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Role of the Human Heat Shock Protein hsp70 in Protection against Stress-Induced Apoptosis

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Resistance to stress-induced apoptosis was examined in cells in which the expression of hsp70 was either constitutively elevated or inducible by a tetracycline-regulated transactivator. Heat-induced apoptosis was blocked in hsp70-expressing cells, and this was associated with reduced cleavage of the common death substrate protein poly(ADP-ribose) polymerase (PARP). Heat-induced cell death was correlated with the activation of the stress-activated protein kinase SAPK/JNK (c-Jun N-terminal kinase). Activation of SAPK/JNK was strongly inhibited in cells in which hsp70 was induced to a high level, indicating that hsp70 is able to block apoptosis by inhibiting signaling events upstream of SAPK/JNK activation. In contrast, SAPK/JNK activation was not inhibited by heat shock in cells with constitutively elevated levels of hsp70. Cells that constitutively overexpress hsp70 resist apoptosis induced by ceramide, a lipid signaling molecule that is generated by apoptosis-inducing treatments and is linked to SAPK/JNK activation. Similar to heat stress, resistance to ceramide-induced apoptosis occurs in spite of strong SAPK/JNK activation. Therefore, hsp70 is also able to inhibit apoptosis at some point downstream of SAPK/JNK activation. Since PARP cleavage is prevented in both cell lines, these results suggest that hsp70 is able to prevent the effector steps of apoptotic cell death. Processing of the CED-3-related protease caspase-3 (CPP32/Yama/apopain) is inhibited in hsp70-expressing cells; however, the activity of the mature enzyme is not affected by hsp70 in vitro. Caspase processing may represent a critical heat-sensitive target leading to cell death that is inhibited by the chaperoning function of hsp70. The inhibition of SAPK/JNK signaling and apoptotic protease effector steps by hsp70 likely contributes to the resistance to stress-induced apoptosis seen in transiently induced thermotolerance.

Cellular stress impairs numerous physiological functions, damages cellular structures, and can lead to cell death. Death often occurs through an ordered pathway of self-destruction termed apoptosis (reviewed in references 23, 25, 73, and 78). This physiological mechanism of cell removal is essential during embryogenesis and for the maintenance of appropriate cell numbers in all multicellular organisms but is also triggered when cells are confronted with such nonphysiological conditions as exposure to mild hyperthermia, anoxia, UV irradiation, or certain chemotherapeutic or noxious drugs. Deregulated apoptosis is the underlying basis of a number of human pathologies. For example, impaired apoptosis contributes to the accumulation of neoplastic cells in many cancers whereas several neurological and immunological disorders are characterized by excessive cell elimination. Consequently, there has been intense interest in the signaling mechanisms, effector molecules, and essential targets of this evolutionarily conserved process.

The apoptotic process is characterized by a distinct sequence of morphological and biochemical events. Contacts between cells are broken, water is extruded, and cytoplasmic shrinkage occurs. Chromatin condenses, and the nucleus is partitioned into multiple fragments. Subsequently, the cell fragments into several membrane-enclosed apoptotic bodies that are recognized and removed by phagocytic cells. Among the biochemical changes are increased Ca^{2+} ion concentrations, cellular acidification, oligonucleosomal DNA fragmentation, and the degradation of specific nuclear proteins. Proteolysis is mediated by a conserved group of cysteine proteases that are related to the interleukin 1β-converting enzyme (ICE). ICE was implicated in cell death when it was found to be homologous to the *Caenorhabditis elegans* death gene 3 (*ced3*) (81). Genetic studies have shown that *ced3* is essential for the programmed cell deaths that occur during the development of this nematode worm (16). A protein resembling ICE (prICE) is present in extracts prepared from apoptotic cells and cleaves substrates known to be targeted during apoptosis but does not cleave pro-interleukin-1β (39). This enzyme (caspase-3, previously designated CPP32, Yama, or apopain [3]), is responsible for the cleavage of poly(ADP-ribose) polymerase (PARP), the 70-kDa subunit of the U1 small (70-kDa) ribonucleoprotein (U1-70K), and the catalytic subunit of DNA-dependent protein kinase (6, 55, 71). Each of these substrates has as its recognition site an Asp residue in the P_1 and P_4 positions. A tetrapeptide corresponding to this site (*N*-acetyl-Asp-Glu-Val-Asp-CHO [aldehyde] [Ac-DEVD-CHO]) is effective at blocking the activity of caspase-3 and can prevent apoptosis (55). The caspases exist as proenzymes that are proteolytically processed to their active forms in response to an apoptosis-inducing stimulus (reviewed in references 34, 54, 61, and 79).

For most inducers of cell death, the pathway that links the death signal to the activation of the caspases is not clear. Signaling through the tumor necrosis factor alpha-1 (TNF-α1) or the CD95/Fas receptor recruits a receptor-interacting protein (FLICE) that contains an ICE-related protease domain and can initiate the caspase cascade directly (4, 52). TNF-α and Fas binding, like a number of environmental stresses, also activate the sphingomyelin signaling pathway (reviewed in references 26 and 31). Hydrolysis of the phospholipid sphingomyelin by a membrane-associated sphingomyelinase generates the second-messenger molecule ceramide. A direct role for

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ceramide in mediating cell death is supported by the observation that the exogenous addition of ceramide or bacterial sphingomyelinase induces apoptosis (32, 57) and that the ceramide metabolite, sphingosine-1-phosphate, prevents apoptosis (12). The stress-activated protein kinase (SAPK/JNK) signaling pathway is also stimulated by many of the same treatments that result in ceramide production (74, 77). This ERK (extracellularly regulated kinase) subfamily responds to inflammatory cytokines and such stresses as UV light, ionizing radiation, oxidative stress, and heat shock (36) (reviewed in reference 35). Targets for the SAPKs are the transcription factors c-Jun, ATF2, and Elk1, and their phosphorylation and activation are associated with either cell cycle delay or apoptosis (8, 59, 74, 80, 82). Expression of nonphosphorylatable mutants of c-Jun or kinase-inactive mutants of either MEKK1 or SEK1 prevents apoptosis (74, 80, 82). A link between the sphingomyelin and SAPK/JNK pathways has been proposed based on the observation that exogenously added ceramide activates the SAPK/JNK pathway (12, 36, 74, 77) and that apoptosis can be prevented in ceramide-treated cells expressing a dominant negative c-Jun mutant (74).

Cells respond to stress by adaptive changes that either limit or repair damage and thereby prevent cell death. In all organisms, a mild hyperthermic exposure elicits a transient state of thermoresistance known as thermotolerance (24, 60). The observation that the level of thermotolerance during the course of its development and decay is quantitatively related to the absolute levels of a group of heat-induced proteins, particularly hsp70, first suggested that the heat shock proteins (HSPs) play a protective role in cell survival (37, 42, 70). Mild hyperthermia is able to induce apoptosis, and this is prevented in thermotolerant cells (43, 51, 69). Cell lines that have been stably transfected with HSP-encoding genes are protected from hyperthermia as assessed by colony formation assays (38, 40) and by measuring indicators of apoptosis (43, 65). Overexpression of hsp27 protects L929 cells from the protein kinase C inhibitor staurosporine and from Fas-mediated apoptosis (45). Also, down-regulation or disruption of hsp70 expression results in apoptosis (14, 75). Little is known about how HSPs protect cells, particularly because it is not known which of the numerous heat-induced cell perturbations is the critical target that gives rise to cell lethality and is protected in thermotolerant cells. The realization that apoptotic cell death represents an ordered biochemical pathway permits an investigation of which events are affected by HSPs. We have examined resistance to stress-induced apoptosis by using cell lines in which the expression of hsp70 either is constitutively elevated or is regulated by a tetracycline-regulated transactivator. The role of hsp70 in blocking the apoptotic process was examined by studying its effect on activation of the caspase protease cascade and on signaling through the SAPK/JNK pathway.

MATERIALS AND METHODS

Plasmid construction and establishment of transfected cell lines. The human acute lymphoblastic leukemia T-cell line PEER and cell clones with high levels of constitutive hsp70 expression (F2 and F3) have been described previously (50). In these transfected cells, hsp70 expression is under the control of the β-actin promoter. To generate PEER cells with inducible expression of hsp70, a tetracycline-responsive dicistronic plasmid (pTR-DC/HSP70-GFP) that encodes hsp70 as the first cistron and the green fluorescent protein (GFP) as the second cistron (see Fig. 4A) was constructed. The plasmid was assembled by first ligating a *Bam*HI fragment containing hsp70 coding sequences from pGEM-hsp70-Bam (1) into *Bam*HI-cut pTR/tpl to yield pTR/tpl-hsp70. The pTR/tpl plasmid includes a PCR fragment containing the adenovirus tripartite leader with the adenovirus major late enhancer bracketed by splice donor and acceptor sites located downstream of the minimal cytomegalovirus TATA box (CMV*-1) of the tetracycline-regulated promoter within the pUHD10-3 tTA response plasmid (64). A fragment from pTR/tpl-hsp70 which includes the Pcmv*-1 promoter, tpl, and hsp70 sequences was subsequently ligated with a fragment from pTR-CD/GFP (49) that includes the encephalomyocarditis virus internal ribosome entry site and coding sequences for the S65T mutant of the GFP (28). A tTA-expressing PEER cell line (PEER-tTA) (49) was cotransfected with the dicistronic pTR-DC/HSP70-GFP plasmid together with the plasmid ptk/NEO. The ptk/NEO plasmid consists of a *Bst*EII fragment from pREP9 (Invitrogen, San Diego, Calif.) containing the neomycin resistance gene. Plasmid DNA for transfection was purified with Qiagen plasmid kits (Chatsworth, Calif.). Transfections were carried out by electroporation with a Bio-Rad Gene Pulser (Hercules, Calif.). The cells (10^7 in 0.4 ml of complete medium) were mixed with 20 μg of linearized plasmid (4:1 ratio of pTR-DC/HSP70-GFP to ptk/NEO) and exposed to an electric pulse of 230 V with a capacitance of 960 μF at room temperature. G418-resistant cells (400 μg/ml) were selected in batch culture, and cells with tetracycline-regulatable expression of hsp70 were selected by removing tetracycline from the medium followed by flow cytometric cell sorting of the GFP-positive cells with an EPICS ELITE ESP cell sorter (Coulter Corp., Hialeah, Fla.) as described previously (49).

