimensional SDS-PAGE and Western blotting. Exposure of K562 cells and other mammalian cell lines (data not shown) to either proteasome inhibitor resulted in the elevated synthesis of Hsp70 and Hsp90 (Fig. 7A), which corresponded to a 20-fold increase in Hsp70 levels and a 12-fold induction of Hdj-1 (Fig. 7B and C). These results are supported by recent observations with yeast, where inhibition of proteasome function resulted in induction of heat shock gene expression (31). Analysis by two-dimensional PAGE revealed that treatment by MG132 or heat shock led to the induced synthesis of a common set of proteins (Fig. 7E and F). In addition, MG132 treatment specifically induced the synthesis of two proteins of approximately 35 kDa which were not detected following heat shock.

DISCUSSION

Activation of HSF2 upon down-regulation of the ubiquitin-proteasome pathway not only reveals that the inducible expression of heat shock genes responds to changes in protein turnover but establishes a novel role for HSF2. Although previous studies on HSF2 activation suggested a role as a development- and differentiation-specific factor, a puzzling feature was the ubiquitous expression of HSF2 in different mammalian tissues or tissue culture cell lines, where it was maintained in an inert state (37, 38, 65). The evidence that HSF2 was inducibly regulated was based on observations in human K562 cells that HSF2 DNA-binding and transcriptional activities were induced by hemin (55, 56, 58). Since hemin is known to cause nonterminal erythroid differentiation of K562 cells, these observations were interpreted to provide support for a role of HSF2 in differentiation rather than as a redundant stress-regulated HSF. However, since hemin was also known to have the biochemical property of inhibiting proteasome activity, and since we have demonstrated here, using proteasome inhibitors, that HSF2 activation occurs in a cell type-independent manner, these results suggest a common regulatory pathway for HSF2, perhaps similar to the universal activation of HSF1 by heat shock. Ubiquitination and proteasome activities are themselves modulated during stress (13, 22, 24, 52, 62) and during development and differentiation (25, 53, 54, 62). Furthermore, activation of HSF2 in the mouse ts85 cell line, which carries a conditional mutation in E1, reveals that even though ubiquitination is not inhibited completely at the nonpermissive temperature in these cells (8), the down-modulation of ubiquitination obtained is sufficient to regulate HSF2 activity. In addition, our results explain the previously observed expression of the hsp70 and hsp105 heat shock genes in ts85 cells at the nonpermissive temperature (5, 21).

HSF2 is a short-lived protein, and activation of HSF2 is accompanied by its increased synthesis and decreased degradation, the consequence of which is the accumulation of HSF2. These features suggest that the regulation of HSF2 has features reminiscent of the *Escherichia coli* heat shock promoter-specific σ^{32} subunit of RNA polymerase. Under normal conditions of cell growth, σ^{32} is a short-lived protein (59); upon heat shock, σ^{32} levels increase, principally due to decreased degradation by the FtsH protease, and hence increased protein stability, which ensures that the heat shock genes are induced (23, 59, 60). The posttranscriptional regulation of HSF2 presented here distinguishes this member of the HSF family from its long-lived counterpart, HSF1. Although we have not detected polyubiquitinated forms of HSF2 when proteasome activity is inhibited, nevertheless, this is suggested by the activation of HSF2 in the ubiquitination-deficient ts85 cell line. Arrest of proteasome activity also causes an increase in HSF2 protein synthesis, while HSF2 message levels are unaffected (reference 55 and data not shown). The correlation between HSF2 protein accumulation and activation is also observed when HSF2 is overexpressed by transient transfection (14).

