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A Role for Heat Shock Protein 27 in CTL-Mediated Cell Death

Paul J. Beresford, Madhuri Jaju, Rachel S. Friedman, Margaret J. Yoon, and Judy Lieberman

CTL exocytosis of granules containing perforin and granzyme proteases induces apoptotic cell death. Either granzyme A or B can act with perforin to trigger apoptosis. Granzyme B activates a ubiquitous apoptotic cascade induced by caspase cleavage, but the granzyme A pathway is largely unknown. Using affinity chromatography with recombinant mutant inactive granzyme A, we previously isolated two granzyme A-binding proteins, PHAP (putative HLA-associated protein) I and II. PHAP II, a substrate of granzyme A, is degraded within minutes of CTL attack. Two additional cytoplasmic proteins of 27 and 53 kDa bind strongly to the mutant granzyme A column, requiring 6 M urea to elute. Sequencing identified these as the monomer and dimer of hsp27, a small heat shock protein up-regulated by stress and cellular activation. Hsp27 coprecipitates with granzyme A from cytoplasmic lysates and is not a substrate of the enzyme. Hsp27 translocates to the detergent-insoluble fraction of target cells and relocates from diffuse cytoplasmic staining to long filamentous fibers, especially concentrated in a perinuclear region, within minutes of CTL attack. Hsp27 may participate in morphologic changes during granule-mediated lysis. Low or absent levels of hsp27 expression in T lymphocytes, even after heat shock, may play a role in CTL resistance to granule-mediated lysis.


Cytotoxic T lymphocytes induce programmed cell death or apoptosis, a stereotypic sequence of events, characterized by cellular membrane blebbing, nuclear condensation, and DNA degradation (1). CTL can use at least two distinct pathways to lyse target cells. The \( \text{Ca}^{2+} \)-dependent granule exocytosis pathway initiated by specific Ag recognition is thought to be the dominant mechanism for CTL lysis of virally infected cells (2–5). After TCR engagement, cytolytic granules migrate to the region of target cell apposition, fuse to the CTL plasma membrane, and release their contents into the intercellular space between the CTL and its target (6–8). The CTL granules contain a pore-forming protein perforin and a group of serine proteases termed granzymes contained in a proteoglycan matrix (9–14). Granzyme A and B and perforin are coexpressed only in cells with cytolytic activity, predominantly NK and CD8\(^+\) T cells, but also in cytolytic CD4 and \( \gamma\delta \) T cells. Recently, the molecular pathways involved in the induction of cell death by granzyme B, a protease with Aspase activity, have begun to be identified. Granzyme B cleaves members of the caspase family of cysteine proteases, thereby activating a ubiquitous apoptotic cascade (15).

Evidence from transfection experiments and knockout mice rendered deficient in perforin, granzyme A, or B suggests that granzyme A or B can independently with perforin induce target cell apoptosis (2, 16–21). Granzyme A-deficient mice are defective in their ability to protect against hepatic failure and death from ecromelia, a cytopathic mousepox virus. The functional importance of granzyme A was also demonstrated in a murine CTL line, stably transfected with granzyme A antisense cDNA, which had 3- to 10-fold reduced granzyme A activity and induced 50 to 70% less cytolysis and DNA fragmentation in target cells (22).

Granzyme A, the most abundant of the proteases in human CTL granules, is a trypic protease that cleaves synthetic substrates with Lys or Arg at the P1 position (11, 23). Granzyme A is unique among the granzymes in forming a disulfide-linked homodimer of approximately 50 kDa (24). Like granzyme B, it is produced in CTL as a proenzyme, which can be activated by dipeptidyl peptidase I cleavage of an activation dipeptide (25). It is stored in its cleaved active form in the acidic granules of cytotoxic cells, at a pH at which it is inactive.

We have produced in Escherichia coli a soluble and active form of granzyme A (rGranA) that recognizes and cleaves known granzyme A synthetic substrates, is inhibited by known inhibitors of native granzyme A, and is recognized by a conformational Ab raised to native granzyme A (26). Using affinity chromatography with a Ser→Ala mutant of granzyme A (S→ArGranA), we previously identified two ubiquitous proteins, PHAP\(^\text{I,PP2A}\) or mapmodulin and PHAP II (set, TAF-1, or L\(_{\text{PP2A}}\)), which bind to granzyme A (27–32). PHAP II is a substrate for granzyme A and is degraded within minutes of CTL attack (26). In this study, we describe the interaction of granzyme A with the monomer and dimer of the small heat shock protein hsp27.

