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Aberrant Regulation and Modification of Heat Shock Factor 1 in Senescent Human Diploid Fibroblasts

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ABSTRACT

Induction of the heat shock response (HSR), determined by hsp70-luciferase reporter and HSP70 protein expression, is attenuated as a function of age of the IMR-90 human diploid fibroblasts. To better understand the underlying mechanism, we evaluated changes in the regulation and function of the HSF1 transcription factor. We show that the activation of HSF1 both in vivo and in vitro was decreased as a function of age, and this was attributable to a change in the regulation of HSF1 as the abundance of HSF1 protein and mRNA was unaffected. HSF1 was primarily cytosolic in young cells maintained at 37°C, and heat shock promoted its quantitative nuclear translocation and trimerization. In old cells, some HSF1 was nuclear sequestered at 37°C, and heat shock failed to promote the quantitative trimerization of HSF1. These changes in HSF1 could be reproduced by treating young cells with H_2O_2 to stunt them into premature senescence. Flow cytometry measurement of peroxide content showed higher levels in old cells and H_2O_2-induced premature senescent cells as compared to young cells. Experiments using isoelectric focusing and Western blot showed age-dependent changes in the mobility of HSF1 in a pattern consistent with its *S*-glutathiolation and *S*-nitrosylation; these changes could be mimicked by treating young cells with H_2O_2. Our results demonstrated dynamic age-dependent changes in the regulation but not the amount of HSF1. These changes are likely mediated by oxidative events that promote reversible and irreversible modification of HSF1 including *S*-glutathiolation and *S*-nitrosylation. J. Cell. Biochem. 106: 267–278, 2009.

KEY WORDS: AGING; HSF1; TRANSCRIPTION FACTOR REGULATION; POST-TRANSLATIONAL MODIFICATION; *S*-GLUTATHIOLATION; *S*-NITROSYLATION

Aging is a near universal phenomenon. While the mechanisms that underlie aging remain to be elucidated and defined, there is good evidence that genes and cell physiology that confer stress resistance prolong life-span [Lithgow and Kirkwood, 1996; Tatar et al., 1997; Kirkwood and Austad, 2000; Kirkwood et al., 2000]. This correlation between stress resistance and life-span suggests that a stress resistance gene may function as a longevity-assurance gene, and that resistance to stress can be used as a screen for isolating long-lived variants of the organism, as has been accomplished in *Saccharomyces cerevisiae* [Kennedy et al., 1995; Smeal and Guarente, 1997] and *Caenorhabditis elegans* [Murakami and Johnson, 1996].

One of the most studied responses to stress is induction of the heat shock transcriptional response [Morimoto, 2008]. We previously reported that induction of the heat shock response is attenuated in aging human diploid fibroblasts [Liu et al., 1989, 1996]. An attenuated response to stress is also observed in aging animal model systems and in cells/tissues derived from them [Heydari et al., 1993, 1996, 2000; Liu et al., 1996; Locke and Tanguay, 1996; Morimoto, 2008]. These considerations suggest that an attenuated heat shock response is a hallmark of aging in the dividing human diploid fibroblasts (replicative senescence), non-dividing post-mitotic cells (cellular senescence), and organisms. Given the well-known function of HSPs in protein homeostasis, including protein folding,

Abbreviations used: EMSA, electrophoretic mobility shift assay; HDF, human diploid fibroblasts; HSR, heat shock response, HSF1, human heat shock factor 1; HSP, heat shock protein; DTT, dithiothreitol; FACS, fluorescent activated cell sorting; IAA, iodoacetate; IAM, iodoacetamide; IEF, isoelectric focusing; ROS, reactive oxygen species; SIPS, stress-induced premature senescence; DCFH-DA, 2′,7′-dichlorofluorescin diacetate.

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trafficking, conformation change, and degradation, the idea that the attenuated induction of HSPs may contribute to the functional decline of the aged seems intuitive.

The present study was undertaken to gain a better understanding of the underlying mechanism of this age-dependent decrease in induction of HSPs. As HSF1 is identified as the transcription factor that mediates the heat shock response in higher eukaryotic cells [Morimoto, 1998; Wu, 1995], and as the decrease in induction of HSPs in senescent cells is correlated with a decrease in the activation of HSF1 [Choi et al., 1990; Heydari et al., 1993, 1996, 2000; Locke and Tanguay, 1996], the focus of this study is on the regulation of the HSF1 protein during aging of the IMR-90 human diploid fibroblasts. We report here that whereas the steady level of HSF1 remains unaffected in the course of aging, the regulation, function and modification of HSF1 undergo dynamic age-dependent changes. Importantly, these changes can be mimicked by treating young IMR-90 cells with a sublethal dose of H_2O_2 that stuns the cells into premature senescence, and likely involve both reversible and irreversible oxidative modification of the HSF1 protein.

MATERIALS AND METHODS

MATERIALS

Cell culture supplies were from Invitrogen, Inc. (Carlsbad, CA). The polyclonal rabbit anti-human HSF1 antibody was as previously described [Yang et al., 2008a]. PVDF membrane and Enhanced chemiluminescence reagents used for the immuno-Western blot detection of HSF1 were from GE Healthcare (Piscataway, NJ). The protein crosslinker, ethylene glycolbis(succinimidylsuccinate) (EGS, dissolved in dimethyl sulfoxide) was from Pierce Chem. Co. (Rockford, IL). The peroxide sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was from Invitrogen/Molecular Probes (Carlsbad, CA). Molecular biology reagents, including restriction enzymes, RNase were from New England Biolabs and Invitrogen, Inc. Ampholine, glutathione disulfide, glutathione, dithiothreitol, iodoacetic acid, iodoacetamide, sodium nitrite, and other chemicals were from Sigma–Aldrich Chem. Co. (St. Louis, MO).

CELL CULTURE AND PREPARATION OF CELL EXTRACTS

Early passage IMR-90 human diploid lung fibroblasts at population doubling levels (PDLs) ranging from 12 to 20 were obtained from the Coriel Institute, Camden, NJ. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were re-fed every 5–7 days and were subcultured at confluency and split at 1:4 to 1:8 ratio in fresh growth medium. Failure to grow to confluence, defined as the inability of cells to fill a 100-mm dish within a 1–2-week period occurred at PDL of approximately 42–50. In this study, cells with a PDL for 12–25 were defined as "young," and cells with PDL >40 were defined as "old."

To induce premature senescence [Toussaint et al., 2000], confluent cultures of IMR-90 cells at PDL of 20–30 were treated with 200 μM H_2O_2 in serum-free medium for 2 h at 37°C, and then refed with fresh serum-containing medium. Cells were subcultured the next day and allowed to grow to confluency or near-confluency over the next 7–10 days. Morphological examination of these stress-induced prematurely senescent cells revealed that they were indistinguishable from replicatively senescent cells with PDLs >40. The standard condition of heat shock used in this study was 42°C for duration as indicated.

Extracts of whole cells, cytosol and nucleus were prepared essentially as previously described [Baler et al., 1993; Manalo and Liu, 2001]. Briefly, for preparation of whole cell extracts (WCE), cells were harvested and the cell pellet was resuspended in 2× packed cell volume of Buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, 10 μg/ml each of leupeptin and pepstatin, and 0.01 U/ml of aprotinin). Cells were homogenized with a Dounce homogenizer (15 strokes, B-type pestle), and the homogenate was centrifuged at 12,000*g* for 15 min at 4°C to yield a whole cell extract supernatant. For preparation of the cytosol and nuclear extracts, the cell pellet was suspended in 2× packed cell volume of Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/ml each of leupeptin and pepstatin, and 0.01 U/ml of aprotinin) and Dounce homogenized (B-type pestle; 15 strokes). The homogenate was centrifuged at 10,000*g* for 10 min to yield the cytosol supernatant. The nuclear pellet was placed at −70°C for 20 min, resuspended in 1× volume of Buffer C, and allowed to thaw on ice for 10 min. This was centrifuged at 10,000*g* for 15 min at 4°C to yield the nuclear extract.

