Activation of Gene Transcription by Heat Shock Protein 27 May Contribute to Its Neuronal Protection*

Meyer J. Friedman†, Shihua Li†, and Xiao-Jiang Li‡

From the †Department of Human Genetics, Emory University School of Medicine, Atlanta, Georgia 30322 and the ‡Department of Medicine, School of Medicine, University of California, San Diego, La Jolla, California 92093

Heat shock proteins are up-regulated as a physiological response to stressful stimuli and generally function as molecular chaperones for improperly folded protein substrates. The small heat shock protein HSP27 (or HSPB1) has multiple cytoplasmic roles. HSP27 also can translocate to the nucleus in response to stress, but the functional significance of this nuclear distribution has not been elucidated. We have previously implicated HSP27 as a genetic modifier of spinocerebellar ataxia 17 (SCA17), a neurological disease caused by a polyglutamine expansion in the TATA-binding protein (TBP). Altered expression of HSP27 is also found in cell models of other polyglutamine diseases, including Huntington disease as well as SCA3 and SCA7. Here, we show that Hsp27, unlike Hsp70, is not detected in mutant TBP aggregates in primary cerebellar granule neurons from transgenic SCA17 mice. Although HSP27 overexpression does not reduce the aggregation of cotransfected mutant TBP containing 105 glutamines, it potentiates activated transcription from both TATA-containing and TATA-lacking promoters. Neither HSP40 nor HSP70 elicits the same transcriptional effect. Moreover, HSP27 interacts with the transcription factor SP1, and coexpression of SP1 and nuclear localization signal-tagged HSP27 synergistically activates reporter constructs for the SP1-responsive neurotrophic receptor genes Ngfrp75 and TrkA. Overexpression of nuclear localization signal-tagged HSP27 also rescues mutant TBP-mediated down-regulation of TrkA in a PC12 cell model of SCA17. These results indicate that nuclear HSP27 can modulate SP1-dependent transcriptional activity to promote neuronal protection.

Protein misfolding is a characteristic feature of multiple neurodegenerative disorders, including the polyglutamine (polyQ) diseases, which are caused by expansion of a CAG or mixed CAA/CAG trinucleotide repeat in the coding region of at least nine unrelated genes. Various heat shock proteins (HSPs), as well as ubiquitin, proteasomal subunits, and autophagic markers, have been detected in intracellular aggregates formed by mutant polyQ proteins expressed in cultured cells, transgenic mice, and patient brains, indicating that these structures engage the cellular response to misfolded protein (1). Intriguingly, the time-dependent increase in polyQ aggregate load negatively correlates with the neuronal expression of certain HSPs in multiple mouse models (2–6). Although the pathological role of polyQ aggregates remains controversial and could be disease-specific, the therapeutic potential of manipulating HSP expression has been staked largely on the possibility of reducing toxicity by limiting the misfolding and/or promoting the clearance of polyQ-expanded proteins.

HSPs contribute to normal cell physiology and are crucial components of the cellular stress response. Many HSPs serve as molecular chaperones to facilitate folding or refolding of protein substrates that otherwise would be targeted for degradation. The HSP70 family, comprising constitutive and inducible members, is prominently involved in the proper folding of both nascent and mature proteins. Co-chaperones, such as HSP40 proteins and BAG1, directly stimulate the intrinsic ATPase activity of HSP70 that is required for these processes (7). As part of its collaboration, HSP40 mediates the initial recognition of conformationally immature (8) or misfolded (1, 8) substrates and delivers these client proteins to HSP70 (8).

Some stress-induced proteins, including HSP27, which is alternatively referred to as Hsp25 in mice or HSB1 in general, can also modulate cell death by antagonizing various steps in apoptotic pathways (9, 10). We have shown previously that polyQ-expanded TBP causes down-regulation of Hsp27, resulting in diminished neurite outgrowth in PC12 cells and neuritic defects in primary neurons cultured from SCA17 transgenic mice (6). Although HSP27 performs critical functions in the cytoplasm that promote neuronal viability and neuritic integrity (11, 12), it also accumulates in neuronal nuclei in response to stress (13–15). However, the role of nuclear HSP27 in neuronal protection remains unclear.