Cell culture and heat shock treatments. Cells were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum (Life Technologies, Gaithersburg, Md.) at 37°C in a humidified 5% CO_2 atmosphere. For the F2 and F3 cells, the medium was supplemented with 250 μg of G418 per ml. Cells with tetracycline-regulated hsp70 expression were maintained in medium containing 200-μg/ml G418 (Life Technologies), 100-μg/ml hygromycin (Sigma Chemical, St. Louis, Mo.), and 20-ng/ml anhydrotetracycline (Spectrum Chemical, Gardena, Calif.). For induction of hsp70, the cells were centrifuged, washed three times by resuspension in 25 ml of phosphate-buffered saline (PBS), and recentrifuged. The cells were then resuspended in medium without anhydrotetracycline. The following day, the cells were collected, washed once with PBS, resuspended in fresh medium, and returned to the incubator for an additional 24 h. In each experiment, the level of induction was measured by monitoring GFP fluorescence in live cells by flow cytometry. The analysis was performed with an EPICS XL flow cytometer (Coulter Corp.) equipped with a 15-mW air-cooled argon ion laser emitting at 488 nm. A 525-nm band pass was used to measure the green fluorescence. For the heat shock treatments, the cells were seeded into flasks at a concentration of 10^6 cells per ml in medium containing 25 mM HEPES buffer and submerged in a circulating water bath maintained at 43.0 ± 0.1°C. The cells were either collected immediately or returned to the 37°C CO_2 incubator for various periods of time after the hyperthermic exposure. Ceramide (*N*-acetyl-D-sphingosine; Sigma) was dissolved in ethanol (10 mM stock solution) and diluted into culture medium at concentrations ranging from 20 to 80 μM. Control cultures received an equivalent amount of ethanol (0.8%).

Apoptosis measurements. The extent of DNA fragmentation in heat-treated cells was determined by the diphenylamine assay as described previously (51). Cell viability and apoptotic index were quantitated by staining cell suspensions with acridine orange and ethidium bromide (11) as described previously (46). Stock solutions (100 μg/ml) of each dye were prepared in PBS and diluted 1:25 into the cell suspensions. Cells were examined by fluorescence microscopy, and the numbers of cells that were viable (excluding ethidium bromide), necrotic (not excluding ethidium bromide), apoptotic, and apoptotic lysed (cells with apoptotic nuclei not excluding ethidium bromide) were determined. Duplicate counts of at least 200 cells were made for each treatment. Flow cytometry was used to measure forward light scattering, an indication of cell size, to assess cell viability. Apoptotic cells lose water and are smaller than viable cells (18). In cells expressing both hsp70 and GFP, viability (forward scatter) was assessed relative to GFP fluorescence.

Western blotting and flow cytometric measurements. hsp70 levels were assessed by Western blotting and by flow cytometry. For Western blotting, cells were collected and lysed and extracts containing equivalent amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Novex, San Diego, Calif.) as described previously (49). Levels of hsp70 were assessed by immunoblotting with the hsp70-specific monoclonal antibody C92 (Stressgen, Victoria, B.C., Canada). For detection, the membranes were incubated with a horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (IgG) (Amersham Corp., Arlington Heights, Ill.) followed by ECL chemiluminescence (Amersham Corp.). Flow cytometric measurements were performed on cells (10^6) that were fixed at −20°C for 16 h in 80% methanol and washed twice with $Ca^{2+}Mg^{2+}$-free PBS. The fixed cells were incubated for 1 h on ice with the C92 anti-hsp70 antibody (2 μg/10^6 cells), washed twice with PBS, and then incubated with an anti-mouse phycoerythrin-conjugated IgG antibody (3 μg/10^6 cells) (Biomeda Corp., Foster City, Calif.). The analysis was performed with an EPICS XL flow cytometer (Coulter Corp.). The red phycoerythrin fluorescence was collected by using a 550-nm dichroic long pass filter and a 575-nm band pass. In cells expressing both hsp70 and GFP, a 525-nm band pass was used to collect the green fluorescence.

Immunoblotting was used to examine the cleavage of PARP and the processing of procaspase-3 in apoptotic cells. Cells were lysed in sample buffer containing urea (62.5 mM Tris-HCl [pH 6.8], 6 M urea, 10% glycerol, 2% SDS, and 5% 2-mercaptoethanol). Extracts containing equal amounts of protein were subjected to SDS-PAGE (Novex) and transferred to nitrocellulose membranes. The intact 113-kDa PARP protein and the 89-kDa proteolytic fragment were detected with a mouse monoclonal antibody, C-2-10 (purchased from G. G. Poirier, Laval University, Sainte-Foy, Quebec, Canada). Caspase-3 was detected with a

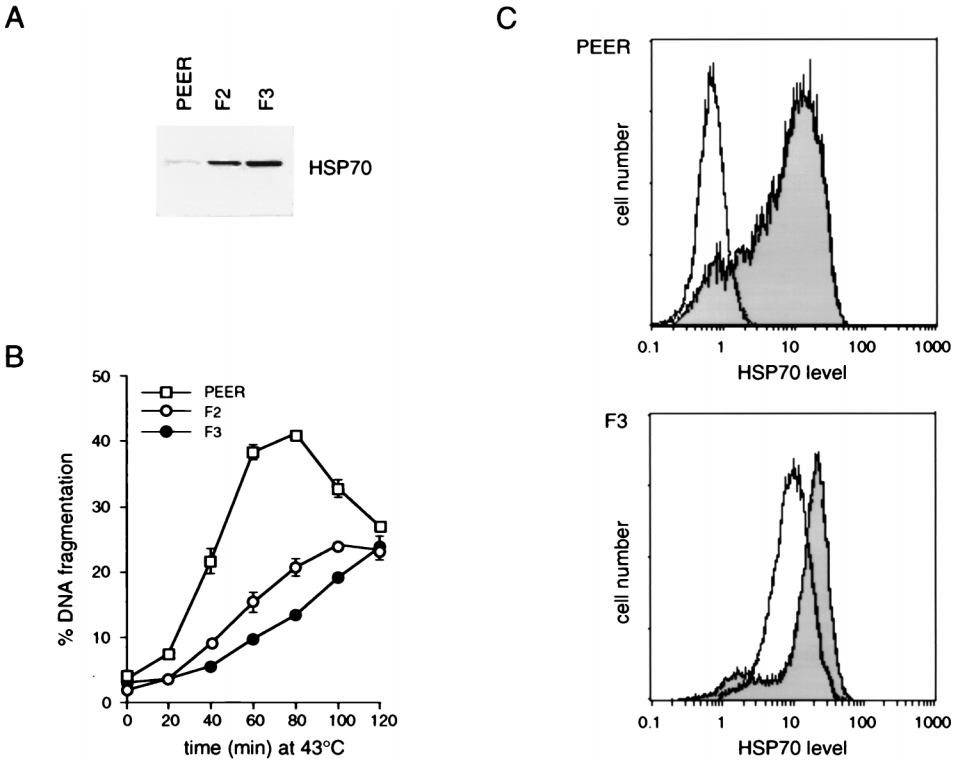

FIG. 1. Resistance to heat-induced apoptosis in cells that constitutively overexpress hsp70. (A) Western blot analysis showing the levels of hsp70 in the parental cell line PEER and the two hsp70-expressing clones (F2 and F3). hsp70 gene expression in these transfected cells is under the control of the constitutively active β-actin promoter. (B) DNA fragmentation was measured in cells that were exposed to 43°C for 20 to 120 min and then returned to 37°C for 12 h. Shown are the means and standard deviations from triplicate determinations. (C) hsp70 levels in control and heat-shocked PEER and F3 cells measured by flow cytometry. Control and heat-shocked (43°C for 60 min followed by 12 h at 37°C) cells were fixed, incubated with an hsp70-specific antibody followed by a phycoerythrin-conjugated anti-IgG antibody, and analyzed by flow cytometry. The profile for the heat-shocked cells is shown in gray.

mouse monoclonal antibody, p17 (provided by D. W. Nicholson, Merck Frosst, Pointe Claire, Quebec, Canada), that recognizes the intact 32-kDa protein and the processed 17-kDa subfragment. Antibody complexes were revealed with a horseradish peroxidase-conjugated sheep anti-mouse IgG and ECL (Amersham).

SAPK/JNK assay. SAPK/JNK activity was measured in vitro by using a bacterially expressed glutathione *S*-transferase (GST)–c-Jun fusion protein as a substrate (36). The pGEX-jun 5-89 plasmid and SAPK assay protocol were provided by J. Woodgett (Ontario Cancer Institute, Toronto, Ontario, Canada). GST–c-Jun was coupled to glutathione-Sepharose 4B beads (Pharmacia Biotech, Uppsala, Sweden) and washed four times with PBST (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 0.1% Triton X-100) before use. Control and heat-treated cells (2.5×10^6 to 5×10^6/sample) were washed with PBS and lysed on ice for 30 min in 0.5 ml of hypotonic lysis solution (150 mM NaCl, 20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 7.0], 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 20-μg/ml leupeptin, and 50 mM NaF). Lysates were cleared by centrifugation (13,000 × *g* at 4°C for 10 min), protein concentration was determined (Bio-Rad DC protein assay), and an equal amount of each extract (250 to 300 μg of protein) was incubated with the GST–c-Jun–Sepharose beads on a rotary shaker for 16 h at 4°C. The complexes were washed four times with PBST and then incubated at 30°C for 20 min in 40 μl of kinase buffer (10 mM $MgCl_2$, 50 mM Tris-HCl [pH 7.5], 1 mM EGTA [pH 7.5], 100 μM ATP, and 16 μCi of [γ-^{32}P]ATP [7,000 Ci/mmol; ICN Pharmaceuticals, Costa Mesa, Calif.] per sample). The kinase reaction was terminated by the addition of an equal volume of 2× sample buffer and heated at 95°C for 5 min. Equal volumes (generally 10 μl of a 1:10 dilution) were loaded onto 12.5% polyacrylamide gels. The gels were stained with Coomassie blue, destained, dried, and exposed to film.

