Journal of Cellular Biochemistry 98:1528–1542 (2006)

Heat Shock Transcription Factor (Hsf)-4b Recruits Brg1 During the G1 Phase of the Cell Cycle and Regulates the Expression of Heat Shock Proteins

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Abstract Human brahma-related gene 1(*Brg1*) is a subunit of the switching/sucrose non-fermenting (SWI/SNF) chromatin-remodeling complex and regulates transcription during cell growth and differentiation and has been found to be mutated in many types of human cancers. Mammalian heat shock factor 1 (Hsf1), which binds conserved sequences on the promoter of the *hsp70* gene when cells are exposed to various stress stimuli, utilizes Brg1–SWI/SNF complexes and stimulates transcription in vitro at the level of initiation and elongation. In contrast to the stress-inducibility of Hsf1, in vitro transcribed/translated Hsf4b binds to the heat shock element (HSE) constitutively and loses its ability to bind HSEs following stress. The regulation of Hsf4b transcriptional activity in vivo remains unclear. Here, we present evidence that Hsf4b recruits Brg1 complexes to the promoters of heat shock proteins (HSPs) under physiological growth conditions. Furthermore, in an asynchronous cell population, the association of Hsf4b with Brg1 complexes is regulated in response to activation/inactivation of the extracellular signal regulated protein kinase 1/2 (ERK1/2) signaling pathway. Since Brg1 is also the target of mitogen-activated protein (MAP) kinases and other protein kinases and it is hyperphosphorylated and inactivated during the G2/M phase of the cell cycle, we tested whether the association of Hsf4b with Brg1 complexes is altered during the cell cycle. The results indicate that association of Hsf4b with Brg1 complexes is undetectable during G2/M; however, an Hsf4b interaction with Brg1 complexes is evident at 1–3 h after progression of cells into G1, where chromatin structure is presumed to be more accessible to transcriptional regulatory proteins. At this time, Hsf4b exhibits increased DNA-binding activity and is detectable on promoters of multiple Hsps. To determine the unique role of Hsf4b in stimulating the expression of Hsps during the cell cycle, experiments were conducted with mouse embryo fibroblasts (MEFs) deficient in individual Hsfs. The results indicate that in the absence of Hsf1 and Hsf2, Hsf4b expression in cells leads to increased ability of Hsf4b to bind HSE during G1, leading to enhanced synthesis of inducible Hsp70. J. Cell. Biochem. 98: 1528–1542, 2006.

Key words: Hsf4b; Brg1; cell cycle; transcription

The heat shock transcription factor (Hsf) family regulates gene expression via binding to conserved sequences present in the promoters of heat shock genes. The function of mammalian Hsfs are unique since Hsf1 is transcriptionally active when cells are exposed to stresses such as heat shock, heavy metals, or hypoxia [Kingston et al., 1987; Wu, 1995; Morimoto, 1998]. Hsf2 is active during spermatogenesis and in the developing central nervous system [Rallu et al., 1997; Kallio et al., 2002; McMillan et al., 2002; Wang et al., 2003, 2004]. Hsf2 protein is in low abundance in cells but accumulates following treatment of cells with inhibitors of the ubiquitin proteosome pathway. These results suggest an inter-relationship between Hsf2 activity and alterations in the

Abbreviations used: Hsf, heat shock factor; HSE, heat shock element; Hsp, heat shock protein; MEF, mouse embryo fibroblasts; Brg1, brahma-related gene 1; Brm, brahma; SWI/SNF, mating type switching/sucrose non-fermenting; MAP kinases, mitogen activated protein kinases; ERK1/2, extracellular signal regulated protein kinase 1/2; JNK, c-Jun-N-terminal kinase; DUSP26, dual specificity phosphatase 26; GST, glutathione-S-transferase; ChIP, chromatin immunoprecipitation assay.

Grant sponsor: National Institutes of Health grants; Grant numbers: GM0707451, CA62130.

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Received 2 September 2005; Accepted 26 January 2006
DOI 10.1002/jcb.20865

ubiquitin proteosome pathway [Mathew et al., 1998]. Hsf2 has also been found to be sumoylated, and this modification leads to increases in its DNA-binding activity [Goodson et al., 2001]. Recent studies indicate that Hsf4 expression and transcriptional activity can be initially detected during the development of the lens and in the lens fiber cells between postnatal (P) days P1–P5 and its activity continues throughout adult life [Fujimoto et al., 2004; Min et al., 2004; Somasundaram and Bhat, 2004]. Consistent with Hsf4 activity in the developing mammalian lens, targeted disruption of the *hsf4* gene in mice leads to the degeneration of the lens and formation of cataracts early during postnatal development [Fujimoto et al., 2004; Min et al., 2004]. Interestingly, missense mutations in the DNA-binding domain of the *hsf4* gene have been detected in humans with Marner and Lamellar cataracts [Bu et al., 2002]. In addition, a splice mutation in intron 12 of the human *hsf4* gene, which is associated with autosomal recessive congenital cataracts, has also been reported [Smaoui et al., 2004]. These results indicate a critical role for Hsf4 during lens fiber cell differentiation. Although the function of the *hsf4* gene is becoming clear, the mode of Hsf4 transcriptional regulation remains unclear. Human cell lines expressing exogenous Hsf4a (the transcriptional repressor isoform) exhibit lower levels of basal and inducible expression of heat shock protein 90 (Hsp90), Hsp70, and Hsp25 [Nakai et al., 1997; Zhang et al., 2001]. Hsf4a interacts with the basal transcription factor TFIIF in an in vitro reconstitution system, suggesting a potential mechanism for transcriptional repression induced by this factor [Frejtag et al., 2001]. In contrast, the Hsf4b isoform is a relatively weak transcriptional activator, and Hsf4b can substitute for survival defects of yeast cells that lack endogenous Hsf [Tanabe et al., 1999]. We have recently found that Hsf4b is phosphorylated by the mitogen-activated protein (MAP) kinases, extracellular signal regulated protein kinase 1/2 (ERK1/2), c-Jun-N-terminal kinase (JNK1), and p38 in vitro. Hsf4b phosphorylation by ERK1/2 coincides with its accumulation in cells and increases its ability to bind heat shock element (HSE) [Hu and Mivechi, 2006].

The human mating type switching/sucrose non-fermenting (SWI/SNF) chromatin remodeling complexes play an important role in controlling gene expression during cell growth, differentiation, and maintenance of genetic stability [Dunaief et al., 1994; Randazzo et al., 1994; Phelan et al., 1999; Muchardt and Yaniv, 2001]. Mammalian SWI/SNF consists of a 1.5–2 mDa complexes containing at least 11 subunits and either Brg1 or Brm ATPase subunits [Wang et al., 1996]. Brg1 or Brm complexes use the energy of ATP hydrolysis to remodel nucleosomes to alter transcription. A number of transcriptional activators or repressors have been identified that recruit Brg1 or Brm and other associated factors to remodel nucleosome and stimulate or repress transcription [Murphy et al., 1999; Strobeck et al., 2000, 2001]. Brg1 appears to be hyperphosphorylated and is excluded from the condensed chromatin while cells are in mitosis [Reyes et al., 1997; Sif et al., 1998]. Using several protein kinase inhibitors or activators, it has been postulated that Brg1 could be phosphorylated by several distinct protein kinases during mitosis [Reyes et al., 1997]. In vitro, Brg1 can be inactivated following phosphorylation by ERK1/2 and reactivated after treatment with general protein phosphatase inhibitors [Sif et al., 1998]. Brg1 exhibits reduction in phosphorylation as cells enter G1. This reduction in phosphorylation has been associated with Brg1's increased ability to stimulate chromatin remodeling activity [Sif et al., 1998]. Among the Hsfs, Hsf1 has been shown to associate with Brg1 in a sub-stoichiometric manner [Sullivan et al., 2001]. Hsf1 also associates with complexes containing INI1/SNF5, which are also conserved components of the SWI/SNF complex [Sullivan et al., 2001]. In the purified system, Hsf1 transcriptional activation domain appears responsible for Brg1 recruitment to the *hsp70* promoter [Brown and Kingston, 1997; Sullivan et al., 2001]. Furthermore, mutational analyses of Hsf1 indicate that amino-acid residues involved in the activation of transcriptional elongation can severely perturb Hsf1 and Brg1 association [Sullivan et al., 2001]. In other experiments, inducible expression of dominant-negative alleles of Brg1 or Brm in mammalian cells have been shown to reduce *hsp70* gene expression following exposure of the cells to metabolic inhibitors or heavy metals, but not following treatment of cells with other stressors such as heat shock [De La Serna et al., 2000].

