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# The Chaperone Function of hsp70 Is Required for Protection against Stress-Induced Apoptosis

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**Cellular stress can trigger a process of self-destruction known as apoptosis. Cells can also respond to stress by adaptive changes that increase their ability to tolerate normally lethal conditions. Expression of the major heat-inducible protein hsp70 protects cells from heat-induced apoptosis. hsp70 has been reported to act in some situations upstream or downstream of caspase activation, and its protective effects have been said to be either dependent on or independent of its ability to inhibit JNK activation. Purified hsp70 has been shown to block procaspase processing in vitro but is unable to inhibit the activity of active caspase 3. Since some aspects of hsp70 function can occur in the absence of its chaperone activity, we examined whether hsp70 lacking its ATPase domain or the C-terminal EEVD sequence that is essential for peptide binding was required for the prevention of apoptosis. We generated stable cell lines with tetracycline-regulated expression of hsp70, hsc70, and chaperone-defective hsp70 mutants lacking the ATPase domain or the C-terminal EEVD sequence or containing AAAA in place of EEVD. Overexpression of hsp70 or hsc70 protected cells from heat shock-induced cell death by preventing the processing of procaspases 9 and 3. This required the chaperone function of hsp70 since hsp70 mutant proteins did not prevent procaspase processing or provide protection from apoptosis. JNK activation was inhibited by both hsp70 and hsc70 and by each of the hsp70 domain mutant proteins. The chaperoning activity of hsp70 is therefore not required for inhibition of JNK activation, and JNK inhibition was not sufficient for the prevention of apoptosis. Release of cytochrome *c* from mitochondria was inhibited in cells expressing full-length hsp70 but not in cells expressing the protein with ATPase deleted. Together with the recently identified ability of hsp70 to inhibit cytochrome *c*-mediated procaspase 9 processing in vitro, these data demonstrate that hsp70 can affect the apoptotic pathway at the levels of both cytochrome *c* release and initiator caspase activation and that the chaperone function of hsp70 is required for these effects.**

Protein-damaging stresses, such as exposure of cells to elevated temperatures, activate an adaptive response leading to the increased synthesis of a group of proteins that regulate protein-folding processes (reviewed in reference 43). Members of the hsp70 family of molecular chaperones recognize nonnative domains that are exposed during protein translation, membrane translocation, oligomerization, and ultimately degradation. The abundant cytoplasmic and nuclear protein hsc70 is assisted in this task by the highly inducible hsp70 protein, whose synthesis is controlled by the level of nonnative protein substrates. Conditions that alter protein structure can result in the exposure of hydrophobic regions that are normally buried within the molecule, leading to their aggregation and loss of function. The ability of hsp70 to compete for binding to these hydrophobic regions coupled with an ATP-driven mechanism for release prevents protein aggregation and assists in the establishment of the native conformation (reviewed in references 4 and 18). Peptide binding is localized to a 15-kDa region near the COOH-terminal end of hsp70. Binding and release are regulated by ATP binding and hydrolysis, which occur in a 44-kDa $NH_2$-terminal ATPase domain. Conformational changes mediated by ATP hydrolysis stabilize a high-affinity association between hsp70 and a substrate. The rate of ATP turnover is regulated by a family of cochaperones that bind to the ATPase domain and to a COOH-terminal 10-kDa region. The COOH-terminal amino acid sequence Glu-Glu-Val-Asp, which is absolutely conserved in all eukaryotic hsc70 and hsp70 family members, is essential for association with the cochaperones hsp40 (hdj1) and Hop (8, 11). Deletion or mutation of these four residues increases the intrinsic ATPase activity and prevents stable association with peptide substrates (11).

An increased demand for the chaperoning function of hsp70 occurs at many stages in the life of a cell, as evidenced by changes in its level of expression during development and through the cell cycle. hsp70 and other stress-induced chaperones also determine the outcome of cells that are faced with death (27, 31). Elevated expression of hsp70 following exposure of cells to a mild heat stress provides an increased level of resistance to a subsequent more severe heat treatment that normally results in extensive apoptosis (36, 47). Stress-induced apoptosis is initiated by the release of cytochrome *c* from the mitochondrial intermembrane space (24, 70). The binding of cytochrome *c*, together with dATP, to the apoptotic protease-activating factor (Apaf-1) in the cytoplasm causes Apaf-1 to expose its N-terminal caspase recruitment domain, leading to association with procaspase 9 (33, 51). Recruitment of procaspase 9 into this complex, the apoptosome, results in procaspase 9 processing, and the apoptosome is then able to process downstream effector caspases, including caspases 3, 6,

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and 7. The inactivation and dismantling of the dying cell are brought about by the action of these proteases through cleavage of specific targets.

Elevated expression of hsp70 can block these death processes. Cleavage of the caspase 3 substrate poly(ADP-ribose) polymerase is inhibited in hsp70-expressing cells (5, 12, 44). This is a result of reduced processing of procaspase 3 and not due to an inhibition of the activity of the processed enzyme (44). Protection by hsp70 has been reported to occur downstream of cytochrome *c* release (22, 29) and in some cases downstream of caspase 3 activation and cleavage of caspase 3 substrates (22). In vitro experiments, using mitochondrion-free cytoplasmic extracts, revealed that purified recombinant hsp70 can inhibit cytochrome c-dATP-mediated caspase activation (1, 29). This effect requires the peptide binding domain and C-terminal EEVD sequence of hsp70.

Stress signaling through the JNK pathway precedes apoptotic death in cells exposed to protein-damaging stresses such as heat shock and ethanol, and also occurs in response to non-protein-damaging signals such as stimulation with cytokines such as interleukin-1 and tumor necrosis factor (TNF) or following UV irradiation (reviewed in references 19 and 26). hsp70 has been shown to block JNK activation and prevent apoptosis in response to both protein-damaging and physiological stimuli (12, 39, 44, 62, 69). However, this has only been observed in cells in which hsp70 was transiently overexpressed using tetracycline-regulated expression in stable cell lines, adenovirus delivery, or a mild heat treatment (reviewed in reference 13). The ability of hsp70 to block JNK activation is not detected in cells that have been selected for constitutive hsp70 expression (5, 22, 44). Although JNK activation was no longer repressed in these cells, they were protected from apoptosis. Inhibition of JNK activation can be accomplished by an hsp70 protein lacking its ATPase domain (63). This protein with ATPase deleted has also been shown to provide protection from extreme hyperthermic exposures (30, 57, 63).

The aim of this study was to determine whether the chaperone function of hsp70 is required for the prevention of heat-induced apoptosis. Since constitutive overexpression of hsp70 is known to affect cell growth and disrupt signaling pathways (10, 44) we were concerned that forced overexpression of hsp70 could lead to the selection of variant cell lines with adaptations affecting apoptotic signaling or effector processes. We therefore used an inducible expression system to generate cell lines expressing various hsp70 domain mutants. Cell lines with tetracycline-regulated expression of hsc70, hsp70, or hsp70 lacking either the ATPase domain or the C-terminal EEVD sequence or with a replacement of the EEVD sequence with four alanines were generated using a dicistronic expression cassette that also encodes the green fluorescent protein (GFP). A cell line with tetracycline-regulated expression of both GFP and the blue fluorescent protein (BFP) was used as a control. The effect of these proteins on cell growth and on survival following heat shock was examined and compared to their ability to affect JNK signaling and apoptotic processes.

## MATERIALS AND METHODS

**Construction of plasmids.** All transfected cell lines were generated using a tetracycline-regulated dicistronic expression vector derived from plasmid pTR-DC/GFP (45). Transcripts from this vector carry a gene of interest in the position of the first cistron and the GFP gene as the second cistron. This plasmid was modified by the addition of the adenovirus tripartite leader sequence and the insertion of the human hsp70 gene to create plasmid pTR5-DC/hsp70-GFP (44). This plasmid was used to create the vectors encoding the hsp70ΔEEVD and hsp70AAAA proteins. Construction of the pTR5-DC/hsp70AAAA-GFP plasmid was described by Shi et al. (56). The pTR5-DC/hsp70ΔEEVD-GFP plasmid was constructed using the same strategy, which involved replacing a *Cla*I-*Eco*RI fragment encoding the wild-type hsp70 sequence with a corresponding fragment from plasmid pMSHsp70ΔEEVD (11). Plasmid pTR5-DC/GFP was modified by the addition of the hygromycin resistance gene, under the control of the thymidine kinase promoter, from pCEP4 (Invitrogen Inc., Carlsbad, Calif.) creating pTR5-DC/GFP*tk/hygro. Plasmid pTR5-DC/hsp70-GFP was also modified in the same way to create pTR5-DC/hsp70-GFP*tk/hygro. The hsp70ΔATPase expression plasmid was created by digesting pTR5-DC/hsp70-GFP*tk/hygro with *Bgl*II and removing the 924-bp fragment to crate an in-frame deletion of amino acids 119 to 426 comprising the ATPase domain. Plasmid pTR5-DC/hsc70-GFP*tk/hygro was created by cloning the hsc70 cDNA as a *Bam*HI fragment from plasmid β-actin-hsc70 into the unique *Bgl*II site of pTR5-DC/GFP*tk/hygro. A dicistronic expression plasmid carrying both the BFP gene and the GFP gene was constructed and used as a control. The BFP gene was amplified with *Bam*HI ends from pQBI50-BFP (Quantum Biotech, Montreal, Quebec, Canada) and ligated into *Bgl*II-cut pTR5-DC/GFP*tk/hygro.

**Generation of transfected cell lines.** A PEER cell line expressing the reverse tetracycline-controlled transactivator (rtTA) was generated by transfection with plasmid pUHD172-1-neo (15). The transfected cells were selected with 400 μg of G418 (Life Technologies, Gaithersburg, Md.)/ml in complete medium, which consisted of RPMI 1640 (Mediatech Inc., Herndon, Va.) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah). Following selection in bulk culture the cells were screened by transient transfection with the tetracycline-regulated GFP expression plasmid pTR/GFP and cloned as described previously (45). A single rtTA-expressing clone (PErTA) was used as the parent cell line for the generation of each of the hsp70-expressing clones. For transfection, cells ($10^7$ in 0.4 ml of complete medium) were electroporated with a BTX T820 electroporator using a single pulse of 200 V at 50 ms (Genetronics Inc., San Diego, Calif.). Transfections were performed with 10 μg of linearized plasmid DNA. The pTR5-DC/hsp70AAAA-GFP and pTR5-DC/hsp70ΔEEVD-GFP plasmids were cotransfected with plasmid ptk/hygro. Transfected cells were selected in bulk culture with 200 μg of hygromycin (Sigma Chemical Co., St. Louis, Mo.)/ml. The hsp70AAAA and hsp70ΔEEVD cells were screened and selected by flow-cytometric sorting of GFP-positive cells after overnight incubation with 1 μg of doxycycline hydrochloride (Sigma)/ml. Clones were obtained using the AUTO-CLONE multiwell automated cell deposition system (EPICS ELITE ESP; Beckman-Coulter, Hialeah, Fla.). The other transfectants were cloned using the Quixell micromanipulator cell transfer system (Stoelting Co., Wood Dale, Ill.), which was mounted on a Leica DM-IRB inverted fluorescence microscope (Leica Canada, Montreal, Quebec, Canada). Complete details on the use of this micromanipulator can be found in Caron et al. (6). All clones were subsequently characterized by monitoring GFP expression by flow cytometry and hsp70 expression by Western blotting as described previously (44). Measurement of both hsp70 and GFP by flow cytometry was performed as described previously (44). The PETA70 cell line, in which hsp70 expression is controlled by the tetracycline-regulated transactivator, tTA, and is induced by the removal of tetracycline has been described (44).

