Heat shock protein 70 (HSP70) has been shown to act as an inhibitor of apoptosis. We have also observed an inhibitory effect of HSP70 on apoptotic cell death both in preheated U937 and stably transfected HSP70-overexpressing U937 (U937/HSP70) cells. However, the molecular mechanism whereby HSP70 prevents apoptosis still remains to be solved. To address this issue, we investigated the effect of HSP70 on apoptotic processes in an in vitro system. Caspase-3 cleavage and DNA fragmentation were detected in cytosolic fractions from normal cells upon addition of dATP, but not from preheated U937 or U937/hsp70 cells. Moreover, the addition of purified recombinant HSP70 to normal cytosolic fractions prevented caspase-3 cleavage and DNA fragmentation, suggesting that HSP70 prevents apoptosis upstream of caspase-3 processing. Because cytochrome c was still released from mitochondria into the cytosol by lethal heat shock despite prevention of caspase-3 activation and cell death in both preheated U937 and U937/hsp70 cells, it was evident that HSP70 acts downstream of cytochrome c release. Results obtained in vitro with purified deletion mutants of HSP70 showed that the carboxyl one-third region (from amino acids 438 to 641) including the peptide-binding domain and the carboxyl-terminal EEVD sequence was essential to prevent caspase-3 processing. From these results, we conclude that HSP70 acts as a strong suppressor of apoptosis acting downstream of cytochrome c release and upstream of caspase-3 activation.

Apoptosis, which is characterized by cell shrinkage, membrane blebbing, nuclear breakdown, and DNA fragmentation, is indispensable for embryo development, tissue homeostasis, and regulation of the immune system (1–4). Malfunctions of apoptosis have been implicated in human diseases including cancer, neurodegenerative disorders, and ischemic stroke (5–7). Apoptosis can be induced by a variety of different signals, including activation of the Fas or tumor necrosis factor receptors, growth factor deprivation, excessive DNA damage, treatment with chemotherapeutic drugs, or stresses such as heat shock, hypotonic shock, or UV irradiation (8–11).

Apoptosis signals such as Fas ligand and tumor necrosis factor activate procaspase-8 through molecular interactions between components of the death-inducing signaling complex (12–13). Activated caspase-8 cleaves Bid (Bcl-2 interacting protein), and the carboxyl-terminal domain translocates to mitochondria where it initiates cytochrome c release (14). The release of cytochrome c triggers the formation of a complex containing Apaf1, a mammalian CED-4 homologue, and procaspase-9, which is then autoprocessed and thereby capable of processing downstream effector procaspases such as procaspase-3 (15). The processing of these caspases is followed by the cleavage of apoptotic substrates, leading to the disruption of important cellular processes, changes in cellular and nuclear morphology, and ultimately to cell death (16–18). Diverse apoptotic signals for caspase activation converge at the mitochondrial level, provoking the release of cytochrome c, which participates in the central control or executioner phase of the cell death cascade (19–21). However, the role of cytochrome c release in apoptosis is still confusing and contradictory, and the mechanism of cytochrome c release has not been elucidated.

Because ischemia and neurodegenerative diseases such as Alzheimer’s and Huntington’s diseases may result from excessive apoptosis, it may be beneficial to limit apoptosis as a way to manage these diseases (22, 23). Cellular and viral proteins such as Bcl-2, CrmA, and IAP1 might serve as therapeutic agents to inhibit apoptosis, because Bcl-2 prevents cytochrome c release, CrmA inhibits caspase-8, and IAP blocks caspase-3 activation (13). In addition, HSP70 has been suggested as a promising molecule for controlling apoptosis because HSP70-overexpressing transgenic mice showed reduced brain and heart ischemia (24, 25).