To examine the transcriptional regulation of Hsf4b by Brg1 chromatin remodeling complexes, we investigated their association in cells

expressing the Hsf4b transcription factor. Our results show that Hsf4b interacts with Brg1 containing complexes during the G1 phase of the cell cycle. Hsf4b and Brg1 do not interact during G2/M when Brg1 is presumably hyperphosphorylated and exhibits reduced chromatin remodeling activity. During the time period that Hsf4b interacts with Brg1, it can be detected on the promoter elements of Hsps, which is associated with increased Hsp levels in cells.

METHODS AND MATERIALS

Plasmids

The expression vector pGEX-Hsf4b was generated by PCR amplification of full-length human Hsf4b cDNA. PCR products were inserted into the pGEX-2T vector (Amersham Biosciences, Piscataway, NJ) using *Bam*HI and *Eco*RI restriction enzymes. The expression construct pcDNA3-Flag-Hsf4b was generated by inserting a Flag tag sequence at the amino terminus of human Hsf4b and subcloning into the pcDNA3 vector. The plasmids containing the constitutively active form of *Xenopus* Mek in the vector pcDL-SRσ 296 was the gift of Dr. M. Iwashima (Medical College of Georgia). The plasmid containing pcDNA3-HA (NH_2-terminal)-DUSP26 was constructed using DUSP26 cDNA from plasmid pMyr-DUSP26 that was isolated from a yeast two-hybrid screening, using the restriction enzymes *Kpn*I and *Xho*I. The characterization of DUSP26 that was found to bind Hsf4b is recently reported [Hu and Mivechi, 2006].

Cell Culture and Synchrony

HeLa cells, a human cervical carcinoma cell line, were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS). *Hsf1, hsf2, hsf1hsf2*, or *hsf4*-deficient mouse embryo fibroblasts (MEFs) were obtained from timed pregnancies at embryonic day (E) 13.5 from mouse lines deficient in these factors [Zhang et al., 2002; Min et al., 2004; Wang et al., 2004]. *Hsf*-deficient MEFs were transformed using SV40 large T antigen and were cultured as above. To synchronize cells, MEFs were cultured to 50% confluency, transiently transfected with expression plasmids as described in the result section, and after 24 h, were treated with nocodazole (300 ng/ml, except for MEFs deficient in *hsf2* or in both *hsf1* and *hsf2* for which 150 ng/ml was used) for 20 h (G2/M block) [Meng et al., 2004]. Cells were then rinsed with phosphate-buffered saline (PBS) and collected and analyzed as indicated in the figure legends.

Transient Transfection Assays

Transient transfections were performed using the Mirus Trans IT-LT1 (Mirus Corp., Madison, WI). Transfected DNA mixes included 3 μg of expression plasmid DNA (for 60-mm dishes) and, when required empty expression vector was used as a control.

Immunoprecipitation, Immunoblotting, and Pull-Down Assays

Cells were co-transfected with the appropriate plasmids, allowed to recover for 48 h, rinsed with PBS, and appropriately treated and harvested. Cells were lysed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5) containing a 1× cocktail of protease inhibitors (Sigma Chemical Co. St. Louis, MO). The protein concentration of the supernatant was estimated using a BCA protein assay kit (Pierce, Rockford, IL). One milligram of each of the cell lysates was mixed with 40 μl of a 50% solution of protein A-agarose and incubated at 4°C for at least 1 h. The protein A-agarose was then centrifuged and the precleared supernatant was incubated with 5 μg of primary antibody and incubated for 2 h at 4°C. 50% protein A-agarose (30 μl) was then added to the cell lysate antibody mixture and incubated at 4°C for an additional 2 h. The protein A complexes were centrifuged at 10,000*g* for 1 min, and the pellet was washed with lysis buffer five times [Hu and Mivechi, 2003, 2006]. 2 × SDS sample buffer (100 μl) was added, and samples were heated for 5 min at 100°C. The samples (35 μl) were fractionated on SDS–PAGE and analyzed by immunoblotting using appropriate antibodies. The membrane was immunoblotted using primary antibodies as described in the text. The corresponding HRP-conjugated secondary antibodies were used, and signals were developed using the enhanced chemiluminescence (ECL) method (ECL kit, Amersham Pharmacia Biotech, Piscataway, NJ) [Dai et al., 2000; Hu and Mivechi, 2003].

For in vitro pull-down assays, 800 μg of cell lysate prepared in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, and 10% glycerol was

incubated with 20 μg of the purified glutathione-S-transferase (GST)-Hsf4b isolated from the transfected *E. coli* BL21 and was incubated for 6 h at 4°C. The GST-Hsf4b proteins were precipitated with 50 μl of a 50% slurry of glutathione-conjugated–Sepharose 4b beads. After rinsing five times with lysis buffer and once in buffer containing 50 mM Tris HCl, pH 7.4, and 200 mM NaCl, beads were boiled in the SDS sample buffer. Proteins were separated on an SDS–PAGE gel, transferred to the membrane, and were immunoblotted [Hu and Mivechi, 2003].

Antibodies to Brg1 and Brm were purchased from BD Biosciences (San Jose, CA). Antibodies to Hsp90α, Hsp70, and Hsp25 were purchased from StressGen (British Columbia, Canada). Antibodies to Flag and β-actin were purchased from Sigma.

In Vivo Cross-Linking and Chromatin Immunoprecipitation (ChIP) Assays

ChIP assays were performed according to the previously published procedures [Takahashi et al., 2000]. HeLa cells or MEFs deficient in *hsfs* were cultured to 60% confluency (100 mm tissue culture dishes) and, following the appropriate treatment, were fixed by the addition of one-tenth volume of formaldehyde (Merck, Whitehouse Station, NJ) in 0.l M NaCl, 1 mM EDTA, 0.5 mM EGTA pH 8, 1 M HEPES, pH 8, directly to the medium and were placed on ice. To terminate the reaction, 2.5 M glycine was added to a final concentration of 0.125 M, and the mixture was incubated on ice for 5 min. Cells were rinsed with ice-cold PBS, re-suspended in 10 ml of lysis buffer 1 (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.25% NP-40, 0.25% Triton X-100, and a cocktail of protease inhibitors (Sigma)). The mixture was rocked for 10 min at 4°C, and nuclei were pelleted by centrifugation. Nuclei were re-suspended in 8 ml of buffer 2 (200 mM NaCl, 1 mM EDTA (pH 8), 0.5 mM EGTA pH 8, 10 mM Tris-HCl pH 8, and a cocktail of protease inhibitors). The lysate was rocked for 10 min at 25°C. Nuclei were pelleted as above and re-suspended in 8 ml of buffer 3 (1 mM EDTA pH 8, 0.5 mM EGTA, pH 8, 10 mM Tris-HCl, pH 8, and a cocktail of protease inhibitors). Nuclei were sonicated in 3–5 ml aliquots in a 15 ml conical tube with a microtip setting of 2.5 and constant power (20–25 s pulses for 10 times). The sonication resulted in average DNA fragment sizes of ~0.5 kb DNA as judged by electrophoresis in a 1.4% agarose gel. Samples were adjusted to RIPA buffer (50 mM HEPES, pH 7.5, 1 mM EDTA, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, and a cocktail of protease inhibitors) by adding a two times strength RIPA buffer stock. The lysate was pre-cleared by incubating with pre-washed protein A beads alone. The lysate was then incubated with 5–10 μg of specific antibody followed by incubation for 16 h at 4°C. Pre-blocked protein A beads (40 μl) (50% slurry) were added and the mixture was incubated for an additional 3 h at 4°C. The precipitates were rinsed seven times with 1 ml of RIPA buffer supplemented with 0.5 M NaCl, incubating for 5 min on ice in between spins. The immune complexes were eluted with 100 μl elution buffer (1% SDS, 0.1 M $NaHCO_3$). To reverse the DNA/protein cross-links, samples were incubated for 6 h at 65°C. DNA samples were ethanol precipitated, centrifuged, washed with 70% ethanol, and dried under vacuum. The precipitates were re-suspended in 120 μl of 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 (TE), buffer containing 200 μg/ml of Proteinase K and was incubated for at least 1.5 h at 55°C. Samples were then extracted once with phenol:chloroform (1:1), and once with chloroform, and ethanol precipitated, and the pellet from each sample was re-suspended in 50 μl TE buffer. Semi-quantitative PCR or real-time PCR was performed on each sample. Primers used following ChIP assays to detect individual Hsps were as follows: Hsp 27 sense: 5′-GAATTCATTGCTTTTCCT-TAACGAG-3′; Hsp 27 anti-sense: 5′-GGCTC-GGGCTGCGCTTTTATGCTCCTCC-3′; Hsp70a sense: 5′-CTTTGCCTGATACAGATGCTACTT-G-3′; Hsp 70a anti-sense: 5′-GCCACTGCACTC-CAGCCTGGGTAGC-3′; Hsp 70b′ sense: 5′-GAGCTAGAACCTTCCCGCGTTTC-3′; Hsp 70b′ anti-sense: 5′-GCGAACCTTCCCGCACCTTC-CCGCCC-3′; Hsp 70b sense: 5′-CGGACCGA-TCCGCCCGAACCTTCTC-3′; Hsp 70b anti-sense: 5′-GGAAAGGTTCGCGAAAGTTCGCG-GC-3′; Hsp 90 sense: 5′-GGGCGGGACCGC-GGGACCGCCGAGACAGGCCTGG-3′; Hsp 90 anti-sense: 5′-CTCGC AGGAGTAG AGGAAG-GGCGGAG-3′; β-actin sense: 5′-GGG GTTG-GGGCCTGGCTTCCTG-3′; β-actin anti-sense: 5′-AGTCCTTAGGCCGCCAGGGGCGCC-3′. The promoter fragments of Hsps contained one or more previously identified HSEs (Genbank numbers for genes used to design primers were