**Cell growth and viability measurements.** Cell growth was measured using a flow-cytometric cell counting method. Cells in log phase growth were resuspended in fresh media with or without doxycycline (1 μg/ml) at a concentration of $1.5 \times 10^5$ cells/ml, and the viable and nonviable cells were counted twice daily over a period of 4 days. Cell suspensions (490 μl) were mixed with 10 μl of a fluorescent bead suspension (Flow-Check fluorospheres; Beckman-Coulter) resulting in a bead concentration of $2 \times 10^4$ beads/ml. The cell population was gated using forward and side light scattering to discriminate viable and nonviable cells on the basis of cell size and granularity. Flow-cytometric sorting of these two populations, followed by acridine orange staining and microscopic examination, confirmed that they corresponded to homogeneous populations of viable and apoptotic cells. The analysis was performed on an EPICS XL-MCL flow cytometer (Beckman-Coulter) using a 488-nm dichroic filter. The acquisition was stopped after counting $10^3$ beads, which permitted a constant sample volume (50 μl) to be analyzed for each sample. The total number of cells per milliliter in each map (viable and nonviable) was then calculated. The apparent growth rates ($\mu_{app}$) were calculated from the time profiles of viable cells plotted against the integral of viable cells. Death rates ($k_d$) were calculated from the time profiles of dead cells against the integral of viable cells (49). A ratio of $\mu_{app}$ and $k_d$ for the induced cells relative to the noninduced cells was calculated. This method was also used to measure the number of viable cells after heat shock.

Cell survival after heat shock was also measured by staining with annexin V-phycoerythrin (PE) (Pharmingen Canada Inc., Mississauga, Ontario, Canada). Cells were induced by incubation with doxycycline (1 μg/ml) for 24 h, except for the hsc70- and BFP-expressing cell lines, which were induced for 48 h. Cells were washed twice with phosphate-buffered saline (PBS) to remove the doxycycline before the start of the heat shock treatment. Cells were resuspended in fresh medium containing 20 mM HEPES buffer (pH 7.4) at a concentration of $10^6$ cells/ml and heated by immersion in a 43°C water bath for 60 min. Control cells were kept at 37°C. Following the heat shock the cells were diluted to $0.5 \times 10^6$ cells/ml with fresh 37°C medium and returned to a 37°C $CO_2$ incubator for 9 h. Preliminary experiments showed that this was the time at which the percentage of annexin V-positive cells reached a maximum and that after this time the cells began to lose membrane integrity. After this recovery period the cells were collected, washed twice in PBS, and resuspended in binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of $10^6$

cells/ml. Annexin V-PE (5 μl) was added to 100 μl of the cell suspensions, and after a 15-min incubation at room temperature an additional 400 μl of binding buffer was added. The cells were then analyzed by flow cytometry using a 488-nm argon ion laser excitation source. The simultaneous analysis of GFP and annexin V-PE was performed using the following filter set: a dichroic 550-nm long pass plus a 525-nm band pass for GFP and a dichroic 600-nm long pass plus a 575-nm band pass for the PE emission. Cell viability was calculated as the percentage of annexin V-negative cells out of the total cell population for the noninduced cells and out of the population of GFP-positive cells for the induced cells. The experiment was repeated three times for each cell line, and the difference in the viability of the induced cells relative to that of the noninduced cells was calculated. A one-tailed *t* test was performed to test for differences between the means of each of these values relative to those for the BFP-expressing cell line.

**Caspase measurements.** Cell extracts were prepared for measurement of caspase activity by incubation in cell lysis buffer (ApoAlert kit; Clontech, Palo Alto, Calif.) at a concentration of $10^6$ cells/50 μl for 10 min on ice followed by centrifugation at 13,000 × *g* for 5 min at 4°C. The supernatants were then stored at −80°C. The protein concentration was determined using the DC protein assay kit (Bio-Rad Laboratories Canada Ltd., Mississauga, Ontario, Canada). DEVDase activity was measured by mixing 20 μl of cell extract with 50 μM *N*-acetyl–DEVD–7-amino-4-methylcoumarin (Ac-DEVD-AMC) (Biomol Research Laboratories, Plymouth Meeting, Pa.) in a total volume of 200 μl of reaction buffer {50 mM HEPES-KOH (pH 7.0), 10% glycerol, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 2 mM EDTA, 5 mM dithiothreitol (DTT)} and measuring the continuous liberation of AMC at 37°C using a Cytofluor multiwell plate reader (PerSeptive Biosystems, Framingham, Mass.) with excitation and emission wavelengths of 380 and 460 nm, respectively. Values for the relative fluorescence units of AMC released per minute per microgram of protein were calculated for each sample and plotted relative to the maximum value for each cell line. Procaspase processing was also measured by Western blotting as described previously (44) using an anti-caspase 3 antibody (provided by D. W. Nicholson; Merck Frosst Center for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada) and an anti-caspase 9 antibody (provided by D. R. Green; La Jolla Institute for Allergy and Immunology, San Diego, Calif.).

**Analysis of JNK activation and JNK phosphatase activity.** JNK activation was measured by Western blotting using a phosphorylation state-specific anti-JNK antibody (Promega Corp., Madison, Wis.). Cells (2 × $10^6$) were lysed in 50 μl of buffer C (25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM HEPES [pH 7.9], 1 mM DTT, 50 mM NaF, 1 mM $Na_3VO_4$, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 20 μg of leupeptin, 5 μg of pepstatin, and 2 μg of aprotinin/ml). The lysate was centrifuged at 16,000 rpm for 15 min at 4°C, and the supernatant was retained. A portion of each sample (20 μl) was mixed with 5 μl of 5× sodium dodecyl sulfate (SDS) sample buffer (250 mM Tris-HCl [pH 6.8], 50% glycerol, 10% SDS, 25% 2-mercaptoethanol, 0.5% bromophenol blue) and heated to 90°C for 5 min. The samples (20 μg each) were subjected to SDS-polyacrylamide gel electrophoresis (Novex, San Diego, Calif.) and transferred to Hybond-C nitrocellulose (Amersham Corp., Arlington Heights, Ill.). Membranes were blocked with 1% bovine serum albumin (BSA) in TBST (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20) and then incubated with a 1:5,000 dilution of the anti-ACTIVE JNK polyclonal antibody in TBST–0.1% BSA for 3 h at room temperature. The membranes were washed three times with TBST and then incubated for 1 h with a 1:10,000 dilution of a horseradish peroxidase (HRP)-conjugated sheep anti-rabbit immunoglobulin G (IgG) (Amersham). Following three washes with TBST the membranes were processed by enhanced chemiluminescence (ECL) (Amersham) and exposed to film. The blots were subsequently stripped by incubation at 50°C for 30 min in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl [pH 6.7]), washed three times with TBST, blocked in TBST–1% BSA, and then incubated overnight at 4°C with a 1:1,000 dilution of an anti-JNK antibody (New England Biolabs Inc., Beverly, Mass.). The blots were then incubated with HRP-conjugated anti-rabbit IgG and processed by ECL. This antibody detects total JNK and was used to verify that equivalent amounts of JNK were present in each sample.

JNK phosphatase activity in adenovirus-infected cells was measured as described previously (39). An adenovirus transfer vector encoding hsp70 containing a replacement of the C-terminal amino acids EEVD with AAAA was constructed by subcloning the hsp70 coding sequence from plasmid pTR5-DC/hsp70AAAA-GFP into the adenovirus transfer vector pAdTR5-K7-$GFP_Q$ (38). Recombinant adenoviruses were generated as described previously (38).

**Examination of cytochrome *c* release.** Cytosolic extracts were prepared by homogenizing 2 × $10^7$ cells in an isotonic buffer [220 mM mannitol, 68 mM sucrose, 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 20 μg of leupeptin, 5 μg of pepstatin, and 2 μg of aprotinin/ml] using a Dounce homogenizer and a type B pestle. Cell disruption was monitored by staining an aliquot of the homogenized cells with trypan blue. The lysates were centrifuged at 800 × *g* for 10 min, and the resulting supernatants were clarified by centrifugation at 100,000 × *g* for 30 min. Equal amounts of protein from each of the 100,000 × *g* supernatants were analyzed by Western blotting using anti-cytochrome *c* (7H8.2C12; Pharmingen) and antiactin (C4; ICN) antibodies. Cell viability was assessed at the time of cell collection by counting apoptotic and viable cells after staining with acridine orange and ethidium bromide (44). Cytochrome *c* release was also measured in cells permeabilized with streptolysin O (66). Cells ($10^6$) were washed with PBS, collected by centrifugation, and resuspended in 50 μl of StrepO buffer (20 mM HEPES-KOH [pH 7.5], 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10 μM z-VAD-FMK (Biomol), 0.1 mM PMSF, and 5 μg of pepstatin, 10 μg of leupeptin, and 2 μg of aprotinin/ml) containing 60 U of streptolysin O (Sigma). After incubation at 37°C for 30 min the permeabilized cells were pelleted by centrifugation at 16,000 × *g* for 30 min at 4°C. The supernatant, containing cytosolic proteins, was mixed with 12.5 μl of 5× SDS sample buffer. The pellet, which contained the permeabilized cells including the mitochondria, was dissolved in 62.5 μl of 1× SDS sample buffer and sonicated. Samples were heated to 90°C for 5 min, and then equal volumes of the pellet and supernatant fractions were analyzed by Western blotting using anti-cytochrome *c* (7H8.2C12; Pharmingen) and anti-cytochrome oxidase subunit II (A-6404; Molecular Probes) antibodies.

For immunofluorescence analysis, cells (3 × $10^4$) were collected onto glass slides in a cytocentrifuge (Cytospin; Shandon Products Inc., Runcorn, Cheshire, United Kingdom) air dried, and fixed in 4% paraformaldehyde for 10 min at room temperature. After being washed in PBS the cells were permeabilized with 0.2% Triton X-100, washed with PBS, and then blocked with 10% fetal bovine serum in PBS. This was followed by an overnight incubation at 4°C with anti-cytochrome *c* antibody (6H2.B4; Pharmingen) diluted 1:200 in blocking buffer. The cells were then washed with PBS and incubated with a 1:100 dilution of a Texas red-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., Bar Harbor, Maine) for 1 h at room temperature. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; Sigma), which was included at a concentration of 1 μg/ml with the anti-mouse antibody. Images were acquired with a Sensys charge-coupled device camera (Roper Scientific, Tuscon, Ariz.) using OpenLab software (Improvision, Coventry, England).