In vitro caspase-3 activity assay. [^{35}S]methionine-labeled PARP was synthesized in vitro with the Promega TNT/T7 coupled transcription-translation kit (Promega Corp., Madison, Wis.). A pBluescript-II SK$^+$ plasmid encoding the full-length PARP cDNA was obtained from D. W. Nicholson (Merck Frosst). Reaction mixtures (25 μl) contained 2.5 μl of the in vitro-synthesized PARP together with 42 pM (10 U) purified caspase-3 (obtained from D. W. Nicholson, Merck Frosst) in assay buffer (50 mM HEPES-KOH [pH 7.0], 10% [wt/vol] sucrose, 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS], 5 mM dithiothreitol, 20-μg/ml leupeptin, 10-μg/ml pepstatin A, 10-μg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride). The mixtures were incubated at 37°C for 1 h, and reactions were terminated by the addition of SDS sample buffer and then analyzed by SDS-PAGE and fluorography. Protease inhibitor experiments were performed by adding either the tetrapeptide aldehyde inhibitor Ac-DEVD-CHO (BIOMOL, Plymouth Meeting, Pa.) or affinity-purified recombinant hsp70 (obtained from R. I. Morimoto, Northwestern University, Evanston, Ill.) to the reaction mixtures for a preincubation period of 20 min at 37°C before addition of the substrate. Experiments with hsp70 were also carried out in the presence of 1 mM ATP or 1 mM ATP-γ-S (Sigma) in reaction mixtures containing 1.5 mM Mg^{2+}.

RESULTS

Constitutive overexpression of hsp70 inhibits heat-induced apoptosis. Previously, we have shown that resistance to heat-induced apoptosis can be transiently acquired in the human T-lymphocyte cell line PEER by prior exposure to a hyperthermic temperature and that the development of this thermotolerant state correlated with the induced synthesis of hsp70 (51). To determine whether elevated levels of hsp70 are sufficient to prevent the onset of the apoptotic pathway, we examined the effect of hyperthermic exposure on transfected PEER cell lines that stably express hsp70 under the control of the constitutive β-actin promoter (50). The levels of hsp70 in the transfected cell lines, F2 and F3, are approximately 10- and 20-fold higher than in the nontransfected cell line, PEER, as measured by Western blotting and densitometry (Fig. 1A). The extent of DNA fragmentation was used to measure apoptotic cell death. The proportion of fragmented DNA relative to total DNA was quantitated in cells that were heated at 43°C for up to 120 min and then returned to 37°C for 12 h (Fig. 1B). In the parental cell line PEER, maximal levels of DNA fragmentation oc-

curred in cells that were heated for 60 to 80 min. Longer treatments resulted in less DNA fragmentation, presumably as a consequence of necrotic cell death in cells that were exposed to extreme hyperthermic conditions. In the hsp70-expressing clones, the same hyperthermic treatments resulted in substantially less DNA fragmentation (Fig. 1B). Exposure to 43°C for 80 min resulted in approximately 40% DNA fragmentation in the PEER cell line but only 20% in the F2 and 12.5% in the F3 cell lines. Therefore the degree of protection towards the initiation of the apoptotic process appears to be dependent upon the absolute level of hsp70.

Flow cytometry was used to quantitate the amount of hsp70 in the control and heat-shocked cells. This was carried out to determine whether the constitutive level of hsp70 in the F3 cell line was similar to that of the heat-shocked PEER cell line and representative of a physiologically relevant level. Control and heat-shocked cells (43°C for 60 min followed by 12 h at 37°C) were fixed with methanol and incubated with the hsp70-specific antibody, C92. This was followed by incubation with a fluorescein isothiocyanate-conjugated anti-mouse antibody and flow cytometric analysis. The level of hsp70 in the heat-shocked PEER cells ranged from a level similar to that of the control cells to a level 16-fold higher (Fig. 1C). The majority of the cells (82%) contained elevated levels of hsp70, indicating that HSP synthesis is not impaired in the dying cells. The level of hsp70 in the nonstressed F3 cells was similar to that of the heat-shocked PEER cell line (15-fold higher than that in control PEER cells), and heat shock increased the level of hsp70 2-fold (30-fold higher than that in control PEER cells). These results indicate that it is the prior synthesis of hsp70 that is essential for preventing the initiation of the apoptotic program and that the level of hsp70 in the F3 cell line, which is resistant to heat-induced apoptosis, is equivalent to that produced in cells that have been exposed to hyperthermia.

Tetracycline-regulated expression of hsp70 from a dicistronic hsp70-GFP construct provides protection against apoptosis. To exclude the possibility that clonal variation might have contributed to the effects observed in the cell lines with constitutive hsp70 expression, we produced a transfected PEER cell line in which the expression of hsp70 is under the control of the tetracycline-regulated transactivator protein tTA (22). A dicistronic expression cassette was assembled in which the first cistron, encoding hsp70, is separated from the second cistron, encoding the GFP, by the encephalomyocarditis virus internal ribosome entry site (Fig. 2A). This facilitated the isolation of transfected cells and provided a useful way to monitor the level of transgene induction during the course of an experiment (49).

A pool of cells with tetracycline-regulatable hsp70 expression (PETA70) was selected by flow cytometric cell sorting of green cells after incubation in medium lacking anhydrotetracycline. Elevated levels of hsp70 were detected in these cells within 16 h after removal of tetracycline, and hsp70 accumulated to high levels by 40 h as detected by Coomassie blue staining (Fig. 2B). However, hsp70 and GFP expression was heterogeneous in the PETA70 cells (Fig. 2C). Forty-eight hours after induction, approximately 50% of the cells had levels of GFP and hsp70 that were approximately 100-fold above those of the noninduced cells. Despite the heterogeneity of hsp70 expression in the induced cells, the coexpression of GFP made it possible to examine the effect of hsp70 on resistance to hyperthermia by comparing the green and nongreen populations by flow cytometry. In the noninduced cells, approximately 45% of the cells were judged to be apoptotic after heat shock based on cell size as measured by forward light scattering (Fig. 2D, quadrant 3). For the induced cells, only

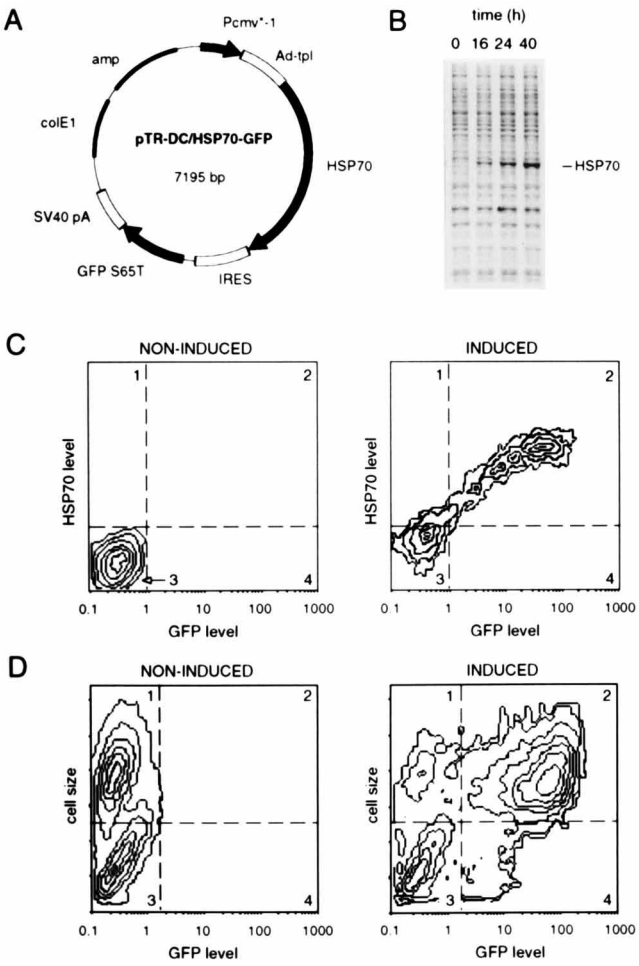

FIG. 2. Tetracycline-regulated expression of hsp70. (A) Schematic representation of the tetracycline-regulated dicistronic expression plasmid. The transcription unit contains coding sequences for hsp70 and the GFP (GFP S65T) separated by the encephalomyocarditis virus internal ribosome entry site (IRES). The transcript also includes the adenovirus tripartite leader sequence (Ad-tpl). The Pcmv*-1 promoter contains seven repeats of the Tet operator and can be positively activated by binding the tetracycline-regulated transactivator protein (tTA). SV40, simian virus 40. (B) Expression of hsp70 following induction was assessed by SDS-PAGE and Coomassie blue staining. Extracts were prepared at 16, 24, and 40 h after removal of anhydrotetracycline. (C) Flow cytometric analysis of hsp70 levels and GFP fluorescence in cells grown in the presence of anhydrotetracycline (noninduced) and 48 h after removal of anhydrotetracycline (induced). The level of hsp70 is proportional to the level of GFP fluorescence. (D) Analysis of viability in the noninduced and induced cells. Cells were heated at 43°C for 1 h and returned to 37°C for 12 h. Flow cytometry was used to measure cell size (forward light scatter) and GFP fluorescence. The apoptotic cells are smaller in size than viable cells (left panel, quadrant 3). For the induced cells, the apoptotic cells are predominantly those with low GFP levels (quadrant 3). Cells with high levels of hsp70 (GFP positive) remain viable after the heat shock treatment (quadrant 2).

about 10% of the green cells were apoptotic (quadrant 4) whereas approximately 68% of the nongreen cells were apoptotic (quadrant 3). Among the green cells (quadrants 2 and 4), the apoptotic cells were those with somewhat lower levels of GFP. The very bright GFP-positive cells appeared to be completely resistant. This suggests that the level of resistance is related to the level of hsp70.