To study the in vitro activation of HSF1, S100 cell extract was prepared. For this, the cytosolic extract prepared as described above was mixed with 0.11 volume of Buffer B (0.3 mM HEPES, pH 7.9, 1.4 mM KCl, and 30 mM $MgCl_2$) and then centrifuged at 100,000*g* for 60 min. The supernatant was then removed and dialyzed against 200 volume of Buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT) for 1–2 h at 4°C. All extracts were stored in aliquots at −70°C until use. Protein determination was done using the BCA kit from Pierce Chem. Co.

ASSAY OF THE HSP70 PROMOTER DRIVEN FIREFLY LUCIFERASE REPORTER

Cells were transfected with the hsp70-firefly luciferase reporter DNA along with the internal control of phRLSV40 (synthetic humanized Renilla luciferase DNA) [Oza et al., 2008; Yang et al., 2008a,b]. Unless indicated otherwise, the amount of each DNA used was 0.5 μg/35 mm plate or 1.5 μg/60 mm plate, and the amount of Lipofectamine 2000 used (in μl) was 3× that of the total amount of DNA (in μg). Six hours after DNA transfection, cells were trypsinized and plated into individual wells of a 96 Stripwell™ plate (Corning/Costar 9102); these identically transfected cells allowed for testing of the effects of different times of heat shock on reporter gene expression. To evaluate heat shock induction of the hsp70-luciferase reporter gene, designated wells of cells were placed in a 42°C incubator for 2 h followed by recovery at 37°C for 4 h prior to harvesting. The assay is robust and allowed for semi-high-throughput screening of the effects of drugs and treatment conditions on hsp70-reporter gene expression.

The Dual-Glo luciferase assay reagent system from Promega Inc. (E2920) was used to assay for the firefly and then the Renilla

luciferase activity according to manufacturer's instructions. Luciferase activity was measured using the Perkin Elmer Victor 2 multiplate reader equipped with dual injectors. Result of the hsp70-firefly luciferase activity was normalized against that of the Renilla luciferase, and to facilitate comparison across experiments for statistical analysis this ratio was set at 1 for the 37°C control. By normalizing the hsp70-firefly luciferase activity against that of the Renilla luciferase internal control, we effectively negated experimental variables such as differences in transfection efficiency, cell number, as well as non-selective and toxic effects of the treatment conditions/reagents on gene expression. Result represents the average of four separate determinations ± standard deviation.

ASSESSMENT OF THE ACTIVATION OF HSF1

The DNA-binding activity of HSF1 was determined by electrophoretic mobility shift assay (EMSA) using a double stranded synthetic oligonucleotide containing four inverted repeats of the NGAAN consensus Heat Shock Element (HSE) sequence as previously described [Choi et al., 1990]. Briefly, the reaction mixture, contained 20–30 μg protein of extracts from control and heat shocked cells, 500 ng of poly dI-dC to block non-specific DNA-binding activity, 0.25–1 ng of [32P]HSE in a DNA-binding buffer of 10mM Tris(pH 7.8), 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT and 5% glycerol. The mixture was incubated at room temperature for 20–30 min. Protein–DNA complex was resolved from the free DNA probe by electrophoresis in a 4% polyacrylamide gel [Choi et al., 1990]. A glycerol-bromophenol blue-xylene cyanol dye solution was added to empty wells to monitor the migration of free probes.

For assessment of the in vitro activation of HSF1, aliquots of S100 cell extract were activated either by heat (42°C, 60 min) or NP-40 treatment (2%, 37°C, 60 min) according to methods previously described [Mosser et al., 1990].

Trimerization of HSF1 was determined by crosslinking of protein subunits with ethylene glycolbis(succinimidylsuccinate) (EGS, dissolved in dimethyl sulfoxide) prior to SDS–polyacrylamide gel electrophoresis and immuno-Western blot detection of HSF1 as previously described [Huang et al., 1994]. In one of the experiments presented (Fig. 4), a pore-limiting polyacrylamide (5–50%) gel electrophoresis system was used to assess the stoichiometry of HSF1 according to methods previously described [Clos et al., 1990].

IMMUNO-WESTERN BLOT DETECTION OF HSF1 AND HSP70

Samples were subjected to analysis by SDS–polyacrylamide gel electrophoresis and immuno-Western blot detection of HSF1 and HSP70 were done as previously described [Yang et al., 2008a,b]. Aliquots of cell extracts containing 10-20 μg of protein were mixed with equal volumes of a 2× sample buffer of 125 mM Tris–HCl (pH 6.8), 20% glycerol, 10 mM DTT, 2% SDS and 25 μg/ml of bromophenol blue. Samples were heated at 100°C for 5–10 min prior to loading onto the sample wells of a BioRad mini-gel apparatus. Gel electrophoresis was done as described using a 4% spacer gel and a 8% separation gel, or a 4% spacer gel and a 5–10% polyacarylamide gradient separation gel [Manalo and Liu, 2001].

After electrophoresis, the polyacrylamide gel was equilibrated at 25°C for 10 min in a buffer of 20 mM Tris, 150 mM glycine, 20% methanol, pH 8.0. Proteins were then transferred electrophoretically onto a PVDF membrane in the same buffer. Non-specific protein binding sites were blocked by incubation of the membrane with 5% nonfat dry milk in a Tris-buffered-saline-Triton buffer [TBST: 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.1% Triton X-100]. The primary antibody for both HSF1 (RTG88, 1:10,000 dilution) and HSP70 (Stressgen SPA 812, 1:10,000 dilution) were prepared in TBST with 3% nonfat dry milk. Signal detection was done using the enhanced chemiluminescence procedure as previously described [Manalo and Liu, 2001; Yang et al., 2008a].

CHEMICAL MODIFICATION OF HSF1 AND ISOELECTRIC FOCUSING GEL ELECTROPHORESIS

S-nitrosoglutathione (GSNO) was prepared by mixing 100 mM glutathione and 100 mM sodium nitrite in 200 mM HCl at room temperature as previously described [Ji et al., 1999]. Concentration of GSNO was determined by OD_{334nm}, using the extinction coefficient 767 $M^{-1}cm^{-1}$. Aliquots of whole cell extract were incubated with various concentrations of glutathione disulfide (GSSG) or freshly prepared GSNO at 37°C for 10 min as previously described [Ji et al., 1999]. Reaction was terminated with 40 mM iodoacetamide (IAM) or iodoacetate (IAA) and incubation at room temperature for 15 min. To test for reversibility of these modification reaction, dithiothreitol (final concentration 15 mM) was added to an aliquot of the reaction mixture and incubated at room temperature for 15 min.

For isoelectric focusing, a gel mixture of 50% urea, 16% acrylamide, 2.4% Ampholine (pH 4–6) was prepared. Polymerization was initiated by the addition of 10% ammonium persulfate and *N,N,N′N′*-tetramethylenediamine, and the gel mixture was immediately poured into a mini-vertical gel apparatus and a comb was inserted. Samples - in an IEF loading buffer of 8 M urea, 2% Triton X-100, 1% 2-mercaptoethanol, 0.4% bromophenol blue, 2.4% and Ampholine (pH 4–6) were loaded onto individual wells of the IEF gel. The anolyte (lower) and catholyte (upper) solution were 10 mM of phosphoric acid and 20 mM sodium hydroxide, respectively. Electrofocusing was conducted at 150 V for 30 min, then 200 V for 2.5 h. After electrofocusing, the gel was processed for transfer of the proteins onto a PVDF membrane for immuno-Western blot detection of HSF1 according to methods described.

