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# Proteasome activity or expression is not altered by activation of the heat shock transcription factor Hsf1 in cultured fibroblasts or myoblasts

**David M. Taylor, Edor Kabashi, Jeffrey N. Agar, Sandra Minotti, and Heather D. Durham**

Montreal Neurological Institute, McGill University, Montreal, QC, H3A 2B4, Canada

**Abstract** Heat shock proteins (Hsps) with chaperoning function work together with the ubiquitin-proteasome pathway to prevent the accumulation of misfolded, potentially toxic proteins, as well as to control catabolism of the bulk of cytoplasmic, cellular protein. There is evidence for the involvement of both systems in neurodegenerative disease, and a therapeutic target is the heat shock transcription factor, Hsf1, which mediates upregulation of Hsps in response to cellular stress. The mechanisms regulating expression of proteasomal proteins in mammalian cells are less well defined. To assess any direct effect of Hsf1 on expression of proteasomal subunits and activity in mammalian cells, a plasmid encoding a constitutively active form of Hsf1 (Hsf1$^{act}$) was expressed in mouse embryonic fibroblasts lacking Hsf1 and in cultured human myoblasts. Plasmid encoding an inactivatible form of Hsf1 (Hsf1$^{inact}$) served as control. In cultures transfected with plasmid *hsf1$^{act}$*, robust expression of the major stress-inducible Hsp, Hsp70, occurred but not in cultures transfected with *hsf1$^{inact}$*. No significant changes in the level of expression of representative proteasomal proteins (structural [20Sα], a nonpeptidase beta subunit [20Sβ3], or 2 regulatory subunits [19S subunit 6b, 11Sα]) or in chymotrypsin-, trypsin-, and caspaselike activities of the proteasome were measured. Thus, stress-induced or pharmacological activation of Hsf1 in mammalian cells would upregulate Hsps but not directly affect expression or activity of proteasomes.

## INTRODUCTION

The cellular response to stress (heat shock response) is mediated by the production of highly conserved families of proteins called heat shock proteins (Hsps) (reviewed in Morimoto and Santoro 1998; Verbeke et al 2001). These molecular chaperones work in tandem with the ubiquitin-proteasome pathway for protein quality control to prevent the accumulation of damaged proteins (reviewed in Glickman and Ciechanover 2002). Of the 3 mammalian heat shock transcription factors (Hsfs) identified, Hsf1 is the member that controls stress-mediated *hsp* gene expression (reviewed in Voellmy 2004). Hsps protect cells from irreversibly misfolded proteins by targeting them to the proteasome for degradation.

The ubiquitin-proteasome system is the primary mechanism for disposal of stress-denatured proteins (Grune et al 1995; Sitte et al 1998; Grune 2000) as well as being responsible for the majority of normal protein turnover (Rock et al 1994). Proteasomes consist of a 20S catalytic core and 2 regulatory subunits capping the core ends that recognize, deubiquitinate, and unfold substrates (reviewed in Kloetzel 2001; Glickman and Ciechanover 2002). The 20S core is a 28-subunit (14 gene product) cylinder consisting of 2 outer heptameric rings of structural α-subunits (α1–7) and 2 inner heptameric rings of β-subunits (β1–7), 3 of which (β1, β2, and β5) harbor the 6 active sites. The 19S regulatory subunit comprises 2 substructures. The base, which attaches to the α-ring of 20S, is composed of 6 adenosine triphosphatase (ATPase) and 2 non-ATPase subunits. The lid, which contains up to 10 non-ATPase subunits, is responsible for substrate binding and has poorly understood regulatory functions. In addition to these constitutive proteasomes, another form, termed the immunoproteasome, is induced and functions

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Correspondence to: Heather D. Durham, Tel: 514 398-8509; Fax: 514 398-1509; E-mail: heather.durham@mcgill.ca.

as part of the immune response through creation of peptide ligands for major histocompatibility complex–class I presentation (reviewed in Rechsteiner and Hill 2005). In this response, cytokines, such as interferon-γ, upregulate 3 alternate, catalytically active β-subunits ($\beta1_i$ or LMP2, $\beta2_i$ or MECL-1, $\beta5_i$ or LMP7) and an alternate regulatory complex (PA28 or llS). Various intermediate or hybrid subtypes of proteasome have been isolated, with difference in subunit composition correlating with different hydrolytic activities (Tanahashi et al 2000; Kopp et al 2001; Shringarpure et al 2001; Cascio et al 2002).

In comparison with regulation of immunoproteasome subunits, the molecular mechanisms controlling constitutive and stress-induced regulation of proteasome gene expression in mammalian cells are less well understood. In yeast, coordinated regulation of genes encoding components of the proteasomal and ubiquitin systems is achieved through binding of the transcription factor Rpn4 to a common proteasome-associated control element (PACE) (Mannhaupt et al 1999; Xie and Varshavsky 2001). The Rpn4 regulates proteasome gene expression through a negative-feedback loop. The transcription factor is itself a proteasomal substrate, accumulating when proteasome levels are insufficient and being quickly degraded when activity is restored (Xie and Varshavsky 2001; Fleming et al 2002). No homologue of Rpn4 or its DNA-binding element has been identified in the mammalian genome. In mammalian cells, treatment with proteasome inhibitors caused upregulation of proteasome subunits in rat vascular smooth muscle cells (Meiners et al 2003) and proteasome activity in cultured neocortical neurons (Lee et al 2004), although gene expression analysis did not detect upregulation of proteasomal subunits in clonal lines of SH-SY5Y subjected to low-level, chronic proteasome inhibition (Ding et al 2004). Indirect antioxidants activate transcription of cytoprotective genes through the antioxidant response element (ARE), an action dependent on the Keap1-Nrf2 signaling pathway (reviewed in Motohashi and Yamamoto 2004; Nguyen et al 2004). Transcription of numerous genes encoding 20S, 19S, and 11S proteasome subunits, as well as some chaperones, is responsive to indirect antioxidants through this pathway (Kwak et al 2003a, 2003b). Similar to Rpn4 in yeast, Nrf2 is catabolized by the proteasome, providing an important mechanism of regulating gene expression in response to stress (Nguyen et al 2003). Peroxisome proliferators also increase the expression of proteasome genes but independent of Nrf2 (Anderson et al 2004) and Hsf1 (O'Brien et al 2002), indicating that proteasome levels can be altered through multiple pathways.