Hsp27, a widely expressed phosphoprotein induced by heat shock and other stressors, has been implicated as a molecular chaperone and as a regulator of the cytoskeleton by binding to the actin cap (33–35). It is rapidly phosphorylated at Ser\(^{78}\) and Ser\(^{62}\) in response to serum, phorbol ester, calcium ionophore, bombesin, thrombin, bradykinin, fibroblast growth factor, leukemia inhibitory

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2 Address correspondence and reprint requests to Dr. Judy Lieberman, The Center for Blood Research, 800 Huntington Avenue, Boston, MA 02115. E-mail address: lieberman@cb.med.harvard.edu

3 Abbreviations used in this paper: PHAP, putative human histocompatibility leukocyte antigen-associated protein; BLCL, B lymphoblastoid cell line; hsp, heat shock protein; PHA-P, phytohemagglutinin P LAK, lymphokine-activated killer cell; MFI, mean fluorescence intensity; pro-rGranA, recombinant human granzyme A before cleavage of engineered propeptide; rGranA, recombinant human granzyme A; S→ArGranA, mutant recombinant granzyme A with active site serine replaced by alanine.
factor, high density lipoprotein, TNF, and IL-1 (35–37). In one report, the overexpression of hsp27 in a breast cancer cell line increased its susceptibility to cytolyis by γδ T cells (38).

Materials and Methods

**Production of active rGranA and inactive S→ArGranA**

Human pro-rGranA was expressed in *E. coli* from a pet26b plasmid (Novagen, Madison, WI) with an enterokinase site 5′ of the predicted first amino acid of the active enzyme (rGranA) for in vitro activation and a C-terminal His tag, as described (26). S→ArGranA was constructed similarly, but without the enterokinase site and with the active site Ser replaced by Ala. Recombinant proteins were purified from bacterial pellets by Ni²⁺ chelation, as described (26).

**Affinity chromatography with S→ArGranA**

Purified S→ArGranA (4 mg) was coupled to 1 ml of Affi-Gel 10 (Bio-Rad, Richmond, CA) to produce an affinity matrix. Cytoplasmic extracts prepared from 1 × 10⁶ K562 cells treated with Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 25 mM KCl, 5 mM MgCl₂, 1 mM PMSF, and 10 mM Tris-HCl, pH 7.6) were applied to the column, washed with 1 M NaCl in lysis buffer, and eluted with 6 M urea. Protein-containing fractions were analyzed by SDS-PAGE. Protein bands at 27 and 53 kDa in the 6 M urea eluate were subjected to tryptic digest and peptide sequencing performed by Harvard Microchemistry Facility.

**Cell lines**

K562, HL60, and Jurkat cells were obtained from American Type Culture Collection (Rockville, MD). YT-Indy was a kind gift of Z. Brahim (Indiana University School of Medicine, Indianapolis, IN). B lymphoblastoid cell lines (BLCL) were produced from EBV-infected PBMC by standard methods. T cell lines were generated by stimulating density-separated PBMC at 5 × 10⁹/ml with 2 µg/ml phytohemagglutinin P (Difco, Detroit, MI) in T cell medium (RPMI 1640 supplemented with 2 mM glutamine, 2 mM Hepes, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM β-mercaptoethanol, and 15% FCS) to which was added 300 IU/ml human rIL-2 (Cetus, Emeryville, CA). Lymphokine-activated killer (LAK) cell lines were produced by culturing PBMC in T cell medium with 1000 IU/ml human rIL-2 for 2 to 3 wk. The LAK and T cell lines are predominantly CD³⁺CD⁸⁺ T cells.

**Immunoblot**

Electrophoresed samples were transferred to nitrocellulose membranes, blocked overnight with 5% nonfat milk and 0.05% Tween in TBS, and incubated for 1 h with a 1/2000 dilution of anti-hsp27 polyclonal rabbit serum (the kind gift of M. Mendelsohn, New England Medical Center, Boston, MA), or mouse mAb G3.1 (Stessgen, Victoria, B.C.), or 1/2000 dilution of anti-hsp27 polyclonal rabbit sera (the kind gift of M. Mendelsohn, New England Medical Center, Boston, MA).

