Anticancer Drugs Up-regulate HspBP1 and Thereby Antagonize the Prosurvival Function of Hsp70 in Tumor Cells*

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The 70-kDa heat shock protein (Hsp70) is up-regulated in a wide variety of tumor cell types and contributes to the resistance of these cells to the induction of cell death by anticancer drugs. Hsp70 binding protein 1 (HspBP1) modulates the activity of Hsp70 but its biological significance has remained unclear. We have now examined whether HspBP1 might interfere with the prosurvival function of Hsp70, which is mediated, at least in part, by inhibition of the death-associated permeabilization of lysosomal membranes. HspBP1 was found to be expressed at a higher level than Hsp70 in all normal and tumor cell types examined. Tumor cells with a high HspBP1/Hsp70 molar ratio were more susceptible to anticancer drugs than were those with a low ratio. Ectopic expression of HspBP1 enhanced this effect of anticancer drugs in a manner that was both dependent on the ability of HspBP1 to bind to Hsp70 and sensitive to the induction of Hsp70 by mild heat shock. Furthermore, anticancer drugs up-regulated HspBP1 expression, whereas prevention of such up-regulation by RNA interference reduced the susceptibility of tumor cells to anticancer drugs. Overexpression of HspBP1 promoted the permeabilization of lysosomal membranes, the release of cathepsins from lysosomes into the cytosol, and the activation of caspase-3 induced by anticancer drugs. These results suggest that HspBP1, by antagonizing the prosurvival function of Hsp70, sensitizes tumor cells to cathepsin-mediated cell death.

Members of the 70-kDa heat shock protein (Hsp70) family play an essential role in quality control of cellular proteins (1–3) and include stress-inducible Hsp70, constitutively expressed Hsc70, mitochondrial Hsp75, and endoplasmic reticulum GRP78 (4, 5). Under normal conditions, Hsp70 proteins function as ATP-dependent molecular chaperones by facilitating the folding of newly synthesized polypeptides, the assembly of multiprotein complexes, and the transport of proteins across cellular membranes. Under stressful conditions, the synthesis of inducible Hsp70 enhances the ability of cells to cope with increased concentrations of unfolded or denatured proteins (1–6).

The Hsp70 proteins undergo a cycle of substrate binding and release that is accelerated by ATP hydrolysis (1–3). The substrate binding domain of Hsp70 is localized to a 25-kDa COOH-terminal region, with substrate access to this domain being controlled by a COOH-terminal “lid” that exposes the domain in the ATP-bound form and allows substrate binding to occur when Hsp70 is in the ADP-bound form. Opening and closing of the lid are governed by conformational changes associated with ATP binding and hydrolysis, which occur within the cleft of the 45-kDa NH2-terminal ATPase domain. The exchange of bound ADP for ATP results in substrate release, thus allowing Hsp70 to enter a new round of substrate binding and release.

The chaperone activity of Hsp70 proteins is regulated by various accessory proteins, known as cochaperones (1–3). For example, Hsp40 binds to the COOH terminus of Hsp70, stimulates its ATPase activity, and thereby stabilizes the substrate-bound form. The cochaperones Hip and Bag-1 bind to the ATPase domain of Hsp70 and function as nucleotide exchange factors: Hip prevents the dissociation of ADP from Hsp70 and thereby stabilizes the substrate-bound form, whereas Bag-1 promotes the release of ADP and rebinding of ATP, thereby triggering the premature unloading of bound substrate proteins from Hsp70.

In addition to their essential role in protein quality control, Hsp70 proteins have been shown to contribute to tumorigenesis. A high level of expression of these proteins thus enhances the tumorigenic potential of rodent cells in syngeneic animals (7) and is associated with a poor therapeutic outcome in several human cancers (8, 9), whereas depletion of Hsp70 promotes tumor regression (10). The tumorigenic potential of Hsp70 is thought to be attributable to an ability to confer a survival advantage on tumor cells through direct interference with several key components of the apoptotic signaling pathway, including JNK (11–13), AIF (14, 15), and apoptotic protease-activating factor-1 (16–18). The prosurvival function of Hsp70 proteins has been suggested to be distinct from their chaperone activity (14). Hsp70 has been localized to membranes of the endosomal-lysosomal compartment and shown to inhibit the cell death-associated release of cathepsins from these vesicles.
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in tumor cells, thus presumably contributing to its prosurvival function (19, 20).