SAPK/JNK is strongly activated in response to hyperthermia in the hsp70-expressing cell line F3, but in the inducible cell line, hsp70 prevents SAPK/JNK activation. A number of

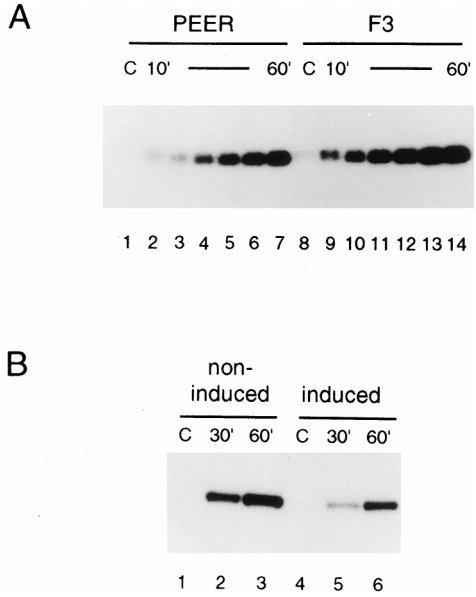

FIG. 3. Heat shock-mediated SAPK/JNK activation. c-Jun N-terminal kinase (JNK) activity was measured in cell extracts with a GST–c-Jun fusion protein as a substrate for γ-^{32}P labeling. (A) PEER and F3 cells were either untreated (lanes 1 and 8, "C") or exposed to a 43°C heat shock for 10, 20, 30, 40, 50, or 60 min (lanes 2 to 7 and 9 to 14). (B) Cells with tetracycline-regulated hsp70 expression were induced by removal of anhydrotetracycline from the medium for a period of 48 h. The noninduced and induced cells were exposed to 43°C for 30 (lanes 2 and 5) or 60 (lanes 3 and 6) min before harvesting for SAPK/JNK activity assay. Control cells (lanes 1 and 4, "C") were maintained at 37°C.

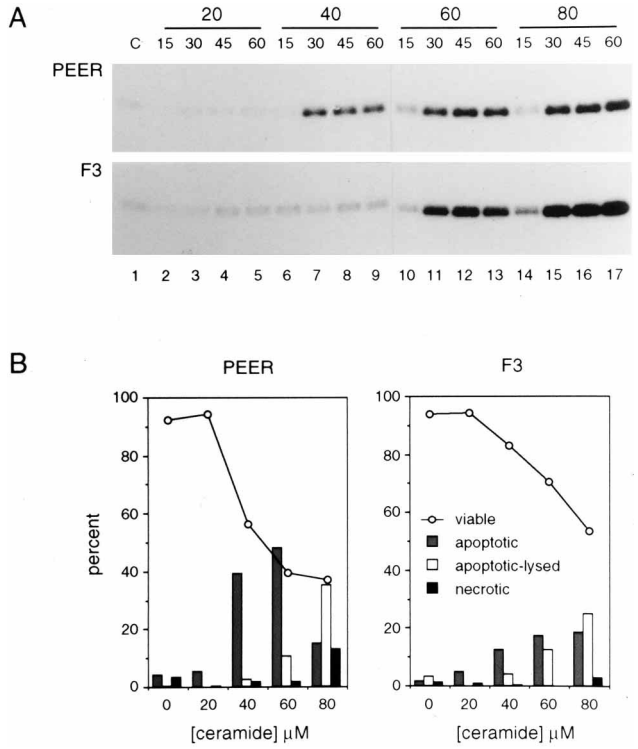

FIG. 4. hsp70-expressing cells resist ceramide-induced apoptosis and contain high levels of active SAPK/JNK in response to ceramide treatment. (A) SAPK/JNK activity was measured in extracts prepared from PEER and F3 cells exposed to 20 (lanes 2 to 5), 40 (lanes 6 to 9), 60 (lanes 10 to 13), or 80 (lanes 14 to 17) μM ceramide for periods of 15, 30, 45, or 60 min. Control cells (lane 1) were exposed to vehicle alone (0.8% ethanol) for 60 min. (B) Quantitation of cell viability and apoptotic index measured 12 h after exposure to ceramide.

cellular stress conditions, including hyperthermia, activate the SAPK/JNK cascade, and this signaling pathway has been implicated in mediating the apoptotic process (8, 59, 74, 80, 82). Potentially, the enhanced survival of the hsp70-expressing cells could be a consequence of reduced SAPK/JNK activation following heat stress. We examined the level of SAPK/JNK activation in PEER and F3 cells that were exposed to 43°C for periods of time ranging from 10 to 60 min (Fig. 3A). SAPK/JNK activity was measured by an in vitro kinase assay using a GST–c-Jun fusion protein as a substrate. In the PEER cell line, increased SAPK/JNK activity could be detected 30 min after hyperthermic exposure and continued to rise throughout the duration of the heat treatment (Fig. 3A). The level of SAPK/JNK activity correlated with cell death. Increased levels of DNA fragmentation were evident following exposure to 43°C for a period of time greater than 20 min (Fig. 1). Rather than a reduced response to hyperthermia, the hsp70-expressing cell line appeared to be somewhat more responsive in that increased SAPK/JNK activity was detected sooner and reached a higher level in these cells relative to the PEER cell line. This difference was consistently seen in three separate experiments. These results suggest that hsp70 protects against apoptosis at some point downstream of SAPK/JNK activation. However, hsp70 was able to prevent the heat-induced activation of SAPK/JNK when this was examined in the cell line with tTA-regulated expression of hsp70 (Fig. 3B). Cells were induced by removal of tetracycline from the medium and then exposed to 43°C for 30 or 60 min. Compared to the noninduced cells, the extent of SAPK/JNK activation was approximately twofold less. Nearly complete inhibition was observed when the experiment was performed with cells that were induced and sorted for high GFP fluorescence by flow cytometry (20). These sorted cells have approximately 100-fold higher levels of hsp70 relative to the uninduced cells. Thus, although inducible expression of hsp70 inhibited SAPK/JNK activation, this was not necessary for protection from apoptosis in the F3 cell line.

Ceramide activates SAPK/JNK in both PEER and F3 cells although the F3 cells are more resistant to ceramide-induced death. Both SAPK/JNK activation and apoptotic death are associated with increased levels of the lipid signaling molecule ceramide (12, 36, 74, 77). As well, the exogenous addition of ceramide induces SAPK/JNK activation and apoptosis, suggesting that ceramide initiates stress-induced apoptosis through the SAPK/JNK cascade (74). We examined whether SAPK/JNK activation could be dissociated from resistance to apoptosis in ceramide-treated cells by comparing these two responses in the PEER and F3 cell lines. Cells were exposed to 20, 40, 60, or 80 μM ceramide, and after 15, 30, 45, and 60 min, the cells were collected for SAPK/JNK activity measurements (Fig. 4A). The effect of ceramide on cell survival was examined 12 h later (Fig. 4B). In the PEER cell line, ceramide activated SAPK/JNK in a dose-dependent manner that correlated with the extent of apoptosis. A dose of 40 μM was required to activate SAPK/JNK and induce apoptosis. At a dose of 20 μM ceramide, SAPK/JNK activity was not stimulated and cell survival was not affected. At the highest dose used (80 μM), most of the apoptotic cells had lost membrane integrity and some necrotic death occurred. In cells that constitutively overexpress hsp70, a higher dose of ceramide (60 μM) was required to activate SAPK/JNK. However, the extent of SAPK/JNK activation was higher in the F3 cell line relative to the PEER cells in the presence of either 60 or 80 μM ceramide. Despite the

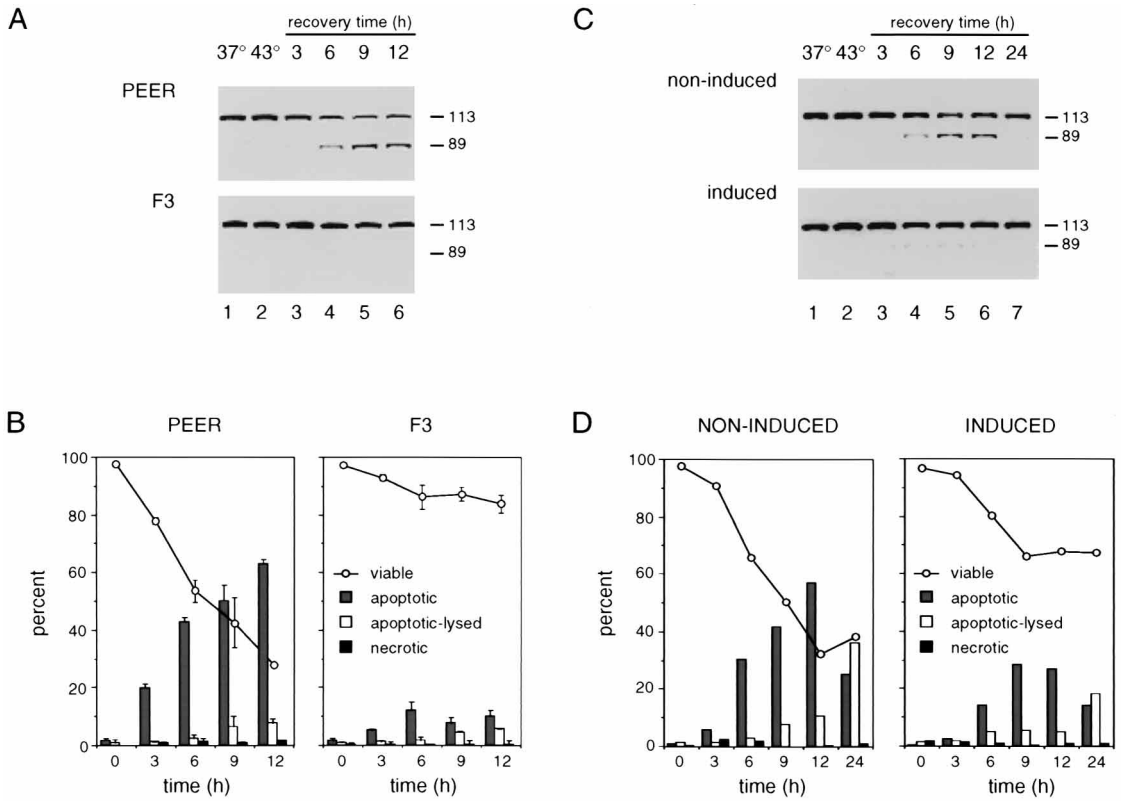

FIG. 5. Resistance to heat-induced apoptosis and inhibition of PARP cleavage in hsp70-expressing cells. PARP cleavage and apoptotic index were examined in PEER cells and the hsp70-expressing cell line F3 (A and B) and in the PETA70 cell line in which hsp70 expression was either noninduced or induced by tetracycline removal (C and D). Shown are results of Western blot analysis of PARP (A and C) in control cells (lanes 1) and cells that were heat shocked for 1 h at 43°C (lanes 2) and then returned to 37°C for recovery periods of 3, 6, 9, and 12 h (lanes 3 to 6) and 24 h for the PETA70 cells (lane 7). The locations of the intact polypeptide (113 kDa) and the cleaved fragment (89 kDa) are indicated. Cells were exposed to 43°C for 1 h and then returned to 37°C. Cell viability (lines) and the percentages of dead cells (bars) that were either apoptotic, late-stage apoptotic (apoptotic lysed), or necrotic were quantitated at various times after the return to 37°C by counting ethidium bromide- and acridine orange-stained cells (B and D). Each data point is the mean ± standard deviation of two experiments.

very strong activation of SAPK/JNK in the F3 cell line, these cells showed more resistance to apoptosis at all doses of ceramide than the PEER cells (Fig. 4B). Therefore, similar to heat shock, ceramide-induced apoptosis is correlated with SAPK/JNK activation and hsp70-expressing cells resist apoptosis but do not contain reduced levels of activated SAPK/JNK, suggesting that hsp70 acts downstream of SAPK/JNK activation to prevent apoptosis.