Here, we provide evidence that nuclear HSP27 interacts with the transcription factor SP1 and stimulates SP1-dependent gene expression. Moreover, in a PC12 cell model of SCA17, overexpression of HSP27 rescues decreased expression of the neurotrophic receptor TrkA (tyrosine kinase receptor A), which is transcriptionally regulated by SP1. These results support a novel role for nuclear HSP27 in transcriptional activation and suggest that down-regulation of HSPs in the polyQ diseases, as has been widely observed, could directly impact gene expression.

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Experimental Procedures

Antibodies and Reagents—Antibodies against TBP included N-12 (Santa Cruz Biotechnology) and 1TBP18 (QED Bioscience, San Diego, CA). Other commercial antibodies utilized were against HSPB1 (C20, Santa Cruz Biotechnology), phospho-HSPB1 (2401, Cell Signaling), HSP70 (W27, Santa Cruz Biotechnology), HSP90 (AC88, Stressgen), hemagglutinin tag (12CA5, Roche Applied Science), γ-tubulin (GTU-88, Sigma), SP1 (PEP2, Santa Cruz Biotechnology), TrkA (06-574, Upstate), His tag (H-15, Santa Cruz Biotechnology), and glyceraldehyde-3-phosphate dehydrogenase (G8795, Sigma). Hoechst 33258 (Molecular Probes) was used for nuclear labeling. Transfections were performed with Lipofectamine 2000 (Invitrogen).

Constructs encoding hemagglutinin-tagged HSP40 and HSP70 have been described previously (5). Human HSP27 cDNA was ligated into the pEBV-HisA mammalian expression vector to generate His-HSP27. His-NLS-HSP27 was derived by in-frame insertion of a fragment encoding the SV40 large T antigen NLS.

Cell Culture—Human embryonic kidney HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. For stably transfected PC12 cells, Dulbecco’s modified Eagle’s medium was supplemented with 10% horse serum, 5% fetal bovine serum, and antibiotics.

Cerebellar granule neurons were cultured from postnatal day 7 transgenic SCA17 mice as described previously (6). After 10 days in vitro, cerebellar neurons were fixed with 4% paraformaldehyde and stained with anti-TBP and anti-HSP antibodies, whereas nuclei were labeled with Hoechst.

Reporter Assays—Minimal (−99 to +13) and proximal (−172 to +13) human HSP27 promoter sequences used to generate DsRed reporter constructs have been described previously (16). A distal rat Ngfrp75 promoter fragment (−1411 to −1720) and a proximal human TRKA promoter fragment (−243 to +20), both of which feature multiple SP1 consensus-binding sites, were also inserted into pDsRed-Express-1 (Clontech). The former was subcloned from a previously characterized luciferase construct (17), and the latter was cloned as a HindIII-EcoRI insert using primers 5′-ATCAAGCTTCACCTCCGA-GGCCGTTCCG-3′ and 5′-GAATTCTCTGTGCGCTCCCAGC-3′. DsRed reporter constructs were cotransfected with the indicated expression vectors in HEK 293T cells. Reporters assays with pEGFP-C3 (Clontech) were performed in HEK 293 cells. Cotransfections were done in triplicate, and assays were carried out as detailed previously (18).

Immunostaining—Immunofluorescence was performed as described previously (6). Fluorescent images were acquired on a Zeiss Axiovert 200 MOT microscope equipped with a digital camera (Hamamatsu Orca-100) and Openlab software (Improvision Inc.). The ×40 objective was used for image acquisition.

Cellular Fractionation and Western Blotting—For whole cell lysates, cells were washed with 1× phosphate-buffered saline and harvested in radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 8.0), 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100) containing protease inhibitor mixture (Roche Applied Science). When relevant, HALT phosphatase inhibitor mixture (Pierce) was included in the lysis buffer. Samples were sonicated for 10 s, placed on a rotating apparatus at 4 °C for 30 min, and ultimately clarified at 3000 rpm for 5 min.

