

# High Threshold for Induction of the Stress Response in Motor Neurons Is Associated with Failure to Activate HSF1

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Heat shock protein 70 (Hsp70) protects cultured motor neurons from the toxic effects of mutations in Cu/Zn-superoxide dismutase (*SOD-1*), which is responsible for a familial form of the disease, amyotrophic lateral sclerosis (ALS). Here, the endogenous heat shock response of motor neurons was investigated to determine whether a high threshold for activating this protective mechanism contributes to their vulnerability to stresses associated with ALS. When heat shocked, cultured motor neurons failed to express Hsp70 or transactivate a green fluorescent protein reporter gene driven by the Hsp70 promoter, although Hsp70 was induced in glial cells. No increase in Hsp70 occurred in motor neurons after exposure to excitotoxic glutamate or expression of mutant *SOD-1* with a glycine→alanine substitution at residue 93 (G93A), nor was Hsp70 increased in spinal cords of G93A *SOD-1* transgenic mice or sporadic or familial ALS patients. In contrast, strong Hsp70 induction occurred in motor neurons with expression of a constitutively active form of heat shock transcription factor (HSF)-1 or when proteasome activity was sufficiently inhibited to induce accumulation of an alternative transcription factor HSF2. These results indicate that the high threshold for induction of the stress response in motor neurons stems from an impaired ability to activate the main heat shock–stress sensor, HSF1.

**Key words:** ALS; amyotrophic lateral sclerosis; heat shock; HSF1; HSF2; Hsp70; motor neuron; proteasome inhibition

## Introduction

The heat shock response is an evolutionarily conserved, cytoprotective mechanism mediated by stress-induced transcription of genes encoding heat shock–stress proteins (HSP) (for review, see Morimoto et al., 1997). Families of stress-induced HSPs and constitutively expressed heat shock cognate proteins (HSC) facilitate nascent protein folding, refolding, or degradation of abnormally folded proteins, protein targeting, and cellular signaling (Gabal et al., 1997; Morimoto and Santoro, 1998; Meriin et al., 1999). Eukaryotic expression of HSPs is mediated by binding and activation of heat shock transcription factors (HSFs) to heat shock elements (HSEs) on *HSP* promoters (Morimoto and Santoro, 1998). Environmental and physiological stresses activate transcription of *HSP* genes in mammals primarily through HSF1 (Morimoto, 1998), whereas HSF2 participates in heat shock gene transactivation developmentally (Min et al., 2000) and in the presence of proteasomal inhibitors (Mathew et al., 1998).

HSPs can protect neural cells from various stresses including hyperthermia, ischemia, oxidative stress, and excitotoxicity (Lo-

wenstein et al., 1991; Rordorf et al., 1991; Uney et al., 1993; Yenari et al., 1998). Findings from our laboratory extended the neuroprotective role of the major stress-inducible heat shock protein 70 isoform (Hsp70) to a culture model of amyotrophic lateral sclerosis (ALS), a neurodegenerative disease characterized by loss of motor neurons in the brain and spinal cord. Approximately 20% of familial ALS (FALS) cases are caused by autosomal dominant mutations in Cu/Zn-superoxide dismutase (*SOD-1*) (Rosen et al., 1993).

Toxicity of mutant *SOD-1* is attributable to a gain of function rather than a loss of the primary enzymatic function, dismutation of superoxide to hydrogen peroxide. One proposed mechanism of disease pathogenesis is that mutations in *SOD-1* confer structural changes in the protein, resulting in altered solubility and aggregation (Deng et al., 1993; Bruijn et al., 1997; Durham et al., 1997; Johnston et al., 2000; Shinder et al., 2001). Consistent with the cellular response to accumulation and aggregation of abnormal proteins, cell lines surviving stable overexpression of mutant *SOD-1* exhibit upregulation of the HSPs, Hsp70, Hsp25, and  $\alpha$ B-crystallin and an increase in chaperoning activity. Expression of mutant *SOD-1* in motor neurons of dissociated murine spinal cord cultures resulted in aggregation of this protein into inclusions and loss of viability (Durham et al., 1997). Both manifestations of mutant *SOD-1* toxicity were delayed by gene transfer of Hsp70 (Bruening et al., 1999), suggesting that an insufficient stress response in motor neurons increases their vulnerability to toxicity. The present study investigated the endogenous heat

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shock response in motor neurons, as monitored by induction of Hsp70 and other HSPs after exposure to hyperthermia and two stresses associated with ALS: glutamate excitotoxicity and expression of SOD-1 with glycine→alanine substitution at residue 93 (G93A SOD-1). The data show a high threshold for induction of the stress response in motor neurons attributable to the lack of activation of the major stress-sensing transcription factor HSF1.

## Materials and Methods

**Spinal cord cultures.** Primary cultures of dissociated spinal cord and dorsal root ganglia (DRG) were prepared from embryonic day 13 CD1 mice and cultured on poly-D-lysine-coated glass coverslips as described previously (Roy et al., 1998). Cultures were used 4–8 weeks after plating and large motor neurons were distinguished from other cell types as described previously (Roy et al., 1998).

**Transgenic mice.** The following lines were maintained in our animal facilities: B6SJL-TgN(SOD1–G93A)1Gur (expressing G93A mutant human SOD-1; hemizygotes become paralyzed in one or more limbs at ~4 months of age), B6.Cg-TgN(SOD1–G93A)<sup>dl</sup>1Gur (hemizygotes develop clinical disease at ~8 months), and B6.Cg-TgN(SOD1)2Gur [expressing wild-type (wt) human SOD-1] (Dal Canto and Gurney, 1994, 1995; Chiu et al., 1995). Nontransgenic littermates were used as controls. Genotyping was performed by PCR analysis of DNA isolated from ear tissue according to protocols provided by the Jackson Laboratory (Bar Harbor, ME). End-stage disease was defined as paralysis in one or both hind limbs. To obtain spinal cords, mice were killed by intraperitoneal injection of ketamine–xylazine (Ketaset, Wyeth–Ayerst, Montreal, Quebec, Canada; Rompun, Chemagro Limited, Etobicoke, Ontario, Canada). All procedures were within the guidelines of the Canadian Council of Animal Care and the Animal Care Committee of McGill University.

**Human spinal cord sections.** Archival paraffin-embedded cervical spinal cord sections were studied from control (two cases; neurologically normal), sporadic ALS (three cases), and autosomal dominant FALS (three cases, one SOD-1 A4V mutation and two without linkage to SOD-1).

**Exposure of spinal cord DRG cultures to thermal and excitotoxic stress.** For heat shock, spinal cord cultures were placed in minimum essential medium enriched with 5 gm of glucose without sodium bicarbonate, pH 7.2, heat shocked in a temperature controlled water bath, and recovered for variable periods at 37°C–5% CO<sub>2</sub> before analysis of HSP expression. To induce excitotoxic stress, an appropriate volume of a 100 mM stock of glutamic acid (Sigma, Oakville, ON) was added.