Cleavage of the caspase-3 substrate PARP is prevented in hsp70-expressing cells exposed to hyperthermia. Among the specific proteolytic substrates that are cleaved during apoptosis is the enzyme PARP. This DNA repair enzyme is cleaved and inactivated by the ICE-related protease caspase-3 (55, 71). We explored the possibility that PARP cleavage occurs during hyperthermia-induced apoptosis and that this might be prevented in cells containing elevated levels of hsp70. Cells were heated at 43°C for 60 min and returned to 37°C for periods of time up to 24 h. Extracts were prepared from the samples and subjected to immunoblotting with a PARP-specific antibody (C-2-10). Cleavage of PARP to produce an 89-kDa fragment was evident by 3 to 6 h after heat treatment of the PEER cells and was increased at 9 and 12 h (Fig. 5A). In contrast, PARP cleavage was suppressed in the heat-shocked F3 cells. In the PETA70 cell line, PARP cleavage was detected by 6 h after the noninduced cells were returned to 37°C and was more extensive at 9 and 12 h (Fig. 5C). In the induced cells, PARP cleavage was minimal with only a small amount of the 89-kDa fragment being detected at 6 and 9 h after the heat treatment.

A quantitative assessment of the percentage of apoptotic cells during the period of recovery from hyperthermia was also carried out. There was a progressive increase in the number of apoptotic cells and a concomitant decrease in viable cells over time for the heat-shocked PEER cells, while the F3 cells were substantially more resistant (Fig. 5B). In the PETA70 cell line, viability progressively decreased to a level of approximately 30% over the 12-h period following the heat shock in the noninduced state; however, in the cells with elevated hsp70, viability was reduced to only about 70% by 9 h after the heat shock and did not change thereafter (Fig. 5D). This corresponds well with the percentage of hsp70-positive cells in the pool after induction (Fig. 2C). Between 12 and 24 h after the heat shock, there was an increase in the number of apoptotic-lysed cells. There was also less of the 89-kDa cleavage product present at 24 h in the noninduced cells than was seen at 12 h. This is likely a result of loss of the cleaved protein from the cells. In summary, these results show that PARP cleavage occurred at a time that corresponded with the appearance of apoptotic cells and that both PARP cleavage and apoptosis are prevented in cells expressing hsp70.

Processing of pro-caspase-3 is inhibited in cells expressing elevated levels of hsp70. Caspase-3 exists as a 32-kDa proenzyme that is processed in cells undergoing apoptosis to an

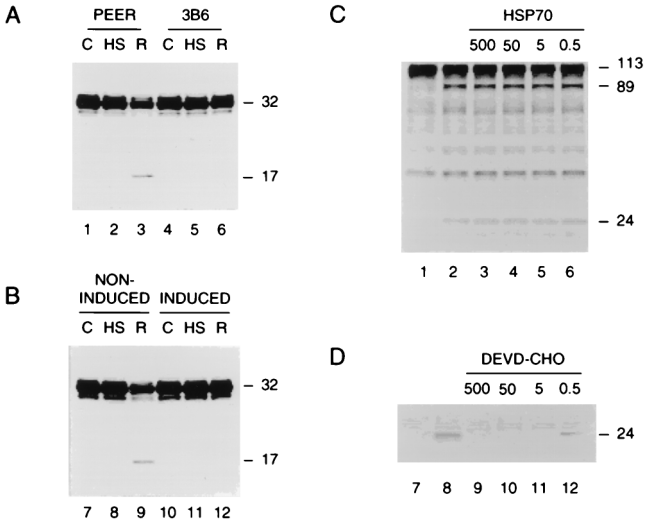

FIG. 6. Pro-caspase-3 processing is inhibited by hsp70 in heat-shocked cells, but the activity of the enzyme is not inhibited in vitro by hsp70. The processing of caspase-3 in heat-shocked cells was examined in PEER and F3 cells (3B6 is a subclone of the F3 cell line) (A) and in the PETA70 cell line grown in the noninduced and induced state (B). Caspase-3 processing was measured by Western blotting of extracts from control cells (C), cells that were heat shocked at 43°C for 1 h (HS), and heat-shocked cells that were returned to 37°C for 12 h (R). The antibody detects the full-length proenzyme (32 kDa) and the processed 17-kDa fragment. Caspase-3 activity was measured in vitro with [^{35}S]methionine-labeled PARP as a substrate (C and D). Caspase-3 (40 pM) was present in each sample except for lanes 1 and 7, which contained labeled PARP only. Purified recombinant hsp70 (C, lanes 3 to 6) and the tetrapeptide DEVD-CHO (D, lanes 9 to 12) were added at concentrations ranging from 0.5 to 500 nM. ATP (1 mM) and Mg^{2+} (1.5 mM) were included in samples 1 to 6. Cleavage of the full-length PARP protein (113 kDa) to produce the 89- and 24-kDa fragments was prevented by the specific inhibitor DEVD-CHO but not by hsp70.

active form consisting of a heterodimer with subunits of 17 and 12 kDa (55). We examined whether hsp70 was able to prevent the processing of caspase-3 or whether it inhibited the activity of the mature enzyme. Western blot analysis revealed that caspase-3 was processed to form the mature p17 subunit in heat-shocked PEER cells and in the PETA70 cells that were maintained in the noninduced state (Fig. 6A and B, lanes 3 and 9). This was evident in cells that were heat shocked and then returned to 37°C for 12 h but not immediately after the heat shock. The appearance of processed caspase-3 corresponded with the time at which PARP cleavage occurred (data not shown). Elevated expression of hsp70 inhibited the processing of caspase-3 both in the F3-3B6 cell line and when hsp70 was induced in the PETA70 cell line (Fig. 6A and B, lanes 6 and 12).

To test whether hsp70 can inhibit the activity of the mature caspase-3 enzyme, we used an in vitro cleavage assay containing affinity-purified caspase-3 and [^{35}S]methionine-labeled PARP (55). The [^{35}S]methionine-labeled PARP protein was generated by a coupled in vitro transcription-translation reaction. The full-length protein (113 kDa) was cleaved to yield fragments of 89 and 24 kDa when incubated for 1 h at 37°C with caspase-3 (Fig. 6C; compare lanes 1 and 2). Addition of purified recombinant human hsp70 at concentrations ranging from 0.5 to 500 nM had no effect on the ability of caspase-3 to cleave PARP in vitro. Caspase-3 activity was unaffected by the presence of hsp70 regardless of whether it was preincubated with the enzyme prior to addition of substrate or whether ATP or the nonhydrolyzable analog ATP-γ-S was included. Purified recombinant human hsp70 that was expressed either in *Escherichia coli* or in the human PETA70 cell line gave identical results. As well, hsp70 did not inhibit caspase-3 activity when measured by a sensitive fluorometric assay with the substrate *N*-acetyl-Asp-Glu-Val-Asp-(7-amino-4-methyl-coumarin) (data not shown). Caspase-3 activity can be effectively inhibited by the tetrapeptide aldehyde Ac-DEVD-CHO (55). PARP cleavage was completely inhibited at tetrapeptide concentrations of 5 to 500 nM and was partially inhibited with 0.5 nM (Fig. 6D). Therefore, these results indicate that hsp70 prevents the activation of caspase-3 in heat-shocked cells but is unable, on its own, to prevent the activity of the active enzyme in vitro.

DISCUSSION

Although a diverse array of signals are capable of inducing apoptosis, it proceeds by a common mechanism involving molecules that sense and execute this biochemical pathway of self-destruction (reviewed in references 23, 25, 73, and 78). The inherent sensitivity of different cells to these signals is equally diverse and can be modified by such factors as the state of differentiation and the cellular environment. Cells which contain elevated levels of HSPs, synthesized in response to a prior exposure to hyperthermia, are more resistant to stress conditions and are spared from the apoptotic pathway. Conceivably, HSPs might either limit cell damage and thereby blunt the death signal or prevent the activation or activity of sensor or effector molecules.

Heat shock activates a number of protein kinase cascades, including the Ras-regulated mitogenic cascade involving ERK1 and the ERK subfamily of kinases (SAPK/JNK and p38 kinase), that are activated by cellular stress (15, 36, 68). Many of the treatments that activate SAPK/JNK, including hyperthermia, inflammatory cytokines, UV and ionizing radiation, oxidative stress, and cytotoxic drugs result in apoptosis (reviewed in reference 35). Apoptosis and SAPK/JNK activation can be induced in the absence of stress by the overexpression of a constitutively active form of the JNK kinase kinase, MEKK1 (33, 80). Furthermore, stress-induced apoptosis can be prevented by the overexpression of a kinase-inactive mutant of the JNK kinase, SEK1, or a nonphosphorylatable dominant negative mutant of c-Jun (74, 80, 82). The heat-induced apoptosis of PEER cells is also correlated with SAPK/JNK activation. Exposure to 43°C resulted in a time-dependent increase in both SAPK/JNK activity and DNA fragmentation.

