

Expression of the cochaperone HspBP1 is not coordinately regulated with Hsp70 expression

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Abstract

Intracellular levels of the heat stress protein Hsp70 are elevated following exposure to elevated temperature. The cochaperone HspBP1 is an intracellular protein that is known to bind to and regulate Hsp70 activity. The purpose of this study was to determine if HspBP1 levels changed when Hsp70 levels were altered. Heat stress resulted in an increase in Hsp70 levels but no change in HspBP1 levels. Treatment of cells with the apoptosis inducing drug camptothecin lowered Hsp70 levels but again had no effect on HspBP1 levels. Cells treated with camptothecin plus heat stress did not exhibit an increase in Hsp70 levels. Over-expression in cells stably transfected with HspBP1 cDNA resulted in a 290% increase in HspBP1 levels without a similar change in Hsp70 levels. These results demonstrate that Hsp70 and HspBP1 are not coordinately regulated but provide evidence that an increase in the ratio of HspBP1 to Hsp70 correlates with apoptosis, in a similar way to reducing the amount of Hsp70. © 2006 International Federation for Cell Biology. Published by Elsevier Ltd. All rights reserved.

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1. Introduction

All eukaryotic cells contain a set of proteins known as heat stress proteins (Hsps) that provide protection from various environmental stresses such as elevated temperatures, exposure to heavy metals and hypoxia. The levels of these proteins increase when cells are exposed to stresses and the increased levels help the cells survive. The most studied has a molecular weight of 70 kDa and is therefore called Hsp70.

Studies have shown that there are other proteins that regulate Hsp70 activity, and therefore this system is more complex (Frydman and Höhfeld, 1997; Mayer et al., 2002). Hsp40 stimulates the ATPase of Hsp70, results in the production of

the ADP form of Hsp70 and facilitates binding to the substrate (Cyr et al., 1994; Silver and Way, 1993). Hip prevents the dissociation of ADP from Hsp70 and thereby stabilizes the binding to the substrate (Höhfeld et al., 1995). A protein named Hop acts as a modulator protein by binding to both Hsp70 and Hsp90 (Johnson et al., 1998), and both Hop and Hsp90 stimulate Hsp70-mediated refolding of a denatured protein. Another regulator of Hsp70 that inhibits Hsp70-mediated refolding is RAP/HAP46 also called Bag-1, which inhibits binding of misfolded proteins to Hsp70 (Zeiner et al., 1997 and Takayama et al., 1997) and causes the release of ADP from Hsp70 (Höhfeld and Jentsch, 1997). Chip (Ballinger et al., 1999) also inhibits the refolding of partially denatured luciferase by binding to the carboxy terminus of Hsp70 and is an Hsp70 dependent ubiquitin ligase (Jiang et al., 2001).

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HspBP1 is an Hsp70 cochaperone that has Hsp70 inhibitory activity (Raynes and Guerriero, 1998). However, it has also been suggested that HspBP1 is a nucleotide exchange factor that could stimulate Hsp70 activity (Kabani et al., 2002; Shomura et al., 2005). The fact that both Hsp70 and HspBP1 levels increase and maintain the same molar ratio in normal vs. tumor tissues suggests that these proteins may be coordinately regulated (Raynes et al., 2003). The experiments presented in this report were designed to determine if HspBP1 and Hsp70 levels change in a similar manner following heat stress, treatment with camptothecin or over-expression of HspBP1.

2. Materials and methods

2.1. Cell culture

Human caucasian promyelocytic cells (HL60, ATCC CCL240, American Type Culture Collection, Rockville, MD, USA) were used for all experiments. Cells were maintained in RPMI 1640 (Invitrogen BV, Groningen, Netherlands) supplemented with 1% non-essential amino acid solution (Invitrogen BV, Groningen, Netherlands), 2 mM L-glutamine (Invitrogen BV, Groningen, Netherlands), 100 units/ml penicillin, 100 µg/ml streptomycin (Invitrogen BV, Groningen, Netherlands) and 15% fetal calf serum (selected batches, Invitrogen BV, Groningen, Netherlands). Cell expansion was performed using 250 ml tissue culture flasks (Falcon, Becton Dickinson Labware, Plymouth, UK). Cells were passaged every 3 days at a density of 0.25×10^6 /ml. Only cells of passages 3–10 were used for the experiments, for better comparability.