RNASE PROTECTION ASSAY OF HSF1 AND HSF2 TRANSCRIPTS

For construction of the hsf1 riboprobe template, pRHSF1, a 307 bp *Hin*dIII–*Pml*I fragment of the pHSF1 plasmid was inserted into *Hin*dIII and *Hin*cII sites of the vector pGEM3Zf(+). For preparation of the [^{32}P]riboprobe, the *Hin*dIII-linearized pRHSF1 template was transcribed in vitro using T7 polymerase in the presence of 50 μCi of [α^{32}P]GTP. Hybridization (overnight at 50°C) of the 347-nt probe with the hsf1 mRNA gave a 295-nt RNase-resistant fragment.

For construction of the hsf2 riboprobe template, pRHSF2, a 600 bp *Kpn*I–*Sph*I fragment from the pHSF2-1 plasmid was subcloned into the pGEM3Zf(+), which was then digested with *Eco*RI and *Hin*dIII to yield a 298 bp fragment (−33 to +265). This 298 bp fragment was inserted into pGEM-2 to create pRHSF2. Linearization of pRHSF2 with *Eco*RI followed by T7 polymerase transcription in vitro gave a 321 nt long riboprobe which, when

hybridized to hsf2 transcript, gave a 265 nt RNase resistant fragment.

Total RNA was isolated from young and old IMR-90 cells according to methods described and hybridized with the [^{32}P]-labeled riboprobes overnight at 50°C [Huang et al., 1994]. After hybridization, samples were digested with RNase T1 and U2, RNA was denatured and resolved on 4% polyacrylamide–8 M urea gel, and the presence of radioactive RNA was detected by autoradiography of the dried gel.

MEASUREMENT OF REACTIVE OXYGEN SPECIES BY FLUORESCENT ACTIVATED CELL SORTER

Intracellular peroxide level was monitored using the H_2O_2-sensitive fluorescent probe 2′,7′-dichloro fluorescein diacetate (DCFH-DA) as previously described [Bass et al., 1983]. Briefly, cells in monolayer culture were trypsinized, washed and resuspended in PBS (106 cells/ml). DCFH-DA was added to the cell suspension to a final concentration of 10 mM and incubated in a 37°C shaking water bath for 30 min. The dye is nonpolar and enters cells rapidly, and once inside the cell, intracellular esterases deacetylate the dye and the polar 2′,7′-dichlorofluorescein is trapped in cells in the reduced non-fluorescent state. Intracellularly produced peroxides oxidize the reduced non-fluorescent dye to 2′,7′-dichlorofluorescein, and the resulting fluorescence is measured by flow cytometry using an EPICS Profile flow cytometer (Coulter Electronics Inc., Hialeah, FL) equipped with an Omnichrome argon laser. Green fluorescence signals were collected using 550 nm long pass dichroic and 525 nm band pass filters. Results were analyzed using EPIC Cytologic software.

RESULTS

AGE-DEPENDENT ATTENUATION IN INDUCTION OF THE HEAT SHOCK RESPONSE

In Figure 1, we used the hsp70-luciferase reporter (A) and HSP70 protein expression (B) to assess induction of the HSR in young (PDL22), middle-age (PDL36), and old (PDL48) IMR-90 cells. Our result showed that heat shock elicited a time-dependent increase in the hsp70-reporter gene activity. Most importantly, we showed that the heat shock induction of the reporter gene was inversely proportional to the "age" of the cells such that after a 2 h at 42°C followed by recovery at 37°C for 4 h there was an average of 12-, 6-, and 3.8-fold increase in reporter gene activity over that of the 37°C control for cells at PDL 22, 36, and 48, respectively.

In Figure 1B, we used Western blot to determine the amount of HSP70 of control (37°C) and heat shocked (42°C followed by recovery at 37°C for 6, 8, 12, and 16 h) IMR-90 cells with PDLs of 22,

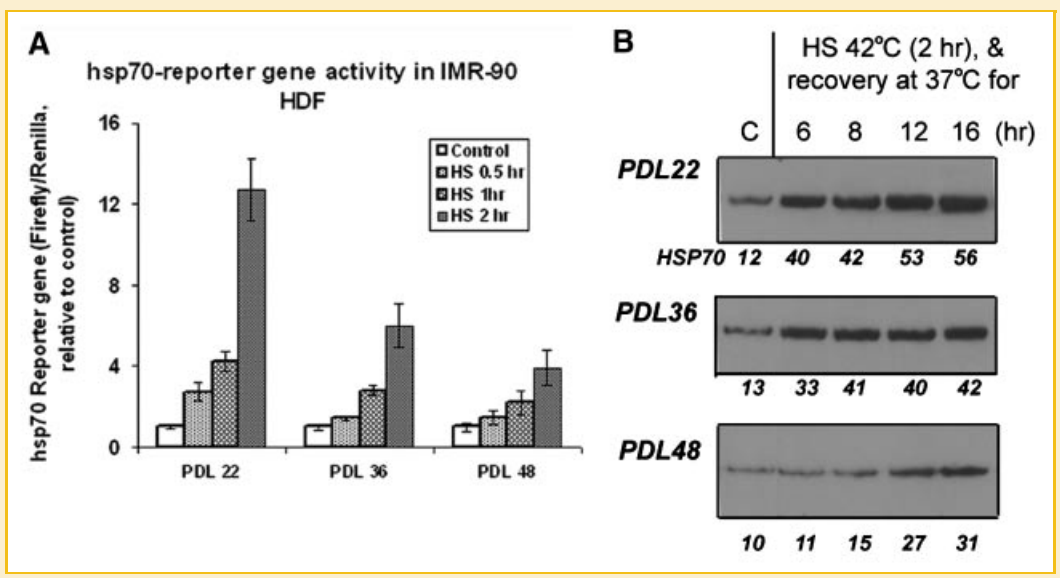

Fig. 1. Age-dependent decrease in induction of the hsp70-luciferase reporter and HSP70 protein. A: Hsp70-luciferase reporter gene activity in young (PDL 22), middle-age (PDL 36), and old (PDL 48) IMR-90 cells under control (37°C) and heat shock (42°C) conditions. IMR-90 cells in 35 mm dishes at PDLs indicated were transfected with the hsp70-firefly luciferase DNA along with the internal Renilla luciferase DNA control according to methods described in the text. Cells were plated into wells of a 96 Stripwell® plate after DNA transfection according to methods described [Yang et al., 2008a]. For heat shock, strips or designated wells of cells were placed in 42°C incubated for times for 0.5, 1, and 2 h followed by recovery at 37°C for a total of 6 h (e.g., cells that were heat shocked for 0.5 h were kept at 37°C for an additional 5.5 h, and cells heat shocked for 2 h were kept at 37°C for 4 h). Assay of Firefly and Renilla luciferase activity were done as described previously [Yang et al., 2008a]. Results, presented as a ratio of firefly/Renilla luciferase relative to that of the 37°C control of 1, represent the average of four separate determination ± standard deviation. B: Immuno-Western blot detection and quantitation of the 72 kDa heat-inducible HSP70 protein of control (C) and heat shocked (HS: 2 h at 42°C followed by recovery at 37°C for 6, 8, 12, and 16 h) young (PDL22), middle-age (PDL 36), and old (PDL 48) IMR-90 cells. The procedure of SDS–gel electrophoresis and Western blot detection of HSP70 are as described in the text. The relative amount of the HSP70 protein, determined by Image J analysis, is shown at the bottom of each panel.