Cells with elevated levels of HSPs might resist apoptosis in response to stress as a consequence of reduced SAPK/JNK activation. When hsp70 levels were increased by using the tetracycline-inducible system, we found that SAPK/JNK activity in response to heat shock was strongly inhibited, indicating that hsp70 blocks signaling events upstream of SAPK/JNK activation. The inhibitory effect of hsp70 on SAPK/JNK activation was also observed in cells that were made transiently thermotolerant by a mild hyperthermic treatment (20). Also, Zanke et al. (82) have shown that SAPK/JNK activation in response to mild heat shock was reduced in a permanently thermoresistant mouse fibroblast cell line. However, SAPK/JNK activation was not inhibited in the cell line that constitutively overexpresses hsp70 and which is highly resistant to heat-induced apoptosis. The inability of hsp70 to affect SAPK/JNK activation in the F3 cell line could be the result of an adaptation to constitutive hsp70 expression. Persistent inhibition of SAPK/JNK activity might not be well tolerated, and therefore this effect by constitutive hsp70 expression may be compensated for by other adaptive changes that restore SAPK/JNK activity in these stably transfected cells.

The observation that the hsp70-expressing F3 cells resist

stress-induced apoptosis in spite of strong SAPK/JNK activation indicates that inhibition of apoptosis by hsp70 can also occur at a level downstream of SAPK/JNK activation. As well, these results might suggest that SAPK/JNK and caspase activation are stimulated through independent pathways. Peptide inhibitors of the caspase family proteases are able to prevent apoptosis of serum or nerve growth factor-deprived PC12 cells without affecting the extent of SAPK/JNK activation, indicating that stimulation of this pathway does not require caspase activity (59). Cross-linking of the TNF receptor 1 (TNF-R1) activates SAPK/JNK and induces apoptosis; however, these two effects can occur independently and are a consequence of interactions between TNF-R1 and distinct TNF-R1-interacting proteins. Induction of apoptosis requires association with TRADD whereas SAPK/JNK activation results from TRAF2 binding (44, 53). The importance of SAPK/JNK signaling in apoptotic events might be dependent upon the apoptosis-inducing stimulus. SAPK/JNK activation in response to TNF-α is mediated by reactive oxygen species inasmuch as it can be prevented by the antioxidant *N*-acetylcysteine. *N*-Acetylcysteine also blocks SAPK/JNK activation resulting from growth factor withdrawal (59) and by UV-C but does not block SAPK/JNK activation by heat shock (2). Elevated levels of HSPs do not inhibit the UV-C-induced SAPK/JNK activation pathway since this was not affected in the thermoresistant cell line TR-4 and these cells are not more resistant to UV-C exposure (82).

The intracellular signals linking cellular stress to SAPK/JNK activation are not known; however, ceramide generation has recently been suggested to play a role. Conditions that lead to ceramide synthesis as well as the exogenous addition of ceramide or sphingomyelinase result in SAPK/JNK activation and increased c-Jun transcriptional activity (77). The closely related dihydro-C_2-ceramide, which differs only by the absence of the 4,5-*trans* double bond, is ineffective in activating SAPK/JNK and does not induce apoptosis (74, 77). Since hyperthermia can cause an increase in ceramide levels (7, 74), the resistance to heat shock displayed by the hsp70-expressing cells could be through a mechanism that limits the extent of ceramide generation or through limitation of the cytotoxic effects of ceramide. We examined this second possibility by comparing the effect of exogenous ceramide on SAPK/JNK activation and cell survival in the PEER and F3 cell lines. Ceramide activated SAPK/JNK in the PEER cell line in a dose-dependent manner that correlated with the extent of apoptosis. Ceramide also strongly activated SAPK/JNK in the F3 cell line; however, a higher dose was required relative to the PEER cell line. This is in contrast to the effects of heat shock, for which the F3 cell line was somewhat more responsive to heat-induced SAPK/JNK activation. This difference in dose response for SAPK/JNK activation by heat shock and ceramide for the PEER and F3 cell lines indicates that stress-induced signals in addition to ceramide are involved in the activation of SAPK/JNK by heat shock.

Similar to what was observed with heat shock, the hsp70-expressing cells were more resistant to the toxic effects of ceramide and yet had high levels of activated SAPK/JNK. This supports the suggestion that hsp70 can protect cells from apoptosis at some point downstream of SAPK/JNK activation. The induction of apoptosis by ceramide or by stress treatments that lead to ceramide accumulation culminates in caspase-3 activation and PARP cleavage, and this can be prevented either by overexpression of Bcl-2 or by addition of peptide inhibitors of caspase-3, indicating that ceramide acts upstream of the caspase protease cascade as a second messenger responsible for signaling cell death processes (47, 67, 83). In the case of apoptosis induced by ligation of the Fas ligand, or expression of the *Drosophila melanogaster* death-domain-containing protein REAPER, ceramide generation was blocked by a specific peptide inhibitor of the caspase family of proteases (21, 62). In these situations, ceramide generation requires an ICE-like protease activity.

The activity of the caspases can be inhibited by the cowpox virus protein CrmA (71), the baculovirus protein p35 (5), and the tetrapeptide aldehyde Ac-DEVD-CHO, which mimics the recognition site in PARP (55). Potent tetrapeptide inhibitors of caspase-3 have an Asp residue in the P_1 position and an anionic residue in the P_4 position (54). Curiously, all of the eukaryotic hsp70 molecules have the conserved carboxyl-terminal sequence-EEVD. Removal or mutation of the terminal four amino acids results in a loss of substrate binding ability (19). Overexpression of a mutant hsp70 protein which lacks the ATP binding domain but retains the carboxyl-terminal peptide binding domain is able to provide heat resistance (41). This might in part be due to the ability of the carboxyl-terminal EEVD sequence to inhibit the caspase protease cascade. For these reasons, we examined whether hsp70 could inhibit the activity of caspase-3 by directly interacting with its active site. We found, however, that hsp70 on its own did not inhibit the activity of caspase-3 in vitro. Since the accessibility of the carboxyl-terminal domain of hsp70 is conformation dependent and influenced by ATP binding, we also performed the inhibitor studies with excess ATP or the nonhydrolyzable analog ATP-γ-S but found that this did not affect the ability of hsp70 to act as an inhibitor of caspase-3 activity.

HSPs act as molecular chaperones, playing essential roles in mediating protein folding, assembly, transport, and degradation (reviewed in references 27 and 48). In cells exposed to hyperthermia, the induced synthesis of these proteins helps to prevent protein denaturation and aggregation and assists in the refolding or removal of damaged proteins. Whether HSPs protect cells by blocking protein denaturation in general or whether a specific heat-sensitive target is protected is not known. HSPs might protect proteins that are essential in controlling the decision to initiate the apoptotic cascade. This decision is in part regulated by protein-protein interactions between death-promoting (Bax, Bad, and Bcl-x_s) and inhibiting (Bcl-2, Bcl-x_L, and Mcl-1) members of the Bcl-2 family (56, 58, 63, 76). Bcl-2-expressing cells resist apoptosis initiated by a number of physiological and stressful conditions including hyperthermia (72). HSPs have been proposed to act by a mechanism independent of Bcl-2 since a thermotolerance-inducing pretreatment can enhance resistance to apoptosis in Bcl-2-expressing cells (69). Bcl-2, like the homologous *C. elegans* protein CED-9 (16), acts upstream of the caspases by preventing their activation (9). The caspases exist as inactive proenzymes that are proteolytically processed in response to apoptotic stimuli to form active heterodimeric proteases. Yama/CPP32 (caspase-3) and ICE-LAP3 (caspase-7) remain in their inactive proforms following apoptosis-inducing treatments in cells expressing either Bcl-2 or Bcl-x_L (9). Similarly, caspase-3 remains in the inactive proenzyme form following stress-induced apoptotic stimuli in cells expressing elevated levels of hsp70. Pro-caspase-3 is not autocatalytic but requires an initial cleavage, mediated by granzyme B, caspase-6, or the TNF-R1/Fas-receptor-activated caspase-10. This initial cleavage reveals other sites which are cleaved autocatalytically by the active enzyme (17). Caspase processing, which is conformation sensitive, may represent a critical heat-sensitive target that is inhibited by the chaperoning function of hsp70.

The ability of hsp70 to prevent stress-induced apoptosis could limit the efficacy of cancer therapy. Expression of hsp70 has been found to be an indicator of poor therapeutic outcome

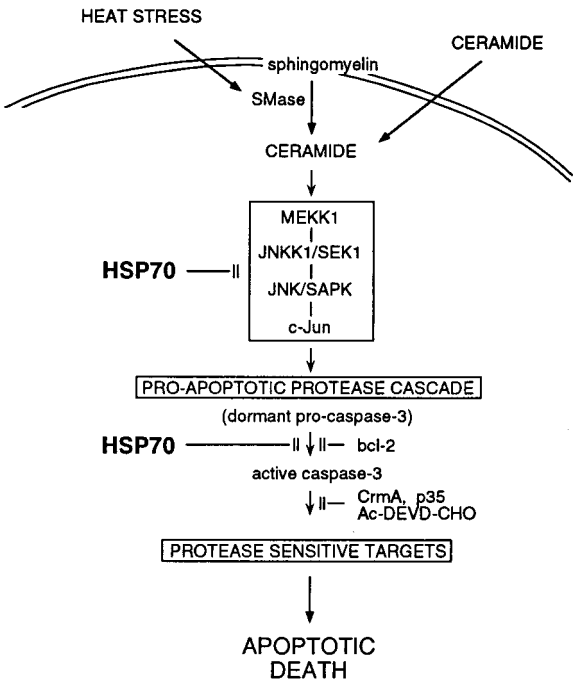

FIG. 7. hsp70 prevents stress-induced apoptosis by interfering with the SAPK/JNK signaling pathway and by blocking the caspase proteolytic cascade. Both heat stress, which can increase intracellular ceramide levels via activation of sphingomyelinase (SMase), and exogenous addition of ceramide lead to activation of the stress-activated protein kinase (SAPK/JNK) signaling pathway. The stress-induced triggering of this pathway is associated with caspase activation and apoptotic cell death. Overexpression of hsp70 prevents apoptosis in cells exposed to hyperthermia or exogenous ceramide. The caspase cascade is blocked in hsp70-expressing cells. Inducible overexpression of hsp70 blocks SAPK/JNK signaling; however, this is not prevented in cells that constitutively overexpress hsp70. See text for details.

in breast cancer (10). Thermotolerant cells have enhanced resistance to several chemotherapeutic drugs, many of which induce apoptotic death (13). A role for hsp70 in tumorigenesis has been suggested based on the observation that many transformed cells have elevated levels of hsp70 and that T-cell lymphoma is an outcome of forced hsp70 gene expression in transgenic mice (66, 75). Elevated expression of hsp70 can protect cells from cytotoxicity mediated by monocytes and from TNF-induced cell death and therefore could allow precancerous cells to escape immune surveillance (29, 30). This effect is likely a consequence of the ability of hsp70 to prevent apoptosis. Antisense inhibition of hsp70 expression induces apoptosis in the acute T-lymphocytic leukemia cell line Molt-4 (75). The targeted disruption of hsp70 expression in the male germ line of transgenic mice also results in apoptosis (14). The ability of hsp70 to interfere with key signaling and effector steps in the apoptotic pathway could provide a growth advantage to tumor cells and account for resistance to cytotoxic treatments.