**Microinjection of plasmid vectors into cultured motor neurons.** To study the effect of G93A mutant and wild-type human SOD-1 on Hsp70 induction in motor neurons, cDNAs were subcloned into pCEP4 and microinjected into motor neuron nuclei at 200 μg/ml in Tris-EDTA as described previously (Durham et al., 1997). pcDNA3.1 plasmids encoding the following forms of human HSF1 were provided courtesy of Dr. R. Voellmy (University of Miami, Miami, FL) and injected at 0.5 μg/ml; HSF1<sup>wt</sup>, constitutively activated HSF1 (HSF1<sup>(+)</sup> or BH-S; resulting from deletion of amino acids 202–316, which regulates transactivational competence of HSF1), and nonactivatable HSF1 (HSF1<sup>(-)</sup> or AV-ST; resulting from deletion of amino acids 453–523, which spans the transcriptional activation domain) (Zuo et al., 1995). Plasmid containing the green fluorescent protein (GFP) gene under the control of the inducible human Hsp70 promoter [pEGFP (enhanced GFP)-HSE-GFP; courtesy of Dr. R. I. Morimoto, Northwestern University, Evanston, IL] was injected at 0.1 μg/ml, and 70 kDa dextran fluorescein or dextran tetramethylrhodamine (TMR) (20 μg/μl; Molecular Probes, Eugene, OR) was included to mark injected cells. After microinjection, coverslips were transferred to 5% CO<sub>2</sub>-equilibrated culture medium supplemented with 0.75% gentamycin and placed in a 37°C–5% CO<sub>2</sub> incubator. Cultures were either immunolabeled with antibody to Hsp70 or imaged for GFP expression in real time using MetaVue (GFP exposure time, 2 sec; TMR exposure time, 0.625 sec; Universal Imaging Corporation, Downingtown, PA).

**Treatment with proteasomal inhibitors.** Spinal cord cultures were transferred to CO<sub>2</sub>-equilibrated medium containing either MG132 (at final

concentrations of 0.1, 1, 2.5, or 5 μM; Peptide Institute, Minoh, Japan) or DMSO carrier and were incubated overnight.

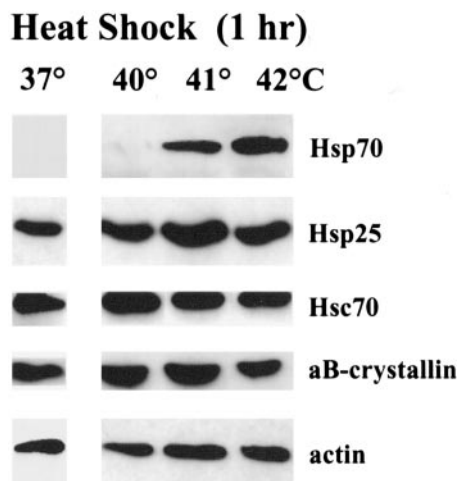
**Antibodies.** Primary antibodies were used against the following: SOD-1 (clone SD–G6, 1:300, Sigma; SOD-100, 1:100, Stressgen Biotechnologies, Victoria, BC), Hsp70 (SPA-810 for inducible Hsp70, 1:1000 for blots, 1:100 for immunolabeling of cultures and mouse tissue sections, 1:500 for human tissue sections, Stressgen Biotechnologies; W27 for both inducible and constitutive Hsp70, 1:100 Santa Cruz Biotechnology, Santa Cruz, CA), Hsc70 (K19, 1:1000 for blots; Santa Cruz Biotechnology), Hsp25 (SPA-801 for rodent Hsp25 with no cross reaction to Hsp27 primate isoform, 1:500/1000 for blots; Stressgen Biotechnologies), Hsp27 (M20, recognizes primate Hsp27 and rodent Hsp25, 1:100 for immunolabeling of cultures, 1:1000 for sections; Santa Cruz Biotechnology), αB-crystallin (SPA-222 and SPA-223, 1:1000 for blots, 1:100 for immunolabeling; Stressgen Biotechnologies), ubiquitin (1:1000; Dako, Carpinteria, CA), HSF1 (SPA-950, 1:100; Stressgen Biotechnologies), HSF2 (1:10; courtesy of Dr. R. I. Morimoto, Northwestern University, Evanston, IL), actin (clone C4, 1:1000; ICN Biomedicals, Cleveland, OH), glial fibrillary acidic protein (GFAP) (Z0334, 1:100 for immunolabeling of cultures, 1:2000 for sections; Dako), neurofilament heavy chain (N-4142, 1:100; Sigma), nitrotyrosine (1:100; Upstate Biotechnology, Lake Placid, NY), MAC-1 (courtesy of Dr. Sam David, McGill University, Montreal, Canada). For epifluorescence microscopy, cultures were labeled with appropriate secondary antibodies (Alexa-fluor 1:100; Molecular Probes). For Vectastain ABC detection systems, biotin-conjugated secondary antibodies were used (1:100; Vector Laboratories, Burlingame, CA). For blots, secondary antibodies conjugated to horseradish peroxidase (HRP) were used (mouse IgG and rat IgG, 1:3000; Jackson ImmunoResearch, West Grove, PA; rabbit IgG and goat IgG, 1:5000–1:10000, Dako).

**Sample preparation for Western blotting.** Spinal cord cultures were harvested in lysis buffer containing the following: 20 mM HEPES, pH 7.5, 100 mM KCl, 5% glycerol, 0.1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride (PMSF). After 30 min of incubation on ice, cell lysates were centrifuged at 12,700 × g for 15 min at 4°C. Lumbar spinal cords from mice were homogenized in 500 μl of the following lysis buffer: PBS, pH 8.0, containing 0.5% Nonidet-P40, 0.2% digitonin, and 0.23 mM PMSF. Cellular debris was pelleted by centrifugation at 15,400 × g for 15 min at 4°C. Supernatant fractions of culture or spinal cord homogenates were collected and protein concentrations were determined using the DC Protein Assay (Bio-Rad, Mississauga, Canada).

**Western blotting.** For spinal cord culture samples, 10 μg of protein were mixed with an equal volume of sample buffer (130 mM Tris, pH 6.8, 20% glycerol, 2% SDS, 10% β-mercaptoethanol, 0.08% bromophenol blue), boiled for 2 min, electrophoresed on 10% SDS–polyacrylamide gels, and transferred to Bio-Rad *trans*-blot transfer medium. Membranes were blocked for 1 hr with 5% milk in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) and then incubated for 2 hr at room temperature (RT) or overnight at 4°C with primary antibody diluted in TBST containing 5% milk and 0.02% NaN<sub>3</sub>. After washing in TBST, blots were incubated in HRP-conjugated secondary antibody–5% milk–TBST for 2 hr at RT. Protein bands were visualized on x-ray film after incubation of the blots in the Renaissance Western blot chemiluminescence reagent (NEN, Boston, MA). Films were scanned using Microtek (Torrance, CA) Scan Wizard. Expression of HSPs was normalized to actin by quantitating band density on films using ImageQuant software (Amersham Biosciences, Baie D'Urfe, Quebec, Canada).

For lumbar spinal cord homogenates, 10 μg of protein were mixed with an equal volume of sample buffer, boiled for 5 min, electrophoresed on 12.5% SDS–polyacrylamide gels, and transferred to nitrocellulose. Membranes were blocked overnight at 4°C in TBST-milk. Incubations with primary and secondary antibodies were 1 hr at RT. Protein bands were detected as described for culture samples. Hsp25 and αB-crystallin levels were normalized to tubulin by quantitating bands using NIH Image or ImageQuant software.

**Immunocytochemistry.** Immunocytochemistry using fluorescent dye-conjugated secondary antibodies was performed as described previously (Roy et al., 1998). Biotin-conjugated secondary antibodies were visual-



**Figure 1.** Heat shock induces Hsp70 in spinal cord cultures. Spinal cord cultures were subjected to increasing heat shock temperatures (40–42°C) for 1 hr, followed by 2 hr recovery at 37°C. Western blots were probed with antibodies to HSPs [Hsp70 (SPA-810), Hsp25 (SPA-801), Hsc70 (K19), and  $\alpha$ B-crystallin (SPA-222)] and actin as loading control. Blots shown are representative of three different experiments. Only Hsp70 was upregulated.

ized using the Vectastain ABC peroxidase kit (Vector Laboratories) and 0.05% DAB.