The Rpn4, ubiquitin, an E3 ligase, and a ubiquitin-conjugating enzyme were identified in a genome-wide screen for Hsf1-regulated genes in yeast (Hahn et al 2004), raising the possibility that Hsf1 could affect expression of components of the ubiquitin-proteasome system in stressed cells, in addition to the well-characterized upregulation of *hsp* gene expression (Kawazoe et al 1998; Meriin et al 1998; Kim et al 1999; Pirkkala et al 2000). This raises the question whether similar mechanisms of coregulation occur in mammalian cells.

Exposure to environmental stresses that are known to activate Hsf1 have had variable effects on expression of proteasome subunits in mammalian cells, depending on the experimental conditions and cell type being investigated. Oxidative stress had no effect on proteasome expression in liver epithelial cells (Grune et al 1995) but did increase expression of proteasome subunits in neuroblastoma cells (Ding et al 2003). Repeated, mild heat shock resulted in significant upregulation of proteasomal catalytic activities and 11S subunit levels in human fibroblasts (Beedholm et al 2004), but acute, mild heat shock led to decreased proteasome messenger RNA (mRNA) levels, inhibition of proteasome complex assembly, decrease in chymotrypsinlike activity, and reorganization of the intracellular distribution of proteasomes (Kuckelkorn et al 2000). The mRNAs for proteasomal components also were reduced in HeLa cells and human lung fibroblasts subjected to multiple stresses (Murray et al 2004).

Our laboratory has been studying the role of the heat shock response and ubiquitin-proteasome system in the neurodegenerative disease, amyotrophic lateral sclerosis (ALS). In a transgenic mouse model of familial ALS caused by dominantly inherited mutations in the gene encoding Cu/Zn-superoxide dismutase, proteasome levels were substantially decreased in motor neurons of lumbar spinal cord, the tissue most affected by the disease (Kabashi et al 2004; Cheroni et al 2005), and proteasome activity of this tissue as a whole was reduced (Kabashi et al 2004). These findings suggest an inability of vulnerable cells and tissues to upregulate proteasomes to meet the increased demand imposed by the disease process. Motor neurons also have a high threshold for activation of Hsf1 (Batulan et al 2003). If Hsf1 has a role in stress-induced regulation of ubiquitin-proteasome genes, cells that fail to activate Hsf1 could be disadvantaged in both protein chaperoning and protein degradation capabilities.

The protein chaperone and ubiquitin-proteasome pathways are implicated in several neurodegenerative diseases (reviewed in Sherman and Goldberg 2001; Ciechanover and Brundin 2003; Petrucelli and Dawson 2004; Ross and Poirier 2004), including ALS (Bruening et al 1999; Kabashi et al 2004), and upregulating the heat shock response has therapeutic potential (reviewed in Morimoto and Santoro 1998; Muchowski and Wacker 2005). It is important to know whether therapies targeting Hsf1 directly increase the cellular capacity for protein degradation or just reduce the substrate load through upregulation of protein chaperones. On the other hand, inappropriate increase in

proteasome activity could have negative consequences. A clinical trial of recombinant human ciliary neurotrophic factor (CNTF) in ALS patients was halted because of dramatic loss of lean body mass (ALS CNTF Treatment Study Group 1996). CNTF was recently reported to increase 20S proteasome mRNA and proteolytic activity (Jho et al 2004).

In this study, the role of Hsf1 in the expression of proteasomal subunits and activity was assessed directly by overexpressing a constitutively active form of Hsf1 in mouse embryonic fibroblasts (MEF) lacking Hsf1 and in cultured human myoblasts. No increase in the level of expression of proteasomal proteins or proteasomal activity was attributed to activation of Hsf1, and intracellular distribution was unaltered.

## MATERIALS AND METHODS

### Cell cultures

The Hsf1$^{-/-}$ MEFs were a kind gift from Dr I.J. Benjamin, University of Utah School of Medicine (McMillan et al 1998). Cells were maintained in medium consisting of Dulbecco modified Eagle medium (DMEM) (GIBCO, Burlington, Ontario, Canada) buffered with 3.7 grams per liter $NaHCO_3$ (BDH Inc, Toronto, Ontario, Canada), 10% fetal bovine serum, 1× antibiotic-antimycotic solution, 0.1 mM Minimum Essential Medium (MEM) nonessential amino acids solution, 1 mM sodium pyruvate, and 0.001% 2-mercaptoethanol. Fetal bovine serum was purchased from Hyclone (Logan, UT, USA); the latter 4 ingredients were purchased from GIBCO. The cultures were maintained at 37°C in 5% $CO_2$.

Primary human myoblast cultures were established from repository stock of cells cultured from healthy muscle biopsy, generously provided by Tim Johns in the lab of Dr Eric Shoubridge (McGill University, Montreal, QC, Canada). Cells were maintained in medium consisting of Clonetics® Skeletal Muscle Cell Basal Medium (SkBM®) (Cambrex, Walkersville, MD, USA) supplemented with bovine serum albumin, bovine fetuin, insulin, dexamethasone, recombinant human epidermal growth factor, and gentamycin sulfate with amphotericin B (all supplied with SkBM® as SingleQuots® according to manufacturer's instructions), plus 15% fetal bovine serum.

### Transfection of Hsf1 constructs

For Western blot analysis, Hsf1$^{-/-}$ cells were cultured in Multiwell™ 6-well dishes (Beckton Dickinson and Company, Franklin Lakes, NJ, USA). Transfections were performed at 90–100% confluency to ensure adequate protein levels for blotting. Transfection efficiencies were 50–80% between experiments but consistent within each experiment. Plasmid vectors encoding a constitutively activated form of human Hsf1 (Hsf1$^{act}$) and an inactivatible HSF1 (Hsf1$^{inact}$) were generously provided by Dr R. Voellmy (University of Miami, Miami, FL, USA). Transfections (including mock transfections as procedural control) were carried out using Plus™ reagent (Invitrogen, Carlsbad, CA, USA) and Lipofectamine 2000™ (Invitrogen). Cells for experimentation were harvested 48 hours after the start of the transfection procedure. For immunocytochemistry, cells were grown on glass coverslips (Fisher Scientific Inc, Nepean, Ontario, Canada) in Multiwell™ 12-well dishes (Beckton Dickinson and Company).