36, and 48. The results show that the induction of HSP70 protein was reduced by about 50% in the old cells (PDL 48) when compared to that of the young cells (PDL 22). These results corroborate earlier observation in various aging models, from cells to mammals, to support the thesis of an age-dependent decrease in induction of the HSR [Liu et al., 1989; Choi et al., 1990; Heydari et al., 1993, 1996, 2000; Locke and Tanguay, 1996; Jurivich et al., 1997; Gutsmann-Conrad et al., 1999; Lee et al., 1999b].

AGING ADVERSELY AFFECTED THE ACTIVATION BUT NOT THE EXPRESSION OF HSF1

Previous studies from our laboratory demonstrated that the heat-induced activation of HSF1 DNA-binding activity was inversely proportional to the age of the IMR-90 human diploid fibroblasts [Choi et al., 1990], and similar results have been reported using cells and tissues of young versus old laboratory animals [Heydari et al., 1993, 1996, 2000; Locke and Tanguay, 1996]. In Figure 2A, we show

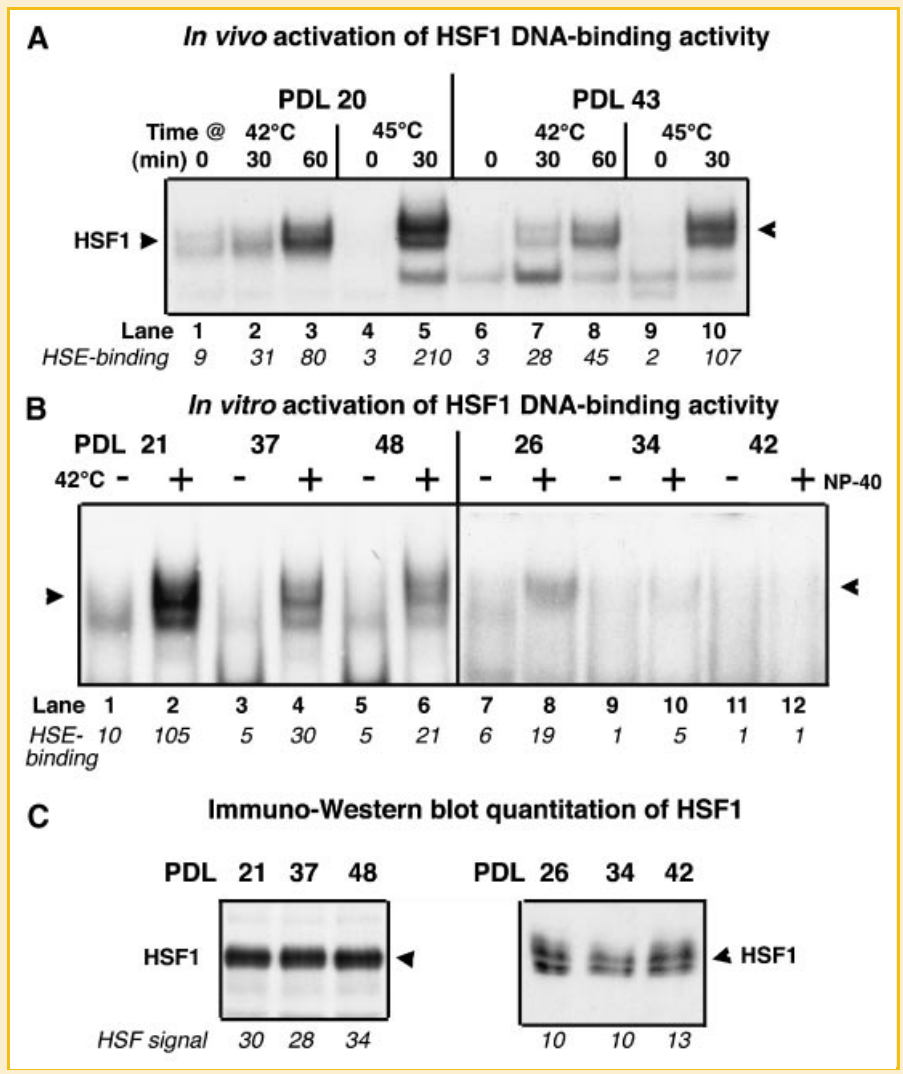

Fig. 2. Activation of HSF1 is attenuated as a function of age of the IMR-90 human diploid fibroblasts. A: In vivo activation of HSF DNA-binding activity. Confluent cultures of IMR-90 at PDL of 20 and 43 were placed in incubators set at 37, 42, and 45°C for time periods indicated. Cells were harvested and whole cell extracts prepared according to method described in the text. Aliquots of whole cell extracts containing 20 μg proteins were used to assay for protein binding to [^{32}P]HSE. The position of the specific HSF-HSE complex is as indicated. HSE-binding activity, quantitated by densitometric scanning of the autoradiogram is indicated at the bottom of the figure. B: In vitro activation of HSF DNA-binding activity by heat and NP-40. Confluent cultures of IMR-90 cells grown at 37°C with PDLs indicated (PDL 21 = young; PDL 37 = middle age; PDL 48 = old) were harvested and S100 extracts prepared. Aliquots of the cell extracts containing the same amount of HSF1 as determined by immuno-Western blotting (see Fig. 2C) were used to assess the "activatability" of HSF1 by heat and NP-40. For in vitro heat activation (lanes 2,4, and 6 of Fig. 2B), cell extracts were incubated at 42°C for 60 min. For NP-40 activation (lanes 8, 10, and 12 of Fig. 2B), extracts were incubated with 2% NP-40 at 37°C for 60 min. Parallel samples incubated at 37°C without NP-40 served as controls (the "−" samples). The position on the gel of the specific HSF-HSE complex is as indicated. The relative HSE-binding activity, quantitated by densitometric scanning of the autoradiogram, is indicated at the bottom of the figure. C: Immuno-Western blot probing for HSF1. Aliquots of the S100 extract, identical to that used in the experiment shown in Figure 2B, were used for immuno-Western blot probing for HSF1. The position on the gel of the HSF1 protein is as indicated. (*Note*: The HSF1 band was resolved into two in the right panel of Fig. 2C because of the use of a lower % acrylamide separation gel.) The relative abundance of the HSF1 protein, quantitated by densitometric scanning of the fluorogram, is indicated at the bottom of the figure.

that activation of the HSF1 DNA-binding activity was dependent on the time (30 and 60 min) and severity (42°C vs. 45°C) of heat stress and was inversely proportional to the "age" of the cells used. To evaluate if the decreased activation of HSF1 in senescent cells may be due to age-dependent changes in the heat shock signal transduction events and required the integrity of cell or if it is due to a change in the inherent ability of the HSF1 to be activated, we used an in vitro activation assay to test for the "activatability" of HSF1 [Mosser et al., 1990]. Result in Figure 2B show that the in vitro activation of HSF1 by heat (42°C, 60 min; lanes 1–6) or NP-40 (2%, 37°C. 60 min; lanes 7–12) was inversely correlated with the age of cells from which the extracts were derived. As shown in Figure 2C, the relative concentration/abundance of HSF1 in the S-100 cell extracts used for the in vitro activation assay in Figure 2B was similar.

Figure 3 shows that neither the amount of the HSF1 protein nor the transcript encoding for HSF1 changed as a function of age.