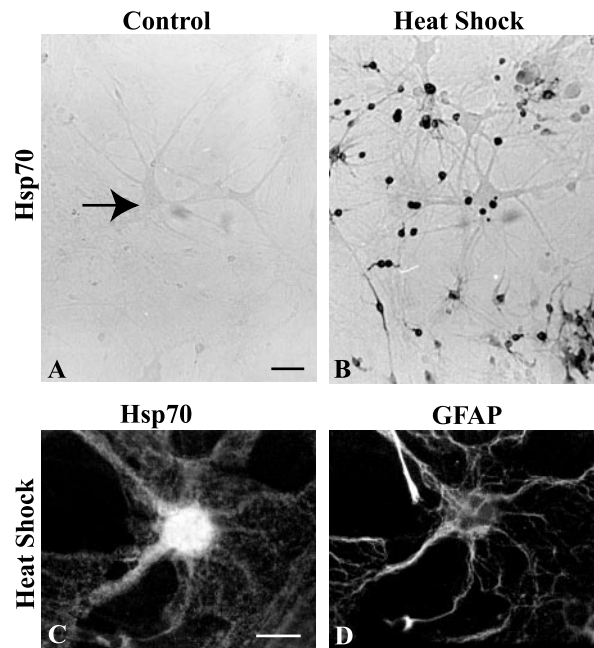
**Immunohistochemistry.** Lumbar spinal cords from transgenic mice were placed in Histo-Prep frozen tissue-embedding media (Fisher Scientific, Ottawa, Canada) and frozen in isopentane. Twenty micrometer cross sections were mounted onto glass slides and fixed in 3% paraformaldehyde for 30 min, permeabilized in 0.5% Nonidet-P40–PBS for 5 min, fixed in 3% paraformaldehyde for 2 min, and blocked for 3 hr at RT. To prevent nonspecific labeling with mouse monoclonal primary antibodies, F<sub>AB</sub> fragment anti-mouse IgG H+L (Jackson ImmunoResearch) was added for 30 min. Endogenous peroxidase activity was quenched by 5 min incubation with 2% H<sub>2</sub>O<sub>2</sub>. Primary antibodies were incubated overnight and detected using biotinylated secondary antibodies (1 hr at RT), Vectastain ABC peroxidase system, and 0.05% DAB. Sections were dehydrated in ethanol, dipped in xylene, and mounted in Permount (Fisher Scientific).

Human ALS spinal cord sections were deparaffinized using routine techniques. Antigen retrieval was performed at pH 10.0, and slides were incubated with 3% H<sub>2</sub>O<sub>2</sub> for 5 min, 5% BSA for 20 min, and 0.1% trypsin–0.1 M PBS, pH 7.2, for 15 min at 37°C. Sections were labeled with either SPA-810 monoclonal antibody recognizing Hsp70 (1:500) or a goat antibody recognizing Hsp27 (M20; 1:1,000) overnight at 4°C and detected using HRP-conjugated secondary antibodies and the Elite Vectastain ABC peroxidase system (Vector Laboratories) with 0.05% DAB.

## Results

### Heat shock-induced Hsp70 expression in glial cells but not motor neurons

Because induction of Hsp70 is a well characterized feature of the cellular response to hyperthermia, Hsp70 expression was used as the primary marker to investigate the heat shock–stress response in spinal cord cells. Primary spinal cord cultures were exposed for 1 hr to increasing temperatures (40–42°C) and then incubated for 2 hr at 37°C to allow for protein synthesis. Initially, HSP expression was examined by Western blotting to screen for thermally induced changes (Fig. 1). Inducible Hsp70 was minimal to nondetectable in cultures maintained at the 37°C control temperature, but increasing levels were observed in cultures exposed to temperatures above 40°C. When normalized to levels of actin, an approximate twofold increase in Hsp70 occurred at 42°C compared with 41°C. Expression of other HSPs, Hsp25, Hsc70, and  $\alpha$ B-crystallin was evaluated by immunoblotting, but no consis-



**Figure 2.** Glial cells, but not motor neurons, upregulate Hsp70 after heat shock. *A–D*, Spinal cord cultures were subjected to 37°C control temperature (*A*) or 42°C heat shock (*B–D*) for 1 hr, followed by 2 hr recovery at 37°C. Hsp70 induction was assessed by immunocytochemistry using biotin-conjugated secondary antibodies and DAB as substrate (*A, B*) or by fluorescence-conjugated secondary antibodies (*C*). Heat-shocked spinal cord cultures were also double labeled with antibodies against GFAP (*D*) to verify that glial cells induced Hsp70. The arrow in *A* points to a motor neuron. Scale bars: *A, B*, 50  $\mu$ m; *C, D*, 20  $\mu$ m.

tent increases were observed after heat shock at 40–42°C relative to levels at the control temperature of 37°C.

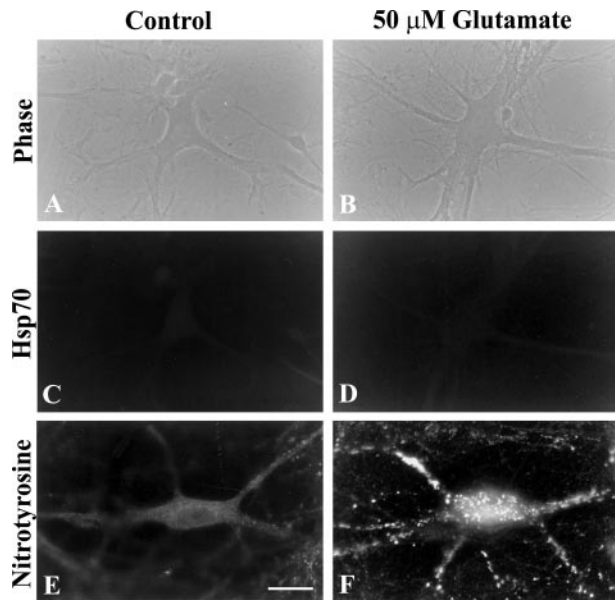
To determine the cell types in spinal cord cultures that upregulated Hsp70 after heat shock, immunocytochemistry was performed (Fig. 2). After heat shock for 1 hr at 42°C and 2 hr of recovery at 37°C, numerous non-neuronal cells expressed Hsp70, whereas motor neurons did not (Fig. 2*B*). Double labeling of cultures with antibodies to Hsp70 and the high molecular weight neurofilament protein confirmed the lack of Hsp70 in large neurons with motor neuron morphology as well as DRG and other neurons in the culture at this heat shock condition (our unpublished data). Double labeling with antibody against the astrocytic marker GFAP confirmed that astrocytes expressed Hsp70 after heat shock (Fig. 2*C, D*).

The absence of Hsp70 induction under these conditions of heat shock could be attributable to sufficient levels of constitutively expressed HSPs, such as Hsc70 or Hsp25, thereby obviating induction of the *Hsp70* gene. Thus, different paradigms of heat shock were examined, including higher temperatures (43°C and 44°C), increasing durations of hyperthermia (up to 8 hr), and prolonged recovery times (up to 6 hr). Some of these extreme heat shock conditions caused motor neuron death; however, surviving motor neurons still failed to induce Hsp70, although expression was detected in other neurons, including DRG neurons, under these conditions (our unpublished data). Together, these results indicate that motor neurons have either a weak or nonexistent stress response after exposure to thermal stress.