## RESULTS

**Inducible expression of hsc70, hsp70, and hsp70 domain mutant proteins.** A PEER cell line expressing the reverse tetracycline-controlled transactivator (PErTA) was established and used to generate each of the hsp70-expressing cell lines. The tetracycline-regulated expression cassette used to generate these cell lines encodes a dicistronic message in which the second cistron is the GFP gene (45). The first cistron encodes either hsc70, hsp70, hsp70 lacking the ATPase domain, hsp70 lacking the four C-terminal amino acids EEVD, or hsp70 in which the EEVD sequence has been replaced by AAAA (Fig. 1A). To control for possible effects of GFP expression, we generated a cell line with the dicistronic vector in which the first cistron is the BFP gene. When induced this cell line expresses both BFP and GFP. Stable cell lines were obtained, after transfection and selection for hygromycin resistance, by transient induction and selection of individual GFP-positive cells with a micromanipulator (6) or by flow-cytometric cell sorting (45). Individual clones were subsequently screened by flow cytometry, and those with similar levels of induced GFP fluorescence were selected. Figure 1B shows flow-cytometric profiles of GFP fluorescence in each of the selected clones before and after induction with doxycycline. Each of the PErTA clones was induced for 24 h except for the HSC70 and BFP-expressing cell lines, which were induced for 48 h. A single peak of GFP-positive cells was observed after induction of the HSC70, HSP70ΔATPase, and HSP70ΔEEVD clones. For the other three clones the distribution was bimodal with approximately 70% of the cells showing high levels of GFP fluorescence.

The levels of the induced proteins in each of the clones was assessed by Western blotting (Fig. 1C). Although each of the clones had similar levels of induced GFP fluorescence, the levels of expression of the proteins encoded from the first cistron were not identical. In the PErTA-HSC70 cell line the level of hsc70 after 48 h of induction was 2.4-fold above the endogenous level. For the other clones the induced levels of expression relative to that of the endogenous hsc70 were 3.9-fold for HSP70, 1.8-fold for HSP70ΔATPase, 9.4-fold for HSP70ΔEEVD, and 3.3-fold for HSP70AAAA. With the exception of that for HSP70ΔEEVD all of the induced levels

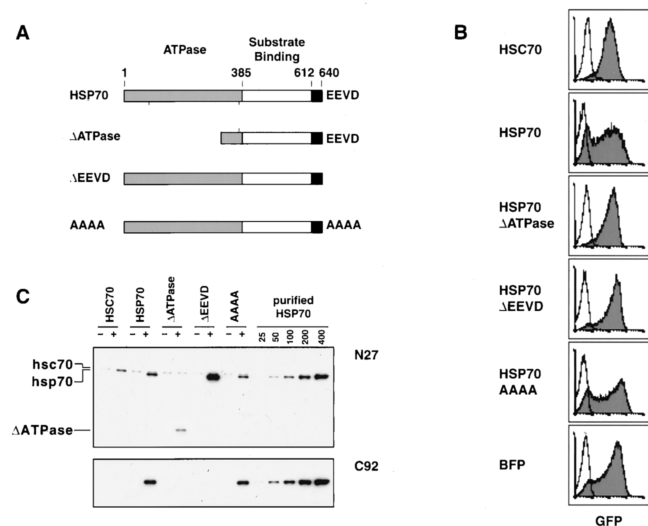


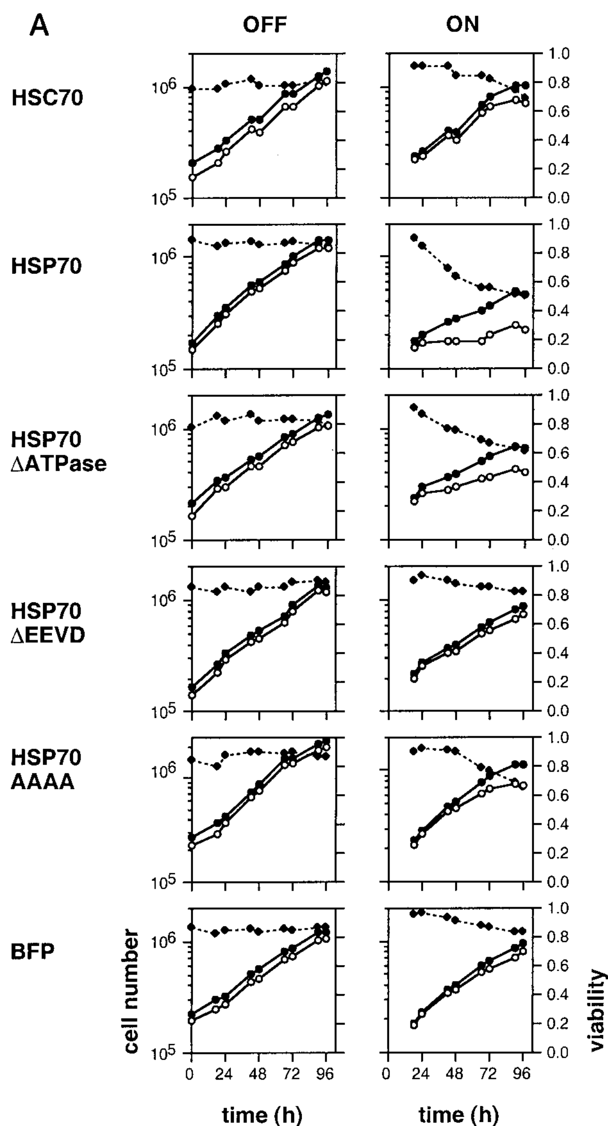

FIG. 1. Stable cell lines with tetracycline-regulated expression of hsc70, hsp70, or hsp70 domain mutant proteins. (A) Schematic representation of hsp70 functional domains. hsp70 and hsc70 have an amino-terminal ATPase domain followed by a peptide binding domain and the C-terminal sequence EEVD. (B) Flow-cytometric profiles of GFP expression in each of the clones grown in the presence (shaded profile) and absence (open profile) of doxycycline. The expression cassette is dicistronic and carries both the gene of interest and the GFP gene. In the BFP-expressing cell line the dicistronic transcript carries both the BFP and GFP genes. All of the cell lines were induced for 24 h except for the hsc70 and BFP-expressing cell lines, which were induced for 48 h. (C) Western blot analysis showing the levels of expression of the proteins encoded by the first cistron. Cells were grown with or without doxycycline as described for panel B. The upper portion shows the results with antibody N27, which recognizes both hsc70 and hsp70. The bottom portion shows the results for hsp70-specific antibody C92. Purified hsp70 (25 to 400 ng) was included to insure linearity of the signals.

were in the range of approximately two- to fourfold above the endogenous level of hsc70. Detection of each of the mutant hsp70 proteins required the use of antibody N27, which detects a common epitope on hsc70 and hsp70. The hsp70-specific antibody C92 recognized the hsp70AAAA protein but was not able to detect the hsp70ΔEEVD protein. The conformation of hsp70 has been shown to be altered by deletion of the EEVD sequence (11). Replacement of this sequence with AAAA does not appear to produce this effect. Although the level of the hsp70ΔEEVD protein was substantially higher than those of the other hsp70 proteins and although hsp70ΔEEVD is likely present in an altered conformation, the protein remained entirely soluble. In all of the clones the induced proteins were found almost exclusively in the soluble fraction of Triton X-100 extracts after centrifugation (data not shown). As well, there was no evidence of cytoplasmic granule formation by either phase-contrast or immunofluorescence examination of any of the cell lines after induction (data not shown).

**Continuous expression of hsp70 affects cell growth and viability.** Constitutive overexpression of hsp70 has been shown to decrease the growth rates of various transfected cell lines (21, 31, 46). This was also observed in a *Drosophila melanogaster* cell line in which the expression of hsp70 was regulated by the metallothionein promoter (10). In order to determine whether a specific domain of hsp70 was responsible for this growth inhibition, we compared the growth rates of each of the clones in the induced and noninduced states (Fig. 2A). Cell counts were performed by flow cytometry, which allowed us to quantitate the numbers of viable and nonviable cells in both the total cell population and the GFP-positive cells. Induced expression of hsp70 had a rapid and dramatic effect on cell growth. Differences in the rates of growth for the HSP70 cell line were evident after 24 h of induction. The $\mu_{app}$ of the induced cells (GFP-positive cells) was 28% of that of the noninduced cells (Fig. 2B). A similar effect on cell growth was seen in cells expressing the hsp70ΔATPase and to a lesser extent in the cells expressing hsp70ΔEEVD or hsp70AAAA. The calculated $\mu_{app}$ decreased to 49% for hsp70ΔATPase-expressing cells and to 67% for hsp70ΔEEVD- and hsp70AAAA-expressing cells. In the control cell line expressing BFP the $\mu_{app}$ of the induced cells was 90% of the value for the noninduced cells. The effect of hsc70 on cell growth was similar to what was observed in the BFP-expressing cell line. The $\mu_{app}$ of the HSC70 cell line when induced was 85% of that for the noninduced cells. However, hsc70 protein levels increased more slowly in this cell line than induced accumulation of hsp70 in each of the PErTA-HSP70 cell lines. If the analysis of growth rates is restricted to only the last 2 days of culture, the $\mu_{app}$ of the hsc70-expressing cells was reduced to 37% of that of the noninduced cells. These results reveal that expression of each of the hsp70 proteins reduced cell growth and that the peptide binding domain has a greater influence on growth inhibition than does the ATPase domain. Presumably this is due to multiple effects, such as titration of the cochaperones Hip or Bag1 by the ATPase domain and by interactions with rate-limiting substrates or the cochaperones Hop or hdj1 by the COOH domain.

Each of the cell lines accumulated approximately the same number of dead cells in both the noninduced and induced states over the course of the 4 days in culture. However, as a result of the effect of hsp70 on cell growth the percentage of viable cells decreased (Fig. 2A). For the HSP70 cell line viability was reduced from 89 to 49% after 4 days of induction. In the noninduced state viability was relatively unaffected, being reduced from 88 to 84%. The $k_d$ for the induced HSP70 cells was 4.7-fold higher than that for the noninduced cells (Fig. 2B). The $k_d$ for the induced BFP cells was 1.6-fold higher than that for the noninduced cells; by comparison, the $k_d$ for the induced hsp70 cells was 2.9-fold higher than that for noninduced cells. The HSP70ΔATPase and HSP70AAAA cell lines also had elevated $k_d$ compared to the BFP-expressing cell line. The ratios of $k_d$ for cells grown in the presence of doxycycline ($k_d$ ON) to $k_d$ for cells grown in the absence of doxycycline ($k_d$ OFF) for these cells were, respectively, 1.5- and 1.8-fold higher than that of the BFP-expressing cell line. The $k_d$ was unaffected in the HSP70ΔEEVD cell line, which had a $k_d$ ON/$k_d$ OFF value similar to that of the BFP-expressing cell line. For the HSC70 cell line the ratio of $k_d$ for the induced cells to that for the noninduced cells was also similar to that for the BFP-expressing cell line. However, if the analysis is restricted to the last 2 days of culture, the $k_d$ is found to be increased 2.8-fold. Therefore, continuous overexpression of hsp70 inhibits cell growth, and this leads to an accelerated rate of cell death. The full-length protein and the protein with ATPase deleted, which had more-significant effects on cell growth, caused higher $k_d$ than the C-terminal domain mutants.