The HT-1080 human fibrosarcoma cell line was obtained from ATCC (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's Media (DMEM) containing defined calf bovine serum (CBS) (Hyclone, Logan, UT, USA) at 10% (v/v), streptomycin (Sigma Chemical Co., St. Louis, MO, USA) at 0.1 mg/ml and penicillin G (Sigma) at 100 U/ml. Establishment of the HT-BP1 cell line was done by stably-transfecting a subclone of HT-1080 cells (HT-TR71) that expresses the tetracycline repressor protein (tetR) with the tetracycline-regulated expression vector (pCDNA4/TO) (T-Rex Expression system, Invitrogen, Inc.) encoding the HspBP1. Antibiotic-resistant subclones were selected using DMEM selection media containing 8% tetracycline-free serum (Hyclone, Logan, UT, USA), 50 µg/ml zeocin and 3.0 µg/ml blasticidin as previously described (Chauhan et al., 2004). Subclones were screened for tetracycline-inducible expression of the HspBP1 protein by culturing in 0.1 µg/ml doxycycline (Dox) for 24 h.

2.2. Heat stress

The cells were heat stressed for 30 min in an incubator set at 44 °C in 100% relative humidity, 5% CO₂/95% air. After heat stress, the cells were cultivated at 37 °C for 0–4 h and processed as described.

2.3. Induction of apoptosis

Apoptosis was induced by incubating the cells with 1 or 10 µM camptothecin (Sigma, Deisenhofen, Germany) for 2–4 h at 37 °C. The cells were subsequently stained for HspBP1 and/or Hsp70, then subjected to a caspase-3 assay or processed for western blot analysis.

2.4. Combined heat stress and camptothecin treatment

Camptothecin was added to the culture medium and the cells were placed in a heat stress incubator for 30 min. The samples were then cultured for 2 additional hours at 37 °C.

2.5. Caspase-3 assay

The caspase-3 assay was performed using a caspase-3 colorimetric kit from R&D Systems (Minneapolis, MN, USA) and the supplied protocol. Briefly, camptothecin induced cells and appropriate controls (5×10^6 cells) were incubated with lysis buffer on ice for 10 min followed by centrifugation for 1 min at $10,000 \times g$. The resulting supernatant was used for the assay (50 µl) or kept at –20 °C until use. For the reaction, 50 µl reaction buffer (containing 10 ml DTT) and 5 µl DEVD-pNA-substrate were added. After incubation for 2 h at 37 °C the samples were quantified by absorption measurement at 405 nm.

2.6. Staining of intracellular Hsp70, HspBP1 and DNA counterstain

Staining of intracellular Hsp70 was performed according to Gottwald et al. (2003). After heat-shock or control incubations, cells were incubated as described above for 0–4 h. Cells, 5×10^6 were stained with 1 µM nucleic acid stain Syto 16 (Molecular Probes Inc, Eugene, OR, USA) for 15 min. After staining, the cells were washed twice with $1 \times$ Perm/Wash solution from the Cytotfix/Cytoperm-Kit (Pharmingen, San Diego, CA, USA) and centrifuged for 5 min with $600 \times g$ at 4 °C. The pellet was resuspended in 300 µl Cytotfix/Cytoperm solution and incubated for 15 min at 4 °C in the dark. The cells were then washed as before. The resulting pellet was resuspended in 300 µl of a 1:100 dilution of the primary HspBP1 antibody (Novus Biologicals, Littleton, CO, USA) or 1:500 dilution of Hsp72 antibody (SPA-810, Stressgen Biotechnologies Corp., Victoria, BC, Canada) in $1 \times$ Perm/Wash solution and incubated for 30 min at 4 °C in the dark. After incubation, the cells were washed as before.