### Glutamate excitotoxicity did not induce Hsp70 in motor neurons

Glutamate excitotoxicity has been implicated in the pathogenesis of ALS (for review, see Cleveland and Rothstein, 2001) both





**Figure 3.** Motor neurons failed to express Hsp70 in response to glutamate. *A–F*, Spinal cord cultures were treated with 50  $\mu\text{M}$  glutamic acid (*B, D, F*) or vehicle (*A, C, E*) for 30 min and then assessed for Hsp70 induction by immunocytochemistry (SPA-810). Nitrotyrosine labeling demonstrated that glutamate treatment had an effect on motor neurons (*E, F*). Scale bar, 20  $\mu\text{m}$ .

through loss of the astrocytic glutamate transporter GLT-1 and consequent exposures to higher levels of glutamate (Rothstein et al., 1995; Cleveland and Rothstein, 2001) and through increased sensitivity to normally nonlethal levels of glutamate (Roy et al., 1998; Ludolph et al., 2000). In previous studies, neuronal cells were protected from glutamate excitotoxicity by a preconditioning mild heat stress, which induced HSPs (Lowenstein et al., 1991; Rordorf et al., 1991). However, the involvement of Hsp70 specifically has depended on the experimental model. Glutamate induced Hsp70 in neurons of hippocampal slices (Sato and Matsuki, 2002) but not in cerebellar granule neurons (Lowenstein et al., 1991). Overexpression of Hsp70 failed to protect cultured cortical or hippocampal neurons from glutamate excitotoxicity (Snider and Choi, 1996; Fink et al., 1997); yet, knockdown of Hsp70 using antisense oligonucleotides sensitized neurons of hippocampal slices to glutamate (Sato and Matsuki, 2002). To determine whether glutamate can affect the heat shock response of motor neurons, spinal cord cultures were exposed to excitotoxic levels of glutamate. In preliminary experiments, motor neuron death began 30 min after the addition of 50  $\mu\text{M}$  glutamate with  $\sim 40\%$  cell loss occurring 4 hr later. Spinal cord cultures were treated for 30 min with 25 or 50  $\mu\text{M}$  glutamate and analyzed immediately or after a 2 hr recovery by immunocytochemistry. No induction of Hsp70 was observed in motor neurons under these conditions (Fig. 3*C, D*). However, the absence of Hsp70 expression was not because of unresponsiveness of motor neurons to glutamate, because strong anti-nitrotyrosine labeling, an indicator of glutamate toxicity (for review, see Doroudchi et al., 2001), was observed (Fig. 3*E, F*). Thus, like heat shock, glutamate excitotoxic stress failed to upregulate Hsp70 in motor neurons. By Western blot analysis, glutamate also failed to induce Hsp70 or alter total levels of Hsp25, Hsc70, and  $\alpha\text{B}$ -crystallin in spinal cord cultures (our unpublished data).

**Table 1. Expression of mutant SOD-1 fails to induce Hsp70 in cultured motor neurons**

| Expression construct | Percentage of motor neurons expressing Hsp70 (mean $\pm$ SD) |               |
|----------------------|--|---------------|
|                      | Day 3  | Day 5         |
| pCEP4                | 0  | 3.3 $\pm$ 5.7 |
| wild-type SOD-1      | 2.3 $\pm$ 4.0  | 1.7 $\pm$ 2.9 |
| G93A SOD-1           | 6.2 $\pm$ 4.3  | 5.5 $\pm$ 4.9 |

Motor neurons in dissociated spinal cord cultures were microinjected with dextran-fluorescein marker and pCEP4 empty vector, pCEP4/wild-type SOD-1, or pCEP4/G93A SOD-1. Cultures were double labeled with antibodies against human SOD-1 (SOD-100) and Hsp70 (SPA-810 or W27) and viewed using epifluorescence microscopy. Values indicate mean percentage of motor neurons immunopositive for both human SOD-1 and Hsp70 at 3 and 5 d after microinjection; average of results from three cultures, with 15–40 human SOD-1-positive neurons per culture.

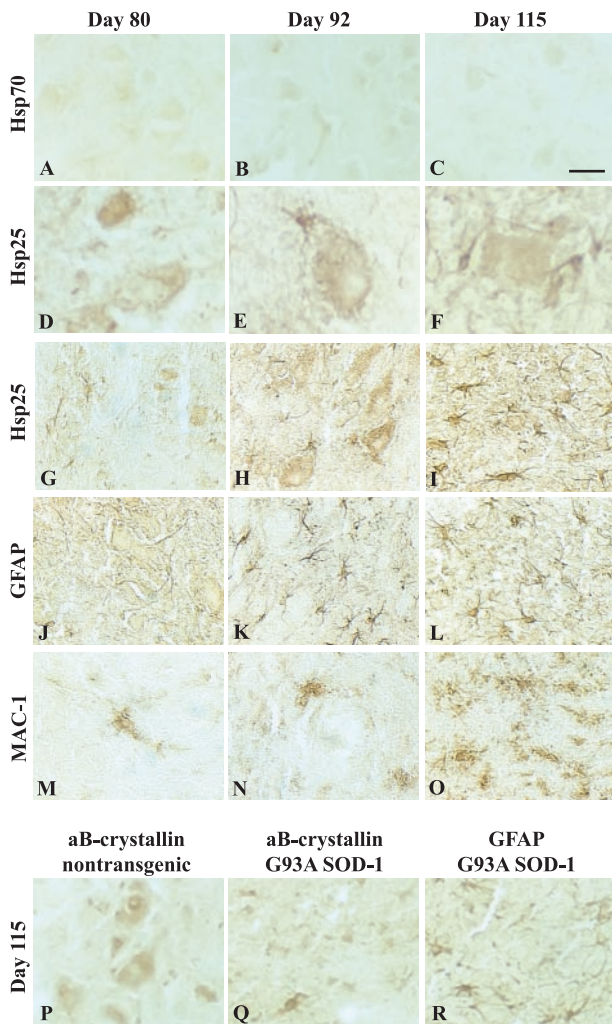
### Expression of G93A SOD-1 in motor neurons did not induce Hsp70

To determine whether expression of the disease-causing mutant protein G93A SOD-1 results in Hsp70 induction in cultured motor neurons, plasmid expression vectors encoding wildtype or G93A SOD-1 were microinjected into the nuclei of motor neurons in primary spinal cord cultures. In this model, motor neuron loss begins  $\sim 3$  d after microinjection of G93A SOD-1 expression plasmid and reaches  $\sim 50\%$  at day five (Durham et al., 1997; Roy et al., 1998). At these times, Hsp70 expression in motor neurons expressing wildtype or G93A SOD-1 was compared by double-labeling cultures with antibodies against Hsp70 and human SOD-1. Hsp70 was detected at low levels in a small percentage of cells, but there was no significant difference among those coexpressing wildtype and G93A SOD-1 (Table 1).

Hsp70 expression in motor neurons was also investigated in B6SJL-TgN(SOD1-G93A)1Gur transgenic mice. Similar to findings in the FALS culture model, Hsp70 was not detected in motor neurons in lumbar spinal cord sections by immunohistochemistry at 80, 92, or 115 d of age (Fig. 4*A–C*). Obvious loss of motor neurons in the ventral horn was apparent between 92 and 115 d as documented in previous studies of these mice (Dal Canto and Gurney, 1994, 1995; Chiu et al., 1995; Hall et al., 1998; Kong and Xu, 1998). Rarely, an immunoreactive background cell was observed, indicating that the antibody could recognize Hsp70 under these experimental conditions.