To obtain nuclear extracts, cells were washed and harvested in 1× phosphate-buffered saline. Cell pellets were resuspended in cold lysis buffer (10 mM Tris-HCl (pH 7.4), 3 mM CaCl2, 2 mM MgCl2, and 1% Nonidet P-40) containing protease inhibitor mixture and passed 15 times through a 27-gauge 0.5-inch needle. After 5 min of incubation on ice, samples were centrifuged at 2307 rpm for 5 min. The supernatant was removed and clarified at 16,000 rpm for 30 min to yield the cytoplasmic fraction.

Nuclear pellets were washed three times with lysis buffer and ultimately resuspended in radioimmunoprecipitation assay buffer. Subsequent processing of nuclear extracts was the same as for detailed above whole cell extracts. Samples were resolved on 4–20% Tris glycine-polyacrylamide gels (Invitrogen), and Western blotting was performed with the indicated primary antibodies.

Immunoprecipitation—Non-transfected HEK 293 cells were washed with 1× phosphate-buffered saline and then harvested in 1× phosphate-buffered saline containing 0.1% Triton X-100 and protease inhibitor mixture. Subsequent processing of
lysates was the same as detailed above for whole cell extracts except that samples for immunoprecipitation were clarified at 12,000 rpm for 10 min. Immunoprecipitation was performed with anti-SP1 antibody or rabbit IgG (2 μg) using 1 mg of precleared lysate. Immunocomplexes were allowed to form overnight on a rotating apparatus at 4 °C and then precipitated following incubation with protein A-agarose (20 μl, 1:1 dilution) for 1 h. Beads were washed twice with lysis buffer prior to immunoblotting. Input in the Western blot was 50 μg of lysate.

Semiquantitative Reverse Transcription-PCR—RNA was isolated from HEK 293 cells using TRIzol reagent (Invitrogen). Transcripts of transfected TBP and the endogenous human TBP gene were simultaneously amplified using PCR primers that flanked the CAG/CAA trinucleotide repeat (forward, 5'-CCACAGCCTATTGCTGAACC-3'; and reverse, 5'-ACTGAAAATCAGTGGCGTGGTTC-3'). Densitometric analysis was performed with the NIH ImageJ 1.42q program.

Statistics—Student's t test and one-way analysis of variance with Tukey's post hoc test were used to determine statistical significance between groups. p < 0.05 was considered significant in all cases.

RESULTS

Hsp27 Does Not Colocalize with polyQ-expanded TBP Aggregates—Nuclear inclusions in SCA17 patient brains are immunoreactive for ubiquitin (19–21) and the HSP40 family member HDJ-2 (20). As cerebellar granule neurons cultured from SCA17 transgenic mice expressing TBP-105Q feature
ubiquitin-positive inclusions (6), we first tested for colocalization of Hsp27 with these nuclear structures. However, we were unable to detect the presence of Hsp27 in TBP-105Q inclusions (Fig. 1A). An antibody against phosphorylated Hsp27 (human Ser82) also failed to label the nuclear inclusions formed by mutant Tbp in granule neurons (data not shown). Meanwhile, staining with a monoclonal antibody for Hsp70 revealed the presence of this HSP in mutant TBP inclusions (Fig. 1B).

Given the differential recruitment of endogenous HSPs to TBP inclusions, we next determined the effect of HSP overexpression on the levels of soluble and aggregated polyQ-expanded TBP. Consistent with our earlier findings regarding chaperone-mediated suppression of mutant huntingtin aggregation (22), Hsp40 but not Hsp70 overexpression dramatically reduced the amount of soluble and aggregated Tbp-105Q in HEK 293T cells (Fig. 2). However, overexpression of His-tagged Hsp27 markedly increased the level of soluble mutant TBP in a concentration-dependent manner (Fig. 2A). An increase in the amount of aggregated TBP was also seen in Hsp27-transfected cells, potentially as a consequence of the elevated protein level of mutant TBP. A similar effect of this small HSP on the solubility and total cellular content of polyQ-expanded versions of huntingtin and androgen receptor has been reported previously (23). As His-Hsp27 is predominantly cytoplasmic in HEK 293 cells (Fig. 2A, lower panel), we asked whether nuclear Hsp27 could recapitulate this phenomenon. Indeed, targeting of Hsp27 to the nucleus via an NLS (Fig. 2B, lower panel) also resulted in elevated levels of soluble and aggregated TBP-105Q in cotransfected cells (Fig. 2B).