In summary, our results show that hsp70 can prevent apoptosis by affecting both upstream signaling (SAPK/JNK activation) and downstream effector (caspase-3-mediated) events (Fig. 7). These effects of hsp70 likely account for its ability to provide resistance to stress-induced apoptosis in thermotolerant cells. The identification of key events in the apoptotic pathway that are affected by adaptive cellular responses, such as elevated expression of hsp70, could facilitate the identification of targets for effective antitumor therapy.

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REFERENCES

1. **Abravaya, K., M. P. Myers, S. P. Murphy, and R. I. Morimoto.** 1992. The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. Genes Dev. **6:**1153–1164.
2. **Adler, V., A. Schaffer, J. Kim, L. Dolan, and Z. Ronai.** 1995. UV irradiation and heat shock mediate JNK activation via alternate pathways. J. Biol. Chem. **270:**26071–26077.
3. **Alnemri, E. S., S. J. Livingston, D. W. Nicholson, G. Salvesen, N. A. Thornberry, W. W. Wong, and J. Yuan.** 1996. Human ICE/CED-3 protease nomenclature. Cell **87:**171.
4. **Boldin, M. P., T. M. Goncharov, Y. V. Goltsev, and D. Wallach.** 1996. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell **85:**803–815.
5. **Bump, N. J., M. Hackett, M. Hugunin, S. Seshagiri, K. Brady, P. Chen, C. Ferenz, S. Franklin, R. Ghayur, P. Li, P. Licari, J. Mankovich, L. Shi, A. H. Greenberg, L. K. Miller, and W. W. Wong.** 1995. Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35. Science **269:**1885–1888.
6. **Casciola-Rosen, L., D. W. Nicholson, T. Chong, K. R. Rowan, N. A. Thornberry, D. K. Miller, and A. Rosen.** 1996. Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. J. Exp. Med. **183:**1957–1964.
7. **Chang, Y., A. Abe, and J. A. Shayman.** 1995. Ceramide formation during heat shock: a potential mediator of αB-crystallin transcription. Proc. Natl. Acad. Sci. USA **92:**12275–12279.
8. **Chen, Y. R., C. F. Meyer, and T. H. Tan.** 1996. Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in γ radiation-induced apoptosis. J. Biol. Chem. **271:**631–634.
9. **Chinnaiyan, A. M., K. Orth, K. O'Rourke, H. Duan, G. G. Poirier, and V. M. Dixit.** 1996. Molecular ordering of the cell death pathway: Bcl-2 and Bcl-x_L function upstream of the CED-3-like apoptotic proteases. J. Biol. Chem. **271:**4573–4576.
10. **Ciocca, D. R., G. M. Clark, A. K. Tandon, S. A. Fuqua, W. J. Welch, and W. L. McGuire.** 1993. Heat shock protein hsp70 in patients with axillary lymph node-negative breast cancer: prognostic implications. J. Natl. Cancer Inst. **85:**570–574.
11. **Cohen, J. J.** 1992. Morphological and biochemical assays of apoptosis, p. 3.17.1–3.17.16. *In* J. E. Coligan (ed.), Current protocols in immunology. John Wiley & Sons, Inc., New York, N.Y.
12. **Cuvillier, O., G. Pirianov, B. Kleuser, P. G. Vanek, O. A. Coso, J. S. Gutkind, and S. Spiegel.** 1996. Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. Nature **381:**800–803.
13. **Dive, C., and J. A. Hickman.** 1991. Drug-target interactions: only the first step in the commitment to a programmed cell death? Br. J. Cancer **64:**192–196.
14. **Dix, D. J., J. W. Allen, B. W. Collins, C. Mori, N. Nakamura, P. Poormanallen, E. H. Goulding, and E. M. Eddy.** 1996. Targeted gene disruption of hsp70-2 results in failed meiosis, germ cell apoptosis, and male infertility. Proc. Natl. Acad. Sci. USA **93:**3264–3268.
15. **Dubois, M. F., and O. Bensaude.** 1993. MAP kinase activation during heat shock in quiescent and exponentially growing mammalian cells. FEBS Lett. **324:**191–195.
16. **Ellis, R. E., J. Yuan, and H. R. Horvitz.** 1991. Mechanisms and functions of cell death. Annu. Rev. Cell Biol. **7:**663–698.
17. **Fernandes-Alnemri, T., R. C. Armstrong, J. Krebs, S. M. Srinivasula, L. Wang, F. Bullrich, L. C. Fritz, J. A. Trapani, K. J. Tomaselli, G. Litwack, and E. S. Alnemri.** 1996. *In vitro* activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. Proc. Natl. Acad. Sci. USA **93:**7464–7469.
18. **Fraker, P. J., L. E. King, D. Lill-Elghanian, and W. G. Telford.** 1995. Quantification of apoptotic events in pure and heterogeneous populations of cells using the flow cytometer. Methods Cell Biol. **46:**57–76.
19. **Freeman, B. C., M. P. Myers, R. Schumacher, and R. I. Morimoto.** 1995. Identification of a regulatory motif in hsp70 that affects ATPase activity, substrate binding and interaction with HDJ-1. EMBO J. **14:**2281–2292.
20. **Gabai, V. L., A. Meriin, D. D. Mosser, A. W. Caron, S. Rits, V. Shifrin, and M. Y. Sherman.** 1997. Hsp70 prevents activation of stress kinases. A novel pathway of cellular thermotolerance. J. Biol. Chem. **272:**18033–18037.
21. **Gamen, S., I. Marzo, A. Anel, A. Piñeiro, and J. Naval.** 1996. CPP32 inhibition prevents Fas-induced ceramide generation and apoptosis in human cells. FEBS Lett. **390:**233–237.
22. **Gossen, M., and H. Bujard.** 1992. Tight control of gene expression in mam-