**Prevention of heat-induced apoptosis by transient expression of hsp70 or hsc70.** The cytoprotective properties of hsp70 have been well documented, although its mechanism of protection is not clear. Since the major role of hsc70 and hsp70 is in protein folding it was of interest to determine whether hsp70 protection is mediated through a chaperoning mechanism. For these experiments the cells were induced for only 24 h, except for the HSC70 and BFP-expressing cell lines, which were induced for 48 h. Induction kinetics are slower for these two cell lines, and so the additional 24 h of induction was needed in



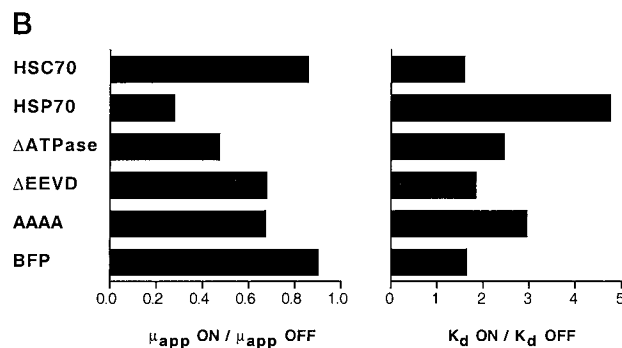




FIG. 2. Effect of hsc70 and hsp70 expression on cell growth. (A) Time profiles of the accumulation of viable cells (open circles), total cells (solid circles), and cell viability (diamonds) for cells grown in the absence (OFF) or presence (ON) of doxycycline. (B) $\mu_{app}$ and $k_d$ were calculated from the data shown in panel A, and the ratios of these values for the cells grown in the presence or absence of doxycycline were calculated. The calculated values for $\mu_{app}$ ON and $\mu_{app}$ OFF for cells expressing the indicated proteins are, respectively, as follows: hsc70 0.0176 and 0.0206 $h^{-1}$; hsp70, 0.0057 and 0.0203 $h^{-1}$; hsp70ΔATPase, 0.0084 and 0.0178 $h^{-1}$; hsp70ΔEEVD, 0.0155 and 0.0229 $h^{-1}$; hsp70AAAA, 0.0149 and 0.0222 $h^{-1}$; BFP, 0.0172 and 0.0191 $h^{-1}$. Corresponding calculated values for $k_d$ ON and $k_d$ OFF are, respectively, as follows: hsc70, 0.0071 and 0.0045 $h^{-1}$; hsp70, 0.0161 and 0.0034 $h^{-1}$; hsp70ΔATPase, 0.01 and 0.0041 $h^{-1}$; hsp70ΔEEVD, 0.004 and 0.0022 $h^{-1}$; hsp70AAAA, 0.0091 and 0.0031 $h^{-1}$; BFP, 0.0044 and 0.0027 $h^{-1}$.

order to achieve levels of expression similar to those for the other cell lines. The levels of expression at these times are shown in Fig. 1C. The induction times that were used were also selected to minimize the effects of expression on growth inhibition or cell death. Analysis of cell cycle distribution by flow cytometry showed that at the end of the induction times there were no differences in the percentages of cells in $G_1$, S, and $G_2$ phases of the cell cycle (data not shown). The cells were extensively washed to remove doxycycline and resuspended in fresh media to shut off expression in the induced cells prior to the heat treatment.

The extent of heat-induced apoptotic cell death was quantitated in each of the clones by annexin-PE binding to externalized phosphatidylserine. The translocation of this inner-membrane phospholipid to the outer leaflet of the membrane is a general feature of apoptotic cell death (37). The analysis of annexin-PE binding by flow cytometry allowed us to measure the percentage of annexin-positive cells in the GFP-positive population. First, it was necessary to demonstrate that the GFP-positive population corresponds to those cells that are also hsp70 positive. Figure 3A shows flow-cytometric profiles of cells that were fixed and processed for hsp70 immunodetection. The bottom panel, which shows the results for the induced cells, demonstrates that the level of GFP fluorescence corresponds to the level of hsp70 immunoreactivity. Therefore, restricting the analysis of annexin-PE binding to only the GFP-positive cells can be used to measure the response of the hsp70-positive cells.

The results of annexin binding after heat shock show that only the full-length hsp70 and hsc70 proteins are able to protect cells from heat-induced apoptosis. Figure 3B shows the results of annexin-PE binding with the HSP70 cell line. The left two panels show control cells that were either noninduced (top) or induced for 24 h (bottom). By this analysis the noninduced cells are 90% viable (annexin negative; quadrant 3) and the induced cells are 84% viable (quadrants 3 and 4). Following a heat shock (43°C for 60 min) and return to 37°C for 9 h (right panels) the viability of the noninduced cells was reduced to 34% whereas the viability of the total population of induced cells was 56%. However, by restricting the analysis of the induced cells to only the GFP-positive population (quadrants 2 and 4), the viability after heat shock was 73%. This same experiment was performed on each of the cell lines, and the results are shown graphically in Fig. 3C. Also included are data for the PETA70 cell line, in which the expression of hsp70 was induced by the removal of tetracycline (44). The viability of the total population of each of the noninduced cells is shown together with the viability of the GFP-positive population of the induced cells. For all of the cell lines the induced cells had higher viabilities after heat shock than the noninduced cells. This is because gating on GFP-positive cells excludes from the analysis those cells that were nonviable at the start of the experiment and which are not excluded from the analysis of noninduced cells. To test for the significance of the difference, the means of the differences in viabilities between the induced and noninduced cells were calculated (Fig. 3C, right) and compared to those of the BFP-expressing cell line. Only the

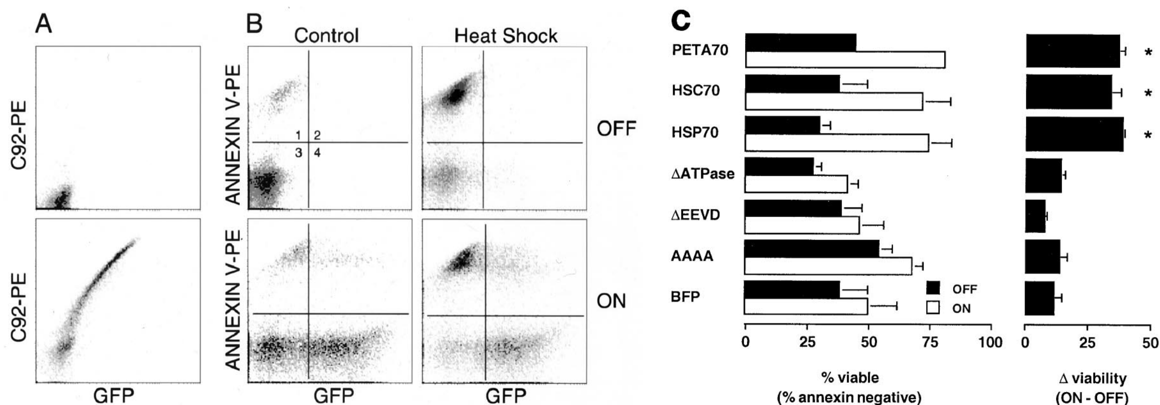



FIG. 3. Protection from heat-induced apoptosis by hsc70 and hsp70 proteins. (A) Demonstration that GFP fluorescence intensity correlates with levels of hsp70. Noninduced (upper panel) and induced (lower panel) cells were fixed and processed for immunocytochemical detection of hsp70 using the hsp70-specific antibody C92 and a PE-conjugated antimouse antibody. (B) Analysis of cell viability by annexin-PE staining in the hsp70-expressing cell line. Control and heat-shocked (43°C for 60 min followed by 9 h at 37°C) cells were incubated with annexin-PE and then analyzed for PE and GFP fluorescence by flow cytometry. Quadrants 1 to 4 correspond to cells that are annexin$^+$ GFP$^-$, annexin$^+$ GFP$^+$, annexin$^-$ GFP$^-$, and annexin$^-$ GFP$^+$, respectively. (C) Results of annexin staining for each of the cell lines after heat shock. The left panel shows the means and standard errors ($n = 3$) for the noninduced (OFF) and induced (ON) cells. For the induced cells the viabilities were calculated for the GFP-positive cells only. The right panel shows the means plus standard errors of the differences between the viabilities of the ON and the OFF cells. Comparison of each of these means to that of the BFP-expressing cell line shows that only hsc70 and the intact hsp70 protein provide protection against apoptosis (*, $P$ values of a one-tailed $t$ test are 0.0002 for the HSC70 cell line, 0.001 for the HSP70 cell line, and 0.0008 for the PETA70 cell line; all other $P$ values are greater than 0.05). The PETA70 cell line has tTA-regulated expression of hsp70.

PETA70, the HSC70, and the HSP70 cell lines had differences in the induced-cell and noninduced-cell viabilities that were significantly different from that of the BFP-expressing cell line ($P < 0.003$). This result demonstrates that the chaperone function of hsp70 is required to protect cells from heat-induced apoptosis since this only occurred in cells expressing the full-length hsp70 protein.

Similar results were obtained by counting the number of viable cells 24 h after the heat shock. Flow cytometry was used to count viable and nonviable cells (gated on cell size versus granularity) in both the total population of noninduced cells and the GFP-positive population of the induced cells (Fig. 4). It was necessary to carry out the cell count 24 h after the heat shock in order to insure that apoptotic cells were no longer present in the viable-cell gate. However, this measurement now assesses the effect of the heat shock on both cell survival and cell growth. Cell numbers are plotted relative to the number of cells in the culture before the heat shock. The relative cell numbers of induced and noninduced cells for control cultures that were not heat shocked are also shown. Only the cells expressing the full-length hsp70 or hsc70 proteins showed significant protection from heat shock. After heat shock, there were more viable induced cells than noninduced cells for the HSC70 and HSP70 cell lines but not for any of the others. For the HSP70 cell line over 90% of the induced cells were viable after the heat shock relative to the number of viable induced cells that were not heat shocked.