as follows: Hsp27, L39370; Hsp70a, AB018045; Hsp70b, X51757; Hsp90, J04988; β-actin, M10277).

Flow Cytometric Analyses

Cells were rinsed two times with PBS and re-suspended in 500 μl of PBS followed by the addition of 5 ml of methanol. The mixture was incubated for at least 2 h at 4°C. Cells were rinsed with PBS and re-suspended in 400 μl of PBS containing 20 μl propidium iodide (1 mg/ml) and 2 μl of RNase (50 mg/ml). Following 30 min incubation at 25°C, flow cytometric analyses were performed using CellQuest™ Pro with luminescence spectrophotometer (excitation at 480 nm and emission at 510 nm).

Gel Mobility Shift Assays

After each treatment, cells were rinsed with PBS and nuclei were isolated using NE-PER (Pierce). The protein concentration of samples was estimated by the bicinchoninic acid method (Pierce). Equal amounts of protein (10 μg) in extraction buffer (volume not exceeding 15 μl) were added to the reaction mixture, which contained 4 μl of binding buffer (37.5 mM NaCl, 15 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.5 mM DTT, 5% glycerol), 10 μg of yeast tRNA, 1 μg of sheared *E.coli* DNA, 10 μg of poly dI:dC, and 1 ng of ^{32}P-labeled HSE oligonucleotide. The mixture was incubated for 15 min at 25°C and resolved on a 4.5% non-denaturing polyacrylamide gel. After electrophoresis, gels were fixed in 7% (v/v) acetic acid for 5 min, and were exposed to X-ray film and then PhosphorImager analyses for quantitation. The nucleotide sequence used for HSE was: 5′-GTCGACGGATCCGAGCGCCTCGAATGTTCTAGAAAAGG-3′. The double-stranded oligonucleotide was labeled using Klenow fragment of DNA polymerase I, deoxynucleotide triphosphates, and α^{32}P-dCTP [Dai et al., 2000].

RESULTS

Human Brg1 Chromatin Remodeling Complex Associates With Hsf4b In Vitro and In Vivo

The *hsf4* gene generates two alternative splice variants: Hsf4a, which lacks transcriptional activity, and Hsf4b, which possesses transcriptional activator properties [Nakai et al., 1997; Tanabe et al., 1999; Frejtag et al., 2001; Zhang et al., 2001]. To determine whether Hsf4b interacts with the chromatin remodeling complexes containing Brg1, which has been shown to regulate both transcriptional activation and repression, we performed co-immunoprecipitation experiments with HeLa cell extracts that were transiently transfected with expression constructs encoding Hsf4b. Immunoprecipitation experiments were performed using antibody to Flag followed by immunoblotting to detect endogenous Brg1, which is present in HeLa cells. The results show that Hsf4b can immunoprecipitate Brg1 in cells cultured under physiological growth conditions (37°C, 5% CO_2) (Fig. 1A, upper panel, lane 3). In reverse experiments, Brg1 can also immunoprecipitate Hsf4b (Fig. 1A, middle panel, lane 3). In addition, in vitro GST pull-down assays followed by immunoblotting showed that GST-Hsf4b and not the GST alone were capable of pulling down endogenous Brg1 from cell lysates of HeLa cells (Fig. 1B). Thus, our results show that the Brg1 subunit of the SWI/SNF chromatin remodeling complexes associates with the transcriptional activator Hsf4b.

Dynamic Association Between Hsf4b and Brg1 Complexes Following Changes in Activity of the MAP Kinase Signaling Pathway

We have recently determined that Hsf4b expressed in cells is phosphorylated on both serine and threonine residues when cultured under physiological growth conditions (37°C, 5% CO_2). In addition, we have shown that purified Hsf4b protein can be phosphorylated by the MAP kinases, ERK1/2, JNK1, and p38, using immune complex kinase assays in vitro [Hu and Mivechi, 2006]. Furthermore, exogenously expressed Hsf4b accumulated in cells following activation of the ERK1/2 or p38 signaling pathways, suggesting that Hsf4b phosphorylation by these protein kinases may increase the level of this protein in cells. To examine whether any alterations in the association between Hsf4b and Brg1 complexes could be detected following activation of the MAP kinase signaling pathways, we performed the following experiments: HeLa cells were transiently co-transfected with Hsf4b plus expression plasmids encoding the constitutively active form of Mek. Expression of the constitutively active form of Mek in cells leads to phosphorylation and increase in ERK1/2 protein kinase activity, which in turn leads to an increase in phosphorylation of downstream target genes [Bogoyevitch and Court, 2004]. Transiently

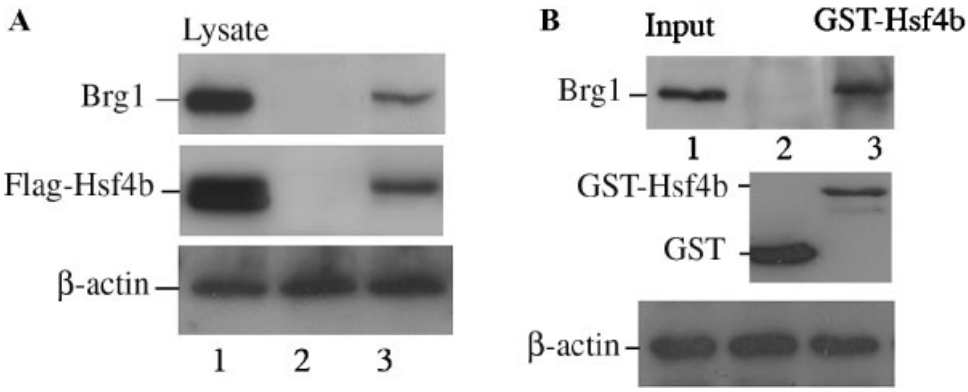

Fig. 1. Brg1 is associated with Hsf4b in vivo and in vitro. **A**: HeLa cells were transiently transfected with expression plasmids encoding Flag-tagged Hsf4b. Forty-eight after transfection, cell lysate from each sample was subjected to immunoprecipitation using antibody to Flag (**upper panel**) or antibody to Brg1 (**middle panel**). Immunoprecipitated materials were immunoblotted using antibody to Brg1 (upper panel) or Flag (middle panel). **Lane 1**: Cell lysate (30 μg) was loaded as a positive control to show endogenous Brg1 and expressed Flag-Hsf4b levels. **Lane 2**: Untransfected cell lysate plus pre-absorbed protein A was incubated with antibody to Flag (upper panel) or non-specific antibody (middle panel). **Lane 3**: Appropriately transfected cell lysates plus pre-absorbed protein A was incubated with antibodies to Flag (upper panel) or Brg1 (middle panel). Membranes were immunoblotted using antibody to Brg1 (upper panel) or Flag-Hsf4b (middle panel). Level of β-actin in the HeLa cell lysate is presented as control. **B**: Purified GST-Hsf4b or GST alone were incubated with nuclear extracts, the pull-down products were detected by immunoblotting using antibody to Brg1 (**upper panel**). **Lane 1**: Marked as ''input,'' represents 10% of the total amount of cell lysate used in each reaction. In **lanes 2** and **3**, GST only and GST-Hsf4b, respectively were used to pull-down Brg1. Levels of purified GST and GST-Hsf4b are presented in the **middle panel**. The levels of β-actin in the cell lysate are presented as controls.

