Evidence That a Rapidly Turning Over Protein, Normally Degraded by Proteasomes, regulates hsp72 Gene Transcription in HepG2 Cells*

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Heat shock protein 72/73 (Hsp70) is a cytosolic molecular chaperone that carries out fundamental roles under both normal and stress situations. There is great interest in delineating the mechanisms whereby Hsp70 levels are regulated. We observed that N-acetyl-leucyl-leucyl-norleucinal (ALLN), a synthetic aldehydic tripeptide that inhibits proteasomes, markedly induced Hsp70 levels (up to 30-fold above base line in HepG2 cells and human endothelial cells). Induction of Hsp70 by ALLN was dose-dependent and not related to cell toxicity. ALLN selectively increased Hsp70 levels without affecting Hsp25, Hsp27, Hsp60, Hsp90, Hsp104, or Bip (immunoglobulin heavy chain binding protein) in HepG2 cells. ALLN induced Hsp70 not only by stabilizing the protein but also by dramatically increasing its synthesis. The modulation of Hsp70 synthesis by ALLN resulted from a rapid and marked increase in transcription of the hsp72 gene, since the induction of hsp72 mRNA was blocked in cells co-treated with actinomycin D. hsp72 mRNA levels were affected in a time-dependent manner by exposure to ALLN; significant elevations occurred within 60 min of treatment, and a decline to background levels was observed by 7 h of recovery. The ALLN-induced increase in hsp72 gene expression was associated with trimerization of the heat shock transcriptional factor (HSF1). ALLN did not affect the steady-state level of HSF1 protein. The effects of ALLN appeared to require de novo protein synthesis, since the induction of both HSF1 trimerization and hsp72 transcription was blocked by co-treatment with cycloheximide. When we tested a series of protease inhibitors, only the related aldehydic tripeptides, N-acetyl-leucyl-leucyl-methioninal and the proteasome inhibitor, Cbz-leucyl-leucyl-leucinal, induced Hsp70 levels. The specific proteasome inhibitor, lactacycin, which has a different structure, also induced Hsp70 levels. Overall, our results suggest that a rapidly turning over protein that is normally degraded by proteasomes may be involved in the regulation of Hsp70 synthesis via effects on the hsp70 transcriptional factor, HSF1.

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Induction of heat shock (stress) proteins (Hsps), a class of molecular chaperones, is a physiological and biochemical response to an abrupt increase in temperature (1, 2) or exposure to a variety of other metabolic insults (3, 4), including heavy metals, amino acid analogs, toxins, and oxidative stress. This response is found in all prokaryotic and eukaryotic cells and is characterized by repression of normal protein synthesis together with the rapid initiation of transcription of several Hsp-encoding genes (2). Among these highly conserved Hsp family members are two nearly identical, cytosolic heat shock proteins, Hsp72 (the inducible form) and Hsp73 (the constitutively synthesized form). These two proteins, commonly referred to as cytosolic Hsp70, function as molecular chaperones and play fundamental roles in a number of important biological processes. Under nonstressed conditions, Hsp70 interacts transiently with nascent polypeptides to facilitate proper folding and maturation and promote protein translocation across mitochondrial and endoplasmic reticulum (ER) membranes (5–8). During stress conditions, Hsp70 is suspected to form a complex with proteins that misfold or unfold, either “rescuing” these proteins from irreversible damage or degradation (9–12) or increasing their susceptibility to proteolytic attack (13).

Recently, elevated expression of Hsp70 and other Hsps has been observed in cells and tissues under conditions potentially relevant to human diseases, including ischemia, oxidant injury, atherosclerosis, and aging (14–16). The increased expression of these stress proteins could represent an acute response to altered physiological states as well as chronic adaptation to particular diseases. The primary function of these stress responses is thought to be cytoprotective. For example, overexpression of Hsp70 alone was demonstrated to protect cells from thermal injury and increase cell survival (17, 18). Elevated levels of inducible Hsp70 have been associated with both improved post-ischemic recovery (19) and tolerance to ischemia in gerbil hippocampal neurons (20). It has also been reported that both heat shock-induced and exogenous Hsp70 can protect smooth muscle cells from serum deprivation-induced cell death (16). Overexpression of Hsp70 also protects murine fibroblasts from both UV light injury and proinflammatory cytokines released during UV exposure (21). A protective role of Hsp70 was demonstrated clearly by several recent studies with transgenic