Hsp70-binding protein 1 (HspBP1) was originally identified as a protein that interacts with Hsp70 and inhibits its chaperone activity (21). It was subsequently shown to bind to the ATPase domain of Hsp70 and to inhibit its ATPase activity (22). However, HspBP1 has also been shown to function as a nucleotide exchange factor for Hsp70 and Hsc70 (23) and to stimulate their chaperone activities (24). Although HspBP1 appears to represent a new member of the class of nucleotide exchange factors for Hsp70/Hsc70, its biological significance has remained poorly understood.

We have now investigated the physiological function of HspBP1, focusing on its possible interference with the prosurvival activity of Hsp70. Our results suggest that anticancer drugs up-regulate the expression of HspBP1, which then specifically binds to Hsp70 and antagonizes its ability to inhibit the cell death-associated permeabilization of lysosomes. HspBP1 thus contributes to the induction of cathepsin-mediated programmed cell death by anticancer drugs in tumor cells.

EXPERIMENTAL PROCEDURES

Materials—Vincristine and paclitaxel were obtained from Sigma, and etoposide was from Wako Pure Chemical (Osaka, Japan). CA-074 Me was obtained from Biomol (Plymouth Meeting, PA). Rabbit polyclonal antibodies to HspBP1 were generated in response to a peptide corresponding to residues 342 to 359 (EKLQQTCEFSSPADSMD) of the human protein. Other antibodies used included those to β-actin (A4700) from Sigma, Bcl-2 (clone 22) from BD Biosciences (Bedford, MA), cathepsin B (CA10) from Calbiochem (La Jolla, CA), green fluorescent protein (GF200) from Nacalai Tesque (Kyoto, Japan), Hsp70 (sc-24) from Santa Cruz Biotechnology (Santa Cruz, CA), and Hsc70 (SPA-815) from Stressgen Bioreagents (Victoria, British Columbia, Canada). Other chemicals and reagents were of the purest grade available.

Cell Culture and Heat Shock Treatment—The human cell lines MKN1 (gastric adenocarcinoma), MKN28 (gastric adenocarcinoma), NUGC-3 (gastric adenocarcinoma), Hep3B (hepatocellular carcinoma), HLE (hepatocellular carcinoma), and HLF (hepatocellular carcinoma), with the underlined sequence corresponding to an EcoRI site, and either 5'-GGGCTCGAGTTCACCGGACCCGCTGGAC-3' (forward), with the underlined sequence corresponding to a XhoI site. The PCR products were digested with EcoRI and XhoI, and the resulting fragments were cloned into the EcoRI- and Sall-digested pEGFP-C1 expression vector (Clontech, Palo Alto, CA) and verified by sequencing. To generate expression plasmids for EGFP-HspBP1(ΔM) or EGFP-HspBP1(ΔMC), we amplified DNA fragments corresponding to COOH-terminal portions of HspBP1 (amino acids 196 to 359 for HspBP1(ΔM) and 196 to 313 for HspBP1(ΔMC)) by PCR with the primers 5'-GGAGTACCTGGACAGCAGCCTGGAC-3' (forward), with the underlined sequence corresponding to a KpnI site, and either 5'-GCAGGATCTACCGAGCCACGCTGGAC-3' (reverse) for HspBP1(ΔM) or 5'-GCCAGATGCCCTACGGGCTCCCAGACACTC-3' (reverse) for HspBP1(ΔMC), with the underlined sequences corresponding to a BamHI site. The PCR products were digested with KpnI and BamHI, and the resulting fragments were cloned into the EGFP-HspBP1 expression vector that had been digested with KpnI and BamHI (to delete the DNA sequence encoding amino acids 154 to 359) and were verified by sequencing. Transfection of HeLa S3 cells with expression plasmids was performed with the use of the Lipofectamine 2000 reagent (Invitrogen). For establishment of HeLa S3 cells stably expressing EGFP or EGFP-HspBP1, the cells were subjected to selection in culture medium supplemented with Geneticin (400 μg/ml) after transfection, and individual resistant colonies were isolated to obtain cell clones.

Recombinant Proteins—The HspBP1 coding sequence was inserted into the pET24b vector (Novagen, Madison, WI), and the resulting construct was introduced by transformation into Escherichia coli strain BL21(DE3). Expression of His6-tagged HspBP1 was induced by the addition of isopropyl β-D-thiogalactopyranoside to the bacterial culture. Bacterial cells were lysed by ultrasonic disruption in 20 ml of a solution containing 20 mM Tris-HCl (pH 8.0) and 100 mM NaCl. Cell lysates were centrifuged at 12,000 × g for 30 min at 4 °C, and the resulting supernatant was applied to a Talon metal affinity column (Clontech). Recombinant His6-HspBP1 was eluted from the column with the column lysis solution supplemented with 100 mM imidazole and subjected to dialysis against phosphate-buffered saline. Recombinant human Hsp70 (NSP-555) and recombinant bovine Hsc70 (SPP-751) were obtained from Stressgen Bioreagents.