HSPs can be induced by various stresses such as ethanol, amino acid analogues, infection, inhibitors of energy metabolism, and heavy metals (26). In addition to its chaperoning function for folding, transport, and assembly of newly synthesized polypeptides (27, 28), HSP70 protects cells from a number of apoptotic stimuli, including heat shock, tumor necrosis factor, growth factor withdrawal, oxidative stress, chemotherapeutic agents, ceramide, and radiation (29–33). HSP70 prevents caspase-3 and SAPK/JNK activation in heat shock- or ceramide-induced apoptosis (34, 35). Despite recent advances, the anti-apoptotic mechanism of HSP70 is still controversial.
Apoptosis Inhibition by HSP70

(35, 36). Therefore, the aim of this study is to elucidate which step in the apoptosis pathway is affected by HSP70. We used an in vitro apoptosis system as well as preheated and HSP70-overexpressing cells. The results demonstrate that HSP70 can inhibit apoptosis downstream of cytochrome c release, but upstream of caspase-3 cleavage, and that the carboxy-terminal region containing the peptide-binding domain is sufficient to inhibit caspase-3 activation.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—The deoxynucleotidetriphosphates' peptidstatin A, leupeptin, and N-acetyl-leucyl-leucyl-norleucine (ALLL) were purchased from Roche Molecular Biochemicals. Phenylmethylsulfonyl fluoride, aprotonin, and bovine heart cytochrome c were obtained from Sigma. The Cpg2/caspase-3 colorimetric protease assay kit was obtained from Medical & Biological Laboratories Co. (Nagoya, Japan). The secondary antibodies, goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase, were obtained from Pierce. The molecular weight standards for SDS-polyacrylamide gel electrophoresis were obtained from Bio-Rad. Enhanced chemiluminescence (ECL) reagent for Western blotting detection was purchased from Amer sham Pharmacia Biotech.

Antibodies for immunoblotting were purchased from the following sources. Anti-cytochrome c (THS2C12) and cytochrome oxidase II were purchased from PharMingen (San Diego, CA) and Molecular Probes (Eugene, OR), respectively. Anti-caspase-3 (CPP32) and anti-PARP were obtained from Transduction Laboratories (Lexington, KY) and Roche Molecular Biochemicals, respectively. Anti-HSP70 and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Cultures and Heat Shock Conditions—U937 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 50 μg/ml penicillin-streptomycin in a 5% CO2 incubator. Culture plates containing cells were wrapped tightly with paraffin and immersed in a water bath at the desired temperature. Mild heat shock conditions to induce HSP70 synthesis was 42 °C for 30 min, and lethal heat shock conditions were 43 °C for 60 or 80 min.

Stable Transfection—To establish a retrovirus-producing cell line, 10 μg of MFG.HPS70.puro and MFG.puro plasmids (37) were transfected into the PA317 retrovirus packaging cell line (ATCC; CRL 9078) containing 2 mM DTT. The transfected cells were selected and used for retrovirus production. The viral infection was essentially as described (40) with several modifications. Briefly, the selected cells were maintained as a population and used for retrovirus production. The viral infection was calculated as follows: % DNA fragmentation determined by liquid scintillation counting. The percent DNA fragmentation was quantitated as described previously (41) with some modifications. Briefly, exponentially growing cells (2.5 × 10⁶ cells/ml) were incubated with blocking buffer (10% non-fat milk, 0.1% Tween 20, 0.05% sodium EDTA, 1 mM sodium EGTA, 250 μM sulfoxide, and 1 mM DTT) at 85 °C × 10⁷ nuclei/ml and stored at −80 °C in multiple aliquots.

Isolation of Mouse Liver Nuclei—Liver nuclei were prepared according to the method of Liu et al. (40). Livers from four FVB strain male mice were rinsed with ice-cold PBS and homogenized in buffer B (20 mM Hepes-KOH, pH 7.6, 2.4 mM succinate, 15 mM KCl, 2 mM sodium EDTA, 0.15 mM spermine, 0.15 mM spermidine, 0.5 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride) by 10 strokes of a tissue-teaser. The homogenates were centrifuged through 10 ml of buffer B at 25,000 rpm for 1 h in a SW 28 rotor (Beckman) at 4 °C. The nuclei pellets were resuspended in buffer C (10 mM PIPES, pH 7.4, 80 mM KCl, 20 mM NaCl, 5 mM sodium EGTA, 250 mM sucrose, and 1 mM DTT) at 85 °C × 10⁷ nuclei/ml and stored at −80 °C in multiple aliquots.