For treatment with the corresponding secondary antibody (rabbit- α -sheep-Cy5, 1:500, Chemicon International, API147S, or rat- α -mouse-APC, 1:500, BD 550874, Pharmingen, San Diego, CA, USA) cells were incubated in 300 µl $1 \times$ Perm/Wash for 30 min at 4 °C. Again, the cells were washed as before. The resulting pellet was resuspended in cell buffer (Cell Fluorescence Assay Kit, Agilent Technologies, Waldbronn, Germany) for microfluidic chip analysis at a concentration of 2×10^6 cells per ml. The microfluidic chip analysis makes use of a microfluidic device that contains 6 sample capillaries. One after another, samples of 500–1000 cells are taken out of a reservoir, transported through the capillaries by negative pressure and are hydrodynamically focused prior to detection by laser-induced fluorescence. For both antibody combinations (sheep- α -HspBP1–rabbit- α -sheep-Cy5 and mouse- α -HSP72–rat- α -mouse-APC) titrations with dilutions ranging from 1:10 to 1:1000 for the primary and 1:20 to 1:2000 for the secondary antibody were made to detect the optimal sensitivity range. All microfluidic assays were performed using the Agilent 2100 bioanalyzer with Cell Assay Extension Kit. Samples of 500–1000 cells were analyzed for 4 min per sample.

2.7. Western blots

The cells were washed with PBS and the supernatant aspirated. Lysis of the cells was achieved by addition of 100–200 µl SDS sample buffer (30 mM Tris–HCl (pH 6.8 at 25 °C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromphenol blue or phenol red). Lysates were kept at –20 °C until use. Prior to blotting, the lysates were sonicated for 10–15 s (60 W, 50% duty cycle) to shear DNA and reduce sample viscosity. A 40 µl sample was then heated to 95–100 °C for 5 min, cooled on ice and centrifuged with $14,000 \times g$ for 5 min. A 30 µg total protein per sample was loaded onto an SDS-PAGE gel (Criterion 4–15% gradient gel, Bio-Rad, München, Germany) and run with 60 mA and 200 V. Electrotransfer to PVDF-membranes (Millipore, Schwabach, Germany) was accomplished using 200 mA and 70 V for 1 h at room temperature in transfer buffer (25 mM Tris, 192 mM glycine, 20% MeOH). PVDF-membranes were activated by wetting with methanol and washed with H₂O before transfer.

The membrane was incubated with blocking buffer (PBS + 0.05% Tween-20 (=PBST) + 2% w/v non-fat dry milk) for 30 min at room temperature. The membranes were washed 3 times with 15 ml PBST on a shaker for 5 min. Next, the membranes were incubated with the primary antibody (1:100 dilution for sheep- α -HspBP1 (Novus Biologicals, Littleton, CO,

USA) and 1:1000 dilution, SPA-810 for HSP72, Stressgen Biotechnologies Corp., Victoria, BC, Canada, respectively) in blocking buffer for 1 h and washed 3 times for 5 min with 15 ml PBST. The membranes were incubated with an alkaline phosphatase-conjugated secondary antibody (A130-101AP, Bethyl Laboratories, Montgomery, Texas, USA) at a 1:500 dilution for HspBP1 and Sigma A3562 (München, Germany) at a 1:2000 dilution for Hsp70, respectively) for 1 h at room temperature. Finally, the blots were washed 3 times for 5 min with PBST. For detection the blots were incubated in BCIP/NBT (Sigma, Steinheim, Germany) staining solution for 2 min. The blots were dried and underwent densitometrical analysis using analySIS 3.1 software (Soft Imaging System GmbH, Münster, Germany).

Stably transfected cells were induced with 1.0 $\mu\text{g}/\text{ml}$ doxycycline (Dox) for 48 h and whole cell extracts were prepared in PBS/TDS cell lysis buffer (phosphate buffered saline containing 12 mM Na deoxycholate, 1% Triton X-100, 0.1% SDS and Sigma Protease inhibitor cocktail for mammalian cells) and analyzed by western blot with standards for quantification of Hsp70 and HspBP1 levels (Raynes et al., 2003).

2.8. Statistics

All experiments were performed in triplicate or greater, unless otherwise indicated. The Mann–Whitney *U*-test was used for statistical comparison.

Results with *p*-values of <0.05 were considered as being statistically different from controls.

3. Results

3.1. HspBP1 levels are not changed when Hsp70 increases or decreases

It is well established that following heat stress Hsp70 levels increase in cells and tissues. Initial experiments were conducted to determine if the Hsp70 cochaperone HspBP1 was regulated in a similar manner using antibody staining and a microfluidic chip assay. Cells were heat stressed for 30 min at 44 °C and analysed for HspBP1 and Hsp70 levels. Fig. 1a and b display the Hsp70 and HspBP1 fluorescence profiles after heat stress, respectively. The HspBP1 fluorescence does not change after treatment whereas the Hsp70 fluorescence increases by a factor of 5.68 ± 0.54 ($p = 0.021$).