**Co precipitation with nickel resin**

K562 cells (1 × 10⁶ cell equivalents) were lysed in 1 ml of 0.1% Nonidet P-40, 1 mM PMSF, and 50 mM Tris-HCl, pH 8, for 30 min at 4°C and microfuged for 10 min at 12,000 rpm. The soluble lysates were incubated with 5 µg of either the proenzyme or the enterokinase-treated active form of rGranA and 20 µl of a 70% slurry of charged nickel resin in nickel resin-binding buffer (5 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9) at 4°C for 2 h. Samples were washed five times with nickel resin-binding buffer, boiled in SDS sample-loading buffer, separated by SDS-PAGE on 15% polyacrylamide gels, and analyzed by immunoblot.

**Heat shock of cell lines and separated PBMC**

Approximately 5 to 10 × 10⁶ cells were incubated for 1 h at 42°C, and then returned to 37°C for the indicated times. Cytoplasmic lysates were prepared as above at 5 × 10⁶ cells/µl lysis buffer. For flow cytometry, cells were fixed and permeabilized using the Caltag Laboratories (Burlingame, CA) Fix and Perm Kit, according to the manufacturer’s protocol. Fixed cells were incubated for 15 min at RT with either 1/200 anti-hsp27 mAb G3.1 or 1/25 mouse IgG1 isotype-matched control Ab (Coulter, Hialeah, FL). After washing with 5 ml HBSS, cells were stained with 1/25 PE-conjugated F(ab’), goat anti-mouse IgG (Dako, Carpenteria, CA). After two further washes, PBMC were resuspended in PBS and labeled with FITC- or Cy5-conjugated mAb to cell surface markers CD8, CD4, CD14, CD16, and CD20 (Immunotech, Westbrook, ME). The washed cell lines and the contained PBMC were resuspended in FACS buffer (2% FCS, 0.2 mg/ml Na₂ EDTA) in PBS with 1% formaldehyde before analysis. Flow-cytometry analysis was performed on tightly gated lymphocyte and monocyte populations using FACS calibur (Becton Dickinson, Mountain View, CA).

**LAK assay**

Effector LAK cell lines (1 × 10⁶ per condition) were incubated in 200 µl RPMI medium supplemented with 10% FCS and 1 mM EGTA in Eppendorf tubes for 30 min at 37°C, then mixed with an equal number of target cells (K562) and pelleted. After gentle addition of 1 µl 1 M CaCl₂ to a final concentration of 5 mM Ca²⁺, cells were harvested at subsequent time points and lysed at 4°C for 30 min in 20 µl Nonidet P-40 lysis buffer, to which was added 0.5 mg/ml EDTA, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 µg/ml aprotinin, 1 mg/ml Pefabloc, and 10 µg/ml EDTA (Boehringer Mannheim, Indianapolis, IN). The insoluble precipitate, including nuclei, was washed twice in Nonidet P-40 lysis buffer plus 1% Triton X-100 and resuspended in 20 µl 0.1% Nonidet P-40 and 10 mM Tris·HCl, pH 8 (containing the same inhibitors as above), before adding 5 µl 5X SDS sample buffer. Samples were boiled for 5 min before electrophoresis and blotted with anti-hsp27 antibody. Control samples, in which CaCl₂ or LAK cells were not added, were analyzed in tandem. Blots were stripped and reprobed with polyclonal rabbit antisera to moesin (a gift of E. Remold-O’Donnell, The Center for Blood Research, Harvard Medical School, Boston, MA).

**Immunofluorescence microscopy**

COS cells, plated at subconfluence and grown for 1 to 2 days on slides (Nunc), were incubated with 5 µg/ml Con A (Sigma, St. Louis, MO) before adding LAK cells at a 1:1 ratio. In some experiments, LAK cells were preincubated for 20 to 30 min in 200 µl complete medium containing 1 mM EGTA. After 20 to 30 min, 1 µl 1 M CaCl₂ was added gently by pipetman so as not to disrupt LAK cell/target cell conjugates to initiate granule-mediated cytolyis. Twenty minutes later, cells were fixed and permeabilized using the Caltag Laboratories Fix and Perm Kit, according to the manufacturer’s protocol. In other experiments, LAK cells were added in complete medium without EGTA, and the slides were centrifuged for 5 min at 866 × g to hasten conjugate formation and incubated for an additional 15 to 20 min at 37°C before fixation. In parallel, adherent COS cells on replicate slides were labeled with ³¹Cr for 1 h and washed before adding LAK cells, as above. Percentage of specific cytotoxicity was assayed from culture supernatants harvested at 15 min and 30 min after centrifugation. To visualize hsp27, cells were stained as above. On some slides, cells were stained with a 1/100 dilution of rhodamine phalloidin (Molecular Probes, Eugene, OR). Stained slides were analyzed using a Zeiss Axioskop fluorescent microscope.