transfected cells were subjected to immunoprecipitation using antibody to Flag, and immunoprecipitated materials were analyzed by immunoblotting using antibody to Brg1 (Fig. 2A). Other groups of cells were treated with sorbitol following the expression of Hsf4b in order to activate the p38 signaling pathway. Consistent with our previous observations [Hu and Mivechi, 2006], Hsf4b accumulates in cells when either the ERK1/2 or the p38 signaling pathway is activated (Fig. 2A, middle panel). Interestingly, the results show that Hsf4b exhibits an increase in association with complexes containing slower migrating form of Brg1 following the expression of active forms of Mek, but not following treatment of cells with sorbitol (Fig. 2A, compare lanes 2, 3, and 4). Hsf4b expressed in cells often appears as two different apparent molecular weights in SDS–PAGE. This at least in part, is related to the Hsf4b phosphorylation status [Hu and Mivechi, 2006].

Using a yeast two-hybrid screening strategy, we have recently isolated a dual-specificity tyrosine phosphatase that interacts with Hsf4b. This phosphatase (DUSP26) inactivates MAP kinases ERK1/2 in vivo. Furthermore, we have shown that co-expression of DUSP26 and Hsf4b abrogates the effects of ERK1/2 on Hsf4b, since DUSP26 inactivates ERK1/2 and prevents ERK1/2 from phosphorylating Hsf4b. The downstream effects of DUSP26 on Hsf4b is a reduction in its phosphorylation and reduced accumulation in cells, leading to decreased ability of Hsf4b to bind DNA [Hu and Mivechi, 2006]. To further examine the role of Hsf4b phosphorylation on its interaction with Brg1 complexes, co-immunoprecipitation experiments were performed using HeLa cells transiently transfected with expression vectors encoding Hsf4b and co-transfected with plasmids encoding either the constitutively active form of Mek, both Mek and DUSP26, or DUSP26 alone. Cell lysates were subjected to immunoprecipitation using antibody to Flag-Hsf4b, and the immunoprecipitated materials were immunoblotted by using antibody to Brg1 (Fig. 2B, upper panel). The results show that while association of Hsf4b with Brg1 complexes is increased once Mek is expressed in cells, the association of Brg1 with Hsf4b is decreased when DUSP26, or Mek and DUSP26 are co-expressed with Hsf4b (Fig. 2B). These results indicate that active form of MAP kinase ERK1/2 enhances Hsf4b's association with Brg1. In contrast, inactive form of ERK1/2 decreases Hsf4b's association with Brg1. To confirm that cells expressing Mek, DUSP26, or cells pre-treated with sorbitol presented in Figure 2A

exhibited phosphorylated and thereby active forms of ERK1/2 and p38 protein kinases, cell lysates were analyzed by immunoblotting using phospho-amino acid-specific antibodies (Fig. 2C). As predicted, the data show that cells expressing Mek express phosphorylated form of ERK1/2 and cells pre-treated with sorbitol show phosphorylated form of p38. In addition, cells expressing DUSP26 exhibit lower levels of phospho-ERK1/2 compared to control in cells that do not express this phosphatase (Fig. 2C).

We had previously determined that phosphorylation of Hsf4b correlated with an increase in Hsf4b protein levels in cells followed by an increase in its ability to bind to the HSE [Hu and Mivechi, 2006]. Thus, we tested whether changes in Hsf4b phosphorylation, which enhanced its interaction with Brg1 complexes (Fig. 2A–D), also correlated with its increased ability to bind to the promoters of several of the Hsps. HeLa cells were transiently transfected with expression plasmids encoding Flag-Hsf4b and co-transfected with expression plasmids encoding Mek, DUSP26, or with both. ChIP assays were performed using antibody to β-actin or Flag, and immunoprecipitated DNA was used in PCR reactions using primers that detected the HSE-encoding promoter fragments of human Hsp27, Hsp70a, Hsp70b, and Hsp90α. The results indicate that Hsf4b was recruited at higher levels to the promoters of these Hsps in cells co-expressing Mek compared to cells that expressed Hsf4b alone (Fig. 2D, compare lanes 5 and 4). In contrast, Hsf4b recruitment to the promoters of Hsps was reduced when cells co-expressed Hsf4b and DUSP26 expression plasmids (Fig. 2D, compare lanes 3 and 4). HeLa cells expressing Hsf4b, Mek, or DUSP26 showed approximately the same amounts of Hsf4b on promoters of Hsps than cells expressing Hsf4b alone (Fig. 2D, compare lanes 6 and 4). Semi-quantitation of the ChIP assays were performed relative to Hsf4b (Fig. 2D, lane 2) and the data is presented in Figure 2E. Real-time PCR was also performed, with results comparable to that presented in Figure 2E.

Association of Hsf4b With Brg1 Complexes During the Cell-Cycle

Since Hsf4b activity appears to be positively controlled by MAP kinase signaling pathways (Fig. 2) and [Hu and Mivechi, 2006], we speculated that Hsf4b phosphorylation and activity could be regulated in a cell-cycle-dependent manner. In addition, although specific phosphorylation sites on Brg1 have not been determined yet, previous data from other laboratories indicate that Brg1 is hyperphosphorylated during G2/M, as shown by retardation of its mobility in

Fig. 2. Phosphorylated form of Hsf4b associates with Brg1 complexes in vivo. **A**: HeLa cells were transiently transfected with Flag-Hsf4b (**lanes 2**, **3**, and **4**) or co-transfected with Flag-Hsf4b and expression plasmids encoding the constitutively active form of Mek (lane 3) to activate ERK1/2. In lane 4, cells were incubated in medium containing 400 mM sorbitol for 30 min to activate p38 prior to harvesting. Forty-eight hours after transfection, cell lysate prepared from each group was subjected to immunoprecipitation using antibody to Flag and immunoblotted to detect Brg1 (**upper panel**). **Lane 1** represents negative control where pre-absorbed protein A was incubated with untransfected cell lysate and antibody to Flag. **Middle panel** presents the level of Flag-Hsf4b expression in cells using antibody specific to Flag. **Lower panel** shows level of β-actin as a control. **B**: HeLa cells were transiently transfected with Flag-Hsf4b (**lanes 2**, **3**, **4**, and **5**) or co-transfected with carrier DNA (lane 1) or with Flag-Hsf4b and expression plasmids encoding the constitutively active form of Mek (lanes 3 and 4) to activate ERK1/2, or co-transfected with plasmids encoding the dual specificity tyrosine phosphatase, HA-DUSP26 (lanes 4 and 5). Forty-eight hours after transfection, cell lysate prepared from each group was subjected to immunoprecipitation using antibody to Flag and immunoblotted to detect Brg1 (**upper panel**). Immunoblotting to detect expression levels of overexpressed Flag-Hsf4b, Mek, HA-DUSP26, and β-actin have been shown following immunoblotting of 30 μg of protein from each cell lysate using antibodies specific to Flag, Mek, HA, or β-actin. **C**: Cell lysates (30 μg) from samples presented in panel B were used in immunoblotting experiments to detect the level of phospho-ERK1/2, total ERK1/2, phospho-p38, Mek or HA-DUSP26 in cells using appropriate antibodies. **Lanes 1–4** are the same as **lanes 2–5** in panel B. **D** and **E**: Hsf4b occupation of Hsps promoters. HeLa cells were transiently transfected using expression plasmids encoding Flag-Hsf4b, Flag-Hsf4b, and constitutive active form of Mek or DUSP26 as in panel B. Forty-eight hours after transfection, cells were harvested and subjected to ChIP assays using antibody to β-actin (**lane 2**) or Flag (**lanes 3–6**). To detect individual promoter sequences immunoprecipitated with Flag-Hsf4b, specific PCR primers were used to amplify regions surrounding the HSE. **Lane 1** represents PCR of individual genes using total DNA extract. Lane 2 represents PCR of the immunopreciptiated DNA using primers specific to β-actin used as a negative control. Lanes 3–6 represent PCR of individual Hsp genes following expression of Hsf4b, Mek, or HA-DUSP26 as indicated. PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining, and the bands were quantitated using densitometry (panel E). Bars 3, 4, 5, 6 in panel E represent fold increase relative to Hsf4b. Bar 3 corresponds to cells expressing Flag-Hsf4b and HA-DUSP26. Bar 4 corresponds to cells expressing Flag-Hsf4b. Bar 5 corresponds to cells expressing Flag-Hsf4b and Mek. Bar 6 corresponds to cells expressing Flag-Hsf4b, Mek and HA-DUSP26.