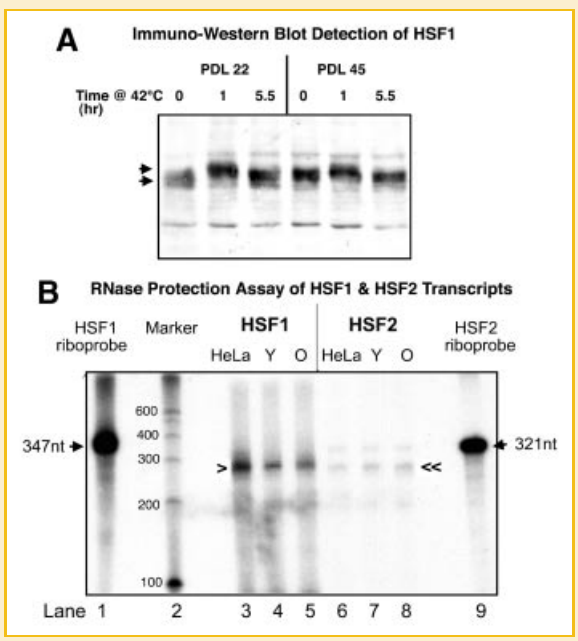

Fig. 3. Quantitation of the steady state level of HSF1 protein and transcript in young and old IMR-90 cells. A: Immuno-Western blot quantiation of HSF1 present in whole cell extracts of young (PDL 22) and old (PDL 45) IMR-90 cells incubated under control (37°C) and heat shocked (42°C) condition. Confluent cultures of IMR-90 cells were heat shocked at 42°C for 1 and 5.5 h. Cells were harvested and whole cell extracts prepared. Aliquots of the whole cell extract containing 20 μg protein were used for SDS–polyacrylamide gel electrophoresis and immuno-Western blot probing for HSF1. The positions on the fluorogram of the normal and the heat-induced hyperphosphorylated HSF1 protein (P-HSF1) are as indicated. B: Quantitation of the HSF1 and HSF2 transcripts in young and old IMR-90 cells by RNase protection assay. Confluent cultures of young (Y; PDL 20) and old (O; PDL 48) IMR-90 cells were used to isolated total RNA according to methods described. Twenty-five micrograms of the RNA was used in the RNase protection assay. Hybridization of the HSF1 and HSF2 mRNA with the 347 nt HSF1 (lane 1) and 321 nt HSF2 (lane 9) [^{32}P]riboprobe resulted in the protection, respectively, of a 295- and 265-nt RNA fragment, and these are indicated by a single and a double arrow head in the figure. For control, total RNA from HeLa cells were included in the analyses of HSF1 and HSF2 transcripts (lanes 3 and 6).

Figure 3A of Western blot detection and quantitation of HSF1 in whole cell extracts and showed that the abundance of HSF1 was not significantly affected by either heat shock or age of the cells. This observation is consistent with results previously published [Fawcett et al., 1994]. The upward shift (retarded mobility) of HSF1 after 1 h (but not 5.5 h) heat shocked was the result of a transient, heat induced hyperphosphorylation of HSF1, and there was no significant difference in this hyperphosphorylation event between the young and old cells (Fig. 3A, lanes 2 and 5). Analysis of the steady state level of HSF1 and HSF2 transcripts in young and old IMR-90 cells by RNase protection assay in Figure 3B revealed no significant difference. In this experiment, RNA isolated from HeLa cells was included as a positive control (Fig. 3B, lanes 3 and 6), and we note that the level of HSF1 transcript in HeLa cells was significantly higher than that of the IMR-90 cells, a result consistent with quantitation of the HSF1 protein by Western blotting (data not shown). Together, the results in Figures 2 and 3 show that aging of human diploid fibroblasts adversely affected the activation of HSF1 without affecting the expression of HSF1 transcript and protein.

HSF1 IS SEQUESTERED AS AN INACTIVE MONOMER IN THE NUCLEAR COMPARTMENT OF AGING CELLS

Activation of HSF1 is a multistep process that includes nuclear translocation, trimerization, and phosphorylation, and each of these steps may be independently regulated [Baler et al., 1993; Sarge et al., 1993; Zuo et al., 1994, 1995]. As the nuclear translocation of HSF1 appears to be a stress-regulated event and as aberrant subcellular localization of transcription factors can have important biological consequences [Chen et al., 1995], we determined the cytosolic versus nuclear distribution of HSF1 in control- and heat shock-young and old IMR-90 cells. In Figure 4A, we showed that while HSF1 was present in both the cytosol and nuclear fractions of the young and old IMR-90 cells, the relative abundance of HSF1 in the nuclear compartment was increased in the old cells (Fig. 4A, lanes 3 and 4). Analysis of the stoichiometry of this nuclear targeted HSF1 in unstressed cells by protein cross linking showed that it was primarily monomeric (Fig. 4B, sample 4). While heat shock promoted the nuclear targeting of >90% of the HSF1 in both young and old cells (samples 7 and 8, Fig. 4B), the trimerization of HSF1 was reduced by >60% in the old cells as compared to that of the young cells (Fig. 4B: samples 7 and 8, 43 versus 125). The quality of the cytosol versus nuclear extracts was validated by the presence of a nuclear-specific 55 kDa HSF1 signal (Fig. 4A,B) as we have previously observed [Huang et al., 1994]; the identity of this protein is not known. Analysis of the stoichiometry of HSF1 of young and old cells by pore-limiting gel electrophoresis in Figure 4C corroborated results obtained by protein-crosslinking of Figure 4B: that heat shock promoted the trimerization of HSF1 in the young cells and this trimerization was reduced in the old cells.

TREATMENT OF CELLS WITH HYDROGEN PEROXIDE REPRODUCED AGE-DEPENDENT CHANGES IN HSF1

We are interested in the underlying cause and the biological relevance of these changes of HSF1 in senescent IMR-90 cells. We

note that while the central cause of replicative senescence remains to be elucidated, it nevertheless has become clear that damaging agents or conditions, notably oxidants [Chen and Ames, 1994; Chen et al., 1998; Chen, 2000; Dumont et al., 2000; Toussaint et al., 2000], inhibitors of histone deacetylase [Ogryzko et al., 1996] and PI3-kinase [Tresini et al., 1998] can induce features of senescence in early passage young diploid fibroblasts. This phenomena is termed stress-induced premature senescence (SIPS) [Chen and Ames, 1994; Chen et al., 1998; Chen, 2000; Dumont et al., 2000; Toussaint et al., 2000]. In the case of hydrogen peroxide induced SIPS, this involves the development of multiple features that include cell cycle arrest, cell enlargement, reduced saturation density, inability to induce ornithine decarboxylase and thymidine kinase upon serum stimulation, activation of the senescent maker β-galactosidase, etc.; namely, features that are indistinguishable from replicative senescent cells [Chen, 2000].

Due to the compressed and relatively well defined time frame for cells to develop this prematurely senescent phenotype, the H_2O_2-treated HDF provides a useful experimental system to probe the underlying mechanism of senescent-dependent changes in regulation and function of HSF1. This being given, we treated young IMR 90 cells (PDL < 25) with 200 μM H_2O_2 for 2 h at 37°C. Morphological observation of the treated cells revealed no visible signs of cell death. The treated cells were then replenished with fresh medium, subcultured and allowed to grow at 37°C to near confluency (7–10 days) prior to experimentation. Figure 5 showed that this H_2O_2-treatment caused an attenuation in the heat-induced activation of HSE-binding activity (Fig. 5A), and mobilized HSF1 to locate to the nuclear compartment at 37°C (Fig. 5B, compare lanes 3 and 4). The total amount of HSF1 was unaffected by this treatment (Fig. 5B, lanes 5 and 6; whole cell extract). Analysis of cellular reactive oxygen species using the peroxide sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) in Figure 6 showed a higher peroxide content in old cells compare to young (by 0.5 log unit), and in H_2O_2 treated prematurely senescent cells compare to the untreated control (by 0.45 log unit). Our observation corroborated that of a previous study where it was shown that the level of intracellular H_2O_2 correlates with senescence [Lee et al., 1999a]. Together, the results in Figures 5 and 6 suggest that the aberrant regulation of HSF1 observed in the replicative senescent cells could be reproduced in the H_2O_2-induced premature senescent cells, and that both the replicative senescent cells and the H_2O_2-induced premature senescent cells have a more oxidizing cellular milieu than that of the young cells.