### Reactive astrocytes in spinal cords of mutant SOD-1 transgenic mice upregulated Hsp25

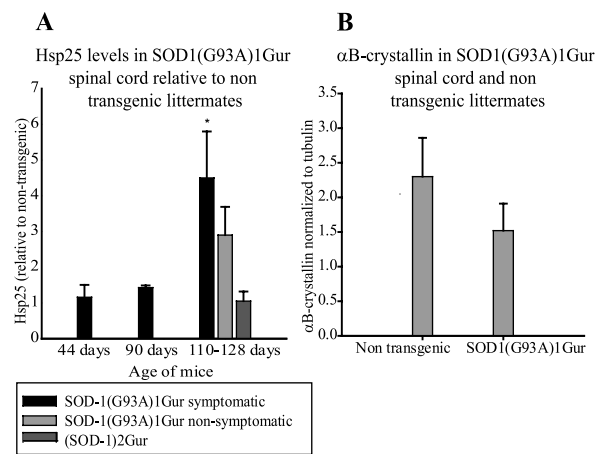
By Western blotting, a significant increase in Hsp25 was observed in lumbar spinal cord homogenates from symptomatic G93A SOD-1 transgenic mice relative to age-matched nontransgenic and wild-type SOD-1 transgenic animals (Fig. 5*A*). The level of Hsp25 was also greater in symptomatic, relative to nonsymptomatic, G93A SOD-1 littermates killed at 115 d of age. Labeling of adjacent spinal cord sections with antibodies against Hsp25 and GFAP determined that Hsp25 was localized to reactive astrocytes (Fig. 4*G–L*). This pattern of Hsp25 labeling was distinct from the microglial marker MAC-1, which also increased with disease progression (Fig. 4*M–O*). The number of Hsp25-expressing astrocytes and intensity of labeling increased between 80 and 115 d of age, paralleling total levels of Hsp25 in spinal cord homogenates (Fig. 5*A*) and the time course of motor neuron loss, microglial infiltration, and clinical progression of disease (*vide supra*). At early stages, astrogliosis was restricted to the ventral gray matter (preclinical phase) and increased progressively until the end stage of disease ( $\sim 4$  months of age). At the end stage, Hsp25 and GFAP-expressing astrocytes were prominent throughout the gray matter. Neither astrocytosis nor microglial activation were



**Figure 4.** Expression of HSPs in lumbar spinal cord of G93A mutant SOD-1 transgenic mice. The 20  $\mu$ m sections of lumbar spinal cord from 80, 92, and 115-d-old mice were labeled with antibodies to Hsp70 (SPA-810), Hsp25 (M20; recognizes primate Hsp27 and rodent Hsp25), GFAP, MAC-1, or  $\alpha$ B-crystallin. No induction of Hsp70 was observed (A–C). Motor neurons constitutively expressed Hsp25 (D–F), but no qualitative changes were noted with disease progression. There was a progressive increase in Hsp25 expressing astrocytes with age (G–I). Motor neurons of 115-d-old nontransgenic mice constitutively expressed  $\alpha$ B-crystallin (P). In lumbar cord from 115-d-old symptomatic G93A SOD-1 transgenics,  $\alpha$ B-crystallin was detected in some but not all reactive astrocytes (Q,R). Reactive astrocytes and microglia were identified by expression of GFAP (J,K,L,R) and MAC-1 (M–O) labeling of adjacent sections, respectively. Scale bars: A–C, G–R, 40  $\mu$ m; D–F, 15.4  $\mu$ m.

observed in spinal cords of nontransgenic littermates or wild-type SOD-1 transgenic mice (our unpublished data). Constitutive expression of Hsp25 was observed in motor neurons as reported previously (Plumier et al., 1997). However, no qualitative difference in expression was noted by immunohistochemistry in motor neurons of G93A SOD-1 mice, either with disease progression (Fig. 4D–F) or in comparison with motor neurons of age-matched wild-type SOD-1 mice or nontransgenic littermates (our unpublished data).

$\alpha$ B-crystallin was detected by immunohistochemistry in motor neurons as well as unidentified cells throughout the neuropil but appeared similar in all lines of transgenic mice (Fig. 4P,Q). In the spinal cord from symptomatic G93A SOD-1 mice, a subpopulation of reactive astrocytes expressed  $\alpha$ B-crystallin (Fig. 4Q,R). However, this was at a much lesser extent than that seen with Hsp25 and was not sufficient to be reflected on Western



**Figure 5.** Increase in Hsp25 with age and disease progression in G93A SOD-1 transgenic mice. *A*, Quantitation of Hsp25 on Western blots of spinal cord homogenates from wild-type and G93A SOD-1 mice. Ratios of Hsp25 to tubulin in samples from transgenic mice were normalized to ratios in matched samples of nontransgenic littermates. Note the threefold increase in Hsp25 in symptomatic G93A SOD-1 mice relative to those at 44 d of age. The mean Hsp25 level in the spinal cord of symptomatic animals was also higher than in samples from nonsymptomatic littermates. No inducible Hsp70 was detected on immunoblots (our unpublished data). Shown are means  $\pm$  SD for three to nine animals per group. The asterisk indicates a significant difference from all other values;  $p \leq 0.05$  (*t* test; one-tailed; unequal variance). *B*, Quantitation of  $\alpha$ B-crystallin immunolabeling relative to tubulin on Western blots of lumbar spinal cord homogenates from 115-d-old G93A SOD-1 mice and age-matched nontransgenic littermates.

blots, which showed similar  $\alpha$ B-crystallin levels among all groups (Fig. 5B).

The above findings in the B6SJL-TgN(SOD1-G93A)1Gur transgenic mouse line were confirmed in mice from the B6.Cg-TgN(SOD1-G93A)<sup>dl</sup> 1Gur lineage which exhibits slower disease progression (our unpublished data).

#### Neither Hsp70 nor Hsp27 are upregulated in spinal cords from familial and sporadic ALS patients

To determine whether the findings in transgenic mice could be extended to ALS patients, HSP expression was assessed by immunohistochemistry in the cervical spinal cord obtained at autopsy from patients with sporadic ALS (three cases), FALS (three cases, one of which harbored the A4V SOD-1 mutation), and two control cases. Motor neurons in sections of the cervical spinal cord obtained from ALS patients (Fig. 6D) were not labeled by anti-Hsp70. Glial immunoreactivity was occasionally observed in other parts of neuropil, indicating that this antibody can recognize Hsp70 when used for this procedure (our unpublished data).

Consistent with observations in transgenic mice, constitutive expression of the human Hsp27 isoform, equivalent to mouse Hsp25, was observed in motor neurons of control (Fig. 6A), sporadic ALS (Fig. 6B), and FALS (Fig. 6C) cases. However, unlike the G93A SOD-1 transgenic mice, high expression of Hsp27 in reactive astrocytes was not detected in spinal cords from either sporadic or FALS patients (Fig. 6C). Hsp27 immunoreactivity appeared localized to motor neuronal cell bodies and neurites rather than coincident with areas of reactive gliosis. Analysis of the background neuropil indicated that Hsp27 labeling (Fig. 6E) did not arise from morphologically typical astrocytes immunolabeled with anti-GFAP (Fig. 6F).

Thus, motor neurons of ALS patients failed to express Hsp70, consistent with findings in primary culture and transgenic mouse experimental models. However, the increase in Hsp25 in reactive



astrocytes in G93A SOD-1 transgenic mice was not replicated in the spinal cord of patients with familial or sporadic ALS.