1] The abbreviations used are: Hsp, heat shock protein; ALLN, N-acetyl-leucyl-leucyl-norleucinal; ALLM, N-acetyl-leucyl-leucyl-methioninal; Hsp70, heat shock protein 72/73; PAGE, polyacrylamide gel electrophoresis; HSF, heat shock transcription factor; ER, endoplasmic reticulum; apoB, apolipoprotein B100; EGS, ethylene glycol bis(succinimidylsuccinate); Bip, immunoglobulin heavy chain binding protein; HepG2, human hepatoma cell line; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; MG132, Cbz-leucyl-leucyl-leucinal; MEM, minimal essential medium; E-64d, N[N-t-transcarboxoxiran-2-carbonyl-1-leucyl]-agmatine.
mice in which overexpression of human inducible Hsp70 protected myocardium from ischemic reperfusion injury (22, 23) and enhanced post-ischemic recovery of the intact heart (24). These potential clinical applications of Hsp70 have stimulated investigators to search for efficient pharmacological means of rapidly and selectively inducing Hsp70.

We have been studying the involvement of molecular chaperones in the assembly and secretion of apolipoprotein-B-100 (apoB)-containing lipoprotein from cultured liver (HepG2) cells. ApoB is a very large, extremely hydrophobic secretory protein that appears to be constitutively translated but inefficiently translocated across the ER membranes (25). As a result, nascent apoB assumes a transmembrane topology with some portion of the nascent protein exposed to the cytosol. Since the extreme hydrophobicity of apoB makes it unlikely that it would maintain a translocation-competent conformation in the cytosol without the "assistance" of a chaperone, we looked for an association of apoB with Hsp70 (26). We found that Hsp70 associated transiently with nascent apoB and that this interaction appeared to be regulated by the translocation status of apoB. We observed that less apoB was bound to Hsp70 in the presence of oleic acid, which facilitates apoB translocation across the ER membranes by stimulating new triglyceride synthesis. In contrast, more apoB was bound to Hsp70 in the presence of a proteasome inhibitor, N-acetyl-leucyl-leucyl-nor-methionyl (ALLN) (27, 28), which protects apoB from degradation without enhancing translocation. During these studies, we observed a marked, unexpected increase in Hsp70 levels in cells treated with ALLN. The studies presented in this report were designed to determine the mechanisms underlying the induction of Hsp70 by ALLN.

EXPERIMENTAL PROCEDURES

Materials—ALLN, leupeptin, mouse anti-human Hsp72/73 monoclonal antibody, a secondary antibody conjugated with horseradish peroxidase (goat anti-rabbit immunoglobulin G (IgG)), and rabbit antirat IgG antibody were from Boehringer Mannheim. Anti-human Hsp25 and anti-human Hsp60 monoclonal antibodies, N-acetyl-leucyl-leucyl-methionyl (ALLN), calpain inhibitor peptide, dimethyl sulfoxide (MeSO), and diithiothreitol were from Sigma. Human Hsp70 oligonucleotide probe was from Oncogene Science. This oligonucleotide was 32P-labeled at the 3' end by T4 kinase, which was from New England Biolabs. 32P was from ICN Pharmaceutical Inc. -[4,5-3H]leucine was from Amersham Corp. Anti-Bip (immunoglobulin heavy chain binding protein, also called Grp78), anti-human Hsp27, Hsp60, and Hsp90 monoclonal antibodies were from Stress Genes. Anti-human Hsp86 and Hsp104 antibodies were from Affinity Bioreagent Inc. Nitricollose transfer and immobilization membranes were from Schleicher & Schuell. RNA extraction kits were from Biotec. The polyclonal antibody against the human HSF1 protein was a generous gift from Dr. Carl Wu of the National Institute of Health (29). Ethylene glycol bis(succinimidylsuccinate) (EGS) was from Pierce. Cbz-leucyl-leucyl-leucinal (MG132) was from Calbiochem. Protein A-Sepharose 4B was from Pharmacia. L-[4,5-3H]leucine was from Amersham-Pharmacia Biotech. Cbz-leucyl-leucyl-nor-leucinal (ALLN) was from ICN Pharmaceutical Inc. L-[4,5-3H]leucine was from Amersham Biotech. 32P was from ICN Pharmaceutical Inc. Cbz-leucyl-leucyl-leucinal (MG132) was from Pierce. 32P was from ICN Pharmaceutical Inc.