Nuclear Hsp27 Stimulates SP1-Mediated Transcriptional Activation—To test if the altered expression of transiently transfected mutant TBP in the presence of exogenous Hsp27 might be transcriptionally mediated, we performed semiquantitative reverse transcription-PCR for TBP in cells that were cotransfected with TBP-105Q and empty vector or His-NLS-Hsp27 (Fig. 3). Using primers that flanked the polyQ-encoding CAG/CAA repeat, we were able to detect cDNAs for normal endogenous and mutant exogenous TBP. In the presence of nuclear Hsp27, we observed an increase in the expression level of polyQ-expanded versions of huntingtin and androgen receptor has been reported previously. As His-Hsp27 is predominantly cytoplasmic in HEK 293 cells (Fig. 2A, lower panel), we asked whether nuclear Hsp27 could recapitulate this phenomenon. Indeed, targeting of Hsp27 to the nucleus via an NLS (Fig. 2B, lower panel) also resulted in elevated levels of soluble and aggregated TBP-105Q in cotransfected cells (Fig. 2B).

**FIGURE 4. Activation of TATA-containing gene promoters by Hsp27 overexpression.** A, reporter assay using HEK 293 cells transiently cotransfected with the cytomegalovirus (CMV)-EGFP reporter and an Hsp (Hsp27, Hsp40, or Hsp70) or empty vector. B and C, reporter assays performed in HEK 293T cells with DsRed reporter constructs featuring the Hsp27 minimal (B) or proximal (C) promoter regions. Western blots in B and C show relative expression of TBP and Hsp27 in lysates of cells harvested for reporter assays. γ-Tubulin served as a loading control. The arrowhead indicates endogenous Hsp27. Annotation of the human Hsp27 promoter elements in schematics is according to a previous characterization (16). *** p ≤ 0.001. ERE, estrogen response element; HSE, heat shock element.

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other HSPs, and it depended on the concentration of exogenous HSP27 (Fig. 2). To further investigate the role of HSP27 in its own expression, we performed reporter assays using minimal or proximal HSP27 promoter fragments (Fig. 4, B and C). Although cotransfected HSP27 did not have any effect on the minimal HSP27 promoter (Fig. 4B), it significantly stimulated expression from the proximal promoter (Fig. 4C). Overexpression of TBP, which is known to activate TATA-containing promoters in a manner that is inversely related to promoter strength (24), served as a positive control in these experiments. Notably, overexpressed HSP27 was more efficient than exogenous wild-type TBP (with 31Q) in inducing transcriptional activation of the proximal promoter construct (Fig. 4C).

There are a limited number of known transcription factor-binding sites, including two for SP1, within the proximal HSP27 promoter fragment used in our reporter assays (Fig. 4C) (16). As SP1 has been implicated in polyQ-induced transcriptional dysregulation (17, 25) and HSP27 has functional attributes reminiscent of a transcriptional cofactor (26), we asked whether the two proteins form an endogenous complex. Indeed, we were able to co-immunoprecipitate HSP27 but not HSP70 or HSP90 from HEK 293 cell lysate with an antibody against SP1 (Fig. 5). Western blotting was performed with antibodies against SP1, HSP90, HSP70, and HSP27.

DISCUSSION

These findings indicate that HSP27, unlike molecular chaperones such as HSP70 and HSP40, is not necessarily involved in the cellular response to aggregation by polyQ-expanded TBP. However, nuclear HSP27 can potentiate SP1-dependent gene transcription, suggesting a novel mechanism for neuronal protection by a small HSP. This nuclear effect is seemingly distinct from cytoprotective roles that HSP27 fulfills in the cytoplasm.