Purification of HSP70 and Its Deletion Mutants—The full-length human HSP70 gene was cloned into pET30a (Novagen). Several deletion mutants of HSP70 were created and ligated into pET30a as follows: hsp70ΔB lacking the BglII-BglII fragment, hsp70ΔS lacking the Smal-Smal fragment, hsp70N lacking the Ncol-Ncol fragment, and hsp70C deleted nucleotides 1756–1932. All recombinant plasmids were transformed into Escherichia coli BL21. Bacteria were grown at 37 °C to an optical density of 0.6, and protease inhibitors (0.1 mM DTT) with 1 mM isopropyl-β-thiogalactopyranoside for 2 h. His-tagged HSP70 and its deletion mutant proteins were purified through a nickel affinity column by passing the soluble fractions of cell lysates. Eluted proteins were dialyzed against buffer A.

DNA Fragmentation Assay—To determine the degradation of chromosomal DNA into nucleosome-sized fragments in the in vitro apoptosis system, aliquots of 50 μl of cytosolic fraction (S-100) and 2 × 10⁷ nuclei were incubated at 37 °C for 1 h in the presence of dATP. A 500-μl aliquot of buffer D (100 mM Tris·HCl, pH 8.5, 5 mM EDTA, 0.2 mM NaCl, 0.2% (w/v) SDS, and 0.2 mg/ml proteinase K) was added to the reaction mixture and incubated at 37 °C overnight. DNA was obtained by ethanol precipitation, separated on a 1.8% agarose gel, and visualized under UV light.

DNA fragmentation was quantitated as described previously (41) with some modifications. Briefly, exponentially growing cells (2.5 × 10⁶ cells/ml) were labeled with 10 μCi/2.5 × 10⁶ cells of [methyl-3H]thymidine for 5 h and washed three times with nonradioactive fresh medium. Labeled cells were exposed to lethal heat shock for different times and returned to 37 °C for 12 h. Cells were harvested in a microcentrifuge tube, washed once with ice-cold PBS, lysed with radioimmunoprecipitation assay buffer, and centrifuged at 105,000 × g at 4 °C, and the supernatant was further centrifuged at 105,000 × g at 4 °C, and the supernatant was further centrifuged at 105,000 × g at 4 °C. The pellets were washed once with ice-cold PBS and resuspended with 5 volumes of buffer A containing 250 mM sucrose. After incubation on ice for 15 min, the cells were homogenized with 8 strokes of a 2-ml Wheaton Dounce homogenizer (Kontes Glass Co.). After the homogenate was centrifuged (750 × g, 10 min at 4 °C) twice, the supernatants were centrifuged again at 12,000 × g for 15 min at 4 °C. The supernatants were stored at 4 °C as cytosolic fractions. The mitochondrial pellets were washed once with PBS, lysed with radioimmunoprecipitation assay buffer, and after 30 min on ice were centrifuged at 14,000 rpm for 10 min. The resulting mitochondrial fractions and the cytosolic fractions were used for Western blot analysis with an anti-cytochrome c antibody.