RESULTS

ALLN Induces Heat Shock Protein 70

Triton X-100, 1% deoxycholic acid, and 0.1% SDS in phosphate-buffered saline (PBS) was used to make the blocking solution. Membranes were then washed four times with 3 x 106 cpm/ml) for 2.5 h at 4°C. Protein A-Sepharose 4B was added afterwards and incubated for another 1.5 h at 4°C to collect the immunocomplexes. After washing four times with 1 x NET buffer (containing 0.5% Triton X-100, 0.1% SDS), the immunocomplexes were mixed with sample buffer and boiled for 5 min. Following centrifugation, the supernatants were aliquoted for the scintillation counting and for 3-15% gradient polyacrylamide gel electrophoresis (SDS-PAGE). The gels were later subject to fluorography. All values were presented as the mean ± S.D. from three individual experiments except as otherwise indicated.

DNA Isolation and Northern Blot Analysis—Total RNA was using the procedure of Chomczynski and Sacchi (30) and a commercially prepared reagent, TRISOLY® (Biotech Laboratories Inc.). Equal amounts of each RNA sample (20 μg) were size-fractionated on a 5% agarose-formaldehyde gel according to the methods described (30) and transferred to a piece of QIAmar Plus nylon membrane. To determine loading and transfer efficiency, RNA was stained with ethidium bromide before and after transfer. The membrane was baked for 30 min at 80°C in a vacuum followed by UV cross-linking. It was then incubated for 4 h at 65°C in a prehybridization mixture containing 10% dextran sulfate, 1% SDS, 1 mM sodium chloride, 50 mM Tris-HCl (pH 7.5), and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out for 18 h at 65°C in the same buffer with a 32P-labeled (1 x 106 cpm/ml) 40-mer oligonucleotide probe to the untranslated 5' region of a human hsp70 gene (Oncogene Science). This probe is specific for the inducible form of hsp72 and does not cross-hybridize to the constitutive form of hsp73, hsp70B or hsp70B′ (31). The membrane was then washed four times with 2 x SSC, 0.1% SDS at room temperature, one time at 65°C for 30 min, and one time at room temperature again for 5 min. After it was rinsed with 2 x SSC at room temperature, the membrane was air-dried and exposed to x-ray film at ~80°C for 48 h.

Preparation of Cell Extracts, Chemical Cross-linking, and Western Blot of HSF—For measurement of the steady-state HSF1 protein levels, whole cell extracts were prepared by lysing the cells directly with 2 x SDS-sample buffer (32). For chemical cross-linking experiments, whole-cell extracts were prepared as described (30). Briefly, the cold phosphate-buffered saline-washed cell pellets were quickly and repeatedly frozen in liquid nitrogen. The pulverized pellet was thawed and resuspended in about 2 packed cell volumes of buffer C (20 mM HEPES, pH 7.9, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM diithiothreitol, 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 0.01 units/ml apronin, 25% glycerol (v/v)). The concentration of NaCl was then adjusted to 0.38 mM. After 10 min of incubation on ice, the extract was clarified by centrifugation at 4°C for 15 min at 10,000 x g. Cross-linking of HSF was immediately performed (33) by adding 1/10 volume of freshly prepared 32P into containing an appropriate concentration of EGS to the whole cell extracts at a final concentration of 1 mM and then incubating the mixture at 22°C for 30 min. Reactions were quenched by the addition of glycine to 75 mM and incubation for 15 min at room temperature. After SDS-PAGE, the proteins were transferred to nitrocellulose, nonspecific protein binding sites were blocked with 5% nonfat dry milk, and the membrane was probed with a 32P-labeled human HSF1 antibody followed by repeated washing and subsequent incubation with the secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG).