**Results**

**Hsp27 binds to granzyme A**

We previously used a S→ArGranA affinity column to identify two ubiquitously expressed proteins, PHAP I and II, as candidate participants in the granzyme A pathway of cell death. We were able to confirm the PHAP II interaction by coimmunoprecipitating PHAP II with inactive granzyme A from cytoplasmic lysates, and demonstrating that PHAP II is a substrate of active rGranA and that it is degraded within minutes of CTL attack (26). Further study of the potential interaction of granzyme A with PHAP II awaits the development of PHAP I Ab and recombinant protein. The PHAP I and II proteins were eluted from the S→ArGranA affinity column by extraction in the presence of 0.2 to 1 M NaCl. To identify additional proteins that might be involved in the granzyme A pathway, we treated the K562 cytoplasmic lysate-Loaded S→ArGranA column with progressively harsher eluting agents. No additional proteins eluted from the column with glycine·HCl or radioimmunoprecipitation assay buffer (data not shown), but 6 M urea eluted two new proteins of 27 and 53 kDa (Fig. 1A). An additional less prominent band migrating with an apparent m.w. of 70 kDa also eluted under these conditions. The 27- and 53-kDa proteins were...
analyzed by tryptic digestion and N-terminal sequencing. The tryptic digest HPLC chromatograms of p27 and p53 were identical. Three analogous peaks of the two proteins were analyzed by mass absorption laser densitometry and found to have equivalent m.w., suggesting that p53 is a dimer of p27. Three p27 peptides (of 5, 8, and 10 amino acids) were identical to sequences of the heat shock protein hsp27. The identification of p27 and p53 as the monomer and dimer of hsp27 was confirmed by immunoblotting with a polyclonal anti-hsp27 rabbit antiserum (gift of M. Mendelsohn) (Fig. 1B). Because of the identification of p27 and p53 with a heat shock protein, we surmised that p70 might correspond to hsp70. In fact, probing the 6 M urea eluate with antisera to hsp70 identifies the 70-kDa band as hsp70 (Fig. 1C).

Hsp27 coprecipitates from cytoplasmic lysates with active and inactive recombinant granzyme A and is not a substrate for the active enzyme

The harsh elution conditions required to strip hsp27 from the granzyme column suggest a high affinity interaction. To confirm the interaction of hsp27 with granzyme A in cells and to determine whether hsp27 might be a substrate of granzyme A, we added His-tagged active rGranA and inactive pro-rGranA (not treated with enterokinase) to K562 cell extracts and precipitated Histagged granzyme A with nickel resin. When the precipitates were analyzed by SDS-PAGE and immunoblotting with hsp27 antiserum, the monomeric and dimeric hsp27 bands were visualized in samples to which rGranA or pro-rGranA was added, but not in control samples to which no granzyme was added (Fig. 2A). To confirm that hsp27 is not a substrate of granzyme A, K562 lysates were incubated with 0.56 μM rGranA, S-ArgrGranA, or pro-rGranA for 1 h at 37°C and analyzed by immunoblot probed with antisera against hsp27 and PHAP II. PHAP II was cleaved by the active rGranA to produce the 25-kDa N-terminal fragment, as previously reported (26), but hsp27 remained unchanged after granzyme A exposure. These results verify the interaction of granzyme A and hsp27 and demonstrate that hsp27 is not a substrate for cleavage by granzyme A.

Hsp27 is not expressed in T lymphocytes before heat shock and is only weakly induced after heat shock

Hsp27 has been reported to be a ubiquitous protein up-regulated in response to heat shock and other stresses. However, as shown in Figure 2, hsp27 was not detected by immunoblot in lysates from LAK cells. We therefore investigated by flow cytometry and

FIGURE 1. Three proteins of 27, 53, and 70 kDa elute from the S-ArgrGranA column with 6 M urea (A). The 27- and 53-kDa proteins were confirmed to be the hsp27 monomer and dimer by tryptic peptide sequencing and immunoblot with hsp27 antisera (B). C. The 70-kDa band in the eluate stains for hsp70 on immunoblot. Lane 1, 6 M urea eluate; lane 2, 1 μg BSA; and lane 3, cytoplasmic lysate of 10⁵ K562 cells.