### Western blotting

After transfection, cultures were rinsed twice with prewarmed, serum-free DMEM and once with phosphate-buffered saline (PBS) at room temperature, then harvested in 100 μL of sample buffer containing 20 mM Tris (EM Science, Gibbstown, NJ, USA), 2 μM ethylenediaminetetraacetic acid (Fisher Scientific Company, Fair Lawn, NJ, USA), and 0.5% Triton X-100 (Pharmacia Biotech AB, Uppsala, Sweden), and then maintained on ice. Cells were manually homogenized and clarified by centrifugation at 13 500 × *g* at 0°C for 10 minutes. Homogenization and centrifugation steps were repeated, followed by sonication for 15 seconds with 2-second pulses at 50% duty, followed by a final centrifugation. Supernatant was removed, and protein concentration was determined using the DC Protein Assay (Bio-Rad, Mississauga, Ontario, Canada). A total of 20 or 40 μg of protein were loaded for sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose for Western blotting. All blots were developed using Western lightning chemiluminescence reagents (PerkinElmer, Boston, MA, USA). Blots were reprobed with a mouse monoclonal antibody to α-actin (C4; ICN Biomedicals, Cleveland, OH, USA) as a loading control. Three cultures per condition (*hsf*1$^{act}$-, *hsf*1$^{inact}$-, mock-, or nontransfected) were assessed on each blot, and each culture was examined on duplicate blots. Pixel density of each band was quantified using Scion Image Beta 4.0.2. and was expressed relative to actin, then expressed as mean ± SD for each of 3 cultures per condition. To compare conditions, the mean actin-normalized value for each subunit in *hsf*1$^{act}$-transfected cultures was expressed relative to the mean calculated for *hsf*1$^{inact}$-transfected cultures, and both *hsf*1$^{act}$- and *hsf*1$^{inact}$-transfected cells were compared separately to mock- and nontransfected cells. The SD of the ratio was calculated as a measure of variability (Taylor 1996). A 2-tailed distribution, 2-sample equal variance *t*-test was used to assess significant difference between the actin-normalized ratios of Hsf1$^{act}$-expressing cultures and each

of the other 3 conditions, as described previously (Kabashi et al 2004).

### Immunocytochemistry

Cells were fixed for 10 minutes in 3% paraformaldehyde (EM Science)/PBS, then permeabilized in 0.5% Nonidet P40 (EM Science)/PBS for 1 minute, followed by an additional 2 minutes in paraformaldehyde and blocking in 3% bovine serum albumin (BSA) (EM Science)/PBS for 30 minutes. Primary antibodies, diluted in BSA/PBS, were applied for 30 minutes followed by 3 rinses in PBS, then exposed to an Alexa-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA), diluted 1:500, for 30 minutes, and finally rinsed in PBS.

### Proteasome activity assay

Transfected cells were harvested in an identical manner as for Western blotting except that lysis buffer was detergent free (detergent activates 20S proteasomes) (Arribas and Castano 1990). Using 96-well opaque plates, protein samples were mixed with amino methyl coumarin (AMC)-conjugated peptide substrates of each of the 3 catalytically active β proteasomal subunits (Peptides International Inc, Louisville, KY, USA). The Suc-LLVY-AMC is cleaved by the chymotrypsinlike activity of 20S β5, Z-LLE-AMC is cleaved by the peptidyl-glutamyl peptide hydrolyzing (PGPH)- or caspaselike activity of 20S β1, and Boc-LRR- is cleaved by the trypsinlike activity of 20S β2. Assays were carried out as described previously (Arribas and Castano 1990; Rock et al 1994; Kabashi et al 2004). In brief, a 50 μL volume of 2× proteasome buffer containing 4 mM adenosine triphosphate, 10 mM $MgCl_2$, and 8 mM dithiothreitol was applied to each well with 50 μL of protein sample, containing 5 μg of total protein. Each sample/substrate combination was assayed in quadruplicate, both in the presence and in the absence of 20 μM of the proteasome inhibitor MG132 (Peptides International Inc.). Substrates at 1 mM were applied to each well in volumes of 2.5 μL, and plates were read immediately on a FX600 multiplate reading fluorimeter (Bio-Tek Instruments Inc, Winooski, VT, USA). Fluorescence was measured at 30-minute intervals for 4 hours. For quantitative evaluation, free AMC was added to protein-free wells at the same concentration as peptide substrates. Values measured as fluorescence units per minute per milligram were divided by the fluorescence units emitted by free AMC at 240 minutes to express activity in ηmol AMC per minute per milligram protein. Proteasome activity results were expressed as means ± SEM, and *t*-tests were carried out as described for Western blot data.

### Antibodies

Primary antibodies used for Western blotting and immunocytochemistry were as follows: rat anti-Hsf1, SPA-950 (clone 10H8, 1:500 for blots, 1:100 for immunocytochemistry, StressGen Biotechnologies, Victoria, British Columbia, Canada), mouse anti-Hsp70, SPA-810 (clone C92F3A-5, 1:10 000 for blots, 1:100 for immunocytochemistry, StressGen Biotechnologies), mouse anti-20S proteasome alpha subunits α1, 2, 3, 5, 6, and 7, PW 8195 (clone MCP231, 1:2500 for blots, 1:50 for immunocytochemistry, Affiniti Research Products, Exeter, Devon, UK), mouse anti-20S proteasome beta subunit 3, PW 8130 (1:2500 for blots, Affiniti Research Products), rabbit anti-19S regulator ATPase subunit 6b (Tbp7), PW 8175 (1:1000 for blots, 1:200 for immunocytochemistry Affiniti Research Products), rabbit anti-11S regulator subunit α (PA28α), PW 8185 (1:5000 for blots, 1:500 for immunocytochemistry, Affiniti Research Products).

## RESULTS

### $Hsf1^{act}$ not $Hsf1^{inact}$ induces expression of Hsp70

Hsf1 was detected in transfected $Hsf1^{-/-}$ cells by Western blotting with a rat monoclonal antibody (10H8) (Fig 1A). Using identical transfection conditions, including DNA concentrations, $Hsf1^{inact}$ was consistently expressed at higher levels than $Hsf1^{act}$, although transfection efficiencies were similar by immunocytochemistry. This would indicate that higher levels of $Hsf1^{inact}$ than $Hsf1^{act}$ were expressed per cell (or unexplained differences in immunoreactivity). Expression of $Hsf1^{inact}$ was tested at earlier times after transfection (8, 12, 24 hours) and using different relative DNA concentrations (data not shown). This figure represents the conditions that led to the smallest difference from $Hsf1^{act}$.