SDS–PAGE, but Brg1 exhibits faster mobility in SDS–PAGE once cells accumulate in the G1 phase of the cell cycle [Reyes et al., 1997; Sif et al., 1998]. Immunoblotting experiments presented in Figure 3A confirmed that Brg1 exhibited retardation in SDS–PAGE and migrated faster when synchronized cells were released from nocodazole treatment (G2/M block). To determine whether there might be changes in the Hsf4b association with Brg1 complexes during the cell cycle, the levels of Brg1 complexes associated with Hsf4b was determined up to 24 h following release of cells from G2/M arrest. The results indicate that the Hsf4b interaction with Brg1 complexes is minimal during G2/M, but the association is increased as cells move into the G1 phase of the cell cycle (Fig. 3B). This demonstrates that the hyperphosphorylated form of Brg1 is unavailable to interact with Hsf4b at 0 and 1 h post-synchronization, but the interaction between these two proteins can be detected beginning at 3 h following cells entering into the G1 phase (Fig. 3B). The level of expression of Hsps as detected by immunoblotting showed increases in the expression of Hsp27 and Hsp70 (Fig. 3C). The expression of Hsp90α

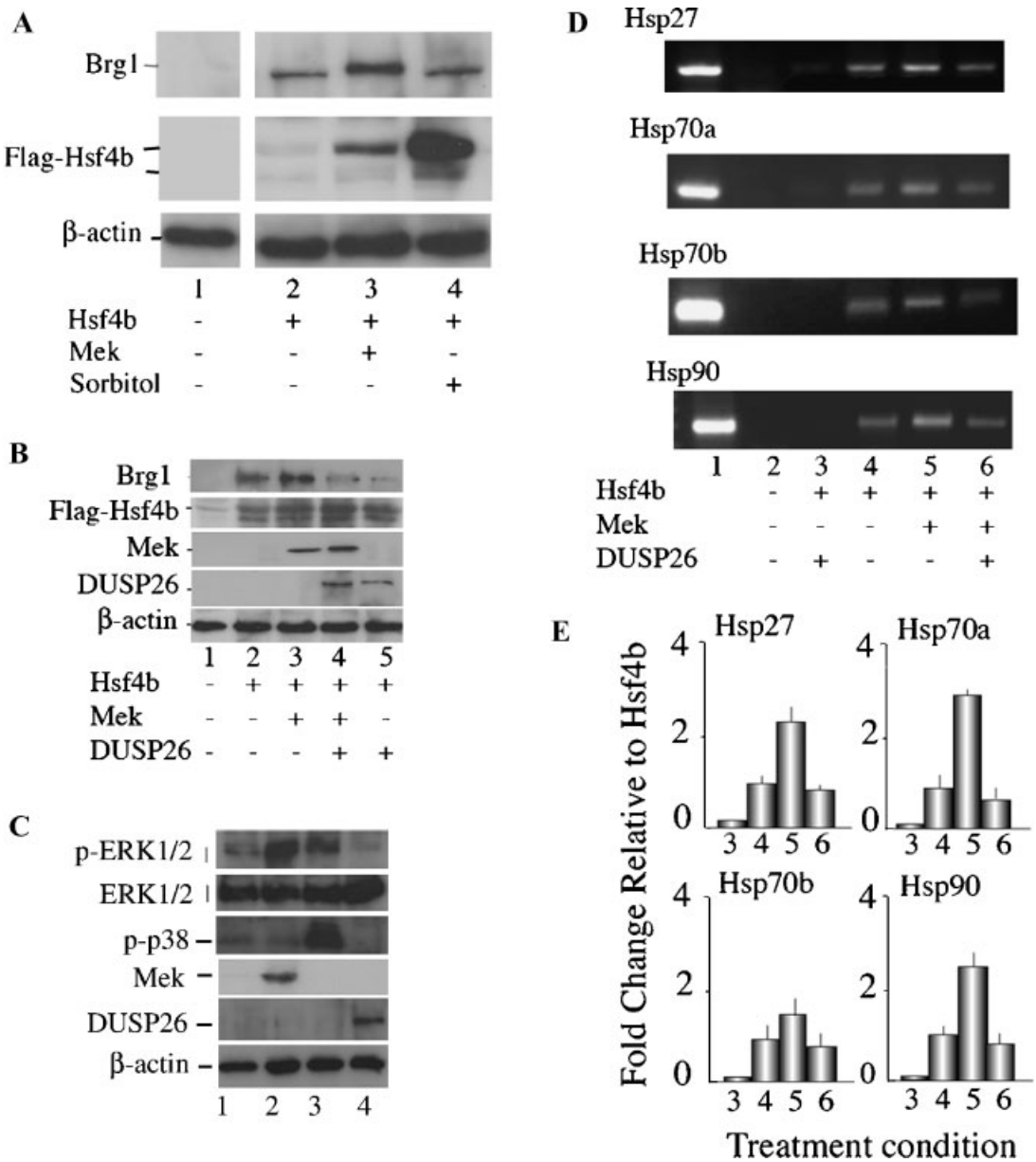

Fig. 2.

appears to remain unchanged during the cell cycle in HeLa cells. We also showed that Hsf4b was associated with promoter fragments of the Hsp27, Hsp70a, Hsp70b, and Hsp90α at approximately 1–3 h following HeLa cells entry into G1 (Fig. 3D). Thus, HeLa cells were transiently transfected with expression plasmids encoding Flag-Hsf4b and synchronized with nocodazole (G2/M block). ChIP assays were performed using antibody to β-actin or Flag, and immunoprecipitated DNA was used in PCR reactions using primers that detected the HSE-encoding promoter fragments of human Hsp27, Hsp70a, Hsp70b, and Hsp90α. The quantitation of the ChIP assays relative to 0 h post-synchrony is presented in the right panel of Figure 3D.

HeLa cells express Hsf1 transcription factor, and a contribution of Hsf1 to increase in Hsp level during the cell cycle cannot be ruled out. Gel mobility shift assays using HeLa cell extracts from synchronized cell populations with or without Hsf4b overexpression indicated that in cells that do not express exogenous Hsf4b, Hsf1 DNA binding activity could be detected at 1, 3, and 5 h post-synchronization. However, there was an additional increase in DNA binding activity in cells expressing Flag-Hsf4b (data not shown). Specific roles of individual Hsfs during the cell cycle was addressed using MEFs deficient in individual Hsfs and the data are presented below. Our results indicate that Hsf1 as well as Hsf4b potentially contribute to the increased expression of Hsps during G1 phase using immunoblotting experiments. Nevertheless, in HeLa cells Hsf4b can be detected on the Hsp promoter elements once expressed in cells, as presented in ChIP assay (Fig. 3D). Both Brg1 and Hsf4b contain putative MAP kinase phosphorylation sites but the precise phosphorylation sites and the extent of phosphorylation of these proteins during mitosis or following expression of Mek (Fig. 2) are unknown. However, the status of ERK1/2 phosphorylation during the cell cycle was investigated, and we found that increases in the phosphorylated forms of ERK1/2 could be detected most strongly 3–5 h after cells entry into G1 phase (Fig. 3E). The quality of the cell synchrony was determined using flow cytometric analyses post-synchrony followed by release of cells from nocodazole treatment (Fig. 3F). The fractions of HeLa cells in different parts of the cell cycle following treatment of cells with nocodazole are presented in Table I.