FIGURE 2. A. Hsp27 immunoblot of K562 cytoplasmic extracts precipitated with Ni²⁺ resin after incubation with nothing (lane 1), pro-rGranA (lane 2), or rGranA (lane 3). K562 (1 × 10⁶ cell equivalents; lane 4) and human LAK cell lysates (1 × 10⁶ cell equivalents; lane 5) were directly loaded without any prior precipitation. Pro-rGranA and rGranA coprecipitate with hsp27. The active enzyme does not cleave it. LAK lysates do not stain for hsp27. B. To verify that hsp27 is not a substrate of granzyme A, K562 cell lysates (10⁷ cell equivalents per lane) were incubated for 1 h at 37°C with nothing (lanes 1 and 6) or 560 nM rGranA (lanes 2 and 7), S-ArgrGranA (lanes 3 and 8), or pro-rGranA (lanes 4 and 9). In lanes 5 and 10, LAK cell lysates (10⁶ cell equivalents) were used as negative control for hsp27 blotting. Electrophoresed samples were analyzed for hsp27 (left) and PHAP II (right) by immunoblot. Only PHAP II was cleaved by the active enzyme.
immunoblot the expression of hsp27 in PBMC and cell lines of various hemopoietic lineages. Analysis of freshly isolated permeabilized human PBMC by flow cytometry revealed abundant hsp27 in monocytes, but no detectable hsp27 above background in B or T lymphocytes (Fig. 3A, Table I). Ten hemopoietic cell lines were also analyzed for hsp27 expression. Hsp27 was expressed abundantly in the myeloid cell line HL60 and in the erythroleukemia cell line K562, as well as in two of three EBV-transformed BLCL. However, in two PHA-stimulated T cell lines, a LAK cell line and Jurkat and YT-Indy cell lines, no hsp27 was detected by immunoblot. To confirm these results, the cell lines were permeabilized and stained for hsp27 and analyzed by flow cytometry (Table II). The mean fluorescence intensity (MFI) corresponded to the immunoblot results. Cells with MFIs less than 46 were negative on immunoblot, whereas cells with MFIs above 97 were positive. The T and NK cell lines had MFIs between 7 and 15, not much above background control Ig levels of 3 to 5. The positive B and myeloid cell lines stained considerably brighter with MFIs of 94 to 390. Therefore, T cells may differ from other hemopoietic cells in expressing barely any hsp27.

Since hsp27 is a heat shock protein, we also analyzed protein levels after heat shock for 1 h at 42°C. As anticipated, hsp27 protein levels increased over 16 h when K562 cells were heat shocked (Fig. 3B). Although no hsp27 was detectable in freshly isolated PBL 1 h after heat shock (data not shown), low levels of protein were detected in both T and B cell populations 14 h after heat

Table I. Hsp27 expression of PBMC before and after heat shock

<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>Mean Fluorescence Intensitya Before heat shock</th>
<th>Mean Fluorescence Intensitya After heat shock</th>
</tr>
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<tbody>
<tr>
<td>CD14+ monocytes</td>
<td>25</td>
<td>117</td>
</tr>
<tr>
<td>CD20+ B cells</td>
<td>4</td>
<td>140</td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>4</td>
<td>83</td>
</tr>
<tr>
<td>CD8+ T and NK cells</td>
<td>4</td>
<td>58</td>
</tr>
<tr>
<td>CD16+ T and NK cells</td>
<td>3</td>
<td>53</td>
</tr>
</tbody>
</table>

a Protein expression was analyzed by flow cytometry of stained permeabilized cells before or 14 h after heat shock for 1 h at 42°C. Background staining with an IgG control Ab gave MFI values in the range of 2 to 4 for gated monocytes and lymphocytes before or after heat shock.