The $Hsf1^{act}$ localized to both the nucleus and the cytoplasm in $Hsf1^{-/-}$ cells, with a slight cytoplasmic predominance. $Hsf1^{inact}$ was primarily nuclear, in accordance with its constitutive binding to *hsp* promoters (Zuo et al 1995), whereas a small percentage of cells exhibited both nuclear and cytoplasmic labeling. Cells that expressed $Hsf1^{act}$ also expressed high levels of inducible Hsp70, whereas no Hsp70 was detected in cells expressing $Hsf1^{inact}$ by Western blotting (Fig 1A) or by immunocytochemistry (Fig 1B).

### Activation of Hsf1 does not upregulate proteasome subunits or alter subunit localization

The levels of 20S, 20Sβ3, a 19S regulator ATPase (S6b), and an 11S regulator subunit (11Sα) were assessed by Western blotting. In comparison with $Hsf1^{inact}$-expressing cells, no significant increase in any of the subunits was detected

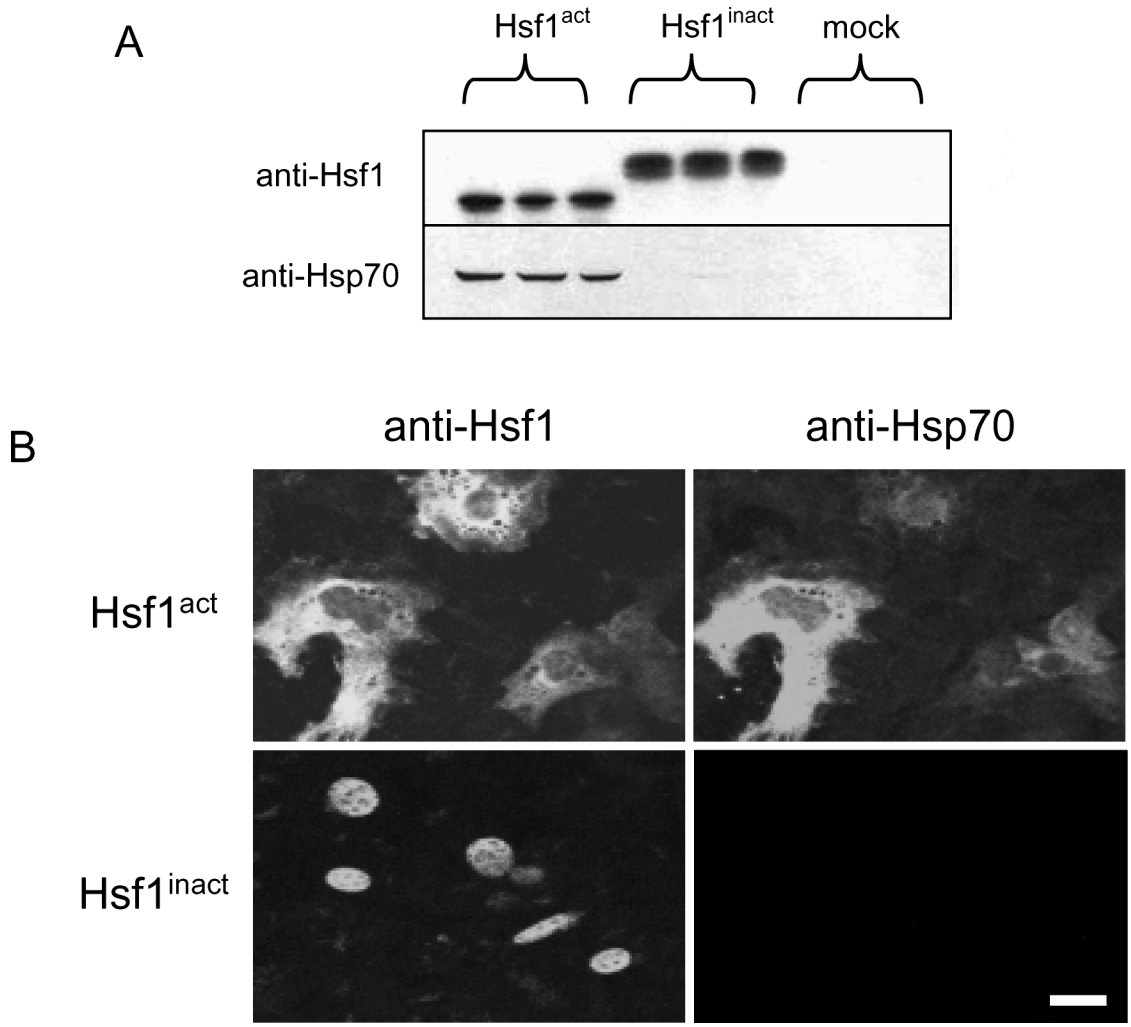


**Fig 1.** Expression of Hsf1$^{act}$, not Hsf1$^{inact}$, induces expression of Hsp70 in the absence of additional stress. The Hsf1$^{-/-}$ MEFs were transfected with *hsf1*$^{act}$ or *hsf1*$^{inact}$ constructs or equivalent volume of vehicle (mock). Expression of Hsf1 and Hsp70 was assessed using rat anti-Hsf1 (10H8), and mouse anti-Hsp70 (C92F3A-5). Only cells expressing Hsf1$^{act}$ expressed Hsp70 by (A) Western blotting or by (B) immunocytochemistry. Scale bar: 20 μm. Hsf1, heat shock transcription factor; MEF, mouse embryonic fibroblasts; Hsp, heat shock protein.

with expression of Hsf1$^{act}$ (Fig 2). The 20Sα, 20Sβ, 19S, and 11S subunits were increased in cells expressing either form of Hsf1 when compared with nontransfected cells, and this was especially pronounced for the 11S regulatory subunit. Although similar results were obtained in comparison with mock-transfected cells, this was not the case for 20Sα, indicating that levels of some proteasomal proteins may increase slightly with transfection.

The effect of Hsf1$^{act}$ on localization of proteasome subunits was assessed by immunocytochemistry. Expression was qualitatively similar regardless of transfection or expression of either Hsf1$^{act}$ or Hsf1$^{inact}$ (Fig 3), whereas the cellular distribution of all 4 proteasome subunits varied from cell to cell within a single culture. The most common distribution is illustrated in Figure 3. The 20Sα subunits and the 19S regulatory particle had nearly identical localization in both the cytoplasm and the nucleus. The 11S labeling was mostly cytoplasmic; however, cells with equal or greater levels in the nucleus were also observed. The state of confluency and position in the cell cycle ($G_0$ vs actively dividing) likely contribute to this variability (Reits et al 1997).