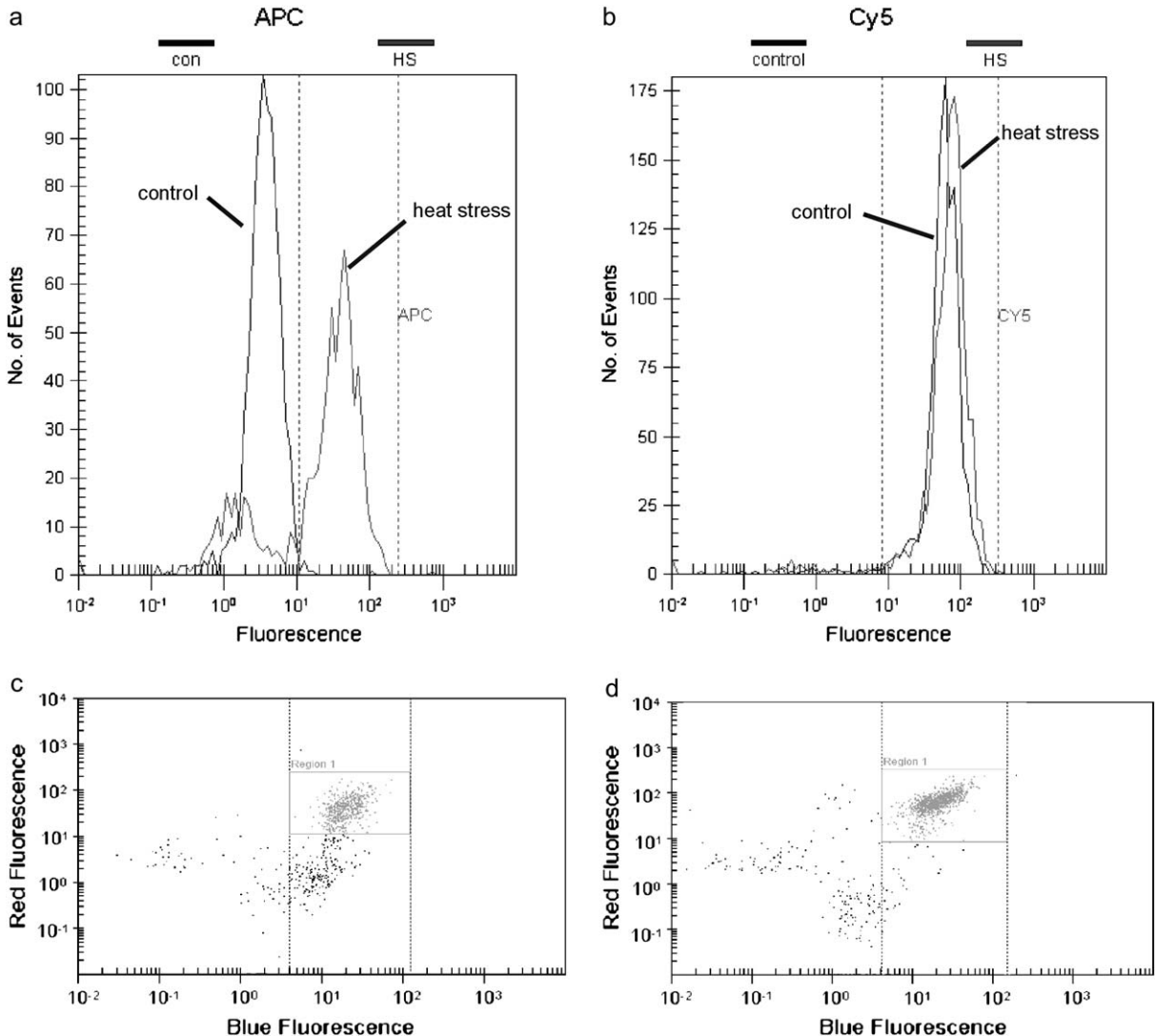


Fig. 1. Histograms of controls and heat stress treated samples obtained after staining HL60 cells (a) for Hsp70 and (b) for HspBP1 after heat stress (30 min, 44 °C, 2 h, 37 °C) induction. Panels (c) and (d) are the corresponding dot plots. The dot plots show the green fluorescence of the nuclear stain Syto16 on the abscissa and the red fluorescence of APC- or Cy5-conjugated secondary antibody on the ordinate.