RNA Interference (RNAi)—Double-stranded small interfering RNAs (siRNAs) specific for human HspBP1 mRNA and a control nontargeting siRNA were obtained from B-Bridge International (San Jose, CA). The sense sequences targeted by

Plasmids and Cell Transfection—An HspBP1 cDNA was isolated from a human heart cDNA library by yeast two-hybrid screening with the ATPase domain of human Hsp70 as the bait (DBD) number AB020592. To generate expression plasmids encoding EGFP-HspBP1 or the deletion mutant EGFP-HspBP1(ΔC), we amplified cDNA fragments from human TIG-3 diploid fibroblast cDNA by polymerase chain reaction with the primers 5'-CCTGGAGTCTAGTCTAGACGACCTGGCCT-3' (forward), with the underlined sequence corresponding to an EcoRI site, and either 5'-CGGCTCAGTCAACGACCTGGCCTGTC-3' (reverse) for HspBP1 or 5'-CGGCTCAGTCAACGACCTGGCCTGTC-3' (reverse) for HspBP1(ΔC), with the underlined sequences corresponding to an XhoI site. The PCR products were digested with EcoRI and XhoI, and the resulting fragments were cloned into the EcoRI- and Sall-digested pEGFP-C1 expression vector (Clontech, Palo Alto, CA) and verified by sequencing. To generate expression plasmids for EGFP-HspBP1(ΔM) or EGFP-HspBP1(ΔMC), we amplified DNA fragments corresponding to COOH-terminal portions of HspBP1 (amino acids 196 to 359 for HspBP1(ΔM) and 196 to 313 for HspBP1(ΔMC)) by PCR with the primers 5'-GGGCTCGAGTTCACCGGACCCGCTGGAC-3' (forward), with the underlined sequence corresponding to a KpnI site, and either 5'-GCAGGATCTACCGAGCCACGCTGGAC-3' (reverse) for HspBP1(ΔM) or 5'-GCCAGATGCCCTACGGGCTCCCAGACACTC-3' (reverse) for HspBP1(ΔMC), with the underlined sequences corresponding to a BamHI site. The PCR products were digested with KpnI and BamHI, and the resulting fragments were cloned into the EGFP-HspBP1 expression vector that had been digested with KpnI and BamHI (to delete the DNA sequence encoding amino acids 154 to 359) and were verified by sequencing. Transfection of HeLa S3 cells with expression plasmids was performed with the use of the Lipofectamine 2000 reagent (Invitrogen). For establishment of HeLa S3 cells stably expressing EGFP or EGFP-HspBP1, the cells were subjected to selection in culture medium supplemented with Geneticin (400 μg/ml) after transfection, and individual resistant colonies were isolated to obtain cell clones.

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the HspBP1 siRNAs were 5’-GTGCAGAAGCTCAAGGTCA-3’ (HspBP1 siRNA-A) and 5’-GAGCTGGAGTCTGTGAAA-3’ (HspBP1 siRNA-B), whereas that targeted by the control siRNA was 5’-ATCCGGCAGATAGTACGTA-3’. Subconfluent cultures of MKN1 cells were transfected with siRNAs (50 nm) for 24 h with the use of Lipofectamine 2000.

**Cell Lysis and Immunoblot Analysis**—Cells were washed twice with ice-cold phosphate-buffered saline, scraped off culture plates into a hypotonic lysis buffer (25 mM Tris-HCl (pH 7.4), 25 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 25 mM p-nitrophophyl phosphate, 20 mM okadac acid, 0.2 mM sodium molybdate, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), aprotinin (10 μg/ml)), and subjected to ultrasonic treatment for 60 s. Cell lysates were centrifuged at 15,000 ×g for 30 min at 4 °C, and the resulting supernatants were fractionated by SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred electrophoretically to a polyvinylidene difluoride membrane and probed with primary antibodies as described previously (26–28). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI) and an enhanced chemiluminescence system (GE Healthcare). Image analysis was performed with Multi Gauge software version 3.0 (Fuji Photo Film, Tokyo, Japan).

**Co-immunoprecipitation Assay**—Cells were lysed in IP lysis buffer (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 25 mM p-nitrophophyl phosphate, 20 mM okadac acid, 0.2 mM sodium molybdate, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), aprotinin (10 μg/ml)), and cell lysates were incubated and gently rocked for 6 h at 4 °C with specific antibodies. Immune complexes were precipitated with Protein A/G Plus-agarose (Santa Cruz Biotechnology), washed three times with IP lysis buffer, and then subjected to immunoblot analysis as described above.