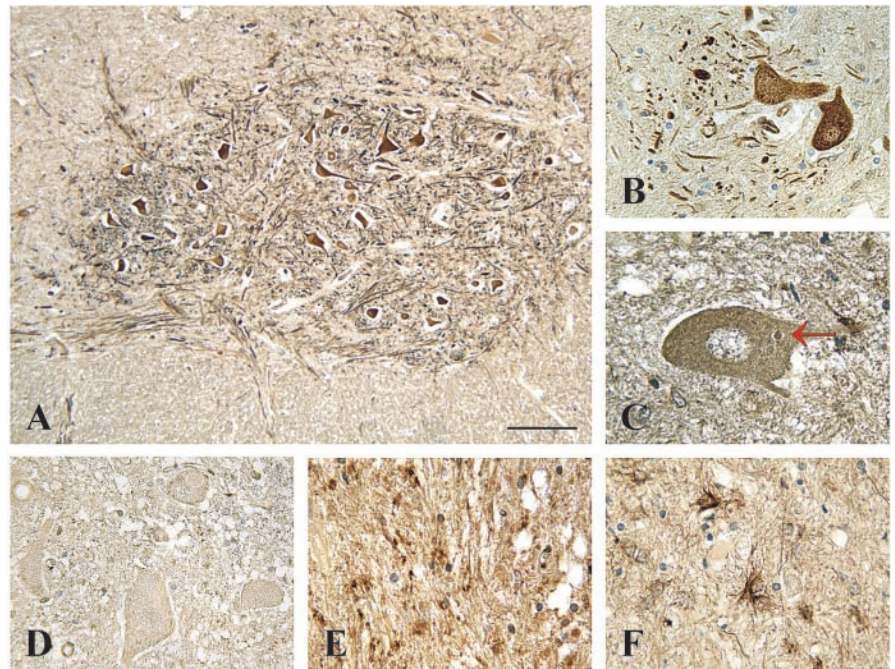
### Motor neurons expressed the major heat shock transcription factors HSF1 and HSF2

To determine whether the failure of motor neurons to induce Hsp70 after heat shock, glutamate, or mutant SOD-1 expression was attributable to lack of HSF, expression of the two major mammalian HSFs, HSF1 and HSF2, was assessed in spinal cord cultures. HSF1 primarily controls transcriptional activation of HSP genes in response to environmental stress by binding to HSEs on HSP promoters as trimers and undergoing hyperphosphorylation (Morimoto, 1998). HSF2 has a high turnover rate because of proteasome-mediated degradation (Mathew et al., 1998). When proteasome activity is inhibited sufficiently, HSF2 accumulates, forms trimers, translocates to the nucleus, and binds to HSEs, events associated with *de novo* transcription of HSP genes (Mathew et al., 1998).

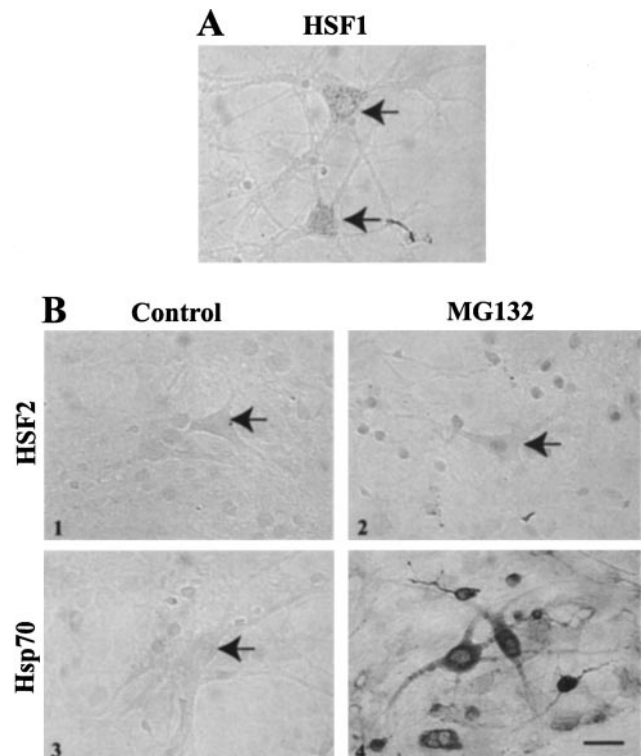
In untreated spinal cord cultures, motor neurons and other cells were labeled by a monoclonal antibody to HSF1, which preferentially labeled nuclei (Fig. 7A). HSF2 was not detected in untreated cultures but accumulated in the nuclei of motor neurons and other spinal cord cells after overnight incubation with proteasome inhibitor MG132 (0.1–1  $\mu\text{M}$ ) (Fig. 7B2) as reported previously in cell lines (Mathew et al., 1998). In addition to increasing levels of HSF2, MG132 induced strong expression of Hsp70 in motor neurons and other spinal cord cells (Fig. 7B4). Similar results were obtained with a more specific proteasome inhibitor, lactacystin (1–10  $\mu\text{M}$ ; Calbiochem, La Jolla, CA) (our unpublished data). Hence, Hsp70 induction can occur in motor neurons under conditions of decreased proteasome activity or via downstream effects of proteasome inhibition.

### Activation of HSF1 caused strong induction of markers of the heat shock–stress response in motor neurons

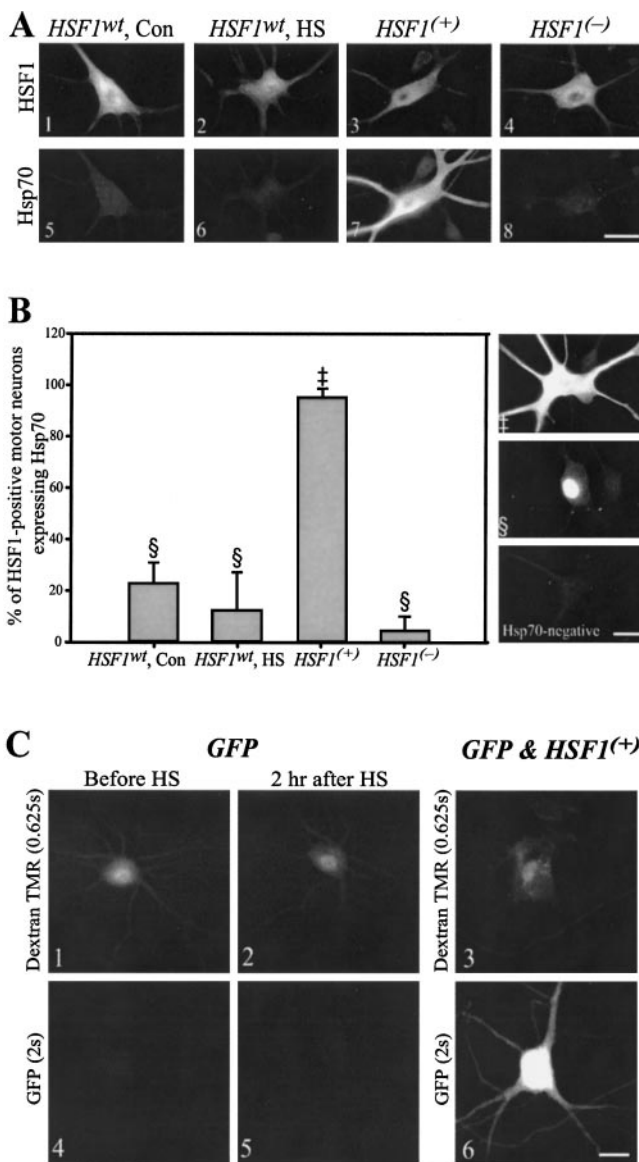
To address the possibility that motor neurons do not have sufficient amounts of HSF1 to mount a heat shock response, HSF1<sup>wt</sup> was overexpressed in motor neurons, and Hsp70 induction was assessed after heat stress. Motor neurons were microinjected with a plasmid-encoding HSF1<sup>wt</sup>, allowed to recover for 48 hr, and incubated at 42°C or 37°C for 1 hr and then 2 hr at 37°C. These conditions of heat shock were the same as those used in experiments shown in Figure 2, but again, no increase in Hsp70 expression was detected before or after heat shock, despite clear overexpression of HSF1<sup>wt</sup> in motor neurons (Fig. 8A1,5). In contrast, robust Hsp70 expression was observed at 37°C in motor neurons injected with plasmid encoding a constitutively activated form of HSF1 (HSF1<sup>(+)</sup>) (Fig. 8A3,7) but not an inactivatable form (HSF1<sup>(-)</sup>) (Fig. 8A4,8). HSF1<sup>(+)</sup> has a deletion of amino acids 202–316, which changes the conformation of the protein rendering HSF1 constitutively active and eliminating the requirement for phosphorylation of serine residues in this region. HSF1<sup>(-)</sup> has a deletion of amino acids 453–523 spanning the transcriptional