Assay for Caspase-3 Activity—Caspase-3 activity was measured according to the manufacturer's protocol. The cells (5 × 10⁶) were lysed with 250 μl of chilled cell lysis buffer on ice for 10 min. After microcentrifugation (10,000 × g, 1 min, 4 °C), the supernatant was used for caspase-3 colorimetric protease assay. 170 μg of protein was diluted to 50 μg of cell lysis buffer and mixed with 50 μl of 2 × reaction buffer (containing 10 mM DTT) and 5 μl of 4 mM Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA) (200 μM final concentration). After incubation at 37 °C for 2 h, samples were analyzed with a spectrofluorometer, and fluorescence was measured with 2 × 10⁴ mouse liver nuclei in the presence of 260 ± 500 μM dATP and incubated for 1 h at 37 °C. At the end of the incubation, samples were centrifuged, and aliquots of supernatant (50 μg of protein) were mixed with 2 × SDS-sample buffer. After boiling for 5 min, samples were subjected to 12% SDS-polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membranes. The membranes were incubated with blocking buffer (10% non-fat milk, 0.1% Tween 20, 0.1% Triton X-100, 1% non-fat milk, 0.1% Tween 20, and 0.1% Triton X-100). The membranes were incubated with blocking buffer (10% non-fat milk, 0.1% Tween 20, and 0.1% Triton X-100). The membranes were incubated with blocking buffer (10% non-fat milk, 0.1% Tween 20, and 0.1% Triton X-100). The membranes were incubated with blocking buffer (10% non-fat milk, 0.1% Tween 20, and 0.1% Triton X-100).
DNA digested with Hin a 12-h recovery, the extent of DNA fragmentation was determined using labeled cells were challenged by lethal heat shock treatments. After a then with anti-caspase-3 for 1 h at room temperature. The membranes fragmentations in control and preheated U937 cells. The [3H]thymidine-labeled cells were challenged by lethal heat shock treatments. After a 12-h recovery, the extent of DNA fragmentation was determined using a 33-counter. C, in vitro cleavage of caspase-3 in extracts from normal and preheated cells. The S-100 fractions (250 mg of protein) were mixed with mouse liver nuclei (2 × 10⁶) and incubated for 1 h at 37 °C in the presence of dATP. Cleaved caspase-3 was detected by Western blotting with a caspase-3 antibody. D, agarase-gel electrophoresis of DNA purified from the extracts treated as described in C. Lane M indicates lambda DNA digested with HindIII as a size marker.

20 mM Tris-HCl, pH 7.4, 150 mM NaCl for 1 h at room temperature and then with anti-caspase-3 for 1 h at room temperature. The membranes were washed with washing buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20, 1% non-fat milk) three times and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse immunoglobulin G. Antibody detection was performed using the ECL detection kit (Amersham Pharmacia Biotech).

To detect PARP cleavages in an intact cell system, whole cell lysate was prepared from cells treated with lethal heat shock, and then immunoblotted with anti-PARP antibody as mentioned above.

RESULTS

Mild Heat Shock Prevents Apoptosis Upstream of Caspase-3 Activation in an in Vitro System—We first examined the effect of elevated HSP expression on heat resistance in U937 cells. A mild heat shock treatment (42 °C, 30 min) that did not induce apoptosis (data not shown) was used to induce HSP70 synthesis. 12 h after the heat treatment, the level of HSP70 in the preheated cells was substantially elevated relative to that of the control cells (Fig. 1A).

When DNA fragmentation was observed in cells exposed to lethal heat shock, the preheated cells, in contrast to the control cells, showed a dramatic level of resistance to DNA fragmentation (Fig. 1B). DNA fragmentation measured in preheated cells was decreased 3.6–4.0-fold when HSP70 was induced by a mild heat treatment.

To elucidate which step in the apoptotic pathway is regulated by HSP70, we employed an in vitro apoptosis system based on the ability of exogenously added dATP to induce apoptosis in an S-100 fraction containing cytochrome c (40). Extracts prepared from control and preheated cells were incubated with exogenous liver nuclei and dATP. The extent of apoptosis activation was determined by monitoring the proteolytic processing of pro-caspase-3 and the intranucleosomal fragmentation of the mouse liver DNA. Processing of procaspase-3 into the p19 and p17 subunits (the small p12 subunit of caspase-3 was undetectable with the anti-caspase-3 antibody used for this study), which occurred in the extract from control cells, was completely inhibited in the extract from preheated cells (Fig. 1C). Additionally, intranucleosomal fragmentation of the mouse liver DNA was almost completely blocked in the extract from preheated cells, whereas extensive DNA fragmentation occurred in the extract prepared from control cells (Fig. 1D).