**Flow Cytometry**—Cells were harvested by exposure to trypsin, fixed with ice-cold 70% ethanol, treated with RNase A (100 μg/ml) (DNase-free, Sigma), and stained with propidium iodide (20 μg/ml) as described previously (27). At least 1 × 10⁶ cells were analyzed for DNA content (excitation at 488 nm, emission at 620 nm) or the expression of EGFP-tagged proteins (excitation at 488 nm, emission at 530 nm) with the use of a FACSCalibur flow cytometer and Cell Quest Pro software (BD Biosciences, San Jose, CA).

**Clonogenic Cell Survival Assay**—Cells seeded in 12-well plates (250 cells per well) were allowed to grow for 48 h before treatment with various drugs for 48 h. They were then allowed to form colonies by incubation in drug-free medium for 5 days. The resulting colonies were fixed with 70% ethanol and stained with 0.5% crystal violet. Colonies consisting of more than 50 cells were counted (29).

**Immunofluorescence Microscopy**—Cells grown on glass coverslips were exposed to various reagents, fixed with methanol at −20 °C, and transferred to blocking buffer (phosphate-buffered saline containing 2.5% bovine serum albumin). Cells were stained with monoclonal antibodies to cathepsin L or cathepsin B (1/200 dilution in blocking buffer), and immune complexes were then detected with Alexa Fluor 546-conjugated goat antibodies to mouse immunoglobulin G (1/200 dilution in blocking buffer) (Molecular Probes, Eugene, OR). Nuclei were stained with Hoechst 33342 (Sigma). Confocal images were obtained with an Axiovert 200M microscope equipped with an LSM 5 Pascal system (Carl Zeiss, Jena, Germany).

**Statistical Analysis**—Data are presented as mean ± S.D. and analyzed where indicated by the two-tailed Student’s t test. A p value of <0.05 was considered statistically significant.

**RESULTS**

**Preferential Association of HspBP1 with Hsp70 in Cells**—To confirm the physiological interaction between HspBP1 and
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In a variety of tumor cell types, HspBP1 exerts a prosurvival effect (17). To determine whether it acted in a similar manner to Hsp70, we examined the association of these proteins in HeLa S3 cells with a reciprocal co-immunoprecipitation assay. Immunoprecipitates prepared with antibodies to HspBP1 were found to contain not only HspBP1 but also a substantial amount of Hsp70 and a small amount of Hsc70 (Fig. 1A). Conversely, HspBP1 was detected in substantial amounts in immunoprecipitates prepared with anti-Hsp70 but was virtually undetectable in those prepared with anti-Hsc70. Essentially identical results were obtained with MKN1 cells (data not shown). Hsc70 is an abundant protein that is constitutively expressed in normal and tumor cells at similar levels; its concentration in most of the cell lines examined was 20 to 30 μg/mg of cellular protein, a value more than 10 times that for Hsp70 (Fig. 2, A and B). These results thus indicated that HspBP1 binds to Hsp70 in cells with much higher affinity than it does to Hsc70. Given that only a small proportion of Hsc70 molecules are associated with HspBP1 in cells, it is likely that HspBP1 exerts little effect on the physiological function of Hsc70. We therefore focused on the interaction between HspBP1 and Hsp70 in subsequent experiments.

HspBP1 is thought to bind to the ATPase domain of Hsp70 through major (including helices α2, α5, α8, and α11) and minor (including helices α14 and α17) interaction sites (24). To confirm these observations, we constructed expression vectors for EGFP-tagged deletion mutants of HspBP1 that lack helix α5 (EGFP-HspBP1(ΔM)), helix α17 (EGFP-HspBP1(ΔC)), or both of these helices (EGFP-HspBP1(ΔMC)) and then introduced the vectors into HeLa S3 cells. Immunoprecipitation with anti-Hsp70 and immunoblot analysis with anti-GFP revealed a marked reduction in the ability of not only EGFP-HspBP1(ΔM) but also EGFP-HspBP1(ΔC) to bind to Hsp70, compared with that of EGFP-HspBP1. EGFP-HspBP1(ΔMC) did not appear to interact with Hsp70 at all (Fig. 1B).