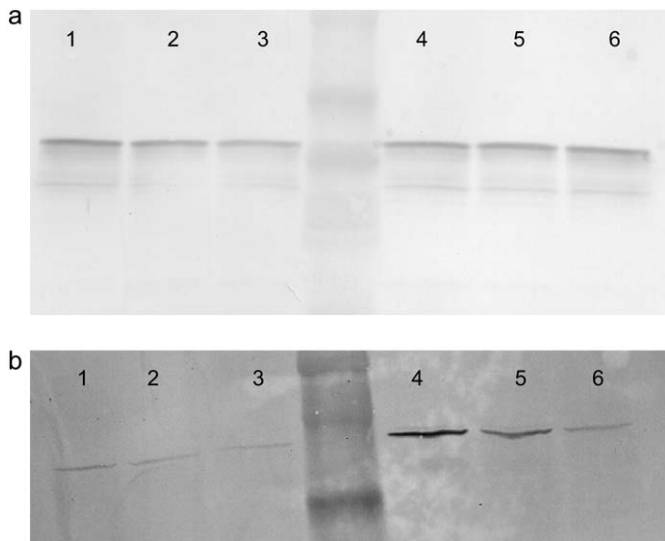


Fig. 2. Western blots of HspBP1 (a) and Hsp70 (b). Controls (lane 1), 1 μ M camptothecin (lane 2), 10 μ M camptothecin (lane 3), heat stress (lane 4), heat stress + 1 μ M camptothecin (lane 5) and heat stress + 10 μ M camptothecin (lane 6).

Western blots were used as an alternative method to confirm that heat stress did not alter HspBP1 levels (Fig. 2a, lanes 1 and 4), but as expected Hsp70 levels increased (Fig. 2b, lanes 1 and 4). Caspase-3 activity was not altered by heat treatment indicating that the cells were not undergoing apoptosis (Fig. 3).

HspBP1 expression did not change after heat stress induction. We therefore, analysed the response of the cells to the apoptotic inducing compound camptothecin, a DNA topoisomerase I inhibitor. The cells were treated with 1 μ M or 10 μ M camptothecin for 4 h and subsequently underwent HspBP1 staining for lab-on-a-chip analysis. There was no statistical difference with 1 or 10 μ M camptothecin compared to controls (Table 1). The caspase-3 assay revealed an increase of caspase-activity by a factor of 3 (Fig. 3) with 10 μ M camptothecin indicating the successful induction of apoptosis. Once again this result was verified using western

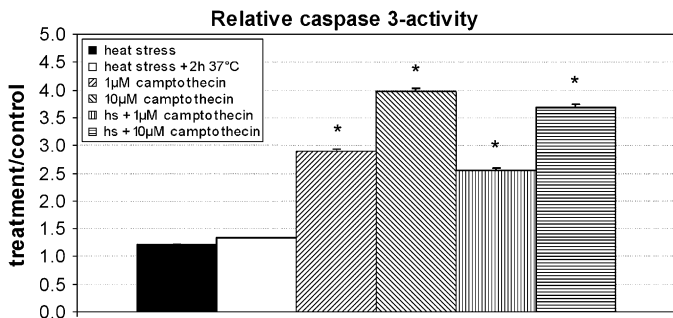


Fig. 3. Caspase-3 activity after treatments. Numbers are the ratio (treatment/control) after heat stress + 2 h at 37 $^{\circ}$ C, after induction of apoptosis by incubation with 1 and 10 μ M camptothecin, combined heat stress and 1 or 10 μ M camptothecin treatment, respectively. Control ratio = heat stress/non-heat stressed (black column). $n = 3$. * $p < 0.05$ Mann–Whitney U -test.