The association between HSF2 and the dynamic state of the proteasome reveals a certain degree of regulatory specificity, although this distinction is not absolute, as HSF1 was also detected in a partially activated state. The level of HSF1 contribution to the total amount of HSF activity induced when proteasome activity is down-regulated varies widely among

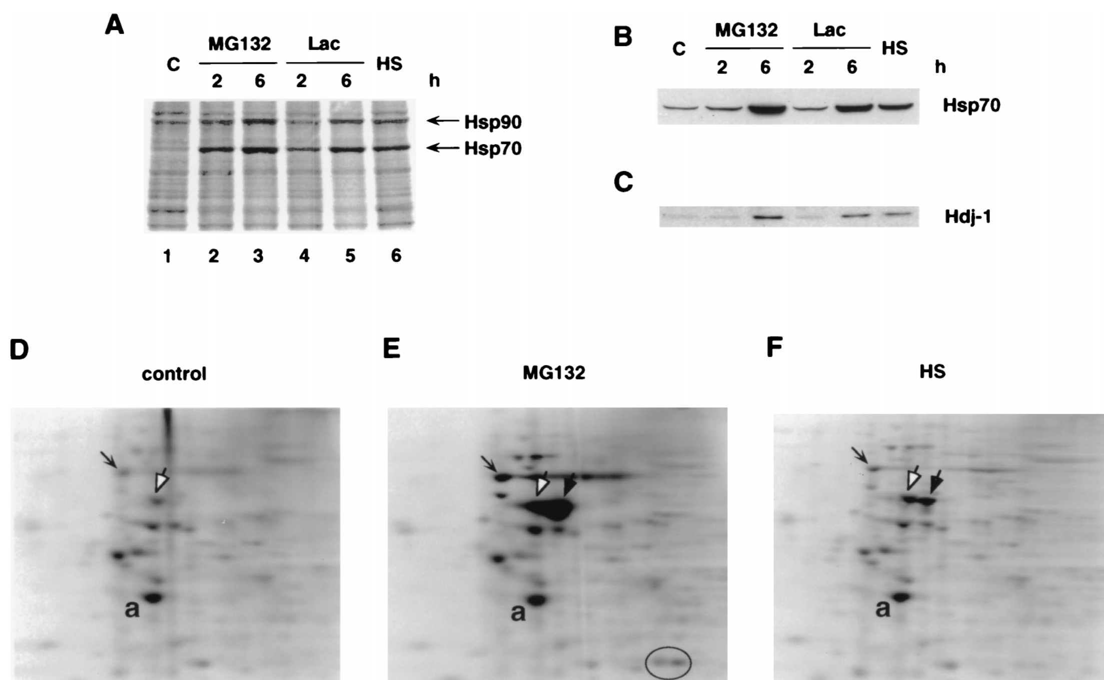

FIG. 7. Induction of heat shock proteins in K562 cells by proteasome inhibitor treatment. (A) ^{35}S-labeled cell extracts from pulse-labeled cells analyzed by SDS–10% PAGE. Cells were left untreated (lane 1), treated with 10 μM MG132 for 2 or 6 h (lanes 2 and 3), treated with 10 μM lactacystin (Lac) for 2 or 6 h (lanes 4 and 5), or heat shocked (HS) at 42°C for 1 h (lane 6). (B) Hsp70 immunoblot of cell extracts using the mouse monoclonal antibody 4G4. From left to right, treatments correspond to those described for panel A, lanes 1 through 6. (C) Hdj-1 immunoblot of cell extracts using rabbit polyclonal sera raised against Hdj-1. From left to right, treatments correspond to those described for panel A, lanes 1 through 6. (D through F) Two-dimensional protein gel analysis of ^{35}S-labeled cell extracts left untreated (D), treated with 10 μM MG132 for 6 h (E), or heat shocked at 42°C for 1 h (F). The first dimension was isoelectric focusing generating a gradient from pH 3 to 10. The second dimension was SDS–10% PAGE. Open arrowhead, location of Hsc70; solid arrowhead, location of Hsp70; arrow, location of Hsp90. The circled proteins of approximately 35 kDa are induced preferentially upon MG132 treatment. The protein spot labeled a corresponds to actin.