Increased Sensitivity of Tumor Cells with a High HspBP1/Hsp70 Molar Ratio to Anticancer Drugs—The molar ratio of HspBP1 to Hsp70 in cells might be expected to be an important determinant of the interaction between these two proteins as well as of the function of the resulting complex. In this regard, an HspBP1/Hsp70 ratio of ~4 has been suggested to be required for inhibition of Hsp70 activity by 50% (30). We therefore next determined the amounts of Hsp70 and HspBP1 in a variety of tumor cell types by immunoblot analysis, with recombinant Hsp70 and His6-tagged HspBP1 (His6-HspBP1) as respective standards. The concentration of Hsp70 in all the tumor cell lines examined was severalfold greater than that in diploid fibroblasts (Fig. 2, A and B). Although the concentration of HspBP1 in many tumor cell types was greater than that in normal fibroblastic cells, the difference in the amount of HspBP1 between tumor cells and normal cells was not as marked as that for Hsp70. Furthermore, no correlation between the expression level of Hsp70 and of HspBP1 was apparent in the tumor cells. The molar ratio of HspBP1 to Hsp70 was thus highly variable

![Figure 2. Expression levels of Hsp70, Hsc70, and HspBP1 in tumor cells.](image)

A, lysates (20 μg of protein) of the indicated cell types were subjected to immunoblot (IB) analysis with antibodies to Hsp70, Hsc70, HspBP1, or β-actin (loading control). The asterisk indicates nonspecific bands. Data are representative of three separate experiments. B, the indicated amounts of recombinant Hsp70 (upper panel), Hsc70 (middle panel), His6-tagged HspBP1 (lower panel), or cell lysates were subjected to immunoblot analysis with the respective antibodies. The blots were subjected to densitometry, and standard curves were constructed for the recombinant proteins for determination of the concentrations of Hsp70, Hsc70, and HspBP1 in the cell lysates. The open arrowhead and asterisk indicate His6-tagged HspBP1 and nonspecific bands, respectively. Data are mean ± S.D. of values from three separate experiments. C, molar ratio of HspBP1 to Hsp70 in each of the cell lines studied. Values are based on a molecular size of 70 kDa for Hsp70 and 39.5 kDa for HspBP1.
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A

B

C

FIGURE 3. Increased sensitivity of tumor cells with a high HspBP1/Hsp70 ratio to anticancer drugs. A, HeLa S3 or MKN1 cells were incubated for 24 or 48 h in the absence (control) or presence of 100 nM paclitaxel, 100 nM vincristine, or 34 µM etoposide. The cells were then fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. B, MKN28, HeLa S3, WiDr, HLF, MKN1, or DLD-1 cells were incubated for 24 or 48 h in the absence (control) or presence of 100 nM vincristine, 100 nM paclitaxel, or 34 µM etoposide and then analyzed as in A for the proportion of cells with a fractional DNA content (sub-G0 phase). C, HeLa S3 or MKN1 cells were incubated first with the indicated concentrations of vincristine, paclitaxel, or etoposide for 48 h and then in drug-free medium for 5 days in a clonogenic cell survival assay. The number of colonies consisting of more than 50 cells was counted and expressed as a percentage of the corresponding value for HeLa S3 or MKN1 cells incubated in the absence of drugs.

among the tumor cell types, ranging from a value similar to that for diploid fibroblasts (more than 10) for HLF, MKN1, and DLD-1 cells to less than 2 for A431, NUGC-3, and MKN28 cells (Fig. 2C).

HspBP1 has been shown to bind to Hsp70 and modulate its activity either negatively (21, 22) or positively (24). In addition, the increased expression of Hsp70 in tumor cells is thought to contribute to the resistance of these cells to the induction of cell death by anticancer drugs (8, 9). To obtain insight into the biological effect of HspBP1 on Hsp70 activity, we compared the drug sensitivities of tumor cell types with a high HspBP1/Hsp70 ratio (MKN1, DLD-1, and HLF cells) and those with a low HspBP1/Hsp70 ratio (HeLa S3, WiDr, and MKN28 cells). The cells were treated with vincristine (100 nM), paclitaxel (100 nM), or etoposide (34 µM) for up to 48 h, and the proportion of dead cells with a fractional DNA content (cells in sub-G0 phase) was then determined by flow cytometry. Tumor cells with a high HspBP1/Hsp70 molar ratio were more susceptible to the anticancer drugs than were those with a low ratio (Fig. 3, A and B). The differences in drug sensitivity between these two groups of tumor cell lines were confirmed by clonogenic cell survival assays performed with much lower concentrations of the anticancer drugs compared with those used for assay of the induction of cell death (Fig. 3C, data not shown). These results suggested that HspBP1 antagonizes the ability of Hsp70 to protect tumor cells from anticancer drug-induced cell death.