Since the level of induced full-length hsp70 was approximately twofold higher than that of hsp70ΔATPase, it is possible that the lack of heat protection by hsp70ΔATPase was due to suboptimal levels of expression. The level of expression can be regulated by altering the concentration of doxycycline; however, since the HSP70 cell line was not uniformly GFP positive after induction, this type of analysis was not possible. Reducing the amount of doxycycline had the effect of reducing the percentage of GFP-positive cells. Consequently, we recloned the HSP70 cell line and were able to isolate a clone that was uniformly GFP positive after induction (clone 8-17). Approximately 94% of the cells were GFP positive when they were incubated for 24 h with 1 μg of doxycycline/ml. This HSP70 clone and the HSP70ΔATPase cell line were incubated with various doses of doxycycline and analyzed for expressed protein levels and resistance to heat-induced apoptosis. Figure 5A shows that the fluorescence index (the product of the percentage of GFP-positive cells and the fluorescence intensity) of the cells increases linearly from 100 to approximately 500 ng of

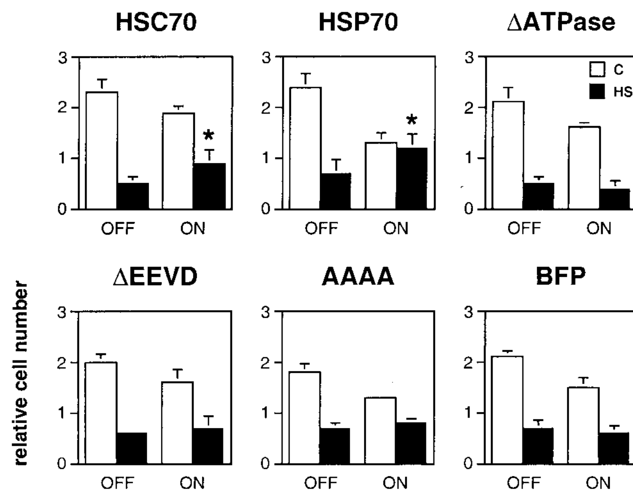



FIG. 4. Cell viability after heat shock is enhanced by hsc70 and hsp70, and this requires the chaperone function of hsp70. Noninduced (OFF) and induced (ON) cells were heated at 43°C for 60 min and returned to 37°C for 24 h. Flow-cytometric cell counts were carried out before the heat shock and after the 24-h recovery period. Separate cultures of nonheated cells were also counted at the same times. Cell numbers at the end of the recovery period are plotted relative to the initial cell count for control (open bars) and heated cells (solid bars). Shown are the means plus the standard errors for three independent experiments. Only the cells expressing hsc70 or hsp70 have significantly higher numbers of viable cells after heat shock (*, $P$ values of a one-tailed $t$ test comparing the relative cell numbers after heat shock for the ON and OFF cells are 0.033 for the HSC70 cell line and 0.022 for the HSP70 cell line; all other $P$ values are greater than 0.1).

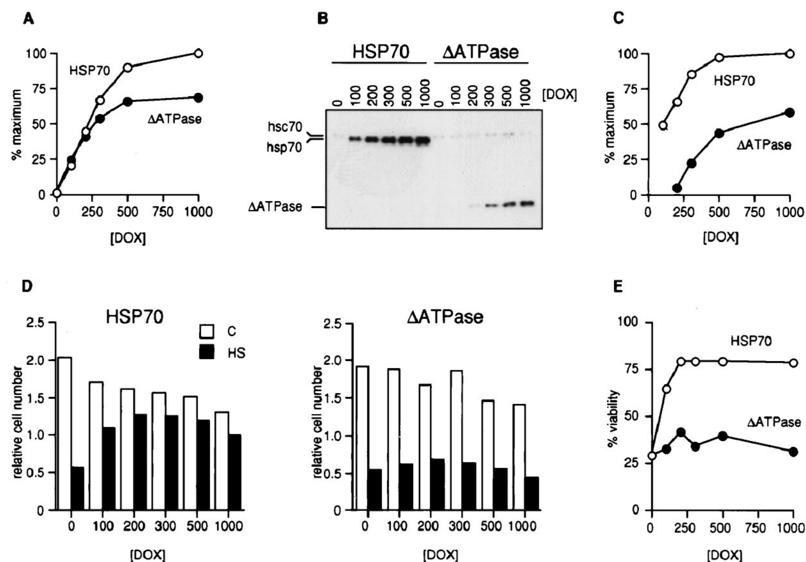



FIG. 5. At equivalent levels of expression the full-length hsp70 protein provides protection whereas the protein with ATPase deleted does not. The HSP70 (clone 8-17) and HSP70ΔATPase cell lines were incubated for 24 h with various doses of doxycycline ([DOX]) ranging from 100 to 1,000 ng/ml and then examined for GFP fluorescence by flow cytometry (A), expressed protein levels by Western blotting (B), and resistance to heat-induced apoptosis (D) as described for Fig. 4. Quantitation of the Western blot (C) and comparison to the percent viabilities after heat shock (E) reveal that the full-length hsp70 protein protects cells from apoptosis when expressed at a level equal to the maximum level attained in the HSP70ΔATPase cell line and that the protein with ATPase deleted does not protect cells at any of the expressed levels.

doxycycline/ml. A higher fluorescence index was attained in the HSP70 cell line at the higher doses of doxycycline. Western blot analysis (Fig. 5B) shows that the level of hsp70 protein expression can be regulated by the concentration of doxycycline in the same manner as the GFP fluorescence index. Quantitation of the Western blot (Fig. 5C) reveals that equivalent levels of expressed proteins are achieved by treatment of the HSP70ΔATPase cell line with 1,000 ng of doxycycline/ml and the HSP70 cell line with 100 to 200 ng of doxycycline/ml. When each cell line was incubated for 24 h with various doses of doxycycline and then exposed to heat shock, only the cell line expressing the full-length hsp70 protein showed resistance to heat-induced apoptosis (Fig. 5D). Maximal resistance was attained at concentrations of 200 ng of doxycycline/ml and greater (Fig. 5E). Although resistance was slightly less at a dose of 100 ng/ml, the percentage of GFP-positive cells was also somewhat less (77% $GFP^+$ at 100 ng/ml and 87% $GFP^+$ at 200 ng/ml). Therefore, the full-length hsp70 protein provided maximal levels of protection when expressed at levels equivalent to that at which hsp70ΔATPase was unable to protect the cells from heat-induced apoptosis.

Although the hsp70-expressing cells were protected from apoptosis, as measured by viability assays performed up to 24 h after the heat treatment, it was possible that they still might eventually succumb to a nonapoptotic death. To determine whether the protected cells were able to proliferate, we measured the growth of control and heat-shocked cells over a period of 5 days after the heat shock exposure (Fig. 6). Not only were there more viable cells 24 h after the heat shock when hsp70 expression was induced, but also the surviving cells proliferated at a rate similar to that of the non-heat-shocked cells. In contrast, the noninduced cells did not recover from a growth-arrested state until about 3 days after the heat shock. This experiment also demonstrates that, when hsp70 is transiently expressed for a period of 24 h and then its expression is shut off by the removal of doxycycline, the growth of the cells is only marginally affected (Fig. 6), which is in contrast to the marked growth inhibition that occurs when hsp70 is continuously expressed (Fig. 2).

**The chaperone function of hsp70 is not required for JNK inhibition.** Since it was clear that the chaperoning function of hsp70 was necessary for prevention of apoptosis, we next examined whether this was also true for inhibition of JNK activation in heat-shocked cells. For this, each of the cell lines were heated at 43°C for 60 min and collected immediately after the heat shock and also after a return to 37°C for 1 h. The amount of activated JNK was measured by immunoblotting with an antibody that specifically recognizes the active phosphorylated forms of JNK1 and JNK2 (Fig. 7). Active JNK was not detected in either the noninduced or induced cell lines in

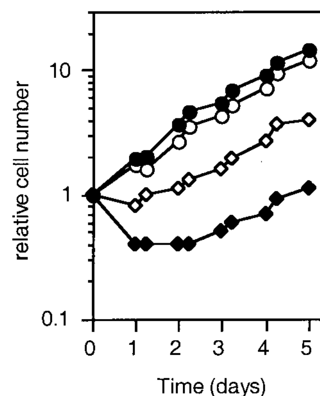



FIG. 6. Cells protected from heat-induced apoptosis by hsp70 retain their proliferative capacity. The HSP70 cell line (clone 8-17), either noninduced (solid symbols) or incubated with 200 ng of doxycycline/ml for 24 h (open symbols), was exposed to 43°C for 60 min and then returned to 37°C (diamonds). The numbers of viable cells were determined, as described for Fig. 4, over a period of 5 days and are plotted relative to the initial cell count. The growth of control non-heat-shocked cells (circles) is shown for comparison.

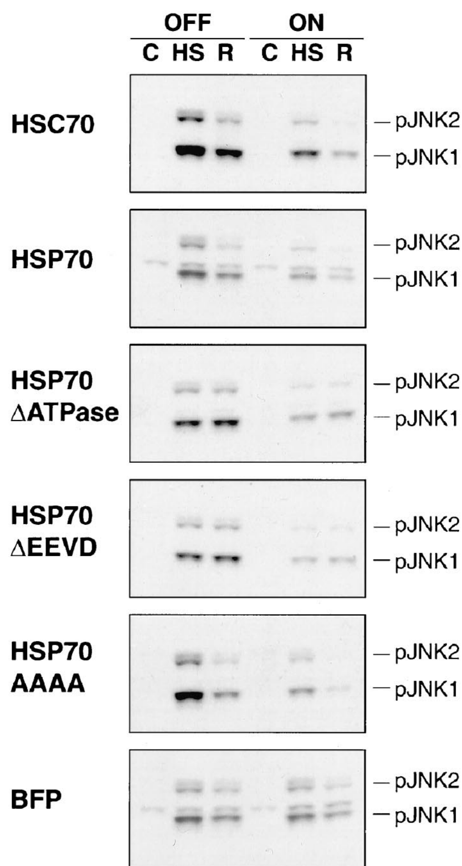



FIG. 7. Inhibition of heat-induced JNK activation in hsc70- and hsp70-expressing cells. Western blot analysis showed levels of phosphorylated JNK1 and JNK2 in noninduced (OFF) and induced (ON) cells. Cells were either not treated (C), heated at 43°C for 60 min (HS), or heat shocked and returned to 37°C for 60 min (R). JNK activation was strongly inhibited in all of the induced cells lines except for the BFP-expressing cell line.