Neuroprotection Conferred by Nuclear HSP27 in an SCA17 Cell Model—Finally, we investigated the potential involvement of nuclear HSP27 in SCA17 pathogenesis. We have reported previously that PC12 cells stably transfected with TBP-105Q show a neurite outgrowth defect in response to nerve growth factor treatment (6), and we have recently detected down-regulation of TrkA in these cells as well as in cerebella from SCA17 transgenic mice (27). Thus, we tested the effect of exogenous nuclear targeted HSP27 on TrkA expression in our PC12 cell model of SCA17 (Fig. 7). NLS-HSP27 significantly increased the level of TrkA in control PC12 cells expressing TBP-13Q (Fig. 7, first lane versus second lane). Importantly, NLS-HSP27 also effectively rescued the reduced expression of TrkA in TBP-105Q cells (Fig. 7, first lane versus third and fourth lanes).

As we have demonstrated previously that Hsp27 is down-regulated in cellular and mouse models of SCA17 (6), we evaluated the nuclear levels of Hsp27 in the presence of polyQ-expanded TBP (Fig. 8). Under basal conditions, little Hsp27 could be detected in the nuclei of control or TBP-105Q PC12 cells by Western blotting. However, following 6 h of recovery from heat shock (1 h at 42 °C), an increase in nuclear Hsp27 was observed in TBP-13Q but not TBP-105Q PC12 cells (Fig. 8A). Meanwhile, nuclear accumulation of Hsp70 in response to heat shock was not noticeably different in recovering TBP-13Q and TBP-105Q cells. This discrepancy in nuclear Hsp27 levels may be due to altered phosphorylation of the small HSP, which is known to correlate directly with its nuclear translocation (28), and/or disruption of its potentially phospho-dependent transport into the nucleus. Under basal conditions, the nuclear level of phospho-Hsp27 (human Ser^82) was higher in TBP-13Q cells than in TBP-105Q cells. Moreover, in contrast to control cells, the SCA17 cell model displayed only a subtle increase in the nuclear concentration of phospho-Hsp27 immediately after heat shock (Fig. 8B). Collectively, these results suggest that a limited nuclear presence of HSP27 in SCA17 may contribute to its own down-regulation as well as that of other proteins that are crucial for neuritic function and neuronal viability.
Like half of the 10-member mammalian small HSP family, HSP27 is constitutively expressed in the brain (29) albeit in a cell-specific manner (30). Down-regulation of Hsp27 by small interfering RNA inhibits neurite outgrowth in cultured primary neurons, which is the opposite effect elicited by its overexpression in the same system (11). Mutation of HSP27 is causative in two related peripheral neuropathies, Charcot-Marie-Tooth disease type 2F and distal hereditary motor neuropathy type 2B, which are characterized by axonal degeneration. Moreover, HSP27 has been implicated in the neuronal response to acute and chronic stress. Among the various tissues in which the small HSP is expressed, the brain is one of maybe only a few that show clear up-regulation of Hsp27 in response to whole body hyperthermic challenge (31). We have previously implicated HSP27 as a genetic modifier of the polyQ disease SCA17 (6), and other studies have suggested that polyQ-dependent toxicity is correlated with a failure to up-regulate (32, 33) or activate (34) HSP27. Thus, altered HSP27 expression or function may contribute to the pathogenesis and/or progression of multiple neurodegenerative diseases.

Several cellular functions have been attributed to HSP27, which is predominantly cytoplasmic in many cell types under steady-state conditions but translocates to the nucleus in response to stressful stimuli. In the cytoplasm, HSP27 binds actin and tubulin to influence the dynamics and structure of the cytoskeleton (9). Under normal conditions, unengaged HSP27 forms tetramers with itself or potentially another small HSP, such as HSPB8 (9, 35), that readily assemble into large oligomers. These high molecular weight complexes, which are capable of binding multiple molecules of a misfolded substrate, have been shown to confer chaperone activity in vitro (36). Given that phosphorylation of HSP27 is necessary for oligomer disassembly and is correlated with its entry into the nucleus (28), nuclear HSP27 might be limited to a non-oligomeric quaternary structure that would preclude a chaperone function.