RESULTS

ALLN Induces Hsp70 Levels in HepG2 Cells in a Dose-dependent Manner—The effect of ALLN on the levels of Hsp70 was analyzed by denaturing immunoprecipitation of extracts of [3H]leucine-radiolabeled HepG2 cells that had been pretreated with ALLN for 4 h at different doses. Denaturing immunoprecipitation was chosen because Hsp70 is a major cytosolic chaperone.
Fig. 1. Dose-dependent induction of Hsp70 by ALLN in HepG2 cells. HepG2 cells were grown up to 95% confluence and then preincubated for 4 h at 37°C with serum-free MEM, followed by radiolabeling with [3H]leucine (150 μCi/ml) dissolved in serum-free, leucine-free medium. Preincubation and labeling medium each contained 1.5% BSA plus different concentrations of ALLN as indicated. After labeling, cells were collected and lysed, and Hsp70 was immunoprecipitated under denaturing conditions as described under “Experimental Procedures.” Cell lysates containing an equal amount of trichloroacetic acid-insoluble radioactivity were used for immunoprecipitation. A, the immunoprecipitates were analyzed by SDS-PAGE and fluorography. Numbers on the left denote molecular markers. B, Hsp70 radioactivity was quantitated by scintillation counting and plotted. All the values were presented as means ± S.D. from three individual culture dishes. The inset shows trichloroacetic acid-perceptible radioactivity at each dose of ALLN.

Hsp70 is co-immunoprecipitated with a number of other proteins under nondenaturing conditions (26, 36). Immunoprecipitation with anti-human Hsp70 antibody showed that untreated cells had low levels of radiolabeled Hsp70 (Fig. 1). This may be due to a low, constitutive level of expression of Hsp73 (Hsc70) in HepG2 cells, similar to that reported for other cell types in the absence of stress (37). Treatment with ALLN was associated with a marked increase in Hsp70 level, which was further confirmed as Hsp72 (the inducible form) by Western blot (data not shown). Hsp70 levels showed a dose-dependent response to ALLN treatment. This induction was not related to ALLN-induced cell toxicity, since at a low concentration of ALLN (10 μg/ml), Hsp70 levels were induced more than 9-fold, while cell total incorporation of radiolabel (trichloroacetic acid-insoluble cpm, an indicator of cell toxicity) did not change significantly (Fig. 1B, inset). When we removed the ALLN-containing medium after a 4-h preincubation and then washed the cells with fresh medium for different periods of time, followed by radiolabeling and immunoprecipitation of cell lysates, we found that Hsp70 levels remained as high as 25-fold at the end of a 2-h wash, 8-fold at the end of a 6-h wash, and 4-fold at the end of an 8-h wash (data not shown). Similar results were obtained from a human microvascular endothelial cell line, which showed a 35-fold increment of Hsp70, with no change in trichloroacetic acid-insoluble cpm, after 4 h of ALLN treatment (40 μg/ml) and 30 min of labeling with [3H]leucine in the presence of ALLN (data not shown). In addition, we found that Chinese hamster ovary cells also respond to ALLN by increasing cellular Hsp70 levels (data not shown).

Hsp70 Is Selectively Induced by ALLN in HepG2 Cells—It was reported (38) that in human diploid fibroblasts, heat shock treatment could induce the synthesis of several members of the Hsp family, including Hsp25, Hsp50, Hsp64, Hsp72, Hsp78, Hsp89, and Hsp98. Short, sublethal episodes of cardiac ischemia increase both Hsp70 and Hsp60 (14). Such complex induction patterns may reflect the need for several of these Hsps to increase in order to protect preexisting proteins from denaturation. It is suggested that molecular chaperones like Hsp70 and Hsp60 may work in tandem to facilitate the folding process (6). To ascertain whether other members of the Hsp family are also induced by ALLN treatment, we compared the whole cell protein pattern of untreated and ALLN-treated HepG2 cells by directly loading [3H]leucine-labeled cell lysates onto SDS-PAGE gel. As can be seen in Fig. 2A, all the proteins were synthesized at similar levels in both ALLN-treated and control cells except Hsp70, which was markedly increased in ALLN-treated cells. To further confirm this result, we selectively determined the protein levels of Hsp25, Hsp27, Hsp60, Hsp86, Hsp90, Hsp104, Bip, and albumin by specific immunoprecipitation of each protein from radiolabeled extracts of cells preincubated with or without ALLN. As can be seen from Fig. 2B, only Hsp70 was induced by ALLN treatment in HepG2 cells.