Hsf4b Becomes Competent to Bind DNA and Drive the Expression of Hsp70 During the G1 Phase in MEFs Deficient in Both *hsf1* and *hsf2* Genes

We performed experiments using wild type MEF or MEF deficient in the *hsf1* gene, to determine the contribution of Hsf4b in enhancing the level of Hsps expression during the cell cycle. Thus, wild-type (+/+) and $hsf1^{-/-}$ MEFs were synchronized and expression of inducible Hsp70 was detected during the cell cycle using immunoblotting. Wild-type cells were efficient in the expression of Hsp70 (Fig. 4A, left panel), while *hsf1*-deficient cells exhibited severe reduction in the level of Hsp70 (Fig. 4A, right panel). To ensure that Hsf4b can indeed enhance the levels of Hsp70 in the absence of the *hsf1* gene, $hsf1^{-/-}$ MEF was transiently transfected with Flag-Hsf4b, cells were then synchronized, and the level of Hsp70 was determined using immunoblotting. The data presented in Figure 4A (right panel) indicate that $hsf1^{-/-}$ cells overexpressing Hsf4b exhibited elevated levels of Hsp70 expression as cells enter the G1 phase of the cell cycle.

The MEFs normally express low levels of endogenous Hsf4b but the activity of endogenous Hsf4b is not known. However, our data clearly indicate that exogenously expressed Hsf4b is capable of inducing Hsp70 expression during G1 phase. To determine whether Hsf4b is capable of binding HSE and drive the expression of Hsp70 in the absence of both *hsf1* and *hsf2* genes (Fig. 4B), gel mobility shift assays and immunoblotting experiments were performed using MEFs deficient in both *hsf1* and *hsf2* genes. The data indicate that in the absence of *hsf1* and *hsf2* genes, no Hsf4b DNA binding activity could be detected (Fig. 4B), however, exogenously expressed Hsf4b binds HSE at 3 and 5 h following release of synchronized MEFs (G2/M block). Increased expression of Hsp70 was also observed at 3 and 5 h post-synchronization (Fig. 4B, lower panel). Asynchronous cell population expressing Hsf4b and synchronized cells in G2/M or 1 h following their progression into G1 phase also exhibit increased expression of Hsp70 compared to cells that do not express Hsf4b, however, no DNA binding activity could be detected (Fig. 4B). The quality of the cell synchrony was determined using flow cytometric analyses (G2/M block) and release (Fig. 4C). The fractions of *hsfs*-deficient MEFs

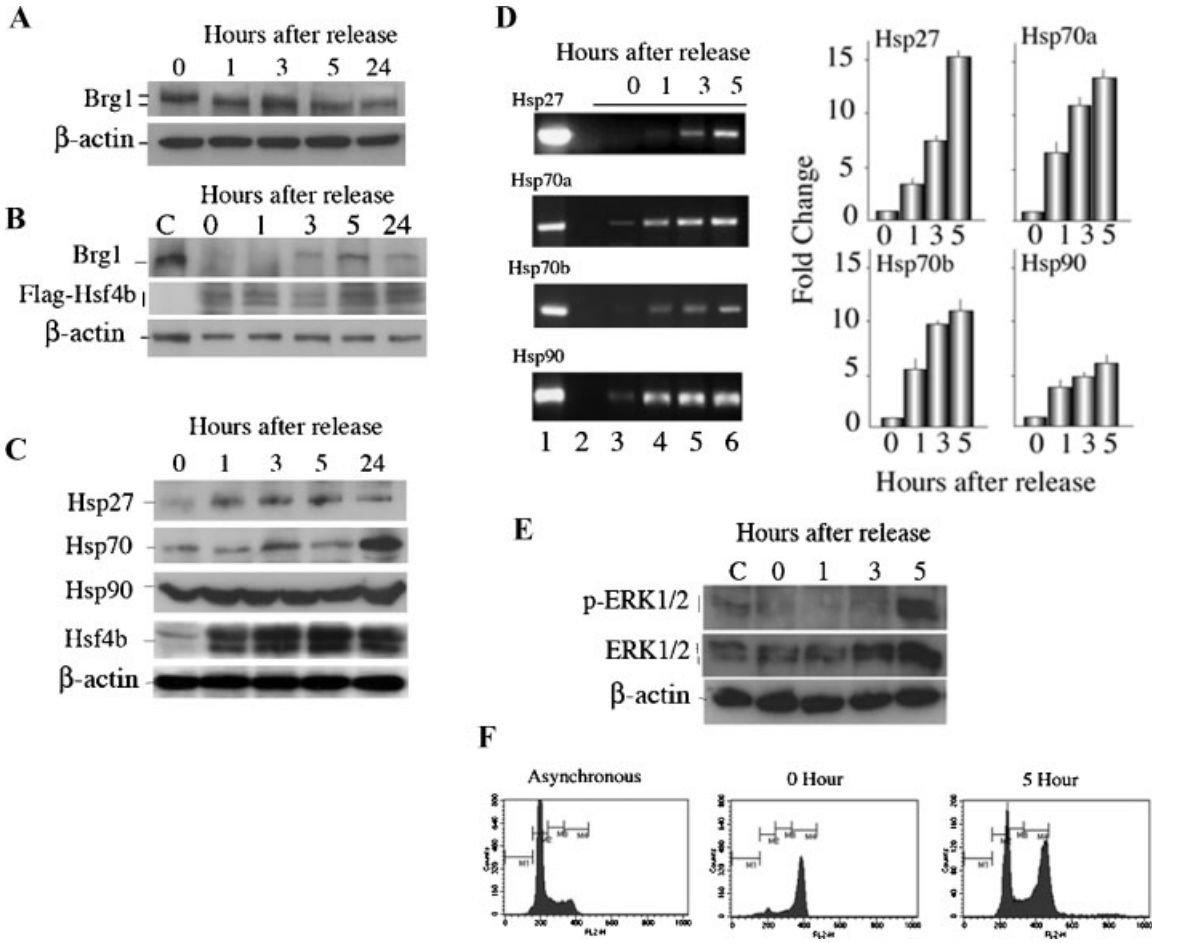

Fig. 3. Reactivation of cell-cycle-dependent Brg1 complexes and association with Hsf4b. **A**: HeLa cells were synchronized in G2/M following treatment of cells with nocodazole. Cells were then rinsed with medium and were harvested at the indicated times. Cell extracts (30 μg) from each sample were analyzed by immunoblotting using antibody to Brg1. β-actin is presented for loading control. **B**: HeLa cells were transiently transfected using expression vectors encoding Flag-Hsf4b. Twenty-four hours after transfection, cells were synchronized in G2/M following treatment of cells with nocodazole treatment. Cells were then rinsed with medium and were harvested at the indicated times. Samples were immunoprecipitated using antibody to Flag and the immunoprecipitated materials were subjected to immunoblotting using antibody to Brg1. Antibody to Flag shows expression of Hsf4b and antibody to β-actin is presented as loading control. ''C'' represents the asynchronous cell population. **C**: To detect expression of Hsps in synchronized HeLa cells expressing Hsf4b, equal amounts of cell lysates (30 μg) from (B) were subjected to immunoblotting using antibodies specific to Hsp27, Hsp70, Hsp90α, and Flag-Hsf4b. Level of β-actin is presented for loading control. **D**: HeLa cells were transiently transfected using expression plasmids encoding Flag-HSF4b. Twenty-four hours after transfection, cells were synchronized as in (B) and subjected to ChIP assays using antibody to β-actin (**lane 2**) or Flag (**lanes 3–6**). To detect individual promoter sequences, specific primers were used to amplify regions surrounding the HSEs. **Lane 1** represents PCR of total DNA extract. Lanes 3–6 represent hours after release from synchrony. PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. Bands were quantitated using densitometry (**right panel**). **E**: Cell lysates from (B) were immunoblotted using antibody to phosphorylated ERK1/2 or total ERK1/2. Level of β-actin was used as loading control. **F**: Flow cytometric analyses of synchronized HeLa cells, before synchrony (asynchronous), at 0 h and 5 h post-synchrony.

in different parts of the cell cycle following treatment of cells with nocodazole are presented in Table I.