Ectopic Expression of HspBP1 Increases the Susceptibility of Tumor Cells to Anticancer Drugs—To elucidate further the biological significance of HspBP1, we established HeLa S3 cells that stably express EGFP-HspBP1. Immunoblot analysis revealed that the expression level of EGFP-HspBP1 was about four times that of endogenous HspBP1 in several of the HeLa-EGFP-HspBP1 cell clones (Fig. 4A). These HeLa-EGFP-HspBP1 cells exhibited an increased sensitivity to the induc-
etoposide or paclitaxel on cell death in both HeLa-EGFP and HeLa-EGFP-HspBP1 cells (Fig. 4B). Clonogenic cell survival assays confirmed these results. Colony formation efficiency (relative to that of corresponding control cells not exposed to drugs) was thus 84.64 ± 1.14 and 46.15 ± 5.81% for HeLa-EGFP cells (clone 3) and HeLa-EGFP-HspBP1 cells (clone 1), respectively, exposed to 1 nM paclitaxel, and these values were increased to 96.33 ± 2.51 and 82.30 ± 3.87%, respectively, for cells first subjected to mild heat shock (data are mean ± S.D. from three independent experiments).

**FIGURE 4.** Ectopic expression of HspBP1 increases the susceptibility of tumor cells to anticancer drugs. A, lysates (40 μg of protein) of HeLa S3 cells stably expressing EGFP (HeLa-EGFP cells, clones 3 and 4) or EGFP-HspBP1 (HeLa-EGFP-HspBP1 cells, clones 1 and 2) were subjected to immunoblot analysis with antibodies to GFP, HspBP1, or Hsp70. The asterisk indicates nonspecific bands. Data are representative of three separate experiments. B, HeLa-EGFP cells (clones 3 and 4) or HeLa-EGFP-HspBP1 cells (clones 1 and 2) were incubated for 48 h in the absence (C) or presence of 100 nM vincristine (V), 100 nM paclitaxel (P), or 34 μM etoposide (E), after which the proportion of cells in sub-G1 phase was determined by flow cytometry. In some experiments, HeLa-EGFP cells (clone 3) or HeLa-EGFP-HspBP1 cells (clone 1) were subjected to mild heat shock (43 °C, 60 min) before treatment with anticancer drugs. Inset, lysates (40 μg of protein) of cells subjected to heat shock were examined by immunoblot (IB) analysis with anti-Hsp70.

C, HeLa S3 cells were transiently transfected for 24 h with expression plasmids for EGFP, EGFP-HspBP1, EGFP-HspBP1(ΔC), or EGFP-HspBP1(ΔMC). The cells were then incubated for 48 h in the absence (control) or presence of 100 nM paclitaxel or 34 μM etoposide, after which the proportion of cells in sub-G1 phase was determined by flow cytometry: only those cells expressing EGFP-tagged proteins were analyzed. Data in B and C are mean ± S.D. of values from three separate experiments. *, p < 0.05; **, p < 0.01 versus corresponding value for HeLa-EGFP cells (clone 3) (B) or cells expressing EGFP (C). #, p < 0.05 versus corresponding value for drug-treated cells not subjected to heat shock.
Transient transfection of HeLa S3 cells with the expression vector for EGFP-HspBP1 also potentiated the induction of cell death by anticancer drugs (Fig. 4C). Transient expression of the EGFP-HspBP1(ΔC) mutant, which exhibits a reduced ability to bind to Hsp70 (Fig. 1B), increased the susceptibility of HeLa S3 cells to etoposide or paclitaxel only slightly (Fig. 4D), whereas transient expression of EGFP-HspBP1(ΔMC), which lacks Hsp70 binding activity (Fig. 1B), did not affect the susceptibility of HeLa S3 cells to these anticancer drugs (Fig. 4D). These results indicated that the biological activity of HspBP1 is completely dependent on its ability to bind to Hsp70.

**Anticancer Drugs Up-regulate HspBP1 in Tumor Cells**—We next examined the possible effects of anticancer drugs on the expression of Hsp70 and HspBP1 in tumor cells. All three anticancer drugs examined (vincristine, paclitaxel, and etoposide) induced a 2.0–2.5-fold increase in the amount of HspBP1 in HeLa S3 cells (representative of tumor cells with a low HspBP1/Hsp70 molar ratio) as well as in MKN1 cells (representative of tumor cells with a high HspBP1/Hsp70 molar ratio), with the highest expression levels being apparent between 6 and 24 h after the onset of drug treatment (Fig. 5). In contrast, the expression level of Hsp70 was not substantially affected by exposure of the tumor cells to these anticancer drugs for up to 24 h.