ALLN Not Only Stabilizes Hsp70 but Also Increases Its Synthesis—To test whether ALLN induces Hsp70 by increasing protein synthesis, we preincubated HepG2 cells with or without ALLN for 4 h at 37°C and subsequently radiolabeled them with [3H]leucine for periods between 10 and 60 min. Anti-Hsp70 immunoprecipitates were collected from cell lysates, proteins were separated by SDS-PAGE (Fig. 3A), and radioactivity was determined by scintillation counting (Fig. 3B). As indicated, the rate of incorporation of [3H]leucine into Hsp70 was increased about 25-fold at every time point in this experiment.

Since ALLN is a protease inhibitor (27, 28, 39–41), we considered the possibility that protection of Hsp70 from proteolytic degradation might also account for the sharp increase in Hsp70 levels induced by ALLN. To determine whether ALLN stabilizes Hsp70, we measured the turnover rate of radiolabeled Hsp70 in the presence or absence of ALLN (40 μg/ml) in a pulse-chase experiment. The result in Fig. 3C shows that without ALLN, newly synthesized Hsp70 disappeared rapidly, with a half-life of about 2 h. This value is comparable with that reported by Landry and co-workers (42) who showed that Hsp70 in Chinese hamster ovary cells displayed a half-life of
Fig. 3. Effects of ALLN on Hsp70 synthesis and turnover rates in HepG2 cells. Confluent culture dishes were preincubated for 4 h with serum-free MEM containing either 1.5% BSA alone (Control) or BSA plus 40 μg/ml ALLN (ALLN). A and B, the preincubated cells were radiolabeled with [3H]leucine (150 μCi/ml) in the presence or absence of ALLN for the indicated periods of time. Cells were then subjected to lysis and immunoprecipitation with anti-Hsp70 antibody as described in the legend to Fig. 1. Immunoprecipitates were analyzed on SDS-PAGE and fluorography (A) and also aliquoted for scintillation counting (B). Each value was presented as mean ± S.D. from three individual dishes. C, the preincubated cells were radiolabeled with [3H]leucine (150 μCi/ml) in serum-free, leucine-free medium with or without ALLN for 20 min, washed three times with medium free of isotope, and then chased in serum-free medium containing BSA with or without ALLN for different periods of time. At the indicated time points, cells were harvested and lysed. Immunoprecipitation of Hsp70 was performed as described above. Data at each time point were presented as means of cpm from duplicate dishes. Similar results were obtained in two separate experiments for both studies.

About 3–4 h. In the presence of ALLN, Hsp70 disappeared very slowly, with a half-life of about 6 h. Thus, stabilization of Hsp70 by ALLN might contribute to the increased cellular level of this protein. However, since treatment with ALLN resulted in an approximate increase of 30-fold of newly synthesized Hsp70 levels after 30-min labeling (data from several experiments) compared with untreated cells, it seemed clear that a 3-fold increase in the half-life of Hsp70 by ALLN could not account significantly for the dramatic induction of Hsp70 we had observed. Thus, increased protein synthesis is the major reason that ALLN dramatically induces Hsp70 levels.