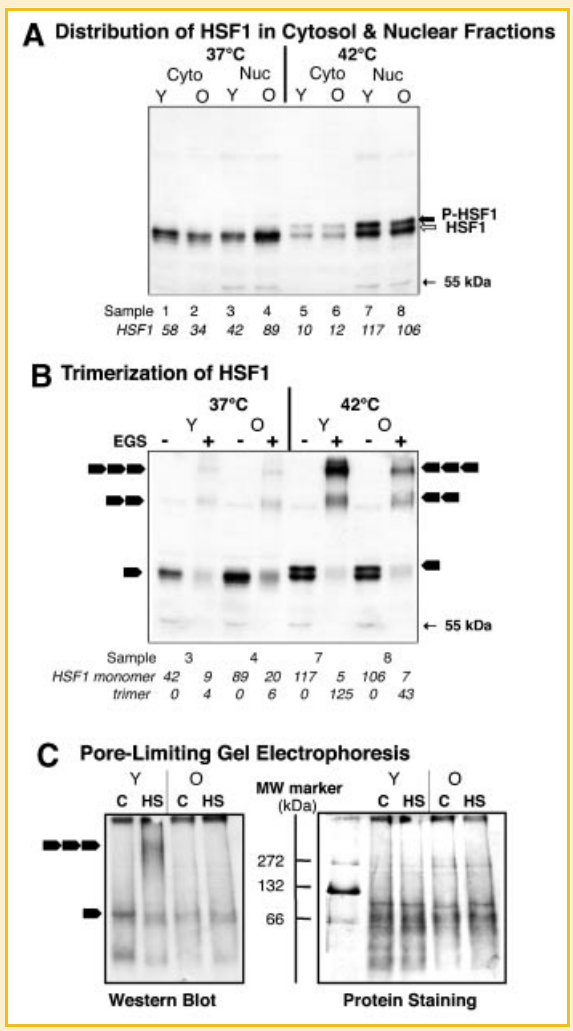

Fig. 4. Subcellular distribution and stoichiometry of HSF1 in extracts of young (Y; PDL 18) and old (O; PDL 46) IMR-90 cells incubated under control and heat shock conditions. A: Distribution of HSF1 in the cytosol and nuclear fractions of control and heat-shocked IMR-90 cells. Confluent cultures of young and old IMR-90 cells incubated under control (37°C) and heat shocked (42°C, 2 h) were harvested and used for the preparation of cytosol and nuclear extracts according to methods described in the text. Aliquots of the cytosol and nuclear extracts containing 20 μg protein were loaded onto a SDS–polyacryladmide (5–10% gradient) gel followed by immuno-Western blot probing for the HSF1 protein. The position of the "normal" and the "hyperphosphorylated" forms of HSF1 are indicated as HSF1 and P-HSF1, respectively. The relative abundance of HSF1, determined by densitometric scanning of the fluorogram, is as indicated at the bottom of the figure. B: Trimerization of HSF1. To assess the stoichiometry of HSF1, aliquots of samples 3, 4, 7, and 8 of Figure 3A were incubated for 30 min at 25°C without and with the protein crosslinker ethylene glycolbis(succinimidylsuccinate) (EGS), and the reaction was then quenched by the addition of 75 mM glyine. The mixture was subjected to analysis by SDS–polyacrylamide (5–10%) gel electrophoresis and immuno-Western blot probing for HSF1. The position on the fluorogram of the HSF1 monomer (both the "normal" and "hyperhosphorylated" forms), dimer, and trimer are indicated by a single, double, and triple arrow heads, respectively. The relative abundance of the HSF1 monomer and trimer is as indicated at the bottom of the figure. *Note*: the lower than expected signal of the HSF1 monomer in the EGS-treated samples is due to crosslinking of amino groups of the HSF1 monomer rendering the protein difficult to detect by the antibody as we have previously reported [Manalo and Liu, 2001]. C: Assessment of the stoichiometry of HSF1 by pore-limiting gel electrophoresis. Aliquots of whole cell extracts from young and old IMR-90 cells containing 25 μg protein were electrophoresed on a nondenaturing 5–50% polyacarylamide gel until the limit of migration was reached [Clos et al., 1990]. Results of immuno-Western blot probing for HSF1 is shown on the left and the pattern of Coomassie blue staining of protein on the right. The positions of the HSF1 monomer and trimer are indicated by a single and triple arrow head, respectively. The positions of marker proteins with apparent molecular weights of 272, 132, and 66 kDa are as indicated.

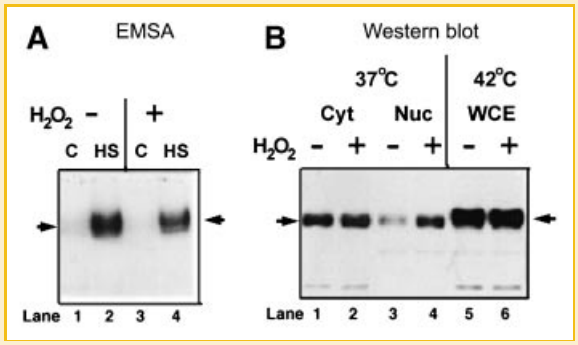

Fig. 5. Treatment of young IMR-90 cells with hydrogen peroxide induced changes in HSF1 similar to that observed in senescent IMR-90 cells. A: HSF DNA-binding activity. To induce the premature senescence of IMR-90 cells, cells at PDL 20 were treated with 200 μM H_2O_2 for 2 h at 37°C followed by recovery in normal serum-containing growth medium for 7 days. Parallel plates of cells not treated with H_2O_2 served as control. Cells were heat shocked at 42°C for 2 h. Aliquots of whole cell extract were used to determine HSF1 DNA-binding activity by EMSA. The specific HSF-HSE complex is indicated by an arrow. The relative HSE-binding activity of the heat shocked cells without and with H_2O_2-pretreatment was 43 and 21, respectively. B: Immuno-Western blot quantitation of HSF1. Control and H_2O_2-pretreated cells, as described in the legend of (A), were used. Cells were harvested and cytosol and nuclear extracts prepared. Aliquots of cell extracts containing10 μg protein were used for HSF1 Western blot analysis. The position of the HSF1 protein is as indicated. Lanes 5 and 6 represent 20 μg aliquots of whole cell extracts of heat-shocked cells without and with H_2O_2-pretreatment (lanes 2 and 4 of A).