Table 1

Ratios of HspBP1 and Hsp70 using intracellular fluorescence following various treatments

Treatment	Fluorescence of sample/control \pm SEM	p
HspBP1 control	1.00	
HspBP1 after heat stress	1.13 \pm 0.02	0.406
HspBP1 after 1 μ M camptothecin	1.08 \pm 0.03	0.643
HspBP1 after 10 μ M camptothecin	1.06 \pm 0.07	0.121
HspBP1 after heat stress + 1 μ M camptothecin	1.10 \pm 0.01	0.439
HspBP1 after heat stress + 10 μ M camptothecin	1.00 \pm 0.04	0.439
Hsp70 control	1.00	
Hsp70 after heat stress	5.68 \pm 0.54	0.021 ^a
Hsp70 after heat stress + 10 μ M camptothecin	1.33 \pm 0.39	0.400, 0.006 ^b

Ratios are expressed as fluorescence of sample/fluorescence control \pm SEM and the corresponding p -values after Mann–Whitney U -test. The ratios of the controls were defined as 1.00.

^a Statistically different from control ($p < 0.05$).

^b Statistically different from heat stress ($p < 0.05$).

blotting and, the levels of HspBP1 were unchanged following camptothecin treatment (Fig. 2a, lanes 1–3). We did find, however, that camptothecin treatment caused a decrease in Hsp70 levels (Fig. 2b, lanes 1–3) with a corresponding increase in caspase-3 activity (Fig. 3). Once again, these results demonstrate that Hsp70 and HspBP1 levels are not coordinately regulated.

3.2. Heat stress treatment combined with camptothecin

The first set of experiments established conditions to either increase or decrease Hsp70 levels while HspBP1 levels remained unchanged. Next, these conditions were combined to determine if a simultaneous induction of heat-shock and apoptosis would have different effects on the HspBP1 expression levels than heat-shock or camptothecin alone. The results from the microfluidic assays are summarized in Table 1. With respect to HspBP1 levels, the cells with the dual stimulus responded in a similar manner to those treated with either stimulus alone, i.e. there were no detectable changes in HspBP1 expression levels. Treatment with camptothecin, however, blocked the heat stress induction of Hsp70. Western blot analysis confirmed these results and verified that camptothecin inhibited the heat stress increase in Hsp70 levels (Fig. 2b, lanes 4–6). Cells treated with both heat stress and camptothecin were apoptotic as demonstrated by an increase in caspase-3 activity (Fig. 3).

3.3. Over-expression of HspBP1

Stable transfection of HT-1080 cells with an expression vector containing HspBP1 resulted in the isolation of a sub-clone that would over-express HspBP1 when treated with doxycycline. Western blot analysis demonstrated that over-expression of HspBP1 in these cells did not alter the

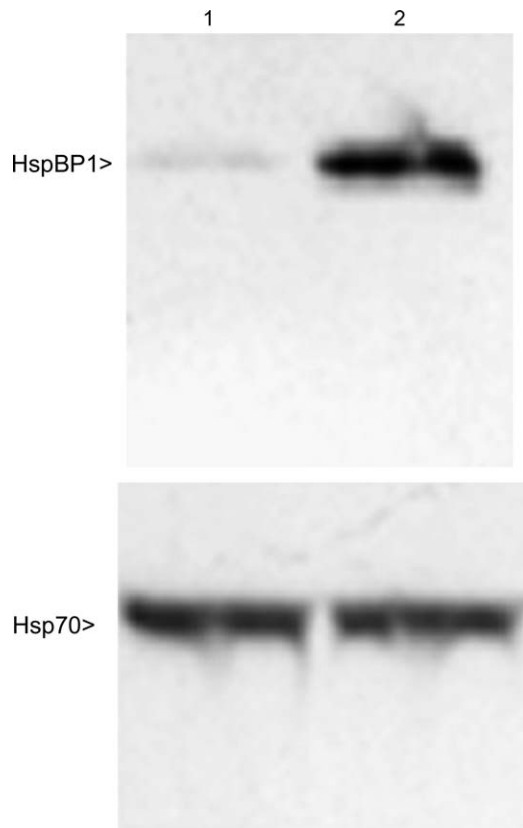


Fig. 4. Over-expression of HspBP1. HT-1080 cells were stably transfected with HspBP1 cDNA as described in Section 2. Cells were incubated without (lane 1) or with (lane 2) 1 µg/ml doxycycline (Dox) for 48 h. Cells were collected and probed for HspBP1 or Hsp70 as indicated using western blots.

expression level of Hsp70 (Fig. 4). The levels of HspBP1 and Hsp70 in these cells were quantified (Table 2) and resulted in a 290% increase in HspBP1 following Dox induction (3.3 ng/µg vs. 12.9 ng/µg), whereas, Hsp70 levels were slightly increased by approximately 16%. The calculated molar ratio of HspBP1 to Hsp70 increased from 0.56 to 1.89 following doxycycline induction. This increase in HspBP1 to Hsp70 ratio did not have any obvious effect on the cells.