**Figure 6.** HSP expression in cervical spinal cord of control, sporadic, and FALS (A4V SOD-1 mutation). Hsp27 was detected in spinal motor neurons and neuritic processes in the anterior horn of control (A), sporadic (B), and FALS (C) patients; the arrow in C points to a spinal motor neuron containing a Bunina body. Hsp70 immunolabeling was not observed in the cytoplasm of motor neurons of FALS (D) or sporadic ALS (our unpublished data). Hsp27 labeling in the neuropil (E) does not correspond to the pattern of GFAP immunoreactivity (F). Scale bars: A, 100  $\mu\text{m}$ ; B, 40  $\mu\text{m}$ ; C–F, 20  $\mu\text{m}$ .



**Figure 7.** Motor neurons express the heat shock transcription factors HSF1 and HSF2. A, Motor neurons and other cells in primary spinal cord cultures were labeled by a monoclonal antibody to HSF1 (SPA-950). B, Overnight treatment with the proteasomal inhibitor MG132 (0.1  $\mu\text{M}$ ) caused HSF2 to accumulate and translocate into the nuclei of spinal cord cells, including motor neurons (1,2). In motor neurons and other cells treated with MG132 (1  $\mu\text{M}$ ), strong Hsp70 (SPA-810) expression was also detected by immunocytochemistry using DAB as substrate (3,4). Arrows indicate motor neurons. Scale bar, 30  $\mu\text{m}$ .



**Figure 8.** Expression of activated HSF1, but not HSF1<sup>wt</sup>, by gene transfer induces HSP70 expression in motor neurons. *A, B*, Motor neurons were microinjected with plasmid expression vectors encoding HSF1<sup>wt</sup>, constitutively activated HSF1 (HSF1<sup>(+)</sup>), or nonactivatable HSF1 (HSF1<sup>(-)</sup>). After 48 hr, motor neurons injected with HSF1<sup>wt</sup> were heat shocked (42°C; 1 hr) or subjected to control conditions (37°C; 1 hr) and recovered at 37°C for 2 hr. All cultures were double labeled with antibodies against HSF1 and Hsp70. *A*, Although motor neurons expressed HSF1<sup>wt</sup> (1,2) at levels comparable with HSF1<sup>(+)</sup> (3), only HSF1<sup>(+)</sup> induced Hsp70 (5–7). *B*, Percentage of motor neurons coexpressing HSF1 and Hsp70 ( $n \geq 4$  cultures per group). Intense Hsp70 immunolabeling (#) occurred only in motor neurons injected with HSF1<sup>(+)</sup>. In the other three conditions, lower intensity labeling was observed frequently in the nucleus (§). Only HSF1<sup>(+)</sup> significantly increased the percentage of motor neurons expressing Hsp70 ( $t$  test; one-tailed; unequal variance;  $p < 0.0001$ ). *C*, HSF1<sup>(+)</sup> also caused strong expression of a GFP reporter gene under the control of the Hsp70 promoter in motor neurons. Left, Motor neurons injected with the dextran TMR marker and GFP vector were imaged before and 2 hr after heat shock. Right, Heat shock failed to induce GFP (5), in contrast to coexpression of HSF1<sup>(+)</sup>, which resulted in strong GFP expression (6). Scale bar, 30  $\mu$ m.

activation domain. Hsp70 induction in motor neurons injected with HSF1<sup>(+)</sup> was consistently intense (Fig. 8A7, *B*, top, #). In the occasional Hsp70-immunopositive motor neuron expressing HSF1<sup>wt</sup> or HSF1<sup>(-)</sup>, labeling was of relatively low intensity and frequently localized to the nucleus (Fig. 8B, middle, §). Not only did HSF1<sup>(+)</sup> cause stronger expression of Hsp70 (Fig. 8A7), it

also caused a significant increase in the number of cells that induced Hsp70 (Fig. 8B).

Similar results were obtained using a GFP reporter gene driven by the human Hsp70 promoter (pEGFP-HSE-GFP). Motor neurons were coinjected with this vector along with the red fluorescent marker, 70 kDa dextran TMR. The following day, motor neurons were imaged for TMR and GFP fluorescence (Fig. 8C) before heat shock (42°C, 1 hr) and (in the same cells) after 2, 10, and 24 hr recovery at 37°C. GFP expression was never observed in motor neurons at any of the recovery times (Fig. 8C4,5). However, when HSF1<sup>(+)</sup> vector was coinjected with pEGFP-HSE-GFP, strong induction of GFP was observed in motor neurons (Fig. 8C6). Thus, even with a high level of expression of wtHSF1, thermal stress failed to activate heat shock gene transcription, but bypassing the normal mechanisms of transcriptional activation resulted in expression of two different proteins under the control of the Hsp70 promoter.

### Discussion

The results demonstrate that motor neurons have a high threshold for induction of the heat shock response. Although glia in primary spinal cord cultures upregulated the major stress-inducible protein Hsp70, after heat shock, motor neurons failed to express Hsp70 when subjected to hyperthermia, glutamate excitotoxicity, or expression of mutant SOD-1. Also, cultured motor neurons microinjected with a GFP reporter gene driven by the Hsp70 promoter failed to induce GFP after heat shock. No induction of Hsp70 was observed in spinal motor neurons in two lines of G93A mutant SOD-1 transgenic mice or in patients with ALS.

Because different stressors can induce expression of different HSPs, Hsp25 and  $\alpha$ B-crystallin were also monitored. Although endogenous expression of both proteins was detected in the cytoplasm of motor neurons in culture and *in vivo*, no qualitative differences were observed after stress treatments in culture, G93A SOD-1 transgenic mice throughout the disease course, or ALS spinal cords. Recently, increased Hsp25 was reported in nuclei of motor neurons in G93A SOD-1 transgenic mice (Vleminckx et al., 2002). This was not observed in the present study, although mice were killed at an earlier stage of clinical disease. Consistent with the findings of Vleminckx et al. (2002), Hsp25 and, to a lesser extent,  $\alpha$ B-crystallin were increased in spinal cord homogenates of G93A SOD-1 mice in parallel with disease progression. By immunohistochemistry, increased Hsp25 was particularly evident in reactive astrocytes, which may represent a common event during reactive astrogliosis or a stress response to mutant SOD-1 toxicity. Studies in other rodent models of injury and human disease have demonstrated increased astrocytic Hsp25 (Manzerra and Brown, 1992; Shinohara et al., 1993; Renkawek et al., 1994; Satoh and Kim, 1995; Marcuccilli et al., 1996; Krueger et al., 1999; Mautes and Noble, 2000). However, the significance of astrocytic Hsp25 to ALS is questioned because upregulation of the human homolog Hsp27 was not observed in the spinal cord of ALS patients, despite demonstration of gliosis by GFAP immunolabeling. Whether the difference is related to species, the HSPs investigated, specific toxicity of the G93A SOD-1 mutation, and disease pathogenesis in mouse models versus human patients remains to be determined.