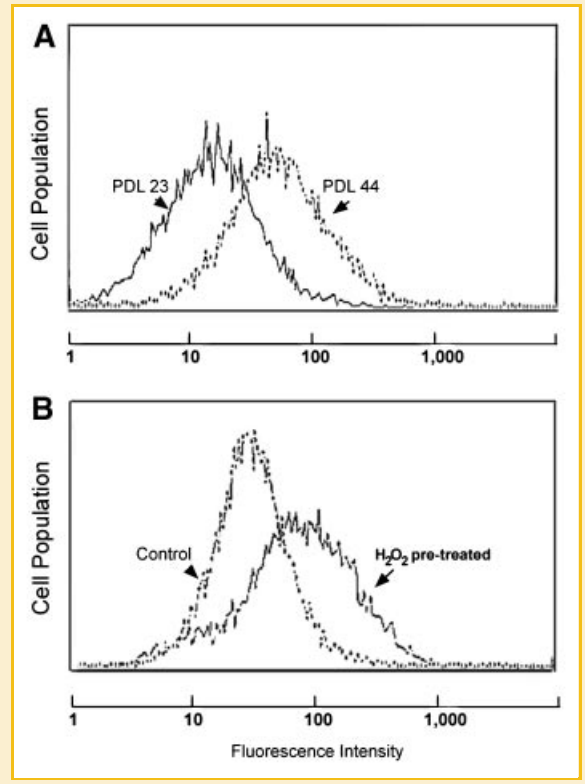

Fig. 6. FACS analysis of peroxide content in IMR-90 cells. Peroxide content was determined using the fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) according to methods previously described [Bass et al., 1983]. A: Peroxide profile in young and old IMR-90 cells. IMR-90 cells with PDL of 22.6 and 44 were trypsinized and loaded with DCFH-DA. Intracellular production of peroxides reduced the non-fluorescent DCFH-DA to the highly fluorescent 2′,7′-dichlorofluorescein (DCF), which was quantitated by a EPICS Profile flow cytometer (Coulter Electronics Inc., Hialeah, FL). B: Peroxide profile in control and H_2O_2 -pretreated IMR-90 cells. Control and H_2O_2-pretreated IMR-90 cells (PDL 24) were used. Cells were trypsinized and loaded with DCFH-DA and the production of ROS determined by flow cytometry. Results were plotted using the EPIC cytologic software.

OXIDATIVE MODIFICATION OF HSF1 IN SENESCENT CELLS

Protein sulfhydryls can be oxidized to several states including protein disulfide, *S*-thiolation, *S*-nitrosylation, and sulfenic acid as well as more highly oxidized (and irreversible) states such as sulfinic and sulfonic acid forms of proteins cysteines. To evaluate if HSF1 may become oxidatively modified in the senescent HDF, we adopted a chemical modification procedure that can be combined with gel isoelectric focusing to detect isoforms of HSF1 [Ji et al., 1999; Hamann et al., 2002]. As schematically illustrated in Figure 7A, modification of protein by *S*-glutathiolation followed by alkylation of free cysteine-SH with the charge neutral iodoacetamide (IAM) would result in an acidic shift in IEF compared to the unmodified protein, whereas modification by *S*-nitrosylation followed by alkylation of free cysteine-SH with the negatively charge iodo-acetate (IAA) would result in an alkaline shift. Figure 7B presents the result of two experiments validating the use of these methods for the detection of *S*-glutathiolated and *S*-nitrosylated HSF1 proteins. We show that the extent of *S*-glutathiolation and *S*-nitrosylation of HSF1 was dependent, respectively, on the dose of GSSG and GSNO used. Further, the reaction could be reversed by the addition of a SH-reducing reagent, dithiothreitol, prior to analysis by IEF and Western blotting (Fig. 7B, lanes 7 and 14). In Figure 7C, aliquots of whole cell extracts from young, old, and H_2O_2-induced premature senescent IMR-90 cells were incubated with iodoacetamide (IAM) and iodoacetate (IAA) to alkylate unmodified protein-SH prior to IEF. First, we note that HSF1 in extract of the young cell alkylated with either IAM or IAA migrated as a doublet in IEF. HSF1 of old IMR-90 cells alkylated with IAM migrated as two bands on IEF, one of which was clearly shifted toward the acidic end of the IEF gel—a pattern consistent with *S*-glutathiolation of HSF1 (Fig. 7C, lanes 1 and 2), and this shift was reversed in part by the addition of 10 mM DTT prior to IEF (Fig. 7C, lanes 2 and 3). HSF1 of old IMR-90 cells alkylated with IAA also migrated as two bands, one of which was shifted toward the alkaline end of the IEF gel (Fig. 7C, lanes 7 and 8). The pattern is consistent with a charge neutral modification of HSF1 in the old cells—such as *S*-nitrosylation. Unlike *S*-nitrosylation, however, the alkaline shift was not reversed by DTT (Fig. 7C, lane 8 and 9). Importantly, treatment of young IMR-90 cells with H_2O_2 in a protocol that promoted the premature senescence of the cells reproduced the HSF1 charge shift observed in extracts of the old cells (Fig. 7C, lanes 4–6 and 10–12), and these changes were completely reversed by the addition of DTT prior to IEF analysis (Fig. 7C, lanes 11 and 12). Together, these results provide evidence of both reversible and irreversible oxidative modification of the HSF1 protein in senescent IMR-90 HDF in a pattern consistent with *S*-glutathiolation and *S*-nitrosylation.