ALLN Up-regulates Hsp70 Transcription in a Time-dependent Manner That Requires de Novo Protein Synthesis—Total RNA was isolated from HepG2 cells that had been incubated for 4 h at 37°C (Fig. 4A) with MEM containing BSA alone (Control, lane 1), BSA plus 40 μg/ml ALLN (lane 2), BSA plus ALLN and 10 μg/ml actinomycin D (A + AD, lane 3), or BSA plus ALLN and 50 μg/ml cycloheximide (A + CXM, lane 4). A radioprobe containing a unique sequence specific for the 5′-untranslated region of hsp72 (inducible form) (33) was used for Northern blot analysis. This probe does not recognize hsp73 (constitutive form). Northern blotting analysis indicated that the amount of hsp72 mRNA was increased dramatically in the ALLN-treated cells. Co-treatment of ALLN with actinomycin D, which blocks RNA synthesis, abolished the effects of ALLN, indicating that ALLN induced hsp72 mRNA by increasing its synthesis rather than protecting it from degradation. It has been reported previously that induction of Hsp70 at the transcriptional level by heat shock, inorganic metals, or metalloporphyrins occurs independently of new protein synthesis (34, 43–45), whereas induction by amino acid analogs requires ongoing protein synthesis (34). Therefore, we examined the effects of the protein synthesis inhibitor cycloheximide at a level (50 μg/ml) that was sufficient to block new synthesis (46). As can be seen, the marked increase in hsp72 mRNA associated with ALLN treatment was abolished by co-treatment with cycloheximide.

As can be seen in Fig. 4B, induction of hsp72 mRNA by ALLN was time-dependent. Although 60 min of ALLN treatment was already associated with a significant increase of hsp72 mRNA levels, treatment with ALLN for 2 or 3 h further increased hsp72 mRNA in proportion to the duration of treatment (Fig. 4B, lanes 1–4). After treatment for 3 h, ALLN-containing medium was removed, and the cells were washed and fed fresh medium containing only BSA. As can be seen from lanes 5–8, hsp72 mRNA levels decreased progressively during recovery. After 7 h of recovery, hsp72 mRNA had declined to background levels (lane 8). This result suggested that...
transcriptional induction of hsp72 mRNA by ALLN was contingent upon the presence of ALLN and that the induction was fully and rapidly reversed upon removal of ALLN.

**ALLN Induces Trimerization of HSF1 without Affecting Its Protein Levels; Trimerization Is Dependent on de Novo Protein Synthesis**—It is known that the heat shock transcriptional response is mediated by the activation of a preexisting 90-kDa protein factor, HSF1, which binds to heat shock elements in the promoters of heat shock genes (47, 48). HSF1 binding to the heat shock elements results in a high level of transcription of hsp72. It was reported that HSF1 is the primary component of HSF-DNA activity present in cells exposed to heat shock, cadmium sulfate, and the amino acid analog l-azetidine-2-carboxylic acid (47). This activation process is achieved by conversion of a latent, non-DNA-binding monomeric form of HSF1 to a DNA-binding trimer (49). We used Western blotting techniques to determine the effects of ALLN treatment on the steady-state level and stoichiometry of HSF1. Fig. 5A demonstrates that the steady-state levels of HSF1 in whole cell extracts were not significantly affected either by heat shock treatment with or without cycloheximide (lanes 4 and 5), or by ALLN treatment with or without cycloheximide (lanes 2 and 3), consistent with the reported data that HSF1 levels are quite stable. The electrophoretic mobility of HSF1 under these conditions, however, was not identical. This may represent different phosphorylation status of HSF1 under different conditions (47, 50). To determine whether HSF1 undergoes a change in size upon treatment with ALLN in vivo, we performed chemical cross-linking of subunit proteins with EGS dissolved in Me_2SO prior to Western blot analysis of HSF1. This technique allowed for assessment of the stoichiometry of the transcriptional factor under different experimental conditions (47). Using this protocol, we showed (Fig. 5B) that under control conditions, most HSF1 is in the monomer state (lane 1); ALLN treatment produced a 270-kDa cross-linked product, suggesting that HSF1 in ALLN-treated cells exists as a trimer in solution (lane 2). This result is comparable with the activation obtained by heat shock (lane 4). Co-treatment with cycloheximide abolished the effect of ALLN on HSF1 trimerization (lane 3). Cycloheximide did not, however, affect heat shock-associated trimerization (lane 5). Combining the results of Fig. 5, A and B, it appears that ALLN activated HSF1 without affecting its steady-state protein levels; in the presence of cycloheximide, however, ALLN treatment was not associated with HSF1 activation to the DNA-binding, trimeric form. These results indicate that the initiation of HSF1 activation by ALLN requires de novo protein synthesis, consistent with results in our transcriptional study (Fig. 4A). In contrast, initiation of HSF1 trimerization by heat shock does not require de novo protein synthesis, consistent with the published data from other laboratories (34, 51).