### Activation of Hsf1 does not increase proteasome activity

Expression of Hsf1$^{act}$ and Hsf1$^{inact}$ was confirmed by Western blotting using 20 μg of total protein from extracts used in the activity assay. As described above, the expression level of Hsf1$^{inact}$ was consistently higher than HSF1$^{act}$ despite a similar transfection efficiency of these 2 constructs (Fig 4A). No significant increase in chymotrypsin-, caspase-, or trypsinlike specific proteasome activity was detected in cultures expressing Hsf1$^{act}$ relative to those expressing Hsf1$^{inact}$ (Fig 4B).

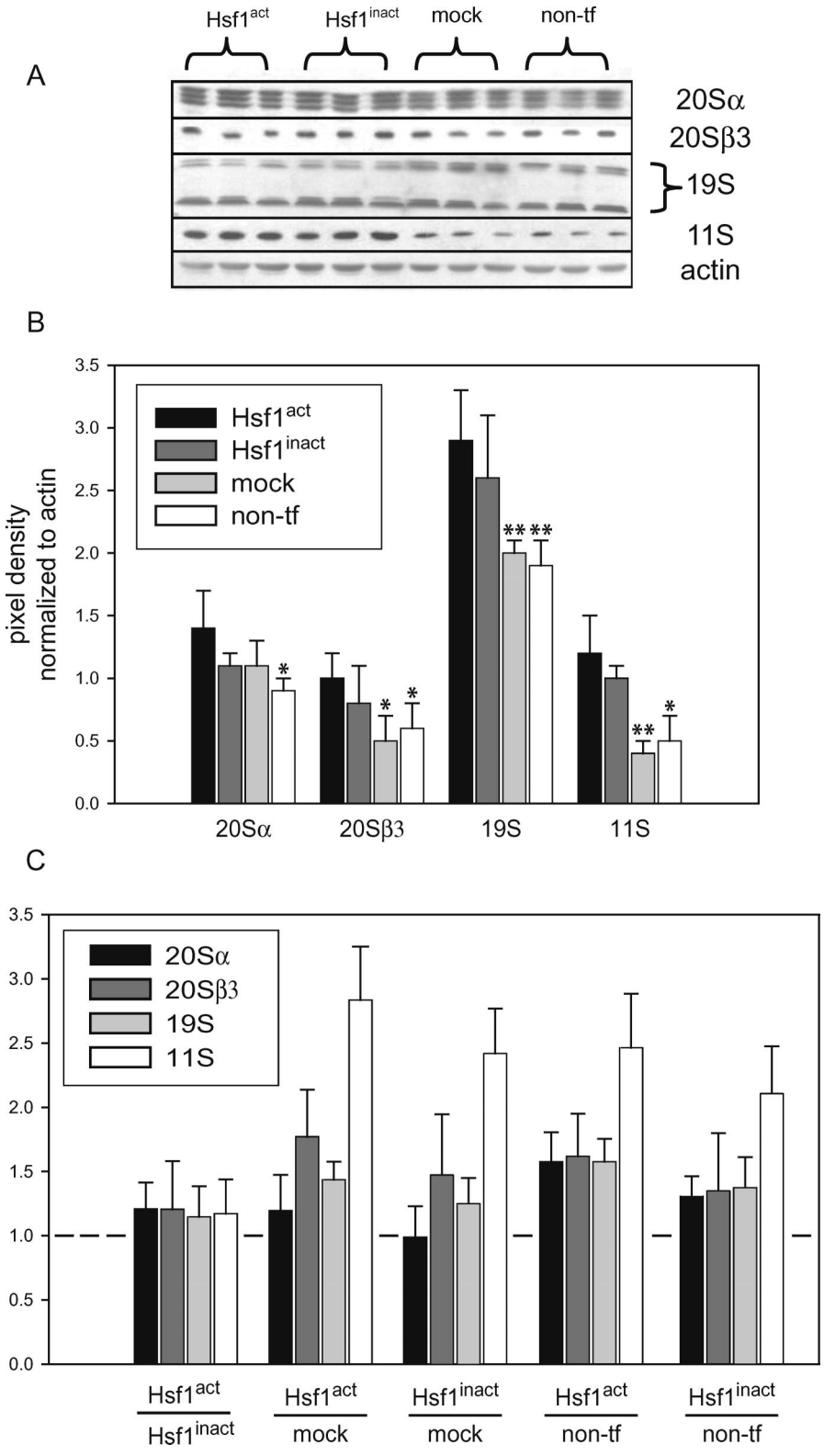


**Fig 2.** Activation of Hsf1 does not alter expression of proteasome subunits. (A) Levels of 20Sα, 20Sβ3, a 19S subunit, and an 11S subunit were examined on Western blots of nontransfected, mock-transfected, *hsf*1$^{act}$- and *hsf*1$^{inact}$-transfected Hsf1$^{-/-}$ MEFs. (B) Blots were reprobed with actin as a loading control. Pixel densities of bands relative to actin were expressed as mean ± SD Significant differences between Hsf1$^{act}$ and other conditions are denoted as $*P < 0.05$, $**P < 0.01$. (C) To compare expression of individual subunits between various experimental conditions, ratios of means shown in (A) were calculated. Error bars represent the SD of the ratio. Although the transfection procedure alone increased the level of the 11S subunit, there was no significant difference in expression of any proteasomal subunit tested in cultures expressing Hsf1$^{act}$ relative to those expressing Hsf1$^{inact}$. Hsf1, heat shock transcription factor; MEF, mouse embryonic fibroblasts.