Table II. Hsp27 expression of cell lines before and after heat shock

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Mean Fluorescence Intensitya Before heat shock</th>
<th>Mean Fluorescence Intensitya After heat shock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonlymphoid cell lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K562</td>
<td>390</td>
<td>585</td>
</tr>
<tr>
<td>HL60</td>
<td>94</td>
<td>137</td>
</tr>
<tr>
<td>B lymphoblastoid cell lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PJB-BLCL</td>
<td>122</td>
<td>329</td>
</tr>
<tr>
<td>DNF-BLCL</td>
<td>118</td>
<td>262</td>
</tr>
<tr>
<td>234-BLCL</td>
<td>16</td>
<td>198</td>
</tr>
<tr>
<td>T and NK cell lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAT-T</td>
<td>7</td>
<td>46</td>
</tr>
<tr>
<td>603-T</td>
<td>8</td>
<td>44</td>
</tr>
<tr>
<td>PJB-LAK</td>
<td>10</td>
<td>97</td>
</tr>
<tr>
<td>Jurkat</td>
<td>15</td>
<td>38</td>
</tr>
<tr>
<td>YT-Indy</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

a Protein expression was analyzed by staining permeabilized cells before or 14 h after heat shock for 1 h at 42°C. Background staining with an IgG control Ab gave MFI values in the range of 3 to 5 for each of the cell lines whether before or after heat shock.
shock. However, expression in cytolytic cell populations expressing CD8 or CD16 was lower than that of B cells or CD4 T cells (Table I). Fourteen hours after heat shock, hsp27 became detectable by immunoblot in the previously negative BLCL and in one of the T cell lines (Fig. 3C). By flow cytometry, hsp27 increased somewhat in all of the T cell lines, but not in the NK cell line YT-Indy (Table II). The T cell lines, however, consistently expressed 2- to 12-fold less hsp27 after heat shock than the other cell lines studied.

Hsp27 coalesces into long filamentous strands within minutes of CTL attack

We have found that another granzyme A-binding protein, PHAP II, translocates to the nucleus and is degraded within minutes of CTL attack (26) (Beresford et al., manuscript in preparation). To determine changes in cellular distribution of hsp27 during CTL attack, we added Ca\(^{2+}\) to K562 targets mixed with human EGTA-preincubated LAK cells. Anti-hsp27 immunoblots of nuclear and cytoplasmic lysates were obtained before and at serial times after Ca\(^{2+}\) was added (Fig. 4A). Hsp27 is present only in the cytoplasmic lysate of K562 cells and is undetectable in the LAK cells. Within 5 minutes of adding Ca\(^{2+}\), hsp27 became detectable in the insoluble pellet and was virtually completely gone from the soluble cytoplasmic lysate within 10 min. However, the control cytoskeletal protein moesin remained in the cytoplasmic fraction during the LAK attack. The addition of Ca\(^{2+}\) without LAK cells to EGTA-treated K562 cells also did not change the localization of hsp27 (Fig. 4B).

From these experiments, we could not distinguish whether hsp27 had migrated into the nucleus or was associated with the cytosolic side of the nuclear membrane or with insoluble cytoskeletal components. In immunofluorescence microscopy experiments with permeabilized cells, we used anti-hsp27 mAb G3.1 staining to examine changes in hsp27 localization during cell-mediated lysis (Figs. 5 and 6). Because immunofluorescence staining is better visualized on adherent cells, we took advantage of the fact that LAK cells lyse Con A-treated targets, such as COS cells, which are not normally LAK targets. Lectin-mediated CTL attack was initiated by adding Ca\(^{2+}\) to EGTA-preincubated LAK cells mixed with adherent Con A-treated COS target cells. Within 20 min of initiating granule exocytosis, the hsp27 coalesced from diffuse cytoplasmic staining to staining in long filamentous strands that concentrate around the nuclear membrane, similar to what occurs in heat shock (39, 40). The addition of Ca\(^{2+}\) without effector T cells did not induce a change in hsp27 localization (data not shown). When Ca\(^{2+}\)-treated COS and LAK cell mixtures were costained with phalloidin, there was a collapse of the actin skeleton into filaments that coincide with those staining for hsp27.