Hsp70 was induced by ALLN and other proteasome inhibitors, but not other proteasome inhibitors—ALLN is a synthetic aldehydic tripeptide that can, in vitro, inhibit the activity of Ca^2+-dependent neutral cysteine proteases. ALLN can also inhibit lysosomal proteases, including cathepsin L, cathepsin B, and calpain D (39). Recently, ALLN has been shown to inhibit proteasome activity (27, 52). We examined the effects of several other protease inhibitors on Hsp70. Cells were pretreated with various protease inhibitors for 4 h prior to radiolabeling, cell lysis, and immunoprecipitation (Fig. 6). Total incorporation of radiolabeled leucine into proteins (trichloroacetic acid-insoluble cpm) was measured as an indicator of both cell toxicity and entrance of the drugs into the cells. Although most of the protease inhibitors, at the given concentrations, showed similar degrees of cell toxicity (Fig. 6, bottom), only ALLN and ALM, among the nine different protease inhibitors tested, were able to significantly induce Hsp70 (Fig. 6, top). ALLN, which is almost identical to ALLN in its structure, showed less ability to induce Hsp70 levels. E-64d (N[ω-trans-carboxyoxiran-2-carbonyl-1-leucyl]-agmatine), which is considered to be a specific inhibitor of cysteine proteases, showed a very weak effect on Hsp70. Leupeptin, an inhibitor of both
serine and cysteine proteases, was ineffective. No induction of Hsp70 was observed with two other serine protease inhibitors, aprotinin and PMSF, or with pepstatin, a metalloprotease inhibitor. It is of interest to note that the effect or lack of effect of these inhibitors on Hsp70 is consistent with their effects on apoB, and on the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Both of these proteins are protected by ALLN and, less effectively, by ALLM; the other protease inhibitors mentioned above do not protect either apoB or 3-hydroxy-3-methylglutaryl-coenzyme A reductase from degradation (40, 41).

Because ALLN and ALLM are both aldehydic tripeptides, a class of compounds found to inhibit proteasomes in several recent studies (27, 52), we determined the effects of MG132, an aldehydic tripeptidyl proteasome inhibitor that reversibly binds to the active sites of the proteasomes (27, 53), and lactacystin, a structurally unique proteasome inhibitor that irreversibly acylates the active site threonine (54). Both molecules induced Hsp70 levels dramatically in HepG2 cells (Fig. 7, A and B) without obvious toxicity to the cells (as indicated by trichloroacetic acid-precipitable radioactivities; data not shown).

**DISCUSSION**

In this study, we demonstrated that ALLN rapidly and selectively induced Hsp70 in HepG2 cells. This effect occurred in a dose- and time-dependent manner and was not related to cell toxicity. In HepG2 cells, ALLN had no effects on Hsp25, Hsp27, Hsp60, Hsp86, Hsp90, Hsp104, Bip, or albumin. ALLN induced Hsp70 mainly by markedly increasing its synthesis. The ALLN-induced increase in Hsp70 synthesis resulted from increased transcription of the hsp72 gene via activation of HSF1; this process appeared to require de novo protein synthesis. Finally, results of experiments with MG132 and with lactacystin, the most specific proteasome inhibitor so far reported, indicated involvement of proteasomes in the regulation of Hsp70 levels in HepG2 cells.

The actual mechanism by which the cell recognizes and responds to a particular stress is still unclear. One common denominator shared by many different agents that induce the stress response is an ability to promote, at least in vitro, the production of unfolded or abnormal proteins (55). These misfolded proteins may generate signals that activate HSF1. An autoregulatory loop model has been proposed by Morimoto et al. (4) and several other investigators (46) to explain the regulation of HSF1 DNA-binding activity by Hsp70 itself. According to this model, HSF1 is maintained in a non-DNA binding form under nonstressed conditions by Hsp70. During heat shock, the appearance of misfolded or aggregated proteins creates a large pool of new protein substrates that compete with HSF1 for association with Hsp70, thereby removing the negative regulatory influence of Hsp70 on HSF1 DNA-binding activity. HSF1 becomes oligomerized, binds to DNA, and acquires transcriptional activity. The result is increased synthesis and accumulation of heat shock proteins, particularly Hsp70. When the pool of free or unassociated Hsp70 increases, as would occur during recovery from heat shock, the heat shock response becomes attenuated. Although several studies support a role for Hsp70 in the autoregulation of HSF1 trimerization and activation, other studies have failed to observe effects of Hsp70 on either the temperature set point or magnitude of HSF activation (50). On the other hand, there is substantial evidence that ongoing protein synthesis is required for HSF activation and Hsp70 induction in cells treated with amino acid analogs, hemin, or 3-hydroxyacetoacetate (34, 56, 57). Protein synthesis does not appear to be required for the Hsp70 response to either heat shock or metal ions (34, 51). These observations raise the possibility that multiple pathways are available, depending on the environmental or chemical perturbation, for stress-induced hsp72 gene activation.