4. Discussion

Hsp70 levels are increased by heat stress and decreased by camptothecin. Heat stress and camptothecin treatment, however, did not alter the intracellular HspBP1 levels. In addition,

camptothecin treatment decreased intracellular Hsp70 levels (70%) when the cells were treated simultaneously with camptothecin during heat stress. We estimated that the HspBP1 to Hsp70 ratio increased by a factor of about 6 and this was accompanied by a stimulation in apoptosis as indicated by an increase in caspase-3 activity.

Elevated levels of Hsp70 have been reported in a number of tumors including breast (Ciocca et al., 1993), lung (Volm et al., 1995), cervical (Ralhan and Kaur, 1995), prostate and renal (reviewed by Jäättelä, 1999; Jolly and Morimoto, 2000). The ability of Hsp70 to help cells survive stress could be a protection mechanism for cancer cells. High levels of Hsp70 have been correlated with shorter survival times for patients. This may be because elevated levels of Hsp70 protect the cells from apoptosis and therefore make cells less responsive to the anti-cancer drugs doxorubicin (Karlseder et al., 1996), topotecan and gemcitabine (Sliutz et al., 1996). Furthermore, drug resistance has been correlated with high Hsp70 expression in breast cancer patients (Vargas-Roig et al., 1998). Studies have shown that lowering the levels of Hsp70 in breast cancer cells (Nylandsted et al., 2000), oral cancer cells (Kaur and Ralhan, 2000) and colon cancer cells (Gurbuxani et al., 2001) results in cell death. If HspBP1 is an intracellular inhibitor of Hsp70 then lowering the level of Hsp70 and not altering the level of HspBP1 would increase the ratio of HspBP1 to Hsp70 and would essentially lower the intracellular level of active Hsp70 causing the cells to undergo apoptosis. This was observed in the experiments reported here and could be used as a new therapeutic strategy for the induction of apoptosis in tumor patients. Previous studies have shown that in tumor cells HspBP1 increases compared to control tissues (Raynes et al., 2003), but this is not harmful to the cells because the Hsp70 levels increase a similar amount and therefore the ratio of these two proteins remains constant in control and tumor tissues.

In experiments reported here, stably transfected cells increased the level of HspBP1 without altering the levels of Hsp70. Prior to induction, the HspBP1 to Hsp70 ratio was 0.56. This is similar to the ratios (0.21–0.42) reported in other cells and tissues (Raynes et al., 2003). Induction of HspBP1 expression in these cells increased the ratio to 1.89 and did not have any observable effect on the cells. We have estimated that an HspBP1 to Hsp70 ratio of approximately 4 is required to inhibit Hsp70 activity by 50% (Raynes et al., 2003). A ratio of 1.89 would inhibit less than 20% of the Hsp70 activity and would be consistent with the lack of an effect in the cells.

The intracellular molar ratios of these two proteins may have an important regulatory function with respect to cell death. The experiments reported here show that this ratio can be altered by lowering the level of Hsp70 and not changing the level of HspBP1 using camptothecin. This would represent a somewhat unusual mechanism for one protein to regulate another. Finally, the fact that neither heat stress nor camptothecin treatment could alter the levels of HspBP1, but did alter Hsp70 levels, indicates different mechanisms for regulating the levels of these two proteins.

Table 2
Amounts of HspBP1 and Hsp70 in HT-1080 cells

Cell Sample	HspBP1 ng/µg	Hsp70 ng/µg	HspBP1/Hsp70 (ng/µg)/(ng/µg)	HspBP1/Hsp70 molar ratio
Control	3.3	10.6	0.31	0.56
1.0 µg/ml Dox	12.9	12.3	1.05	1.89

Cells stably transfected with HspBP1 were grown in the absence (control) or presence of doxycycline (Dox) for 48 h. Cell homogenates were prepared and levels of HspBP1 and Hsp70 were estimated as described in Section 2. Ratios are ng of HspBP1 or Hsp70 per µg of lysate total protein.

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