HSP70 Is a Major Inhibitor of Apoptosis and Caspase-3 Activation—Because the exposure of cells to mild heat shock can induce the synthesis of several different heat shock proteins (33), we prepared HSP70-overexpressing cells (U937/hsp70) to address the question of whether HSP70 itself inhibits apoptosis in the in vitro system. The level of HSP70 accumulation in U937/hsp70 cells was much higher than in control cells (U937/puro) and was very similar to that of preheated cells (Fig. 1A). When the percent of DNA fragmentation was observed in U937/hsp70 cells after exposing to lethal heat shock treatments, the U937/hsp70 cells also showed a dramatic level of resistance to DNA fragmentation in contrast to the U937/puro cells (Fig. 2A). These results are very similar to those obtained with preheated cells.

Fig. 2, B and C, shows the results of the in vitro apoptosis assay using extracts from U937/hsp70 cells. In contrast to U937/puro, caspase-3 activation and intranucleosomal DNA fragmentation were also prevented in cytosolic extracts of U937/hsp70 cells as shown in preheated cells. This result suggests that HSP70 per se blocks caspase-3 cleavage in heat shock-induced apoptosis.

This antiapoptotic function of HSP70 is a general phenomenon, because we have observed similar results in a different cell line, HL60. When these cells accumulated HSP70 by mild heat shock or by HSP70 transfection, they also showed an inhibition of caspase-3 activation, PARP cleavage, and DNA fragmentation in response to a lethal heat shock compared with control HL60 cells (data not shown); this supports the suggestion that HSP70 is a general inhibitor of apoptosis acting upstream of caspase-3 activation.

As a further test of the ability of HSP70 to function as an
inhibitor of apoptosis, the effects of recombinant HSP70 on caspase-3 activation and DNA fragmentation were determined. HSP70 protein was purified using a bacterial expression system. Reaction mixtures containing recombinant HSP70 and cytotoxic extracts from control U937 cells were incubated in vitro and examined for caspase-3 activation. Purified recombinant HSP70 clearly inhibited caspase-3 cleavage in comparison with bovine serum albumin (Fig. 3A). Inhibition of DNA fragmentation was also observed in extracts containing recombinant HSP70 but not in extracts containing added bovine serum albumin (Fig. 3B). These results demonstrate that HSP70 itself prevents the activation of key molecules associated with apoptosis in an in vitro system.

**Heat Shock Induces Cytochrome c Release from Mitochondria, and This Is Not Blocked by HSP70**—Cytochrome c acts as an important molecule at the early stage of apoptosis pathway. Its release from mitochondria leads to the activation of caspase-9, which then converts procaspase-3 into its active form, resulting in apoptosis. To address the question of whether lethal heat shock initiates apoptosis via cytochrome c release, the effect of heat shock on cytochrome c release was determined. After U937 cells were treated with lethal heat shock, cytochrome c release was determined by immunoblot analysis. Cytochrome c was increased in cytosol after lethal heat shock (Fig. 4A). Because HL60 cells also showed cytochrome c release by lethal heat shock (data not shown), this indicates that cytochrome c participates in the executioner phase of apoptotic cell death cascade in response to heat shock.

Since we found that HSP70 blocks apoptosis at some point upstream of caspase-3 activation, we next examined whether HSP70 affects cytochrome c release, which is further upstream in the apoptosis pathway. To address this question, the presence of cytotoxic cytochrome c was measured in preheated U937 and U937/hsp70 cells treated with lethal heat shock. As shown in Fig. 4A, HSP70 had no effect on the release of cytochrome c in lethal heat shocked cells. Because cytochrome c oxidase subunit II, an inner mitochondrial membrane protein (20), was not detected in the cytotoxic extracts, it was determined that there was no contamination of intact mitochondria in the cytotoxic fractions.