ALLN is one of several aldehydic tripeptides that are active against the proteasome (27, 28, 52). It can bind and therefore block the active site in the central cavity of the 20 S proteasome x-ray crystal structure (52). Our demonstration that two other aldehydic tripeptides, ALLM and MG132, and a structurally unique proteasome inhibitor, lactacystin, all induced Hsp70 levels, points to a crucial role for this proteolytic pathway in the regulation of Hsp70 levels in HepG2 cells. Although ALLN and ALLM are potent inhibitors of proteasomes, they also exhibit significant activities against the cysteine proteases, calpain and cathepsin B (27). In contrast, lactacystin is reported to have no detectable effect, even upon extended exposure, on cysteine proteases, serine proteases, trypsin, or chymotrypsin (28, 54). Overall, the results of experiments with these inhibitors strongly support our conclusion that proteasomes are involved in the induction of Hsp70 synthesis in HepG2 cells.

How could inhibition of proteasomal activity result in induction of Hsp70 synthesis? Our results raise the possibility that the link between ALLN and stimulation of both trimerization and activation of HSF1 is a molecule that is sensitive to the status of proteasomal activity. Furthermore, the experiments demonstrating that co-treatment with cycloheximide blocked both the ALLN-associated trimerization of HSF1 (Fig. 5B) and the concomitant increase in hsp72 gene transcription (Fig. 4A) are consistent with the rapid turnover of this molecule. Thus, both de novo protein synthesis and inhibition of proteasomal degradation are required for ALLN to activate HSF1 and thereby induce Hsp70. In contrast to the need for new protein synthesis to see the effects of ALLN, cycloheximide had no effect on heat shock-induced HSF1 activation (Fig. 5B). This is consistent with the report by Mosser et al. (34) that the presence of cycloheximide affected neither heat shock-induced hsp70 transcription nor HSF1 DNA-binding activity and is also consistent with a recent communication (51) demonstrating that heat treatment can directly convert purified HSF1 from the inactive, monomeric form to the trimeric, DNA binding form in vitro. Based on our findings, we hypothesize that for responses to stress other than heat shock, a newly synthesized protein that is normally degraded by proteasomes is involved in HSF1 trimerization and activation. When proteasomal degradation is inhibited, the short-lived protein accumulates, HSF1 trimerizes, and Hsp70 synthesis increases. Our demonstration

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2 M. Zhou, X. Wu, and H. N. Ginsberg, unpublished data.
that removal of ALLN is associated with a rapid return of hsp72 mRNA and Hsp70 protein levels to base line supports a key role for a rapidly turning over protein. Proteasomal degrada-
tion of rapidly turning over proteins has been demonstrated to be important in regulation of the cell cycle (58).

The discovery that proteasome inhibitors are potent inducers of Hsp70 is of interest for a number of reasons. First, the observations presented here imply that a protein normally degraded by proteasomes may be critical for stress responses; identification of this protein will allow for study of the physi-
ological regulation of Hsp70. Second, our results raise the pos-
sibility that other ALLN-associated cellular phenomena, such as disruption of cell cycle (59), decreased cellular cholesterol ester formation (60), and inhibition of the degradation of pro-
teins such as apoB (41) and 3-hydroxy-3-methylglutaryl-coen-
ester formation (60), and inhibition of the degradation of pro-
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ester formation (60), and inhibition of the degradation of pro-

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