To determine whether MEFs deficient in other *hsf* genes respond similarly to exogenously expressed Hsf4b, we used MEFs deficient in *hsf2* or *hsf4* and determined the level of Hsp70 in the absence or presence of transiently expressed Hsf4b. MEFs deficient in each factor were synchronized and collected immediately or following synchronization in G2/M and 5 h after release into G1 phase since the highest Hsp70 expression was observed at this time. The results indicate that in the absence of *hsf2*, no basal level of Hsp70 could be detected, however, exogenous Hsf4b could elevate the level of

TABLE I. Percent Cell Cycle Distribution Following Cell Synchrony

HeLa	Asynchronous	0 h	5 h
% G1	71	6	51
% S	14	13	12
% G2 + M	15	81	37
Mouse embryo fibroblasts	Asynchronous	0 h	5 h
% G1			
+/+	73	8	40
hsf1$^{-/-}$	69	5	45
hsf2$^{-/-}$	69	3	40
hsf4$^{-/-}$	71	11	48
hsf1$^{-/-}$*hsf2*$^{-/-}$	70	6	49
% S			
+/+	12	16	11
hsf1$^{-/-}$	15	11	12
hsf2$^{-/-}$	18	2	25
hsf4$^{-/-}$	9	8	10
hsf1hsf2$^{-/-}$	17	4	10
% G2 + M			
+/+	15	86	49
hsf1$^{-/-}$	16	84	43
hsf2$^{-/-}$	13	95	35
hsf4$^{-/-}$	20	81	42
hsf1$^{-/-}$*hsf2*$^{-/-}$	13	90	41

Hsp70 both in cells that were in G2/M or those in G1 phase (Fig. 5A). In contrast, in cells deficient in *hsf4* gene, in the absence of exogenous Hsf4b, there was a basal level of Hsp70 expression which was further increased in MEFs expressing Hsf4b both at 0 and 5 h post-synchronization (Fig. 5A). These data indicate that Hsf4b is capable of inducing Hsp70 expression during G1 phase of the cell cycle in the absence of other *hsf* genes.

The quality of the cell synchrony was determined using flow cytometric analyses post-synchrony and release from nocodazole treatment (Fig. 5B). The fractions of *hsfs*-deficient MEFs in different parts of the cell cycle following treatment of cells with nocodazole are presented in Table I.

DISCUSSION

In the studies presented here, we investigated the mechanism underlying regulation of Hsf4b transcriptional activity in mammalian cells. In addition, we explored the potential role of SWI/SNF chromatin remodeling complexes in Hsf4b activity. All members of the heat shock factor family (Hsf1, Hsf2, and Hsf4) bind HSE (nGAAn) and activate downstream target genes such as Hsps. However, the mode of activation of the Hsfs differs between each family member. Among the Hsfs, Hsf1 has been shown to depend on SWI/SNF chromatin remodeling factors for its maximal transcriptional activity [Sullivan et al., 2001]. The Hsf1 activation domain is responsible for Brg1 recruitment; however, studies suggest that this recruitment affects Hsf1's ability in both the initiation and elongation steps of transcription [Sullivan et al., 2001]. In other studies, inducible expression of dominant negative alleles of Brg1 or Brm has been shown to reduce Hsp70 inducibility following treatment of cells with cadmium or arsenite but not following treatment of cells with heat shock [De La Serna et al., 2000]. However, the role of Hsf1 in this induction by cadmium or arsenite is unclear, although heat shock most likely leads to an increase in Hsf1 activity. Several studies have suggested that Hsp70 is expressed during the G1 phase of the cell cycle [Wu and Morimoto, 1985; Xing et al., 2005]. However, the Hsf that is responsible for the regulation of Hsp70 is unknown. Stimulation of cells with serum leads to increased expression of Hsp70, which may be due to Hsf1 activity or the activity of transcription factors other than Hsfs [Wu and Morimoto, 1985]. Gel mobility shift assays of synchronized HeLa cells indicate that Hsf1 transcription factor binding activity can be detected at 1, 3, or 5 h after cells have entered into the G1 phase; however, in the presence of exogenous Hsf4b, DNA binding activity of this factor can also be detected at the same time points (data not shown). These results were consistent with the ChIP assays showing the presence of Hsf4b on the Hsp90α, Hsp70a, Hsp70b, and Hsp27 promoters (Fig. 3). In the gel mobility shift assays of HeLa cell extract in vitro, in the presence of endogenous Hsf1, the DNA binding activity of overexpressed Hsf4b was modest perhaps due to the higher affinity of Hsf1 for HSE in vitro (data not shown). We did not detect any Hsf2 DNA-binding activity in HeLa cell extracts during mitosis. Hsf2 was recently shown to bind the Hsp70 promoter in mitotic cells, preventing compaction of this promoter [Xing et al., 2005].

Our results indicate that Hsf4b transcription factor recruits Brg1-containing chromatin remodeling complexes to the promoter of Hsps as cells enter the G1 phase of the cell cycle. There was no interaction between Hsf4b and Brg1 complexes during mitosis, where Brg1 has been shown to be excluded from the chromatin. Interestingly, the Hsf4b interaction with Brg1 changes when MAP kinase activity is manipulated in cells. This data suggest that Hsf4b