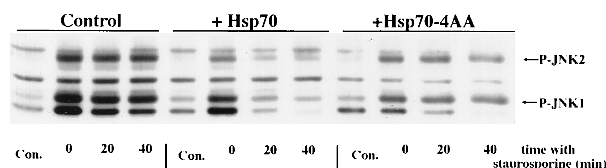



FIG. 8. The C-terminal substitution mutant hsp70AAAA inhibits JNK activation without blocking its rate of dephosphorylation. IMR90 lung fibroblast cells (20 to 30 population doublings) were infected with an adenovirus expressing tTA (AdCMV-tTA) and an adenovirus encoding a tetracycline-regulated expression cassette encoding full-length hsp70 (+Hsp70) or hsp70 with the C-terminal four amino acids EEVD replaced with AAAA (+Hsp70-4AA). Control cells received the same total multiplicity of infection of the hsp70-encoding virus but without the Ad CMV-tTA virus (control). The infected cells were heat shocked for 30 min at 45°C and then incubated at 37°C with the protein kinase inhibitor staurosporine to block new JNK phosphorylation. Extracts were prepared from non-heat-shocked cells (Con.), heat-shocked cells (0), and heat-shocked cells that were incubated with staurosporine for 20 or 40 min at 37°C after the heat shock. Shown is an immunoblot of cytosolic extracts probed with an anti-phospho-JNK antibody.

the absence of stress. Following heat shock high levels of phosphorylated JNK1 (pJNK1) and to a lesser extent pJNK2 were seen in all of the noninduced cells. The amount of each of these phosphorylated proteins decreased during the recovery period at 37°C, except for those produced by the HSP70ΔATPase and -ΔEEVD cell lines, which remained elevated. The extent of both JNK1 and JNK2 activation after heat shock was significantly reduced in all of the induced cells expressing hsc70 and hsp70 proteins, including those expressing hsp70 domain mutants. Expression of BFP had only a small effect on JNK activation. Quantitative analysis of the images revealed that the total pJNK1 and pJNK2 signal for the BFP-expressing cell line was 20% less for induced cells that were heat shocked than for noninduced cells. For the cell lines expressing hsp70 and each of the mutant hsp70 proteins the signals were approximately 70% less. In the hsc70-expressing cell line JNK2 activation was reduced by 75% and JNK1 activation was reduced by 50%. Therefore hsc70, like hsp70, is capable of inhibiting JNK activation. The inhibition of JNK activation by hsp70 occurs independently of its chaperoning activity since the ATPase mutant protein and the C-terminal regulatory domain mutant proteins were equally capable of inhibiting its activation following stress.

Activation of JNK results from an inhibition of its dephosphorylation in cells exposed to protein-damaging stresses with minimal stimulation of its upstream activator, SEK1 (39). Overexpression of hsp70 by adenovirus-mediated gene transfer prevented inhibition of the putative phosphatase, suggesting that hsp70 prevents JNK activation by protecting this phosphatase from heat-induced damage (39). Suppression of JNK phosphatase was also prevented in Rat-1 fibroblasts constitutively expressing an hsp70 protein with ATPase deleted (63). Therefore the chaperone function of hsp70 is not required for this effect. To test whether a functional peptide binding domain was required for the suppression of phosphatase inhibition, we first examined this in the PErTA cell lines expressing the wild-type proteins and C-terminal deletion mutant protein. However, the ATP depletion treatment used to measure phosphatase activity was not efficient in these cells. Consequently, we constructed a recombinant adenovirus providing tetracycline-regulated expression of hsp70AAAA and used this to infect IMR90 fibroblasts (Fig. 8). Cells were coinfected with an adenovirus expressing the transactivator protein tTA. Twenty hours after infection the cells were heated at 45°C for 30 min and collected either immediately or after 20 or 40 min of incubation with staurosporine, which was added to block new JNK phosphorylation (39). As was observed in the PErTA-HSP70AAAA cell line, the expression of this protein reduced the extent of JNK phosphorylation in response to heat shock. However, the rate of dephosphorylation was not affected. Similar results were observed in H9c2 cells infected with this virus (data not shown). Infection with an adenovirus expressing the full-length hsp70 protein results in an accelerated rate of JNK dephosphorylation after heat shock (Fig. 8) (39). Therefore, a functional peptide binding domain is required for hsp70 to promote JNK dephosphorylation in response to heat shock.

**Prevention of procaspase activation by hsp70 requires its chaperone function.** We have previously shown that inhibition of stress-induced apoptosis by hsp70 occurs upstream of caspase 3 activation (44). To determine whether the cytoprotective effects of hsp70 and hsc70 overexpression are associated with an inhibition of caspase activation and whether this required the chaperone function of hsp70, we monitored DEVDase activity in extracts from heat-shocked cells using the fluorogenic substrate DEVD-AMC (Fig. 9). For each of the noninduced cell lines there was a progressive increase in DEVDase activity with time after heat shock. When induced, the HSC70 and HSP70 cell lines showed initial increases in DEVDase activity immediately after exposure to 43°C for 60 min, but this level subsequently decreased in cells that were main-

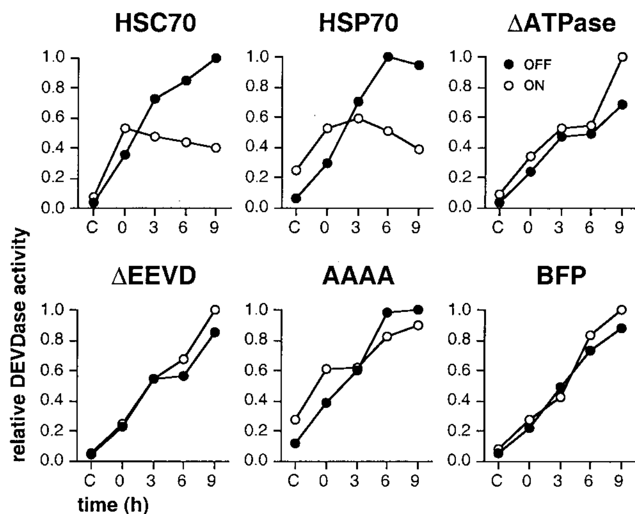



FIG. 9. Inhibition of heat shock-induced caspase activation in cells expressing hsc70 or hsp70 but not in cells expressing the hsp70 deletion mutant proteins. Extracts were prepared from noninduced (solid circles) and induced (open circles) cells that were heated at 43°C for 60 min and collected either immediately after the heat shock (0) or after a return to 37°C for 3, 6, or 9 h. Extracts were also prepared from control non-heat-shocked cells (C). Caspase activity was measured using a fluorometric assay with the substrate Ac-DEVD-AMC. Activities (fluorescence units per minute per microgram of protein) are plotted relative to the maximum activity obtained for each cell line.

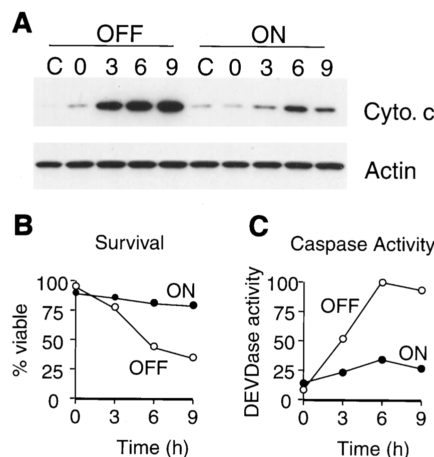



FIG. 11. Release of cytochrome *c* from mitochondria after heat shock is blocked in hsp70-expressing cells. (A) Levels of cytochrome *c* and actin were measured by Western blotting of cytosolic extracts prepared by Dounce homogenization of noninduced (OFF) and induced (ON) HSP70 cells (clone 8-17). Extracts were prepared from nonstressed cells (C) and cells that were exposed to 43°C for 60 min and collected either immediately after the heat shock (0) or following incubation at 37°C for 3, 6, or 9 h. (B) Cell viability was measured at the time of cell collection by counting viable and apoptotic cells after staining with acridine orange and ethidium bromide. (C) Caspase activity in the extracts was measured as described for Fig. 9.

tained at 37°C for 6 to 9 h after the heat shock. However, in the control cell line expressing BFP and in each of the hsp70 mutant-expressing cell lines the increase in DEVDase activity was the same for both the induced and noninduced cells. This reveals that the chaperone function of hsp70 is required to prevent caspase activation.

To determine whether inhibition of DEVDase activity was the result of inhibited processing of either procaspase 3 or its upstream activator caspase 9, we monitored procaspase processing in each of the cell lines after heat shock by Western blotting (Fig. 10). In each of the noninduced cell lines there was a loss of the 32-kDa procaspase 3 protein after heat shock and the appearance of the processed 17-kDa large subunit. The extent of loss of the proform and the abundance of the p17 fragment were reduced in the HSC70 and HSP70 cell lines that were induced prior to the heat shock. However, expression of either of the mutant hsp70 proteins did not inhibit procaspase 3 processing. A similar result was observed for procaspase 9 processing. Heat shock resulted in the loss of the procaspase 9 proform in all of the noninduced cells and in the cells expressing the mutant hsp70 proteins. However, in the cells expressing hsc70 or hsp70 the extent of procaspase 9 processing was substantially reduced. Each of the clones had similar levels of the caspase proforms in the absence of heat shock in both the noninduced and induced states. These data indicate that inhibition of apoptosis by hsp70 is associated with suppression of procaspase activation and that only the intact hsp70 protein is able to accomplish this inhibitory effect.

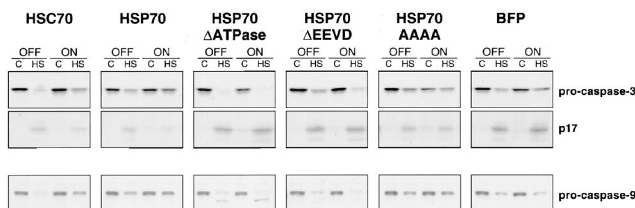



FIG. 10. Inhibition of procaspase 9 and procaspase 3 processing in cells expressing hsc70 or hsp70. Western blot analysis of extracts from noninduced (OFF) and induced (ON) control (C) and heat-shocked (HS; 43°C for 60 min followed by 6 h at 37°C) cells showing levels of the intact procaspase 9 protein, the intact procaspase 3 protein, and its processed large subunit (p17). Protection against loss of the proform of caspase 9 or 3 in the induced state occurs only for cells expressing hsc70 or hsp70.

**Release of cytochrome *c* from mitochondria after heat shock is blocked in hsp70-expressing cells.** Procaspase processing could be inhibited by an effect of hsp70 on apoptosome function or at the level of cytochrome *c* release. Purified hsp70 can block cytochrome *c*-dATP-mediated caspase activation in mitochondrion-free extracts (1, 29). However, this does not exclude the possibility that hsp70 might also block apoptosis upstream of apoptosome formation by preventing cytochrome *c* release. To examine this, cytosolic extracts were prepared by Dounce homogenization of cells in an isotonic buffer followed by ultracentrifugation. The amount of released cytochrome *c* present in the supernatants was assessed by Western blotting (Fig. 11A). A large increase in cytosolic cytochrome *c* was evident by 3 h after the heat shock for the noninduced HSP70 cell line. The amount of released cytochrome *c* increased further at 6 and 9 h. However, substantially less cytochrome *c* was released from cells that were induced to express hsp70 prior to the heat shock. The blot was probed for actin to insure that equivalent amounts of protein were present in each lane. The smaller amount of cytosolic cytochrome *c* in the hsp70-expressing cells correlated with the reduced number of apoptotic cells (Fig. 11B) and the reduced amount of caspase activity in the extracts (Fig. 11C).