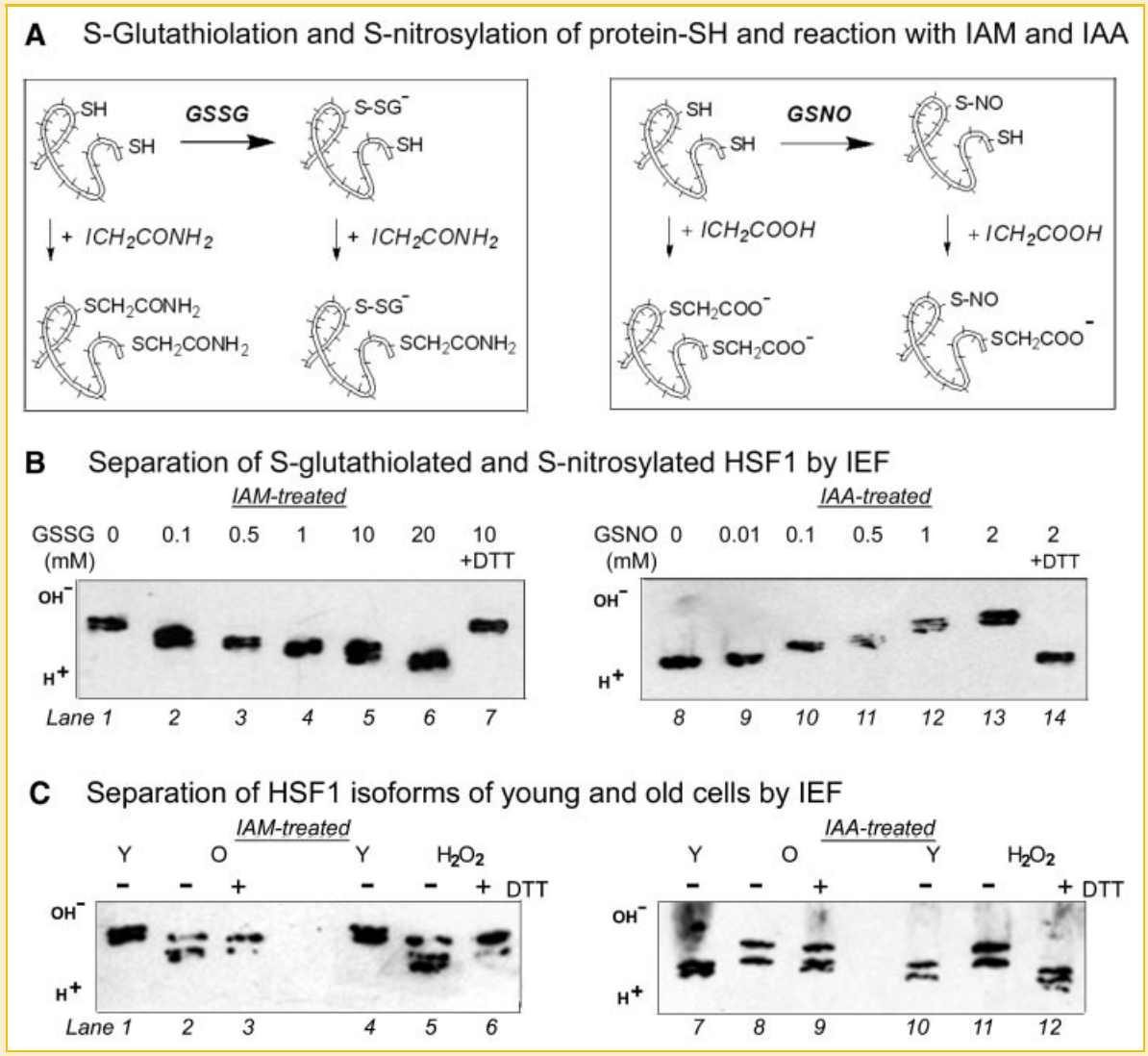

Fig. 7. Chemical modification and detection of charge isoforms of HSF1. A: Schematics illustrating the modification of proteins by *S*-glutathiolation and *S*-nitrosylation followed by the alkylation of free cysteine-SH with iodoacetamide and iodoacetate to generate charge variants of the protein that can be resolved by thin isoelectric focusing. B: Separation and detection of *S*-glutathiolated and *S*-nitrosylated HSF1 by thin isoelectric focusing. Aliquots of a sample of whole cell extract were incubated with the indicated concentrations of glutathione disulfide (GSSG; lanes 1–7) and nitrosoglutathione (GSNO; lanes 8–14) at 37°C for 10 min. The reaction was terminated and free cys-SH alkylated by the addition of iodoacetamide (IAM) or iodoacetate (IAA) according to methods described in the text. DTT was added to a sample to test for reversibility of the modification reaction. Samples were prepared by thin isoelectric focusing and Western detection of HSF1 according to methods described in the text. The basic and acidic end of the IEF gradient is indicated by the OH^- and H^+ symbol, respectively. C: Separation and detection of isoforms of HSF1 in whole cell extracts of young (PDL 22), old (PDL 43), and H_2O_2-induced pre-mature senescent (PDL 24) IMR-90 cells. Aliquots of whole cell extracts were alkylated with IAM or IAA as indicated. To test for reversibility of the modification, DTT was added to an alkylated sample to a final concentration of 15 mM and incubated at room temperature for 15 min. All samples were subjected to analysis by thin IEF and Western blot detection of HSF1 according to methods described in the text.

DISCUSSION

Cell maintenance and stress response mechanisms appear to have primary and important roles in regulating the duration of life. Thus, there is a body of literature evidence that "stress-resistance" is directly correlated with and in fact serves as a predictor of the "longevity" of a number of model systems [Kapahi et al., 1999; Kirkwood and Austad, 2000; Kirkwood et al., 2000]. Furthermore, mutations that confer an extended life-span of *C. elegans* and yeast also afforded a proportional increase in resistance to oxidative stress, thermal stress, and UV radiation [Lithgow and Kirkwood, 1996; Murakami and Johnson, 1996; Smeal and Guarente, 1997; Tatar et al., 1997].

Induction of the heat shock response is a ubiquitous and primary response to stress, the increased production of HSPs function as molecular chaperones and contribute importantly to cellular stress resistance. It has been shown that increased expression of HSPs by either genetic or environmental manipulations increased lifespan in

C. elegans [Lithgow et al., 1995]. Furthermore, while the environmental induction of HSP70 expression is transient, its effect on age-specific survival is persistent. Perhaps, a transient expression of HSPs facilitated the repair, assembly, and degradation of damaged cellular proteins, the accumulation of which would otherwise accelerate aging [Lithgow et al., 1995; Tatar et al., 1997]. The observation of an attenuated HSR in the aging cells and organisms is consistent with the theme of a correlation of stress resistance and longevity, and suggest that the decreased induction of HSPs would contribute to increased morbidity and mortality of the aged.

Induction of the HSR is mediated by the activation of HSF1, and there is good evidence that the attenuated HSR in aging cells is correlated with a decreased activation of HSF1 [Choi et al., 1990; Heydari et al., 1993, 1996, 2000; Fawcett et al., 1994; Liu et al., 1996; Locke and Tanguay, 1996; Jurivich et al., 1997]. In this study, we show that while the steady state level of the HSF1 mRNA and protein do not change with age, its subcellular distribution and ability to undergo activation in vivo and in vitro are dynamically regulated in an age-dependent fashion. In the aging cells, a measurable portion of the HSF1 is sequestered in the nuclear compartment as monomer that appeared to be insensitive to heat-induced activation and trimerization. The possibility that oxidation mediates these changes of HSF1 is supported by the following considerations: (a) The age-dependent changes in the distribution and function of HSF1 can be reproduced by treatment of young cells with a sublethal dose of hydrogen peroxide, a condition known to induce premature senescence of human diploid fibroblasts. (b) There is an increase in the intracellular peroxide content in both the replicative senescent and the hydrogen peroxide-induced premature senescent cells. (c) The use of IEF coupled with Western blot revealed oxidative modification of cysteine-SH of HSF1 from senescent IMR-90 cells.

Our results show that HSF1 is post-translationally modified in the old cells. The modification includes events that add negative charge such as *S*-glutathiolation as well as events that are charge neutral events such as *S*-nitrosylation. Some of the modification appeared reversible in that the addition of DTT erased the charge shift, and others appeared irreversible. This is an important finding as it provides evidence of oxidative modification of a specific transcription factor in cell aging. Protein glutathiolation, which involves the formation of a mixed disulfide between a protein cysteinyl residue and glutathione, can occur in response to oxidative and/or nitrosative stress in intact cells in the presence of a large pool of glutathione; it has been suggested that protein-glutathiolation may serve to transducer the oxidative and nitrosative stimuli into a functional response at various levels of cellular signaling [Klatt and Lamas, 2000]. In aging human lenses, the increase in protein *S*-glutathiolation is correlated with lens nuclear color and opalescence intensity [Lou et al., 1999]. Protein nitrosylation can occur under conditions of ''nitrosative stress'' as in inflammation, neurotoxicity, and ischemia [Stamler et al., 2001; Hess et al., 2005]. In our analysis of HSF1, we note that the charge neutral modification of HSF1 in the senescent IMR-90 cells was not reversed by DTT, suggesting additional modification of the nitrosylated-HSF1. Perhaps, mass spectrometry analysis can provide molecular and chemical details of the modified HSF1 in senescent cells.

The mechanism and biology of the nuclear sequestration of HSF1 monomer in the aging cells are not entirely clear as previous results suggest that oligomerization of HSF1 is required for nuclear transport and there is a close correlation between the subcellular localization and the oligomeric state of the factor [Zuo et al., 1995; Zandi et al., 1997]. One possible explanation is that HSF1 monomer sequestered in the nucleus may represent deactivated HSF1 whose exit from the nucleus is blocked or compromised in the aging cells perhaps as a result of reversible/irreversible oxidative modification. We note that nuclear accumulation of the yeast transcription factor Yap 1, which activates expression of antioxidant genes in response to oxidative stress, is attributed to intramolecular disulfide bond formation, which results in a change of conformation that inhibits nuclear export [Delaunay et al., 2000]. We further note that a recent study on the hypoxia-inducible factor (HIF) provided clear evidence that the trafficking of the factor from the nucleus to the cytosol is intimately linked to the oxygen-dependent ubiquitination of the protein [Groulx and Lee, 2002].

The concept that biological readout is regulated not only by genetic (i.e., transcription and translation) mechanisms that control the profile and level of genes expressed, but also by epigenetic mechanisms such as post-translational modification that change the function, conformation, and localization of individuals proteins is not new. Prions and amyloid-β are just two of best known examples that changes in the conformation, post-translational modification, and aggregation states of proteins can have significant and dire biological consequences [Sherman and Goldberg, 2001; Morimoto, 2008]. We show here that aging affects the function, distribution, and modification but not the steady state cellular level of HSF1. These observations underscore the importance of understanding the function, conformation and localization of proteins, perhaps as much as their expression, in our analysis of biological pathways and regulation, aging included.

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