malian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA **89:**5547–5551.
23. **Green, D. R., and S. J. Martin.** 1995. The killer and the executioner: how apoptosis controls malignancy. Curr. Opin. Immunol. **7:**694–703.
24. **Hahn, G. M., and G. C. Li.** 1990. Thermotolerance, thermoresistance, and thermosensitization, p. 79–100. *In* R. I. Morimoto, A. Tissiéres, and C. Georgopoulos (ed.), Stress proteins in biology and medicine. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. **Hale, A. J., C. A. Smith, L. C. Sutherland, V. E. A. Stoneman, V. L. Longthorne, A. C. Culhane, and G. T. Williams.** 1996. Apoptosis: molecular regulation of cell death. Eur. J. Biochem. **236:**1–26.
26. **Hannun, Y. A.** 1996. Functions of ceramide in coordinating cellular responses to stress. Science **274:**1855–1859.
27. **Hartl, F. U.** 1996. Molecular chaperones in cellular protein folding. Nature **381:**571–580.
28. **Heim, R., A. B. Cubitt, and R. Y. Tsien.** 1995. Improved green fluorescence. Nature **373:**663–664.
29. **Jäättelä, M., and D. Wissing.** 1993. Heat-shock proteins protect cells from monocyte cytotoxicity: possible mechanism of self-protection. J. Exp. Med. **177:**231–236.
30. **Jäättelä, M., D. Wissing, P. A. Bauer, and G. C. Li.** 1992. Major heat shock protein hsp70 protects tumor cells from tumor necrosis factor cytotoxicity. EMBO J. **11:**3507–3512.
31. **Jarvis, W. D., S. Grant, and R. N. Kolesnick.** 1996. Ceramide and the induction of apoptosis. Clin. Cancer Res. **2:**1–6.
32. **Jarvis, W. D., R. N. Kolesnick, F. A. Fornari, R. S. Traylor, D. A. Gewirtz, and S. Grant.** 1994. Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. Proc. Natl. Acad. Sci. USA **91:**73–77.
33. **Johnson, N. L., A. M. Gardner, K. M. Diener, C. A. Lange-Carter, J. Gleavy, M. B. Jarpe, A. Minden, M. Karin, L. I. Zon, and G. L. Johnson.** 1996. Signal transduction pathways regulated by mitogen-activated/extracellular response kinase kinase kinase induce cell death. J. Biol. Chem. **271:**3229–3237.
34. **Kumar, S.** 1995. ICE-like proteases in apoptosis. Trends Biochem. Sci. **20:**198–202.
35. **Kyriakis, J. M., and J. Avruch.** 1996. Protein kinase cascades activated by stress and inflammatory cytokines. Bioessays **18:**567–577.
36. **Kyriakis, J. M., P. Banerjee, E. Nikolakaki, T. Dai, E. A. Rubie, M. F. Ahmad, J. Avruch, and J. R. Woodgett.** 1994. The stress-activated protein kinase subfamily of c-Jun kinases. Nature **369:**156–160.
37. **Landry, J., D. Bernier, P. Chrétien, L. M. Nicole, R. M. Tanguay, and N. Marceau.** 1982. Synthesis and degradation of heat shock proteins during development and decay of thermotolerance. Cancer Res. **42:**2457–2461.
38. **Landry, J., P. Chrétien, H. Lambert, E. Hickey, and L. A. Weber.** 1989. Heat shock resistance conferred by expression of the HSP27 gene in rodent cells. J. Cell Biol. **109:**7–15.
39. **Lazebnik, Y. A., S. H. Kaufmann, S. Desnoyers, G. G. Poirier, and W. C. Earnshaw.** 1994. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature **371:**346–347.
40. **Li, G. C., L. Li, Y. K. Liu, H. Y. Mak, L. Chen, and W. M. F. Lee.** 1991. Thermal response of rat fibroblasts stably transfected with the human 70-kDa heat shock protein-encoding gene. Proc. Natl. Acad. Sci. USA **88:**1681–1685.
41. **Li, G. C., L. Li, R. Y. Liu, M. Rehman, and W. M. Lee.** 1992. Heat shock protein hsp70 protects cells from thermal stress even after deletion of its ATP-binding domain. Proc. Natl. Acad. Sci. USA **89:**2036–2040.
42. **Li, G. C., and Z. Werb.** 1982. Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. Proc. Natl. Acad. Sci. USA **79:**3218–3222.
43. **Li, W. X., C. H. Chen, C. C. Ling, and G. C. Li.** 1996. Apoptosis in heat-induced cell killing: the protective role of hsp-70 and the sensitization effect of the c-myc gene. Radiat. Res. **145:**324–330.
44. **Liu, Z. G., H. Hsu, D. V. Goeddel, and M. Karin.** 1996. Dissection of the TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell **87:**565–576.
45. **Mehlen, P., K. Schulzeosthoff, and A. P. Arrigo.** 1996. Small stress proteins as novel regulators of apoptosis—heat shock protein 27 blocks Fas/APO-1- and staurosporine-induced cell death. J. Biol. Chem. **271:**16510–16514.
46. **Mercille, S., and B. Massie.** 1994. Induction of apoptosis in nutrient-deprived cultures of hybridoma and myeloma cells. Biotechnol. Bioeng. **44:** 1140–1154.
47. **Mizushima, N., R. Koike, H. Kohsaka, Y. Kushi, S. Handa, H. Yagita, and N. Miyasaka.** 1996. Ceramide induces apoptosis via CPP32 activation. FEBS Lett. **395:**267–271.
48. **Morimoto, R. I., A. Tissières, and C. Georgopoulos.** 1994. The biology of heat shock proteins and molecular chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
49. **Mosser, D. D., A. W. Caron, L. Bourget, P. Jolicoeur, and B. Massie.** 1997. Use of a dicistronic expression cassette encoding the green fluorescent protein for the screening and selection of cells expressing inducible gene products. BioTechniques **22:**150–161.
50. **Mosser, D. D., J. Duchaine, and B. Massie.** 1993. The DNA-binding activity of the human heat shock transcription factor is regulated in vivo by hsp70. Mol. Cell. Biol. **13:**5427–5438.
51. **Mosser, D. D., and L. H. Martin.** 1992. Induced thermotolerance to apoptosis in a human T-lymphocyte cell line. J. Cell. Physiol. **151:**561–570.
52. **Muzio, M., A. M. Chinnaiyan, F. C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J. D. Bretz, M. Zhang, R. Gentz, M. Mann, P. H. Krammer, M. E. Peter, and V. M. Dixit.** 1996. FLICE, a novel FADD homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/Apo-1) death-inducing signaling complex. Cell **85:**817–827.
53. **Natoli, G., A. Costanzo, A. Ianni, D. J. Templeton, J. R. Woodgett, C. Balsano, and M. Levrero.** 1997. Activation of SAPK/JNK by TNF receptor 1 through a noncytotoxic TRAF2-dependent pathway. Science **275:**200–203.
54. **Nicholson, D. W.** 1996. ICE/CED3-like proteases as therapeutic targets for the control of inappropriate apoptosis. Nat. Biotechnol. **14:**297–301.
55. **Nicholson, D. W., A. Ali, N. A. Thornberry, J. P. Vaillancourt, C. K. Ding, M. Gallant, Y. Gareau, P. R. Griffin, M. Labelle, Y. A. Lazebnik, N. A. Munday, S. M. Raju, M. E. Smulson, T. T. Yamin, V. L. Yu, and D. K. Miller.** 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature **376:**37–43.
56. **Nunez, G., and M. F. Clarke.** 1994. The Bcl-2 family of proteins: regulators of cell death and survival. Trends Cell Biol. **4:**399–403.
57. **Obeid, L. M., D. M. Linardic, L. A. Karolak, and Y. A. Hannun.** 1993. Programmed cell death induced by ceramide. Science **259:**1769–1771.
58. **Oltvai, Z. N., and S. J. Korsmeyer.** 1994. Checkpoints of dueling dimers foil death wishes. Cell **79:**189–192.
59. **Park, D. S., L. Stefanis, C. Y. I. Yan, S. E. Farinelli, and L. A. Greene.** 1996. Ordering the cell death pathway. Differential effects of BCL2, an interleukin-1-converting enzyme family protease inhibitor, and other survival agents on JNK activation in serum/nerve growth factor-deprived PC12 cells. J. Biol. Chem. **271:**21898–21905.
60. **Parsell, D. A., and S. Lindquist.** 1994. Heat shock proteins and stress tolerance. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
61. **Patel, T., G. J. Gores, and S. H. Kaufmann.** 1996. The role of proteases during apoptosis. FASEB J. **10:**587–597.
62. **Pronk, G. J., K. Ramer, P. Amiri, and L. T. Williams.** 1996. Requirement of an ICE-like protease for induction of apoptosis and ceramide generation by REAPER. Science **271:**808–810.
63. **Reed, J. C.** 1994. Bcl-2 and the regulation of programmed cell death. J. Cell Biol. **124:**1–6.
64. **Resnitzky, D., M. Gossen, H. Bujard, and S. I. Reed.** 1994. Acceleration of the G_1/S-phase transition by expression of cyclins D1 and E with an inducible system. Mol. Cell. Biol. **14:**1669–1679.
65. **Samali, A., and T. G. Cotter.** 1996. Heat shock proteins increase resistance to apoptosis. Exp. Cell Res. **223:**163–170.
66. **Seo, J. S., Y. M. Park, J. I. Kim, E. H. Shim, C. W. Kim, J. J. Jang, S. H. Kim, and W. H. Lee.** 1996. T cell lymphoma in transgenic mice expressing the human hsp70 gene. Biochem. Biophys. Res. Commun. **218:**582–587.
67. **Smyth, M. J., D. K. Perry, J. Zhang, G. G. Poirier, Y. A. Hannun, and L. M. Obeid.** 1996. prICE: a downstream target for ceramide-induced apoptosis and for the inhibitory action of Bcl-2. Biochem. J. **316:**25–28.
68. **Stokoe, D., K. Engel, D. G. Campbell, P. Cohen, and M. Gaestel.** 1992. Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins. FEBS Lett. **313:**307–313.
69. **Strasser, A., and R. L. Anderson.** 1995. Bcl-2 and thermotolerance cooperate in cell survival. Cell Growth Differ. **6:**799–805.
70. **Subjeck, J. R., J. J. Sciandra, and R. J. Johnson.** 1982. Heat shock proteins and thermotolerance; a comparison of induction kinetics. Br. J. Radiol. **55:**579–584.
71. **Tewari, M., L. T. Quan, K. O'Rourke, S. Desnoyers, Z. Zeng, D. R. Beidler, G. G. Poirier, G. S. Salvesen, and V. M. Dixit.** 1995. Yama/CPP32β, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell **81:**801–809.
72. **Tsujimoto, Y.** 1989. Stress-resistance conferred by high level of bcl-2α protein in human B lymphoblastoid cell. Oncogene **4:**1331–1336.
73. **Vaux, D. L., and A. Strasser.** 1996. The molecular biology of apoptosis. Proc. Natl. Acad. Sci. USA **93:**2239–2244.
74. **Verheij, M., R. Bose, X. H. Lin, B. Yao, W. D. Jarvis, S. Grant, M. J. Birrer, E. Szabo, L. I. Zon, J. M. Kyriakis, A. Haimovitz-Friedman, Z. Fuks, and R. N. Kolesnick.** 1996. Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature **380:**75–79.
75. **Wei, Y. Q., X. Zhao, Y. Kariya, K. Teshigawara, and A. Uchida.** 1995. Inhibition of proliferation and induction of apoptosis by abrogation of heat-shock protein (HSP) 70 expression in tumor cells. Cancer Immunol. Immunother. **40:**73–78.
76. **Werner, M. H.** 1996. Stopping death cold. Structure **4:**879–883.
77. **Westwick, J. K., A. E. Bielawska, G. Dbaibo, Y. A. Hannun, and D. A. Brenner.** 1995. Ceramide activates the stress-activated protein kinases. J. Biol. Chem. **270:**22689–22692.
78. **White, E.** 1996. Life, death, and the pursuit of apoptosis. Genes Dev. **10:**1–15.
79. **Whyte, M.** 1996. ICE/CED-3 proteases in apoptosis. Trends Cell Biol. **6:** 245–248.
80. **Xia, Z., M. Dickens, J. Raingeaud, R. J. Davis, and M. E. Greenberg.** 1995.

Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science **270:**1326–1331.

81. **Yuan, J., L. Shaham, S. Ledoux, H. M. Ellis, and H. R. Horvitz.** 1993. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1β-converting enzyme. Cell **75:**641–652.
82. **Zanke, B. W., K. Boudreau, E. Rubie, E. Winnett, L. A. Tibbles, L. Zon, J. Kyriakis, F. F. Liu, and J. R. Woodgett.** 1996. The stress-activated protein kinase pathway mediates cell death following injury induced by *cis*-platinum, UV irradiation or heat. Curr. Biol. **6:**606–613.
83. **Zhang, J., N. Alter, J. C. Reed, C. Borner, L. M. Obeid, and Y. A. Hannun.** 1996. Bcl-2 interrupts the ceramide-mediated pathway of cell death. Proc. Natl. Acad. Sci. USA **93:**5325–5328.