To know exactly the extent of the caspase-3- and PARP cleavages in preheated U937 and U937/hsp70 cells detecting the cytochrome c in the cytosol, we observed caspase-3 and PARP cleavages in lethal heat-shocked cells (Fig. 4A). Despite cytochrome c release by lethal heat shock, the cleavages of procaspase-3 and PARP were protected in both preheated U937 and U937/HSP70 cells in contrast to normal U937 and U937/puro cells, respectively. At the same time, active caspase-3 capable of cleaving DEVD-pNA was analyzed in the same experiment. Caspase-3 activities in both preheated U937 and U937/HSP70 cells upon exposure to lethal heat shock were very low (Fig. 4B). To clarify whether HSP70 protected the cells from dying when caspase-3 activity was blocked even after cytochrome c release, cell viability was also observed in the same experiment. For measurement of cell viability, annexin V binding and PI uptake were analyzed by flow cytometry in control cells and in preheated U937 cells that were exposed in lethal heat shock (Fig. 4C). In the case of preheated cells, the percent of early or late apoptotic/necrotic cells (annexin V+/PI− or annexin V+/PI+ cells) was clearly decreased in comparison to the control. The extent of apoptotic cell death in U937/hsp70 cells was also compared with that of U937/puro cells by flow cytometry analysis. As shown in Fig. 4C, U937/hsp70 cells showed substantially less apoptosis than U937/puro cells. All of the parameters in Fig. 4 were measured in a single experiment with preheated U937 or U937/hsp70 cells, respectively. Taken together, regardless of the inability of HSP70 to block cytochrome c release, HSP70 could interfere with caspase-3 activation and finally cell death.

**DISCUSSION**

HSPs are evolutionarily conserved molecular chaperones that are essential for the proper folding and assembly of proteins. The proteins are structurally and functionally conserved from prokaryotes to mammals. HSP70 provides protection from elevated temperature and contributes to thermotolerance. This ability of HSP70 to protect cells has recently been shown to be a consequence of inhibition of apoptosis (Refs. 29–37; reviewed in Ref. 43). It has been suggested that HSP70 prevents apoptosis by inhibiting the SAPK/JNK signaling cascade (35, 44, 45). However, in some cases, HSP70-expressing cells were found to be resistant to apoptosis without any effect on SAPK/JNK signaling (9, 35, 36). Although survival was shown to correlate with impaired caspase-3 activation, Ja¨a¨ttela¨ et al. (36) recently documented an effect of HSP70 downstream of caspase-3. In that study, HSP70 had no effect on SAPK/JNK or caspase-3 activation but did prevent apoptosis induction. It has also been suggested that other heat-inducible factors might reduce SAPK/JNK activation and that HSP70 might act downstream of SAPK4/JNK (9). Because the anti-apoptotic mechanism of HSP70 is still controversial, we sought to determine where in the pathway between cytochrome c release and caspase-3 activation HSP70 acts. We obtained evidence that apoptosis induced by heat stress was mediated by the cyto-
FIG. 4. The effects of HSP70 on changes of apoptotic molecules and cell death induced by lethal heat shock in preheated U937 and U937/hsp70 cells. A, HSP70 effect on heat-induced cytochrome c release and caspase-3 and PARP cleavages. Preheated U937 or U937/hsp70 cells were treated with a lethal heat shock and returned to 37 °C for 12 h. The presence of cytochrome c in the cytosolic fraction was detected by immunoblot assay. Cytochrome c oxidase subunit II (inner mitochondrial membrane marker) were used to show no contamination of mitochondria in the cytosolic fractions. Caspase-3 and PARP cleavages were also detected by immunoblot assay in a same experiment. An actin protein was used as a control for equal loading of protein. Mit. fr., mitochondrial fractions. B, caspase-3 activity in preheated or U937/hsp70 cells. Caspase-3 activity was measured in the same experiment that detected cytochrome c release. The results presented were reproducible in two separate experiments. C, viability of preheated or U937/hsp70 cells upon exposure to the lethal heat shock. The portion of the cells that was treated with lethal heat shock to determine cytochrome c release and caspase-3 activation was used to measure cell viability. To measure the rate of cell viability, the cells were double-labeled with annexin V and PI and analyzed by a flow cytometry.
HSP70. However, HSP70 can suppress activation of apoptosis by stresses. This model does not specify a unique target for HSP70 by restoring proteins that have been damaged or unfolded (48). The CrmA protein is able to bind to and inhibit the mitochondrial pathway of apoptosis (48). These IAP family members regulate apoptosis signaling by blocking cytochrome c release from mitochondria and the ensuing activation of caspase-9, as well as affecting calcium homeostasis (43). No correlation has been reported between cytochrome c release and apoptosis, since cytochrome c per se does not necessarily lead to apoptosis, and released cytochrome c might have to wait until the cofactor is available. Therefore, the possibility that HSP70 might act on a cofactor molecule could not be excluded.