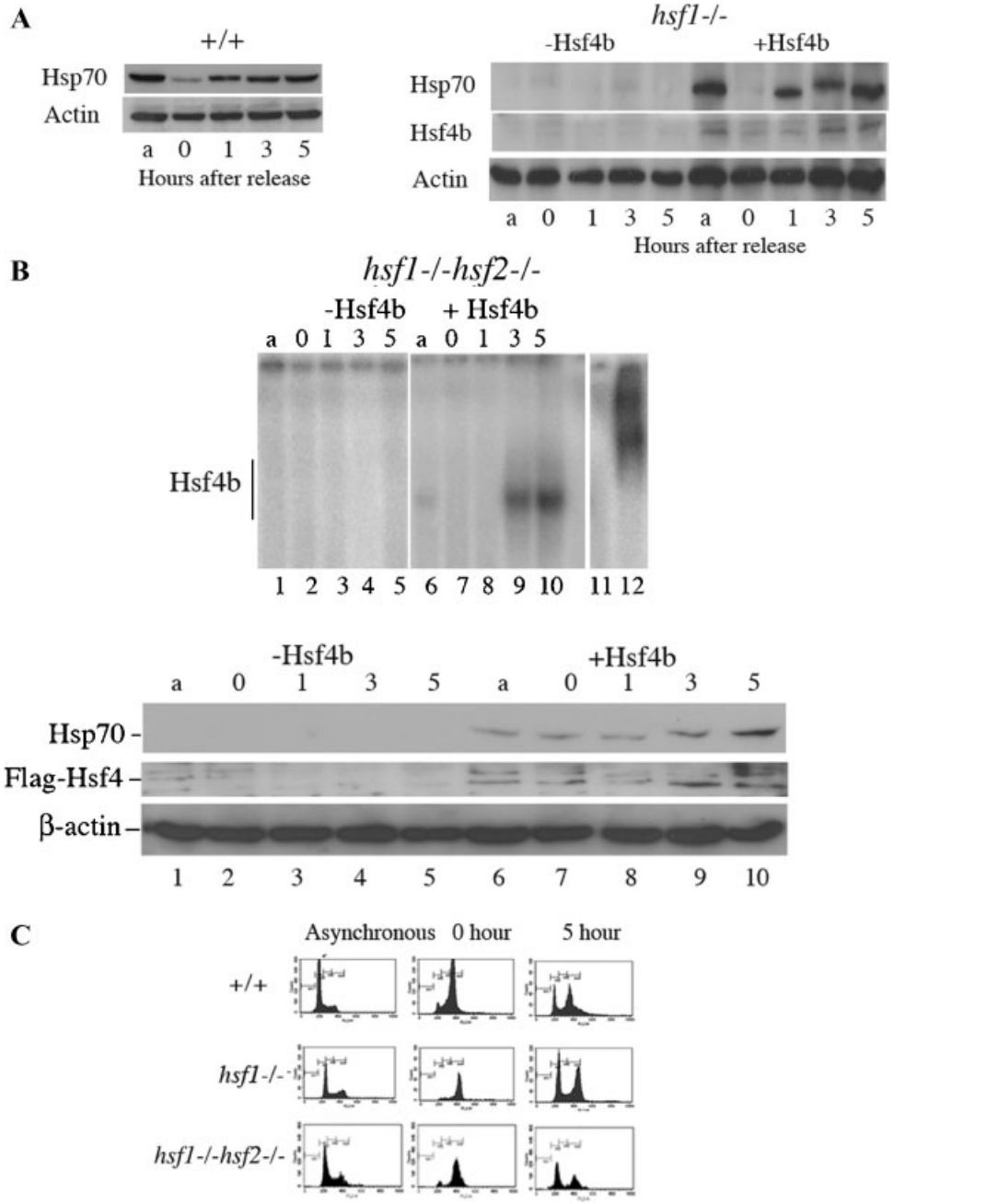

Fig. 4. Hsf4b is activated during the cell cycle and drives the expression of Hsp70. **A**: MEFs from wild type (+/+) or *hsf1*$^{-/-}$ animals were synchronized in G2/M following treatment of cells with nocodazole. Cells were then rinsed with medium and harvested at the indicated times. Cell extracts (30 μg) from each sample were analyzed by immunoblotting using antibody to the Hsp70 or β-actin. Level of β-actin was used as loading control. ''a'' represents asynchronous cell population. **B**: *hsf1*$^{-/-}$*hsf2*$^{-/-}$ MEFs were left untreated or were transiently transfected using expression plasmids encoding Flag-Hsf4b and were synchronized in G2/M with nocodazole treatment. Cells were then rinsed with medium and were harvested at the indicated times and were either analyzed by gel mobility shift assays or 30 μg of cell extracts from each sample was analyzed by immunoblotting using antibody to Hsp70, Flag-Hsf4b or β-actin. ''a'' represents asynchronous cell population. Lanes 1–5 are −Hsf4b and lanes 6–10 are +Hsf4b at indicated times after synchrony, respectively. Lane 11 is the same as lane 10 but plus excess Cdd Hse. Lane 11 is the same as lane 10 plus antibody specific to Flag. **C**: Flow cytometric analyses of synchronized wild type or *hsf*-deficient MEFs, before synchrony (asynchronous), at 0 h and 5 h post-synchrony. Lanes 1–10 as displayed in panel B.

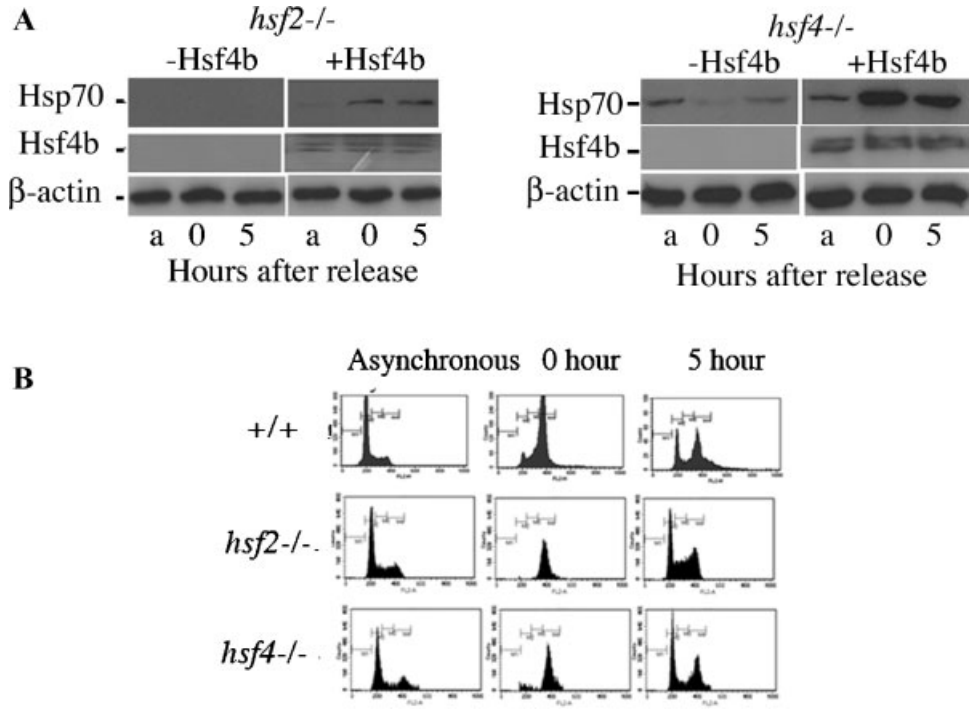

Fig. 5. Exogenously expressed Hsf4b induce expression of Hsp70 in MEFs deficient in *hsf2* or *hsf4* gene. **A**: MEFs deficient in *hsf2* or *hsf4* were synchronized in G2/M with nocodazole. Cells were then rinsed with medium and were harvested at the indicated times. Cell extracts (30 μg) from each sample was analyzed by immunoblotting using antibody to Hsp70 or Flag-Hsf4b. Level of β-actin was used as loading control. ''a'' represents asynchronous cell population. **B**: Flow cytometric analyses of synchronized wild type or hsf-deficient MEFs, before synchrony (asynchronous), or at 0 hour and 5 hour post synchrony.

activity is positively regulated by the MAP kinase signaling pathway. Brg1 also contains multiple phosphorylation sites, and at least the MAP kinases ERK1/2 have been shown to be responsible for hyperphosphorylation of Brg1 during mitosis. The Hsf4b protein level is also increased in response to sorbitol treatment, which mainly activates the p38 signaling pathway, but this did not result in an increase in the interaction between Brg1 and Hsf4b. The reason for this is not understood at this time. Following sorbitol treatment, Hsf4b may accumulate in cells but may require additional signals for full activity or it may not require Brg1 complexes for its activity. In addition, Brg1 may be phosphorylated by multiple protein kinases prior to mitosis since there are at least 13 or more putative MAP kinase or other protein kinase phosphorylation motifs [Reyes et al., 1997; Sif et al., 1998]. This has been postulated by previous studies indicating that HeLa cell nuclei incubated in the presence of inhibitors of serine/threonine protein kinases, such as H7, or inhibitors of tyrosine kinases such as genistein or cdc2 peptide, which inhibits cyclin-dependent protein kinases, inhibited the release of Brg1 or Brm from the nuclei. These data were interpreted to suggest that a cascade of protein kinases could be involved in the release of Brg1 during mitosis [Reyes et al., 1997; Sif et al., 1998]. More studies are needed to clarify how Hsf4b can positively and Brg1 can negatively be regulated by MAP kinases ERK1/2. The role of phosphorylation in the control of gene expression by Hsf4b and how activation of different signaling pathways may control Hsf4b activity or Brg1 chromatin remodeling factor during the cell cycle needs further investigation. Localized changes in the activity of the signaling molecules could be one way, and regulation of Hsf4b activity by ERK1/2 appears to be localized, since we have shown that both ERK1/2 and the dual specificity tyrosine phosphatase DUSP26 that controls ERK1/2 activity can both bind Hsf4b [Hu and Mivechi, 2006].

Most direct result of Hsf4b increased in DNA binding and transcriptional activity was demonstrated in MEFs deficient in *hsf1* and *hsf2* genes. From the data presented in Figure 4, it is clear that Hsf4b is also a

bona fide transcriptional activator and the absence of both *hsf1* and *hsf2* genes can activate transcription of *Hsp70* gene. Our data also indicate that Hsf4b transcription is enhanced following growth stimulation (i.e., in the presence of activated MAP kinases ERK1/2) and during the G1 phase of the cell cycle. We also detected an Hsf4b interaction with Brm (data not shown); however, the condition in which Brm is recruited by Hsf4b to the Hsps promoter is unknown. Furthermore, we also observed that Hsf4a, the repressor isoform of Hsf4, also interacts with Brg1-containing chromatin remodeling complexes (data not shown). Further experiments are required to determine the circumstance where Hsf4a may repress transcription of Hsps and what are the differences between chromatin remodeling complexes recruited by Hsf4a or Hsf4b.

In conclusion, we have shown that Hsf4b binds Brg1 chromatin remodeling complexes. The association of Brg1 and Hsf4b is cell cycle dependent and can be detected on Hsp promoters, leading to increased expression of specific Hsps. We have also shown that Hsf1 and Hsf4b can potentially enhance Hsp70 expression during the G1 phase of the cell cycle. However, there appears to be an interdependency between Hsf1, Hsf2, and Hsf4b in enhancing Hsp 70 expression as cells enter G1.

ACKNOWLEDGMENTS

The authors thank members of our laboratory, Jin-Na Min for providing MEFs and Monica Meyer for maintaining mouse colonies. We also thank Dr. M. Iwashima for providing constitutively active Mek, and Dr. A. N. Imbalzano for providing Brg1 expression vector.

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