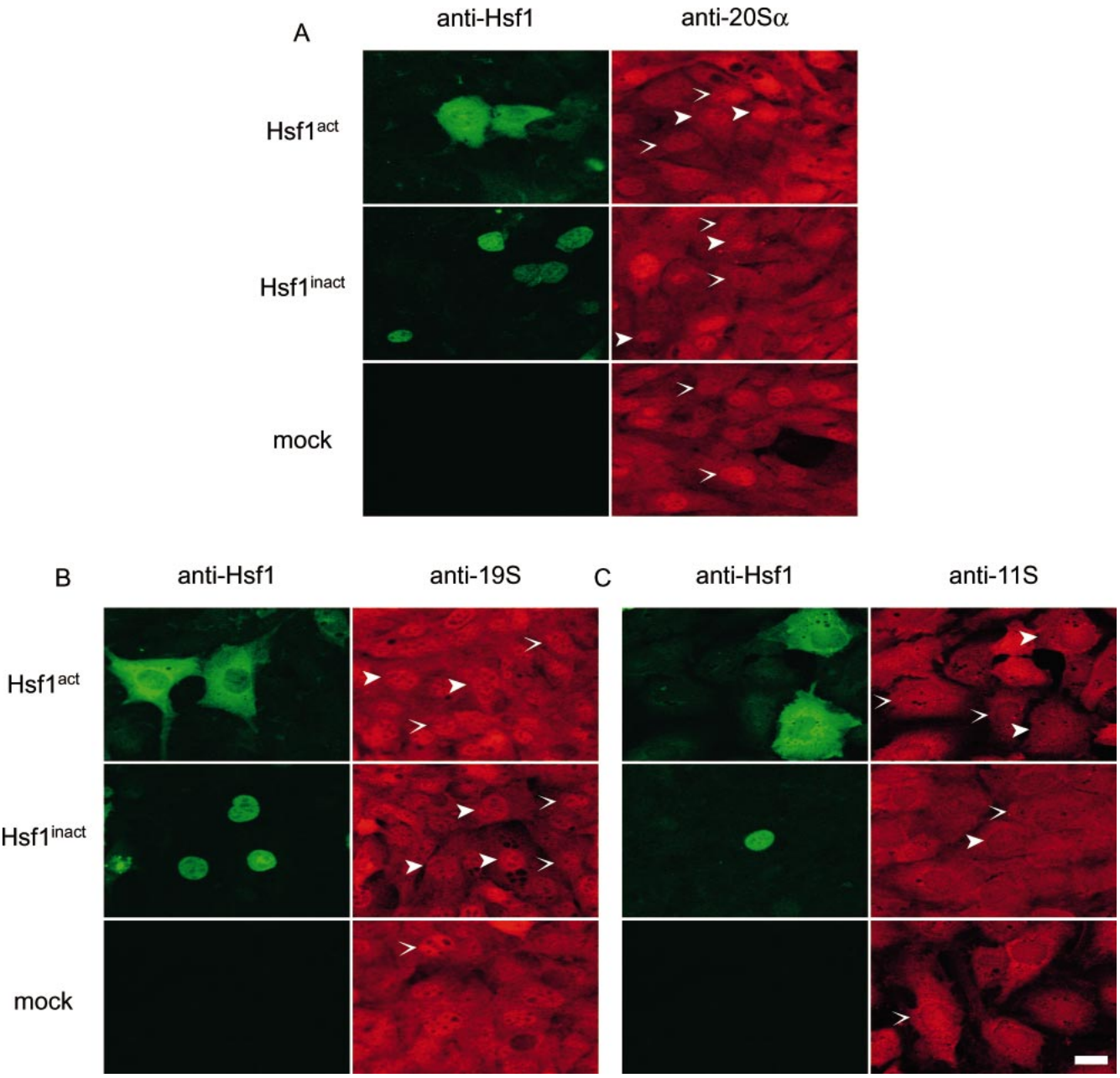


**Fig 3.** Activation of Hsf1 does not alter cellular distribution of proteasomal subunits. Cellular localization of (A) 20Sα, (B) a 19S subunit, and (C) an 11S subunit was assessed by immunocytochemistry. Qualitatively similar labeling was observed for all subunits in *hsf1*$^{act}$-transfected Hsf1$^{-/-}$ MEFs relative to *hsf1*$^{inact}$- or mock-transfected cells, as well as nontransfected cells (not shown). Both (A) 20Sα and (B) 19S subunits display cytoplasmic and nuclear localization with a slight nuclear predominance. (C) 11S labeling is largely cytoplasmic. Solid arrowheads point to cells expressing the corresponding form of Hsf1. Open arrowheads point to cells that lack Hsf1 but have similar labeling as the transfected cells indicated by solid arrowheads. Scale bar: 20 μm. Hsf1, heat shock transcription factor; MEF, mouse embryonic fibroblasts.

### Activation of Hsf1 does not alter expression of proteasome subunits in cultured myoblasts

Similar results were obtained in human primary myoblasts as in MEFs, ie, no change in expression of 20Sα, 20Sβ3, or 11S occurred in myoblast cultures expressing Hsf1$^{act}$ as compared with those expressing Hsf1$^{inact}$ (Fig 5). A small but significantly lower expression of 19S was measured (Fig 5B), but no changes were observed in the other subunits of the proteasome, despite a high level of HSF1 activation.

## DISCUSSION

When proteasome function is compromised, cells coordinately upregulate Hsps with protein chaperoning func-