**Depletion of HspBP1 Reduces the Susceptibility of Tumor Cells to Anticancer Drugs**—We examined the effect of RNAi-mediated depletion of endogenous HspBP1 on the susceptibility of tumor cell to anticancer drugs. For these experiments, we studied MKN1 cells, which express HspBP1 at a relatively high level. Immunoblot analysis revealed that transfection of MKN1 cells with siRNAs (A or B) specific for HspBP1 mRNA, but not that with a control RNA duplex, resulted in a pronounced reduction in the abundance of HspBP1 as well as in marked suppression of the up-regulation of HspBP1 induced by anticancer drugs (Fig. 6A). Depletion of HspBP1 by RNAi reduced the susceptibility of MKN1 cells to anticancer drugs. Clonogenic cell survival assays thus revealed that RNAi-mediated depletion of HspBP1 resulted in a marked increase in the colony formation efficiency of MKN1 cells exposed to vincristine, paclitaxel, or etoposide compared with that observed for drug-treated cells transfected with the control siRNA (Fig. 6B). We confirmed these results by showing that depletion of HspBP1 resulted in marked inhibition of the induction of cell death (cells with a fractional DNA content) by vincristine (100 nm), paclitaxel (100 nm), or etoposide (34 µM) (data not shown).

**Promotion of Anticancer Drug-induced Permeabilization of Lysosomal Membranes and Cathepsin-mediated Cell Death by Ectopic Expression of HspBP1**—Finally, we examined the mechanism by which HspBP1 interferes with the prosurvival function of Hsp70. Hsp70 has been shown to promote cell survival through inhibition of death-associated permeabilization of lysosomal membranes (19, 20). Treatment with anticancer drugs induces both the translocation of lysosomal cathepsins from the lysosomal lumen to the cytosol as well as permeabilization of the mitochondrial outer membrane, events that are followed by caspase- or AIF-mediated programmed cell death (31, 32). We therefore examined the effect of ectopic expression of HspBP1 on anticancer drug-induced permeabilization of lysosomal membranes.

Immunostaining of HeLa S3 cells transiently expressing EGFP, EGFP-HspBP1, or EGFP-HspBP1(ΔC) revealed a predominantly perinuclear and punctate distribution of cathepsin L (Fig. 7A), consistent with the lysosomal localization of this enzyme. Immunostaining with antibodies to cathepsin B yielded essentially identical results (data not shown). Treatment with etoposide for 24 h induced the translocation of these cathepsins from cytoplasmic vesicles to the cytosol in cells expressing EGFP-HspBP1 but not in those expressing EGFP or EGFP-HspBP1(ΔC). Furthermore, prior exposure of the cells expressing EGFP-HspBP1 to mild heat shock to induce up-regulation of Hsp70 (see Fig. 4B, inset) resulted in inhibition of the etoposide-induced translocation of cathepsins. Exposure to etoposide for more than 60 h induced the translocation of cathepsins B and L to the cytosol also in the cells expressing EGFP or EGFP-HspBP1(ΔC) (data not shown). These results suggested that ectopic expression of HspBP1 promotes the anticancer drug-induced permeabilization of lysosomal membranes and the consequent release of cathepsins from lysosomes, and that this action of HspBP1 is sensitive to the up-regulation of Hsp70 induced by mild heat shock.

Cathepsins B and L have been shown to cleave Bid, a proapoptotic BH3-only member of the Bcl-2 family, resulting in the generation of its active ~15-kDa fragment (tBid), which in turn
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induces permeabilization of the mitochondrial outer membrane, the release of cytochrome c into the cytosol, and caspase-3-dependent programmed cell death (31, 33). We found that the cleavage of Bid as well as the activation of caspase-3 (as revealed by the appearance of its active 19- and 17-kDa fragments) induced by paclitaxel or etoposide were enhanced in HeLa-EGFP-HspBP1 cells compared with those in HeLa-EGFP cells. Furthermore, CA-074 Me, a specific inhibitor of cathepsin B, suppressed the cleavage of Bid and the activation of caspase-3 induced by anticancer drugs in both cell types (Fig. 7B). These results suggest that the expression of HspBP1 is dependent on its ability to bind to Hsp70, the expression levels of these two proteins in tumor cells do not appear to be correlated. Similar results suggesting that the expression of HspBP1 is not coordinately regulated with that of Hsp70 were recently described (34).