Because changes in intracellular free Ca\(^{2+}\) could cause cytoskeletal rearrangements, we repeated these experiments without manipulating extracellular Ca\(^{2+}\) concentration by spinning the...
In this pathway is intriguing. A proposed role for PP2A in a third molecule implicated PHAP I and II, which we have identified as participants in the specific PP2A inhibitor, okadaic acid (43). The two other proteins, phosphorylation of hsp27 is increased in cells treated with the ylation state of hsp27 since it dephosphorylates hsp27 in vitro and thought to play a significant role in the regulation of the phosphor-
a nonphosphorylatable mutant form of hsp27 (35, 42). PP2A is of filamentous actin stress fibers is inhibited by overexpression of ing G-actin to polymerize into filamentous F-actin. The formation 
Hsp27 is phosphorylated after cellular activation or stress, allow-
function is thought to be phosphorylation independent (40, 44, 45). A possible role for hsp27 in endonuclease activation needs to be explored. G-actin binding of DNase I, believed to be an important activator of DNA degradation during apoptosis, is known to inhibit its endonuclease activity (46–48). One possible hypothesis is that hsp27, phosphorylated during CTL attack, induces F-actin poly-
merization that liberates DNase I from G-actin inhibition.
Hsp27 might also be involved in the transport of granzyme A or granzyme A pathway proteins such as the PHAPs to the nucleus during CTL attack. We have not yet determined whether there is a direct interaction of hsp27 with either of the PHAP proteins. Interestingly, PHAP I may play a role in other intracellular trafficking changes that occur during apoptosis. PHAP I has been identified recently as a modulator of movement of cytolo-
kinase component and RNA (39, 40). Im-
munelectron microscopy localization of hsp27 after CTL attack or granzyme A loading could determine whether similar structures are observed during CTL attack.
Hsp27 may be involved in the morphologic changes seen with granzyme A loading of target cells. The avian homologue of hsp27 has been shown to regulate the actin cytoskeleton by binding to the cap site of actin (41). The cytoskeletal effect of hsp27 is believed to be regulated by phosphorylation on Ser15, Ser78, and Ser82 in response to a variety of cellular stresses, hsp27 aggregates in so-called stress granules in association with other cytoskeletal components and RNA (39, 40). Im-
munelectron microscopy localization of hsp27 after CTL attack or granzyme A loading could determine whether similar structures are observed during CTL attack.
Hsp27 was identified as a possible granzyme A-interacting protein by elution from a mutant S→ArGranA affinity column. Its binding to the column was so strong that 6 M urea was required for elution. Although we found that hsp27 is not a substrate for granzyme A, we were able to validate a physiologic role of hsp27 in granzyme A-mediated cell death by coprecipitating granzyme A with hsp27 from K562 cell lysates and by showing a change in cellular locali-
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lated form of hsp27 blocks actin polymerization to F-actin. Hsp27 is phosphorylated after cellular activation or stress, allow-
ing G-actin to polymerize into filamentous F-actin. The formation of filamentous actin stress fibers is inhibited by overexpression of a nonphosphorylatable mutant form of hsp27 (35, 42). PP2A is thought to play a significant role in the regulation of the phosphor-
ylation state of hsp27 since it dephosphorylates hsp27 in vitro and phosphorylation of hsp27 is increased in cells treated with the specific PP2A inhibitor, okadaic acid (43). The two other proteins, PHAP I and II, which we have identified as participants in the granzyme A pathway, have been shown to be inhibitors of PP2A (28, 32). A proposed role for PP2A in a third molecule implicated in this pathway is intriguing.
After treatment of cells with some activation or stress stimuli, hsp27 also translocates to or near the nucleus, but the chaperone

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LAK cells onto Con A-treated adherent COS cells to accelerate T cell/target cell binding. Chromium release assay performed with duplicate slides showed 26% specific cytotoxicity 15 min and 66% specific cytotoxicity 30 min after centrifugation. The slides were fixed, permeabilized, and stained for hsp27 before and after 15 min of incubation at 37°C. Identical changes in hsp27 localization were found (Fig. 6).

**Figure 6.** Hsp27 redistributes from diffuse cytoplasmic staining and coalesces into perinuclear aggregates within 15 min of spinning LAK cells onto Con A-treated COS cell targets. Slides were stained either without added LAK cells (A) or after LAK cells are added (B). The LAK cells are not visible since they do not stain for hsp27.

**Discussion**

Hsp27 was identified as a possible granzyme A-interacting protein by elution from a mutant S→ArGranA affinity column. Its binding to the column was so strong that 6 M urea was required for elution. Although we found that hsp27 is not a substrate for granzyme A, we were able to validate a physiologic role of hsp27 in granzyme A-mediated cell death by coprecipitating granzyme A with hsp27 from K562 cell lysates and by showing a change in cellular locali-

**References**


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