cells of different species and tissue origins. In K562 cells, for example, hemin treatment results in negligible levels of HSF1 activation; furthermore, the slow kinetics of HSF2 activation and heat shock gene expression reflects the extended period required for HSF2 levels and proteasome substrates to accumulate. However, in contrast, the proteasome inhibitors MG132 and lactacystin result in an immediate arrest of proteasome activity, which results in the rapid accumulation of abnormal proteins destined for proteasomal degradation. Although these events might be expected to result in the complete activation of HSF1, as a result of the appearance and accumulation of misfolded proteins destined for degradation, we observe that it is principally HSF2 which is activated. Variable levels of HSF1 coactivation are observed in different cell lines, which suggests a regulatory overlap between HSF1 and HSF2 to ensure high levels of chaperones. Consistent with this suggestion, we have observed that stresses, such as amino acid analogs, which lead to the chronic appearance of misfolded proteins result in the complete activation of HSF1 and partial activation of HSF2 (data not shown).

There is a growing body of information to support a role of the heat shock response as a component of the protein-degradative machinery (19, 22, 52). A number of proteases and components of proteolytic pathways are heat shock-induced proteins, including La in *E. coli* (17, 44), eukaryotic ubiquitin (3, 14), and the ubiquitin-conjugating enzymes UBC 4 and UBC 5 (51). Proteasome inhibition results in expression of heat shock proteins (4, 31, 67), and there are several lines of evidence for chaperones in ubiquitin-proteasome-mediated protein degradation. For instance, a ubiquitin-processing enzyme was identified as a suppressor of certain mutations of Hsp70 (7); Hsp90 protects the proteasomal catalytic core against inactivation by oxidative stress, while also modulating its proteolytic activity (6, 61, 64); and mutations in the yeast DnaJ homologs Ydj-1 and Sis affect the ubiquitination of abnormal and short-lived proteins and proteasomal digestion of ubiquitinated proteins, respectively (32, 52). Direct association of chaperones with proteasomal substrates has also been detected and implicated in the determination of substrate fates. For example, the ubiquitin-dependent degradation of certain protein substrates in in vitro reticulocyte lysates was shown to be strongly influenced by the levels of Hsc70, with which these substrates were shown to interact (1). In similar in vitro lysate systems, the nature of the association of selective substrates with the Hsp90 heterocomplex resulted in either of two fates, refolding or proteasomal degradation, with prolonged chaperone association (induced by use of the drug herbimycin A) leading to increased degradation (49, 50). There is also in vivo evidence that chaperone association with a yeast proteasomal substrate, Cln3, is required for efficient phosphorylation, which necessarily precedes the ubiquitination of this substrate (66). These observed molecular chaperone associations with substrates as they undergo polyubiquitination may be important if such extreme forms of posttranslational modifications lead to the accumulation of nonnative proteins. Consistent with this suggestion, we have detected the association of bulk polyubiquitinated proteins with induced Hsp70 and Hsp90 during proteasome inhibition (35a). These interactions are specific and are released in the presence of ATP. Upon reversal of proteasomal inhibition, the levels of polyubiquitinated substrates which associate with Hsp70 and Hsp90 are dramatically reduced, presumably related to the reduction in polyubiquiti-

nated proteins in the cells. The transient association of ubiquitinated substrates with the chaperones suggests that the chaperone-associated proteins are targeted for degradation. In support of this hypothesis, the targeting for proteasomal digestion of a specific polyubiquitinated substrate, apolipoprotein B100, has been shown to be regulated by its association with Hsp70 (16).

An attractive proposal for the HSF family is that the coordinated efforts of multiple HSFs provide chaperone coverage for the diverse cellular events which cause nonnative proteins to appear and ensure that their fates as refolded proteins or degraded products have been determined. We suggest that HSF2 functions as the inducible regulator at the point where misfolded proteins have been marked for degradation, thus ensuring a need for the continued inducible expression of chaperones. These results reveal that HSF activity, and hence chaperones, are required for both the birth and the death of proteins.

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