We obtained results showing that the peptide-binding domain and carboxyl-terminal region containing the last 57 amino acids of HSP70 are indispensable for the inhibition of apoptotic events in a cell-free system. HSP70 contains three functional regions, the ATP-binding domain, the peptide-binding domain, and the EEVD motif, that are conserved in all of the eukaryotic HSP70 at the carboxyl-terminal end of the protein (53). Whereas HSP70 lacking the ATP-binding domain has been shown to provide thermotolerance, the loss of the peptide-binding domain disrupts the protective effect of HSP70 (54). Because it has been reported that the deletion or mutation of the EEVD motif results in a loss of substrate binding ability, the EEVD motif might be important for thermotolerance (50). Our result is consistent with a recent report showing that the HSP70 mutant lacking the ATP-binding domain was still capable of SAPK/JNK suppression (52). These results strongly suggest that the protein refolding activity of HSP70 is unnecessary for its anti-apoptotic function. From these results, we surmised that HSP70 could directly bind to and inhibit the activity of key molecules acting at the execution phase of apoptosis to prevent cell death. It remains to be determined whether HSP70 directly interacts with a component of the pathway leading from cytochrome c release to caspase-3 activation. Currently, we are investigating this possibility.

It has recently been suggested that other heat shock proteins, HSP27, HSP60, HSP10, and HSP90, are also important regulators of apoptosis (55–58). In addition to their protective effects, in some situations various HSPs have been found to accelerate apoptotic processes (56–58). As inappropriate apoptosis is implicated in a number of human diseases, understanding the molecular mechanisms of action of these proteins could offer novel ways of treating apoptosis-related diseases. Several HSPs, especially HSP70, might be useful for the therapeutic treatment of cancer, autoimmune and immunodeficiency diseases, injury after ischemia, and neurodegenerative disorders.

Acknowledgments—We thank Dr. Dick D. Mosser for critical reading of this manuscript.

REFERENCES

1. Steller, H. (1995) Science 267, 1445–1449
2. White, E. (1996) Genes Dev. 10, 1–15
3. Kerr, J. F. R. (1971) J. Pathol. 105, 1–20
4. Wyllie, A. H., Kerr, J. F. R., and Currie, A. R. (1980) Int. Rev. Cytol. 65, 251–305
5. Jacobson, M. D., Weil, M., and Raff, M. C. (1997) Cell 87, 347–354
6. Thompson, C. B. (1995) Science 267, 1462–1465
7. Price, D. L., Sioudia, S. S., and Borchelt, D. R. (1998) Science 282, 1079–1083
8. Martin, S. J., Newmeyer, D. D., Mathias, S., Farschon, D. M., Wang, H. G., Reed, J. C., Kolesnick, R. N., and Green, D. R. (1995) EMBO J. 14, 5191–5200
9. Buzzaard, K A, Giaccia, A. J., Killender, M., and Anderson, R. L. (1998) J. Biol. Chem. 273, 17147–17153
10. Wei, Y. Q., Zhao, X., Kariya, Y., Fukata, H., Teshigawara, K., and Uchida, A. (1994) Cancer Res. 54, 4952–4957
11. Rosette, C., and Karin, M. (1996) Science 274, 1194–1197
12. Imai, Y., Kimura, T., Murakami, A., Yajima, N., Sakamaki, K., and Yonehara, S. (1999) Nature 398, 777–785
13. Salvesen, G. S., and Dixit, V. M. (1997) Cell 91, 443–446
14. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. D. (1998) Cell 94, 491–499
15. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, M. S., Ahmad, M., Alnemri, E. S., and Wang, X. D. (1997) Cell 91, 479–489
16. Lazzalini Y. A., Cole, S., Cooke, C. A., Nelson, W. G., and Earnshaw, W. C. (1993) J. Cell Biol. 123, 7–22