Our present results show that HspBP1 is a relatively abundant protein and is expressed at higher levels than is Hsp70 in both normal and tumor cell types. Moreover, tumor cells with a high HspBP1/Hsp70 molar ratio were found to be more susceptible to anticancer drugs than were those with a low ratio. Ectopic expression of HspBP1 also enhanced the induction of tumor cell death by anticancer drugs; this effect appeared to be dependent on the ability of HspBP1 to bind to Hsp70 and was inhibited by heat shock-induced up-regulation of Hsp70. Conversely, RNAi-mediated depletion of endogenous HspBP1 markedly reduced the susceptibility of tumor cells to anticancer drugs. Together, these results indicate that, under physiological conditions, HspBP1 functions as an inhibitor of the prosurvival function of Hsp70.

Hsp70 protects tumor cells from a wide range of lethal stimuli by various mechanisms, which include prevention of JNK activation, release of AIF from mitochondria, and nuclear import of the released AIF (4–6). However, overexpression of HspBP1 did not substantially affect either the activation of JNK (as determined by immunoblot analysis of the phosphorylation of c-Jun) or the nuclear import of AIF (as examined by immunostaining with anti-AIF) induced by anticancer drugs in HeLa S3 cells (data not shown). Hsp70 localizes to membranes of the endosomal-lysosomal compartment of tumor cells and promotes survival of tumor cells through inhibition of the death-associated permeabilization of lysosomal membranes (19, 20). Furthermore, the expression, secretion, or activity of lysosomal proteases such as cathepsins B, D, and L has been shown to be increased in most human tumor types examined (35, 36). Consistent with these observations, we found that ectopic expression of HspBP1 in HeLa S3 cells promoted anticancer drug-
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induced permeabilization of lysosomal membranes, as reflected by the release of cathepsins B and L from lysosomes into the cytosol, resulting in enhancement of cathepsin-mediated cell death. Furthermore, heat shock suppressed the anticancer drug-induced permeabilization of lysosomal membranes in cells overexpressing HspBP1, likely as a result of up-regulation of Hsp70. These results suggest that the antagonistic effect of HspBP1 on the prosurvival function of Hsp70 is mediated, at least in part, through suppression of the ability of Hsp70 to inhibit the death-associated permeabilization of lysosomal membranes. It remains to be determined whether binding of HspBP1 to Hsp70 interferes with the localization of the latter protein to lysosomal membranes or indeed suppresses its ability to inhibit the death-associated permeabilization of these membranes.

Anticancer drugs induced the up-regulation of HspBP1 in tumor cells, without affecting the expression level of Hsp70. Furthermore, the observed RNAi-mediated abrogation of such up-regulation of HspBP1 was associated with suppression of drug-induced cell death. These observations thus indicate that the up-regulation of HspBP1 by anticancer drugs increases the antagonism of Hsp70 by HspBP1 and thereby sensitizes tumor cells to cathepsin-mediated cell death. The suppression of anticancer drug-induced cell death either by RNAi-mediated depletion of HspBP1 or by cathepsin inhibitors was partial, indicating that the death response was mediated by multiple pathways, including those dependent on and independent of cathepsins (36–38).

The abundance of HspBP1 in cells is regulated at the transcriptional, post-transcriptional, and post-translational levels. Reverse transcription and PCR analysis did not reveal a marked increase in the amount of HspBP1 mRNA in HeLa S3 cells in response to treatment with vincristine or etoposide for up to 24 h (data not shown). The precise mechanism for the up-regulation of HspBP1 induced by anticancer drugs in tumor cells thus remains to be determined.

The amounts of Hsp70 and HspBP1 in normal and tumor cells were previously estimated (30). Although the amounts of Hsp70 in these cells were similar to those determined in the present study, those of HspBP1 were estimated at ~0.1 ng/μg of protein in normal cells and 0.4 to 0.6 ng/μg of protein in tumor cells (30), values that are less than one-tenth of those determined in the present study. Given that an HspBP1/Hsp70 molar ratio of ~4 is thought to be required for inhibition of Hsp70 activity by 50%, the authors of this previous study concluded that global inhibition of Hsp70 activity by HspBP1 in cells is unlikely (30).

We found that tumor cells with a high HspBP1/Hsp70 molar ratio are more sensitive to anticancer drugs than are those with a low ratio. Furthermore, depletion of HspBP1 by RNAi reduced susceptibility to anticancer drugs not only in MKN1 cells (Fig. 6) but also in DLD-1 cells (data not shown). Our present results are thus consistent with the previous observations suggesting that HspBP1 lacks a biological effect in cells. Rather, our results indicate that HspBP1 interferes with the prosurvival function of Hsp70 in tumor cells under physiological conditions. Finally, our finding that anticancer drugs induce the up-regulation of HspBP1 in tumor cells suggests that
this protein, by antagonizing the prosurvival activity of Hsp70, functions to sensitize tumor cells to the cathepsin-mediated cell death induced by anticancer drugs.

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