Although some small HSPs possess molecular chaperone activity in vivo, a capacity for HSP27 to refold a denatured natural substrate has not been demonstrated in cultured cells or mammalian tissues. Coexpression of HSP27 and huntingtin exon 1 with 43Q or of the androgen receptor with 65Q in CCL39 lung fibroblasts results in a noticeable increase in both the SDS-soluble and SDS-insoluble content of the mutant polyQ proteins. Meanwhile, exogenous HSPB8 elicits the opposite effect (23). Overexpression of HSP27 also increases the aggregation of mutant huntingtin exon 1 in a Huntington disease neuronal cell model (37). We observed a similar phenomenon following cotransfection of HEK 293T cells with HSP27 and TBP-105Q. Moreover, we detected recruitment of Hsp70 but not Hsp27 in nuclear inclusions formed by mutant TBP in primary neurons cultured from SCA17 transgenic mice. This finding is consistent with a previous report demonstrating that, unlike HSP70 family members, HSP27 does not colocalize with intranuclear aggregates formed by the mutant version of the polyQ disease.
protein ataxin-3 in cultured cells or patient brains (38). Thus, in contrast to molecular chaperones such as HSP40 and HSP70, it is unlikely that the neuronal protection conferred by HSP27 is due to a direct effect on the accumulation of misfolded polyQ proteins.

Transcriptional roles for certain HSPs, including HSP27, have been proposed previously. HSPs have been implicated in both the assembly (26) and disassembly (39) of transcriptional complexes. HSPs can promote transcriptional activation by regulating transcription factor conformation (40, 41) and stability (26), but genomic mechanisms involving changes in the recruitment of transcription factors to promoter DNA are also plausible.

We found that HSP27 interacts with and potentiates the transcriptional activity of SP1. The interplay between nuclear HSP27 and SP1 could influence the expression of many genes, including the gene that encodes HSP27. To analyze the effect of exogenous HSP27 on its own endogenous expression, we utilized a proximal HSP27 promoter fragment that contained two TATA boxes, two estrogen receptor half-elements, two SP1 sites, and a single AP2 site (16). Exclusion of the upstream heat shock element, which normally recruits members of the heat shock factor family of transcriptional activators, indicates that the effect of HSP27 overexpression was probably independent of a previously described HSF1-mediated mechanism of HSP27 autoregulation (42).

We have also provided evidence for a broader role of HSP27 in SP1-mediated gene expression. We found that, in addition to inducing its own TATA-containing promoter, HSP27, particularly when targeted to the nucleus, stimulated SP1-dependent transcriptional activity from the TATA-less promoters of the neurotrophic receptor genes TRKA and Ngfrp75. Overexpression of either HSP40 or HSP70 failed to activate any of the gene promoters tested, indicating a specific role for HSP27 in transcriptional activation. Our data are consistent with the possibility that nuclear HSP27 functions as a transcriptional cofactor.
for SP1, but additional experiments will be needed to determine whether the interaction between the two proteins is direct. Also, it remains to be demonstrated that the small HSP is present at the promoters of certain SP1-regulated genes. Whether nuclear HSP27 modulates promoter occupancy by SP1, potentially via a cooperative interaction, should be evaluated.

There is already considerable evidence that HSP27 overexpression is protective against different types of neuronal insult or injury (12, 43). With regard to polyQ-mediated pathology, elevated expression of HSP27 attenuates mutant huntingtin toxicity in cultured mammalian cells (37), primary neurons (44), and a mouse model of Huntington disease (44). Given that SCA17 can phenocopy Huntington disease and accordingly is classified as a Huntington disease-like disease (HDL-4), these findings are probably also applicable to the toxic impact of polyQ-expanded TBP. Indeed, we have shown previously that overexpression of HSP27 is protective against neuritic defects induced by TBP-105Q in primary neurons from SCA17 transgenic mice (6). Earlier data and those presented herein indicate that neuroprotection may be conferred by both cytoplasmic and nuclear HSP27. Whereas cytoplasmic HSP27 could provide structural integrity to both the cell body and neuronal processes via interaction with cytoskeletal elements, as has been documented previously (9, 45), nuclear HSP27 may modulate the expression of certain disease-associated genes that are important for neuronal function. In SCA17 and likely other polyQ diseases, reduced availability of HSP27 in neuronal nuclei could exacerbate pathological changes in gene expression mediated by a mutant polyQ protein or fragment (46, 47). Accordingly, augmenting the function or level of nuclear HSP27 may prove beneficial in alleviating polyQ-dependent neuropathology.

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