Neurons, in general, show a higher threshold for induction of the stress response than astrocytes; yet, evidence points to motor neurons having a particularly high threshold for induction of Hsp70. DRG sensory neurons and other neurons in spinal cord–DRG culture expressed Hsp70 after heat shock at temperatures



>42°C but motor neurons did not (our unpublished data). Other studies have shown that spinal motor neurons *in situ* failed to induce Hsp70 after heat shock (Manzerra and Brown, 1992; Brown and Rush, 1999) and spinal cord contusion (Mauter and Noble, 2000) in contrast with other types of neurons, such as cerebellar, cortical, and pyramidal, which upregulated Hsp70 after heat shock (Lowenstein et al., 1991; Bechtold et al., 2000) and in different ischemic paradigms (Krueger et al., 1999; Currie et al., 2000). Although sciatic nerve axotomy increased Hsp25 in adult rat motor neurons (Costigan et al., 1998; Benn et al., 2002), in transgenic models of motor neuron disease, Hsp25 appeared unchanged (in the present study), was increased in nuclei (Vleminkx et al., 2002), or decreased (Pieri et al., 2001).

Exposure of motor neurons to hyperthermia would be expected to activate heat shock gene expression primarily through HSF1 (Morimoto, 1998). Inactive HSF1 can be found: (1) as a monomer in the cytoplasm, forming transient associations with Hsp70 and Hsp90 (Morimoto and Santoro, 1998), and (2) in nuclear granules (Cotto et al., 1997; Jolly et al., 2002). When levels of HSPs are insufficient in stressed cells, HSF1 trimerizes, binds to HSEs, and undergoes phosphorylation at multiple sites. This changes its conformation, recruiting the transcriptional machinery at HSP promoters and culminating in transactivation of heat shock genes (Morimoto and Santoro, 1998; Holmberg et al., 2001). Thus, arrest of HSP induction in motor neurons could occur at the level of HSF1 expression, trimerization of the transcription factor, binding to HSEs, phosphorylation–activation of HSF1, or HSP mRNA stability, processing, and/or translation.

The failure of hippocampal neurons to induce Hsp70 after heat shock was attributed to a lack of HSF1 (Marcuccilli et al., 1996). Motor neurons did express HSF1, but it was still possible that they did not express sufficient levels to mount a heat shock response or that immunolabeling did not reflect the true state. However, when the level of HSF1<sup>wt</sup> was increased in motor neurons, heat shock still failed to induce Hsp70. This experiment demonstrated that the block in stress-induced expression of Hsp70 in motor neurons was not at the level of HSF1 expression.

Previous studies had implicated faster disassembly of hyperphosphorylated HSF1 trimers in the failure of Scrapie to induce Hsp70 in ScN2a neuroblastoma cells (Winklhofer et al., 2001), and the inability of HSF1 to bind to the *Hsp70* promoter in the reduced ability of retinoblastoma Y79 cells to transcribe *Hsp70* after heat shock (Mathur et al., 1994). However, a constitutively active form of HSF1, HSF1<sup>(+)</sup>, but not the inactivatable form, HSF1<sup>(-)</sup>, induced strong Hsp70 when expressed in cultured motor neurons. For HSF1<sup>(+)</sup> to have transactivated gene expression, trimerization, nuclear transport, and binding to HSE must have occurred. Although this could not be assessed directly in single cells of dissociated spinal cord cultures, it has been demonstrated in cell lines that HSF1<sup>wt</sup>, HSF1<sup>(+)</sup>, and HSF1<sup>(-)</sup> oligomerize and bind to HSEs, but only HSF1<sup>(+)</sup> causes transactivation of HSP genes in the absence of stress. Heat shock was required for HSF1<sup>wt</sup> to transactivate HSP gene expression (Zuo et al., 1995).

In addition to Hsp70, HSF1<sup>(+)</sup> induced expression of a GFP reporter under the control of the *Hsp70* promoter. This result and the increase in Hsp70 after HSF1<sup>(+)</sup> gene transfer indicate that once the HSF–HSE complex was activated, transcription and translation proceeded.

The most likely cause of the failure of motor neurons to induce a stress response involves alterations in the signaling pathways that regulate HSF1 activation. HSF1<sup>(+)</sup> has a deletion spanning amino acids 202–316, a region harboring numerous serine residues, the phosphorylation states of which regulate the trans-

activational competence of HSF1 by altering its conformation (Zuo et al., 1995). Deletion of this domain changes the conformation of HSF1, rendering it constitutively active and obviating the requirement for phosphorylation. Within this region, constitutive phosphorylation at serines 303 and 307 by glycogen synthase kinase 3 $\beta$  and extracellular signal-regulated kinase (Chu et al., 1996) represses transcriptional activity of HSF1, implicating dephosphorylation at these sites in HSF1 activation. Stress-inducible phosphorylation of another residue within this domain, serine 230, which lies within a consensus site for calcium-calmodulin kinase II, is a key phosphorylation event resulting in HSF1 activation (Holmberg et al., 2001). Lack of activation or changes in the regulation of these candidate kinases could be responsible for the deficiency in the heat shock–stress response observed in motor neurons.

In contrast to heat shock, glutamate excitotoxicity, and expression of mutant SOD-1, peptide inhibitors of proteasome activity induced robust expression of Hsp70 in motor neurons concomitant with accumulation and nuclear localization of the transcription factor HSF2. This result is consistent with the role for HSF2 in HSP gene transcription (Mathew et al., 1998). Proteasome inhibition has been implicated in disorders characterized by protein aggregation, including trinucleotide repeat diseases (Bence et al., 2001; Sherman and Goldberg, 2001). Mutant SOD-1 also undergoes degradation in proteasomes (Hoffman et al., 1996; Johnston et al., 2000), and recent studies demonstrated decreased proteasome activity in cell lines expressing mutant SOD-1 (Hyun et al., 2002; Urushitani et al., 2002) and lumbar spinal cord from G93A SOD-1 transgenic mice (our unpublished data). Low levels of HSF2 are maintained by proteasomal degradation (see Results). If mutant SOD-1 had abrogated proteasome function, accumulation of HSF2 and transactivation of *Hsp70* would be expected, but neither was observed in cultured motor neurons expressing G93A SOD-1 (our unpublished data) (Table 1) nor in motor neurons in lumbar spinal cord of G93A mice (our unpublished data) (Fig. 5A–C). However, neither the threshold level of proteasome inhibition required for HSF2 accumulation nor the requirement of other factors, such as the HSF2 modulator, SUMO-1 (Goodson et al., 2001), are known. Proteasome inhibition also increases HSF1 activation, presumably by reduced proteolysis of HSF1-targeting kinase(s) (Holmberg et al., 2001). The level of proteasome inhibition by mutant SOD-1 may not be sufficient to induce Hsp70 via either HSF2 accumulation or HSF1 activation.

In conclusion, our results indicate that the impaired ability of motor neurons to mount a heat shock–stress response occurs at the level of activation of HSF1, the major cellular stress sensor. This feature, in addition to other factors, such as neurofilament organization, high level of glutamatergic input, expression of calcium-permeable AMPA receptors, and low levels of free radical scavengers and cytosolic calcium-binding proteins (for review, see Roy et al., 1998), may contribute to their preferential vulnerability to disease. Preliminary studies indicate that expression of constitutively activated HSF1 protects motor neurons from mutant SOD-1 toxicity (our unpublished data), supporting the possibility that pharmacological activation of HSF1 in motor neurons may overcome the deleterious effects of other disease-related and environmental stresses.

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