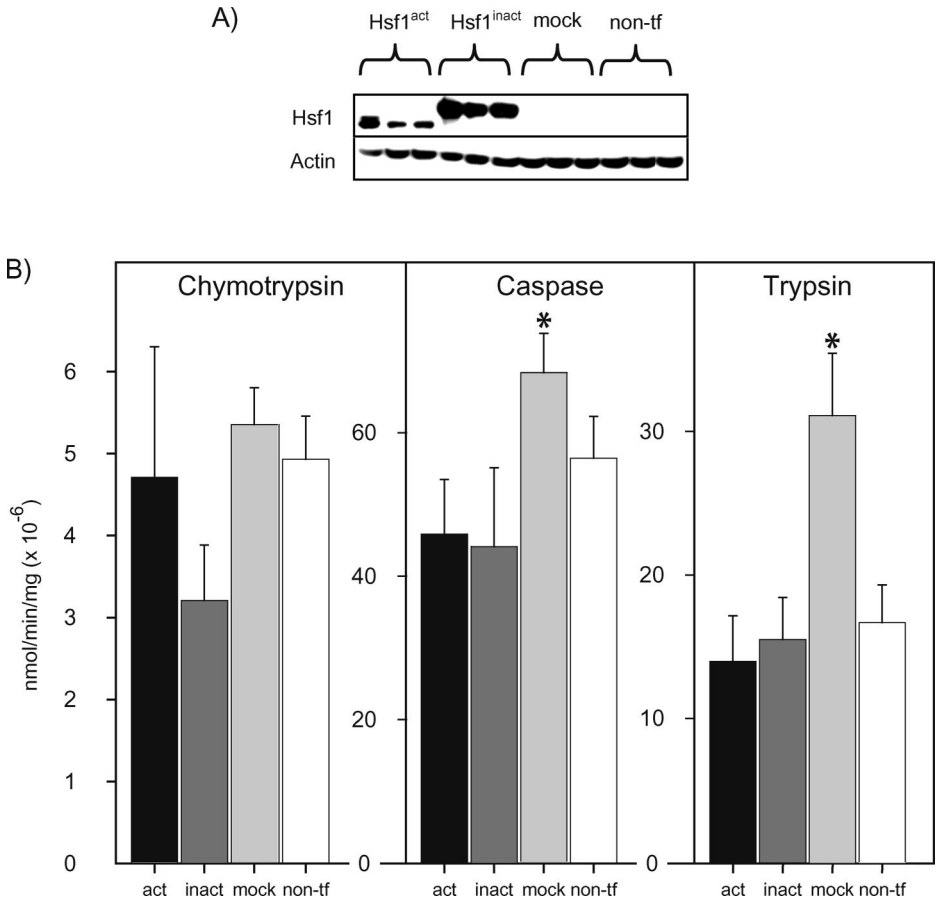


**Fig 4.** Activation of Hsf1 does not alter specific activities of proteasomes. (A) Before conducting activity assays, levels of Hsf1 in transfected, mock-transfected, and nontransfected cells were examined by Western blotting. (B) Using fluorogenic peptide substrates, no significant difference in chymotrypsin-, caspase-, or trypsinlike activities was observed in cells expressing Hsf1$^{act}$ compared with those expressing Hsf1$^{inact}$. Data were expressed as ηmol AMC generated per minute per milligram of protein. Shown are means ± SEM for 3 cultures per condition. * indicates significant difference between *hsf1*$^{act}$- and mock-transfected cells, $P < 0.05$. Hsf1, heat shock transcription factor; AMC, amino methyl coumarin.

tion and components of the ubiquitin-proteasome system to increase proteolytic capacity to meet demand. In yeast, the major transcription factor–regulating genes of the ubiquitin-proteasome system is Rpn4 (Mannhaupt et al 1999; Xie and Varshavsky 2001), but no mammalian homologue of Rpn4 or its DNA-binding element (PACE) has been identified (Meiners et al 2003). Recently, indirect antioxidants and peroxisome proliferators have been shown to increase expression of proteasome subunit genes and proteins through Keap1-Nrf2-ARE (Kwak et al 2003a, 2003b) and peroxisome proliferator–activated receptor (PPAR) pathways (O'Brien et al 2002), respectively; these findings demonstrate control of proteasome levels and activity through multiple pathways in mammalian cells. Hsf1 is the major transcription factor regulating stress-induced expression of Hsps in eukaryotic cells (reviewed in Voellmy 2004). Hsf1 is held in an inactive, monomeric form, complexed in the cytoplasm with other proteins including Hsp90 and Hsp70. When the level of misfolded protein accumulates in the cell because of protein damage, overload of proteasomal degradative capacity, or treatment with proteasome inhibitors, these substrates compete for Hsps, releasing Hsf1, allowing it to trimerize, translocate to the nucleus, bind to DNA, and activate gene transcription (reviewed in Morimoto 1998; Voellmy 2004). In yeast, some members of the ubiquitin-proteasome gene families and their transcription factor Rpn4, are also subject to direct regulation by Hsf1 (Hahn et al 2004). A complete list of mammalian genes that are regulated by direct activation of Hsf1 has yet to be established, but Hsf1 can mediate the transcriptional activity of numerous genes and gene families other than *hsp* (Dewji and Do 1996; Murray et al 2004), many of which lack conventional heat shock elements (Wang and Morgan 1994; Trinklein et al 2003). Activators of the Nrf2 and PPAR pathways increase levels of transcripts for both protein chaperones and proteasomal subunits, indicating that at least some coordinate control of the 2 systems is important for protein quality control in mammalian cells as well as in yeast. The ability of Hsf1 to upregulate proteasome subunits in mammalian cells had not been addressed.

To directly assess the effect of Hsf1 activation on levels of mammalian proteasomal proteins and activity, we expressed a constitutively active form of Hsf1 (Hsf1$^{act}$) that does not require additional stress to initiate transcription of responsive genes (Zuo et al 1995). Transfection of *hsf*1$^{act}$ into Hsf1$^{-/-}$ MEFs or primary human myoblasts failed to induce expression of structural subunits (20Sα), a non-