Cytochrome *c* release was also measured using a nonmechanical method of cell disruption. Cells were permeabilized with streptolysin O to allow extramitochondrial cytochrome *c* to escape (66). The released cytosolic proteins were then separated from the permeabilized cells by centrifugation, and the abundance of cytochrome *c* in each of these fractions was examined by Western blotting (Fig. 12). In nonstressed cells

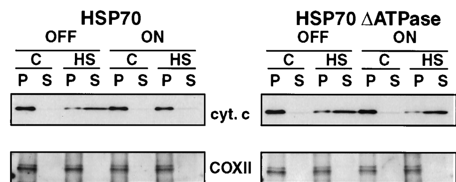



FIG. 12. Cytochrome *c* release from heat-shocked cells measured by streptolysin O permeabilization. Noninduced (OFF) and induced (ON) cells expressing hsp70 (clone 8-17) and hsp70ΔATPase were either not stressed (C) or heat shocked by exposure to 43°C for 60 min and then returned to 37°C for 6 h (HS). Cells were permeabilized by incubation with streptolysin O and then centrifuged to separate the cytosolic proteins (S) from the permeabilized cells (P). Equal volumes from each fraction were analyzed by Western blotting with antibodies to cytochrome *c* (cyt. c) and mitochondrial inner membrane protein cytochrome oxidase subunit II (COXII).

cytochrome *c* is recovered entirely in the pellet. Heat shock (43°C for 60 min followed by 6 h at 37°C) resulted in the release of the majority of the total cytochrome *c* present in noninduced cells. However, when the expression of hsp70 was induced, cytochrome *c* release was almost completely prevented. Expression of the hsp70ΔATPase protein did not prevent cytochrome *c* release. The mitochondrial inner membrane protein cytochrome oxidase subunit II (COXII) was retained in the pellet in all of the samples, indicating that the heat shock and streptolysin O treatments did not lead to general mitochondrial destruction.

To confirm the results observed in disrupted cells, we examined cytochrome *c* localization in fixed cells using immunocytochemical methods (Fig. 13). In control nonstressed cells, cytochrome *c* had a punctate staining pattern indicative of mitochondrial localization (A and E). Heat shock caused the release of cytochrome *c* from the noninduced cells, resulting in a diffuse cytoplasmic staining pattern (C). Many of the cells that had diffuse cytochrome *c* staining also had fragmented nuclei (D). In contrast, cytochrome *c* was retained within the mitochondria of hsp70-expressing cells after heat shock (G) and nuclear morphology was not affected (H). Cells with punctate or diffuse cytochrome *c* staining were counted to determine the percentage of cells with mitochondrion-localized cytochrome *c* (Fig. 14). Approximately 90% of the nonstressed cells had a clearly punctate staining pattern. After heat shock this was reduced to 44% for the noninduced cells; however, it was essentially unchanged in the cells expressing hsp70. This was not the case for cells expressing hsp70ΔATPase (Fig. 14). In fact expression of this protein resulted in an increased number of cells with released cytochrome *c*. These results, together with the results obtained with streptolysin O-permeabilized cells, indicate that the chaperone function of hsp70 is required to prevent cytochrome *c* release in heat-stressed cells.

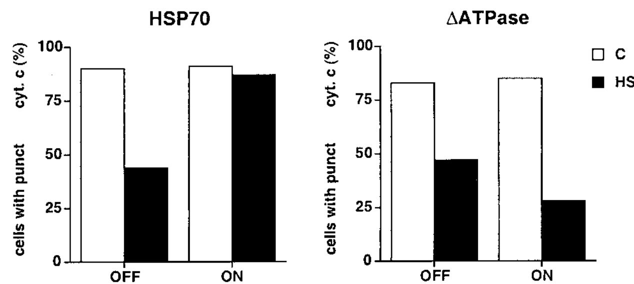



FIG. 14. The chaperone function of hsp70 is required to block cytochrome *c* release. Cytochrome *c* release was analyzed in noninduced (OFF) and induced (ON) cells expressing hsp70 (clone 8-17) and hsp70ΔATPase by immunocytochemistry as described for Fig. 13. The cells with mitochondrion-localized or cytoplasmic cytochrome *c* were counted, and the percentages of cells with mitochondrion-localized cytochrome *c* are plotted. Punct, punctate.

## DISCUSSION

Although it is clear that hsp70 has general cytoprotective properties and can prevent stress-induced apoptosis, it is not known how this is accomplished. hsp70 has been demonstrated

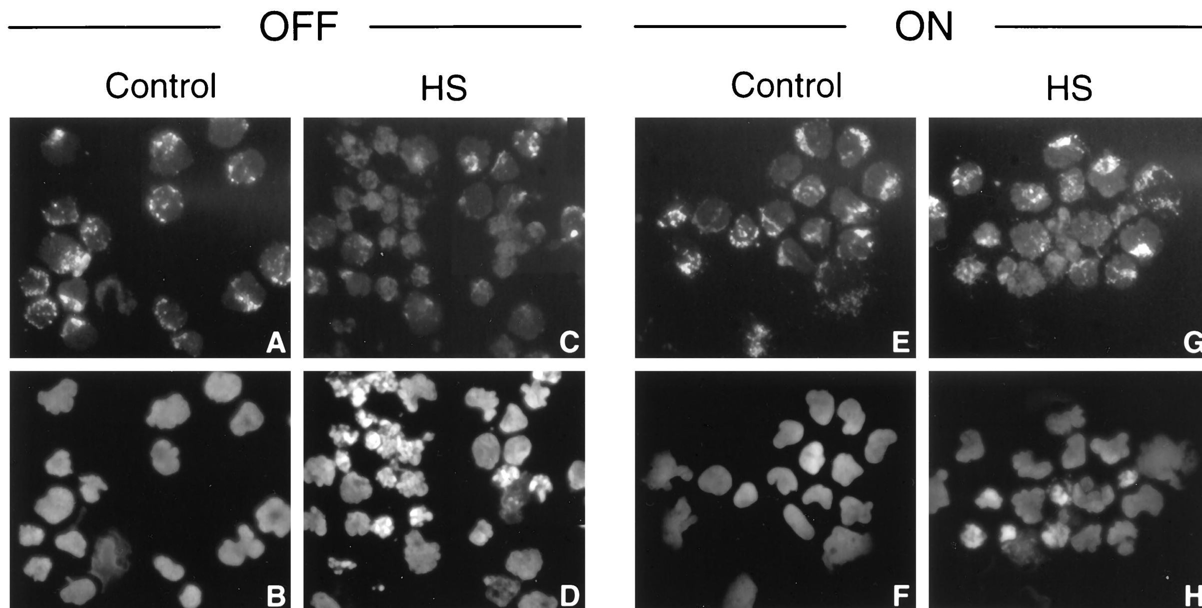



FIG. 13. Immunocytochemical examination of cytochrome *c* release. The localization of cytochrome *c* was analyzed in noninduced (OFF) and induced (ON) HSP70 (clone 8-17) cells before (control) and after (HS) heat shock (43°C for 60 min followed by 6 h at 37°C). Cytochrome *c* has a punctate localization in control cells (A and E). Following a heat shock the mitochondrion-localized immunofluorescence pattern is lost and cytochrome *c* becomes evenly distributed throughout the cytoplasm (C). Cytochrome *c* remains localized to mitochondria in cells expressing hsp70 (G). Nuclei were stained with DAPI (B, D, F, and H).

to affect processes regulating apoptotic signaling, effector molecule activation, and even events downstream of caspase activation. It is possible that hsp70 could affect multiple apoptotic pathways and that cell type-specific differences may account for the various points of intervention by hsp70. However, an important consideration in examining the effect of hsp70 is the manner in which it is expressed. The heat shock response is carefully regulated to ensure that, once an adequate amount of hsp70 is synthesized, transcription of the hsp70 gene is rapidly attenuated (42). This is because continuous high-level expression of hsp70 affects cell growth. In transfected *Drosophila* cells continuous expression of hsp70 from an inducible promoter resulted in growth inhibition and the sequestration of hsp70 into cytoplasmic granules (10). Formation of these granules occurred at a time that corresponded with a resumption of the proliferative capacity of the cells. Cell lines that constitutively overexpress hsp70 have been reported to have prolonged doubling times (21, 31, 46). Also, signaling pathways that are regulated by hsp70 overcome this level of control when hsp70 is constitutively overexpressed (5, 22, 44). In the lymphoid cell line studied here, continuous expression of hsp70 reduced growth rates by about 3.5-fold, and this was associated with an increased rate of cell death. It is likely, then, that in the generation of cell lines expressing hsp70 under the control of constitutively active promoters the isolated clones could result from the selection of variants that have defects in apoptotic signaling or effector processes.

To insure that we were examining direct effects of hsp70 and not effects that were the consequence of adaption to its continuous expression, we used an inducible expression system to generate cell lines expressing hsp70 and various domain mutant proteins. Another advantage of the approach that we have used is that by expressing hsp70 as a dicistronic message encoding the GFP reporter protein it is possible to restrict the analysis to only the GFP-positive cells. This was important because not all of the transfected cell lines express the encoded proteins in all cells after induction. Demonstration of tetracycline-regulated expression by Western blotting alone does not reveal what percentage of the induced cells are expressing the protein under study and how levels of expression vary within the population.

Continuous expression of hsp70 inhibited cell growth. The full-length protein was more inhibitory than were either of the domain mutant proteins. However, the protein with ATPase deleted inhibited growth more than either of the C-terminal mutant proteins. The protein with ATPase deleted is probably as effective at inhibiting cell growth as the full-length protein given that its level of expression was about twofold lower than that of hsp70. This suggests that inappropriate interactions between hsp70 and substrate proteins mediated by the peptide binding domain are responsible for the growth-impaired phenotype. hsc70 overexpression did not affect cell growth over 72 h of induction but was slightly growth inhibitory after this time. However, hsc70 accumulated more slowly than either hsp70 or hsp70ΔATPase. When the analysis was restricted to only the last 2 days, it was evident that inhibition of growth by hsc70 was greater than that of hsp70ΔATPase but not as severe as hsp70. Induced levels of hsc70 were less than that of hsp70 but equal to that of hsp70ΔATPase. Growth inhibition was associated with increased $k_d$. This loss of viability became apparent by 48 h of continuous hsp70 expression. The $k_d$, measured over the entire 96-h period, was highest for full-length hsp70. Both the ATPase mutant and the AAAA substitution mutant had elevated $k_d$ relative to the cell line expressing BFP. However, expression of mutant hsp70ΔEEVD did not increase the $k_d$ above that of the BFP-expressing cell line. Therefore, the growth inhibition observed in the hsp70ΔEEVD-expressing cell line may have been the result of the higher levels of protein expression and not due to a feature that this protein shares with the full-length protein. The absence of an effect of this protein on death rates could be because deletion of the EEVD sequence alters the conformation of the protein such that the domain responsible for this effect is no longer accessible. Bacterially expressed hsp70ΔEEVD yielded a unique set of trypsin cleavage products compared to full-length hsp70, suggesting an altered conformation (11). Also, the fact that this protein was not recognized by hsp70-specific antibody C92, whereas hsp70AAAA was, suggests that hsp70ΔEEVD does not fold properly when expressed in human cells.