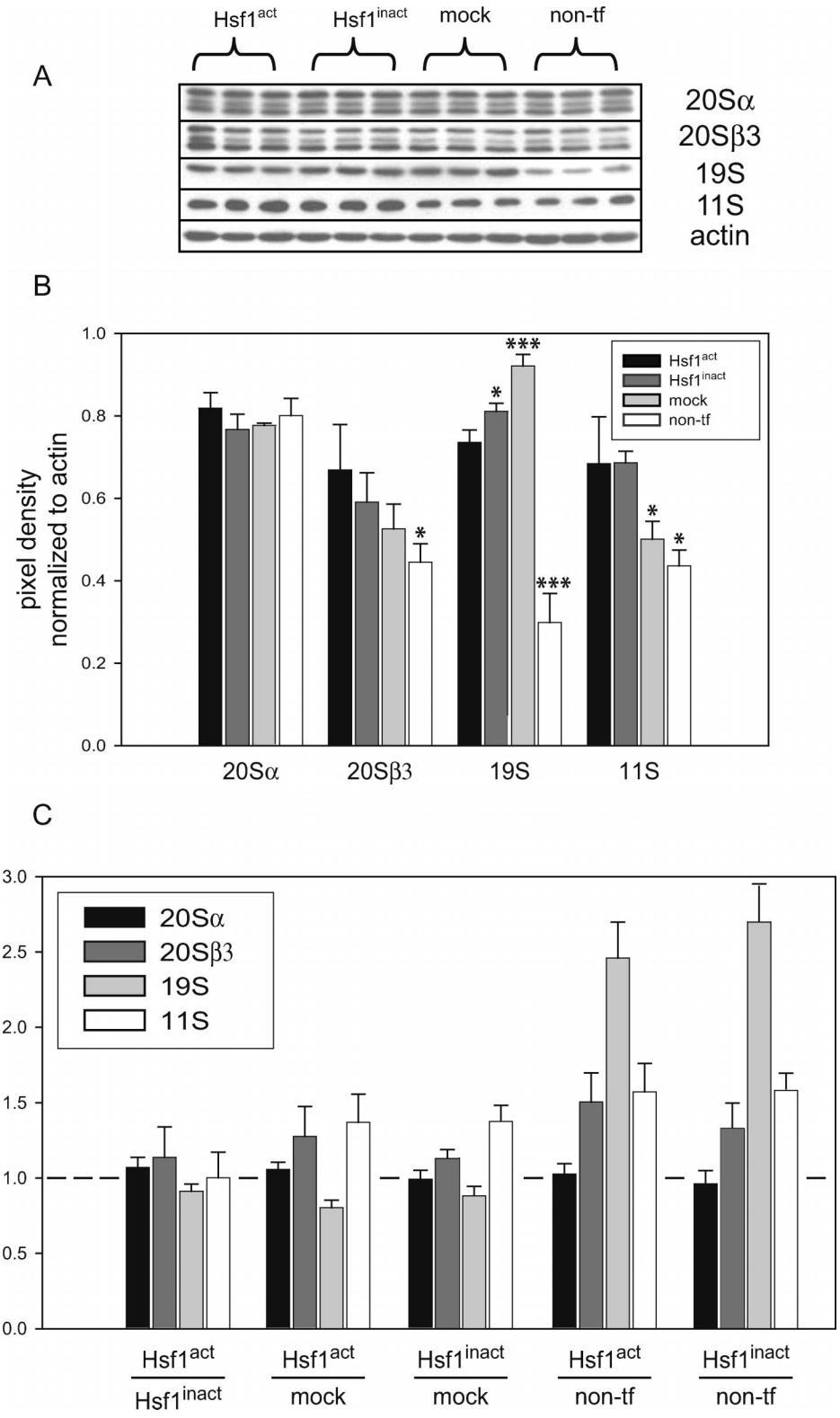


**Fig 5.** Activation of Hsf1 does not alter expression of proteasomal subunits in cultured human myoblasts. (A) Levels of 20Sα, 20Sβ3, a 19S subunit, and an 11S subunit were examined on Western blots of nontransfected, mock-transfected, *hsf1*$^{act}$-, and *hsf1*$^{inact}$-transfected myoblasts. (B) Blots were reprobed with actin as a loading control. Pixel densities of bands relative to actin were expressed as mean ± SD. Significant differences between Hsf1$^{act}$ and other conditions are denoted as $^{*}P < 0.05$, $^{***}P < 0.005$. (C) To compare expression of individual subunits between various experimental conditions, ratios of means shown in (A) were calculated. Error bars represent the SD of the ratio. Hsf1, heat shock transcription factor.

peptidase beta subunit (20Sβ3), 2 regulatory subunits (19S subunit 6b, 11Sα) or to alter proteasome activities compared with control cells expressing the transcriptionally incompetent form, Hsf1$^{inact}$. This lack of effect on proteasome function occurred despite a robust increase in Hsp70, demonstrating Hsf1-mediated transcription of heat shock genes. The inability of HSF1 to alter expression of proteasomal subunits was not likely due to an inability of these cells to induce transcription of proteasomal genes because others have demonstrated their responsiveness to other stimuli: MEFs increase the expression of proteasomal subunits after exposure to indirect antioxidants (Kwak et al 2003a), and induction of proteasomes in muscle cells has been documented in cachexia (see below). Our studies do not rule out the possibility that an initial increase in proteasomal protein expression in *hsf*1$^{act}$-transfected cells is extinguished by activation of repressors or that mRNA levels are increased without increased translation (Meiners et al 2003). In any case, those scenarios would not be significant in the context of long-term therapy to upregulate heat shock genes.

Beedholm et al (2004) reported that enhanced chymotrypsin-, trypsin-, and caspaselike proteasomal activities after repeated, mild heat shock correlated with upregulation of 11S α subunit, which can increase proteasome activity (Whitby et al 2000). The increased 11S subunit expression observed in this study was not specific to cells expressing Hsf1$^{act}$, indicating that it was not a direct effect of Hsf1-mediated gene transcription. We saw no corresponding increase in chymotrypsin-, trypsin-, or caspaselike proteasomal activities. Kuckelkorn et al (2000) reported a redistribution of proteasome complexes away from perinuclear regions after 60 minutes of recovery from mild heat shock as well as a considerable reduction in chymotrypsinlike activity and 20Sα2 mRNA levels. In our study, although minor differences were apparent between nontransfected cells and those expressing Hsf1$^{act}$, they also occurred in cells expressing Hsf1$^{inact}$. Our experimental paradigm is also quite different in not introducing stress subsequent to the transfection procedure.

Yeasts activate a very large transcriptional response to environmental stress, either increasing or decreasing transcription of over 10% of their genome (Gasch et al 2000; Causton et al 2001; Chen et al 2003), with nearly 3% of genes being direct transcriptional targets of Hsf1, including several genes involved in proteolysis (Hahn et al 2004). The response of mammalian cells appears to be more restricted and variable with cell type and can even involve modest downregulation of proteasomal components (Murray et al 2004). The results of this study indicate that activation of Hsf1 would not cause stress-induced expression of representative proteasomal subunits or alter their cellular localization or the overall catalytic activity in mammalian cells. Thus, therapeutic agents that activate or enhance activation of Hsf1, such as arimoclomol (Kieran et al 2004) and celastrols (Westerheide et al 2004), could relieve proteasome dysfunction through increased chaperoning activity but would not be expected to directly influence transcription of proteasome subunit genes to augment proteolytic capacity.

In one sense, lack of effect of Hsf1 on proteasome gene transcription could be an advantage. Another clinical feature of motor neuron diseases is muscle atrophy secondary to denervation. Muscle atrophy (cachexia) occurs in several conditions including disuse, aging, starvation, and cancer. The bulk of myofibrillar protein degradation occurs via the ubiquitin-proteasome pathway and a common transcriptional program is likely activated to increase protein catabolism in these various conditions (reviewed in Jagoe and Goldberg 2001). It will be important that therapies designed to improve catabolism of misfolded proteins in nervous tissue do not inappropriately alter proteasome activity in uncompromised tissue.

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