Forced expression of hsp70 in *Drosophila* cells caused a reduction in growth without affecting viability (10). Recovery from growth inhibition occurred in cells where the distribution of hsp70 changed from diffuse to granular. Formation of hsp70 granules was proposed to be an adaptive response to overcome the suppressive effect that hsp70 has on cell growth. Overexpression of hsp70 in the human lymphoid cell line used in this study did not result in the formation of granules. Perhaps the inability of these cells to sequester hsp70 into cytoplasmic granules, and thereby overcome the effect that hsp70 has on growth inhibition, leads to their eventual loss of viability. The reason for the growth-suppressive effects of hsp70 are not known; however, Feder et al. (10) have suggested that hsp70 might specifically interact with cell cycle regulators. hsp70 accumulates in the nucleus during early S phase and associates with different classes of proteins in a cell cycle-dependent manner (40, 41). hsp70 plays an essential role in regulating the activity of the Wilms tumor suppressor WT1, a transcription factor that controls kidney development and that suppresses growth when overexpressed (35). Overexpression of hsp70 in HL60 cells reduced proliferation and promoted their differentiation (25). Also, the antiproliferative activity of certain prostaglandins has been attributed to their ability to induce the synthesis of hsp70 (54).

In some situations elevated expression of hsp70 has been shown to be associated with increased proliferation. hsp70 is often overexpressed in cancerous cells and can promote oncogenic transformation (20, 64). The Molt4 T-cell line has elevated basal levels of hsp70, and inhibition of its expression by an antisense oligonucleotide inhibited growth and promoted apoptosis (65). Also, in human breast cancer biopsy samples, the expression of hsp70 correlated with elevated proliferative capacity (60). Whether hsp70 will have growth-inhibiting or -promoting effects likely depends on the spectrum of cell cycle regulators that are available for modulation and their relative abundances in various cell types. A cell type-specific effect of hsp70 on growth inhibition was observed in transgenic mice expressing hsp70 under the control of the mouse H-2K promoter (28). These mice displayed severe T-cell hypoplasia with a defect in thymocyte differentiation to the CD4/CD8 double-positive stage (28). This suggests that forced overexpression of hsp70 is not tolerated without other adaptive changes. In fact, other transgenic mouse lines overexpressing hsp70 under the control of the human insulin promoter had generalized malignant T-cell lymphomas (55). Under these situations, the anti-apoptotic function of hsp70 could contribute to the process of tumorigenesis.

Cell lines that have been engineered to overexpress hsp70 have been shown to resist apoptosis in response to a variety of treatments including hyperthermia, ceramide, hypoxia, TNF, ethanol, UV, and several chemotherapeutic drugs (3, 5, 12, 21, 22, 25, 44, 53). Although the mechanism for this inhibition has only recently been addressed, most studies have focused on

caspase cleavage events. Inhibition of apoptosis by hsp70 overexpression was associated with an inhibition of caspase cleavage in cells exposed to hyperthermia, ethanol, or TNF (5, 12, 44). However, Jäättelä et al. (22) found that hsp70 was able to protect cells from staurosporine, TNF, and doxorubicin by acting downstream of caspase 3-mediated cleavage events. These differences could be attributable to the different cell types and inducers used or to the use of transient versus constitutive hsp70 overexpression. We have found that transiently elevated expression of hsp70 or hsc70 can provide protection against heat-induced apoptosis and that this was associated with reduced processing of procaspases 9 and 3. These effects required both the peptide binding and ATPase functions of hsp70, suggesting that protection requires the chaperoning function of hsp70. Purified hsp70 is unable to prevent the cleavage of poly(ADP-ribose) polymerase by purified active caspase 3 in an in vitro assay (44) but can block procaspase 9 and 3 processing initiated by the addition of cytochrome *c* and dATP to cytoplasmic extracts (1, 29). Inhibition of procaspase processing in vitro required both the peptide binding domain and the C-terminal EEVD sequence of hsp70. hsp70 specifically interacts with Apaf-1 and exerts its effect by preventing the recruitment of procaspase 9 to oligomerized Apaf-1 (1). Cells with elevated levels of hsp70 could therefore prevent execution of the apoptotic pathway by blocking the formation of a functional apoptosome.

hsp70 is also able to inhibit apoptosis by preventing the release of cytochrome *c* from mitochondria. This could result from modulating the activity or localization of proapoptotic or antiapoptotic members of the *bcl-2* family. Many forms of stress result in Bax dimerization and relocalization from the cytosol to mitochondria, where it forms ion channels that are believed to disrupt mitochondrial function (reviewed in references 17 and 59). Alternatively, heat stress might directly cause mitochondrial dysfunction, which could ultimately result in the leakage of cytochrome *c* from mitochondria. hsp70 could provide protection from this damage, a task that would be expected to require its chaperone function. Preconditioning heat shock treatments sufficient to induce hsp synthesis and confer thermotolerance provide protection to mitochondria from oxidative or heat shock-induced damage (2, 9, 50). The protected cells retained their mitochondrial membrane potential, mitochondrial ultrastructure was unaltered, and their rate of oxygen consumption was preserved. A role for hsp70 in protecting mitochondrial integrity would provide an additional level of control to prevent the inappropriate activation of apoptosis. Mitochondria play a central role in regulating stress-induced apoptosis (reviewed in reference 16). In addition to cytochrome *c*, other mediators of cell death are released, including the apoptosis-inducing factor, which mediates nuclear disruption (58). As well, portions of procaspases 3 and 6 are localized within the mitochondrial intermembrane space and are released during apoptosis. Following their release these caspases can be purified as a complex containing the mitochondrial matrix chaperone protein hsp60 (52, 67). Maturation of procaspase 3 is accelerated by hsp60 in vitro in an ATP-dependent manner, which has led to the suggestion that hsp60 stabilizes a conformation of procaspase 3 that is sensitive to caspase-mediated processing. Therefore, in addition to preventing apoptosome activation after cytochrome *c* release, hsp70 could limit the release of cytochrome *c* and other apoptogenic molecules from mitochondria. Potentially, hsp70 could restrict the cytoplasmic level of cytochrome *c* by preventing its release from some mitochondria or limit the amount released from each mitochondrion. However, single-cell analysis of cytochrome *c* release has revealed that all mitochondria coordinately release their cytochrome *c* within each affected cell (14). Therefore, hsp70 likely acts to prevent the signal leading to cytochrome *c* release.

Another point in the apoptotic pathway that can be modulated by hsp70 is JNK signaling (reviewed in reference 13). Evidence that activation of this pathway is essential for apoptosis comes from experiments showing that overexpression of a kinase-inactive mutant of SEK1, the JNK kinase, or a nonphosphorylatable dominant-negative mutant of c-jun blocked stress-induced apoptosis (7, 61, 68, 71). However, no protection from a number of stresses, including heat shock, was observed in $sek1^{-/-}$ ES cells and thymocytes in which heat-induced JNK activation is abolished (48). Instead these cells were more sensitive to CD95- and CD3-mediated apoptosis, suggesting that SEK1 activation provides a protective function. Involvement of JNK in stress-induced apoptosis could be cell type dependent or influenced by the severity of the stress. The importance of hsp70-mediated inhibition of JNK activation in suppressing apoptosis is also controversial. The ability of hsp70 to inhibit JNK has been observed in cells in which the expression of hsp70 is transiently induced by tetracycline-regulated expression vectors or by adenovirus-mediated gene delivery (12, 39, 44, 62, 69). However, this ability is lost in cells that have been selected after transfection with constitutively active hsp70 expression vectors (5, 22, 44). Protection from apoptosis in spite of an undiminished activation of JNK in these cells suggests either that hsp70 can act to suppress apoptosis downstream from this essential event or that suppression of JNK activation is not essential for apoptosis inhibition. Our finding that hsp70 proteins that lack their chaperone function and that are unable to protect cells from heat-induced apoptosis are fully competent at suppressing JNK activation reveals that inhibiting JNK activation is not sufficient for protection from heat-induced apoptosis. The role of JNK signaling in cellular events is likely dependent on the state of activation of other signaling pathways and therefore may differ between various cell types (19). In some situations, preventing JNK activation is sufficient to prevent apoptosis; however, in the lymphoid cell line studied here, JNK inhibition alone was not sufficient for protection from heat-induced apoptosis.

hsp70 acts at multiple levels to suppress stress-induced JNK activation. Protein-damaging stresses, such as exposure to hyperthermia, ethanol, or menadione, activate JNK without stimulating SEK1 activity (39). JNK activity is increased as a result of the effect that these stresses have on a putative JNK phosphatase. hsp70 can prevent the loss of phosphatase activity in stressed cells and thereby reduce the extent of JNK activation (39). The hsp70 protein with ATPase deleted constitutively expressed in a transfected Rat-1 cell line is able to accelerate JNK inactivation by increasing its rate of dephosphorylation in heat-shocked cells (63). These cells are protected from extreme hyperthermic exposures (30, 57, 63). In this same cell line, the hsp70 protein with ATPase deleted was found to act in a manner similar to the full-length protein in that it was able to partially prevent heat-induced intranuclear protein aggregation (57). However, it was unable to prevent heat-induced inhibition of protein translation (32) or accelerate the dissociation of heat shock factor HSF1 from its heat shock element binding state (23). The present data suggest not only that the chaperone function of hsp70 is unnecessary for the suppression of JNK activation but also that the substrate binding domain might also be dispensable. The mechanism of JNK suppression by hsp70 containing the C-terminal AAAA substitution is different from that of either full-length hsp70 or hsp70ΔATPase since the C-terminal mutant protein had no effect on JNK phosphatase.

In summary, we have shown that, when transiently induced, hsp70 protects cells against heat-induced apoptosis by preventing cytochrome *c* release, thereby inhibiting procaspase processing. Transiently induced resistance allows cells to cope with minor fluctuations in their environment and prevents unintentional cell loss. Apoptosis plays an essential role in regulating cell numbers, and interference with this process can lead to disease. hsp70 does not affect apoptotic processes initiated by CD3 or CD95 signaling which lead to caspase 8 activation (34). By specifically targeting the mitochondrial pathway leading to procaspase 9 processing, hsp70 could protect against stress-induced death without interfering with apoptotic pathways that regulate the immune response. The present data showing that prevention of cytochrome *c* release and procaspase processing in intact cells require the full-length hsp70 protein strongly suggest that these events are subject to regulation by the cellular chaperone machinery and that this is essential for inhibition of stress-induced apoptosis.

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