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Heat Shock Protein 70 Is Secreted from Tumor Cells by a Nonclassical Pathway Involving Lysosomal Endosomes[1]

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Heat shock protein (HSP)70 can be released from tumor cells and stimulate a potent antitumor immune response. However, HSP70 does not contain a consensus secretory signal and thus cannot traverse the plasma membrane by conventional mechanisms. We have observed HSP70 release from intact human prostate carcinoma cell lines (PC-3 and LNCaP) by a mechanism independent of de novo HSP70 synthesis or cell death. This pathway is similar to one used by the leaderless protein IL-1β. Our studies show that HSP70 release involves transit though an endolysosomal compartment and is inhibited by lysosomotropic compounds. In addition, the rate of HSP70 secretion correlates well with the appearance of the lysosomal marker LAMP1 on the cell surface, further suggesting the role for endolysosomes. The entry of HSP70 into this secretory compartment appears to involve the ABC family transporter proteins and ABC transporter inhibitor glibenclamide antagonizes secretion. Although the cell signals involved in triggering stress induced HSP70 release though this lysosomal pathway are largely unknown, our experiments suggest a regulatory role for extracellular ATP. These mechanisms appear to be shared by IL-1β secretion. Following release, we observed the binding of extracellular HSP70 to the cell surface of the prostate carcinoma cells. These findings suggest that secreted HSP70 can take part in paracrine or autocrine interactions with adjacent cell surfaces. Our experiments therefore suggest a mechanism for HSP70 secretion and binding to the surface of other cells that may be involved in recognition of the tumor cells by the immune system. *The Journal of Immunology,* **2006, 177: 7849–7857.**

Heat shock proteins (HSP)[3] are essential intracellular molecular chaperones (1). However, recent studies show that a fraction of these proteins, normally localized to the cytoplasm or nucleus, can be released from cells and function as intercellular-signaling ligands (2). Indeed, extracellular HSP70 interacts with immune effector cells though high-affinity receptors and can thus orchestrate the immune response (3–5). Such interactions include binding of free extracellular HSP70 to LOX-1 receptors on dendritic cells (DC) or association of cell surface HSP70 with CD94 on NK cells (4, 6). This property of HSP70 is of significance in tumor immunology as HSP70 complexed to tumor Ags is an effective component of antitumor vaccines (7–13). Intriguingly, HSP70 peptide complexes activate both the innate and adaptive immune responses (5, 8, 9). It has been suggested that HSP70 may be sequentially induced in tumor cells in vivo by therapy and then released from cells undergoing subsequent necrosis in association with tumor Ags and thus mediate antitumor immunity (7, 10, 11). We have examined the mechanisms of HSP70 release from tumor cells. The absence of a consensus secretory signal sequence in HSP70 prompted us to examine whether nonclassical secretion mechanisms characterized in other leaderless proteins such as IL-1β and IL-18 were used by HSP70 (12, 13). It has been shown that exposing cells to heat shock causes the release of a number of proteins that lack secretion leaders, including basic fibroblast growth factor 1 (FGF-1) and IL-1α release and this release appears to be unaffected by inhibitors of common secretory pathways such as colchicine and brefeldin A (14–16) (S. K. Calderwood, unpublished data). Although the pathways of secretion of leaderless proteins are not fully defined, a number of contrasting mechanisms have been elucidated. Most notable among these are the pathway of lysosomal exocytosis used by IL-1β release and a plasma membrane translocation mechanism for FGF-1 release after heat shock (12, 15, 17). IL-1β release follows a complex pathway that includes transport of IL-1β to the cell surface in lysosomal endosomes and passage across the plasma membrane by a mechanism involving ABC family transmembrane transporters (12). Secretory lysosomes are acidic organelles sharing similar traits with conventional lysosomes, including a requirement for the maintenance of low pH, that are involved in the secretion of many leaderless proteins (18). ABC family transmembrane-transport proteins are ubiquitous polypeptides found in all cellular organisms, and in unicellular organisms mediate secretion of most leaderless secretory proteins (17, 19). ABC transporter molecules include proteins of fairly large M_r such as toxins secreted by Gram-negative bacteria (20). In mammalian cells, secretion of IL-1β by macrophages also involves an ABC family transporter and is stimulated though the binding of extracellular ATP to cell surface purinergic receptors (21, 22).

Our current experiments show that HSP70 is released from prostate carcinoma cells though an active mechanism independently of either the rate of HSP70 synthesis or cell death. Active HSP70 secretion involves translocation though a lysosomal compartment and can be inhibited by lysomotropic agents. HSP70 release correlates well with the appearance of the lysosomal-associated membrane protein 1 (LAMP1) on the cell surface, suggesting that HSP70-containing lysosomal vesicles may lyse on exit from cells

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[3] Abbreviations used in this paper: HSP, heat shock protein; DC, dendritic cells, FGF-1, fibroblast growth factor 1; LAMP1, lysosomal-associated membrane protein 1; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; HMGB1, high-mobility protein b1.

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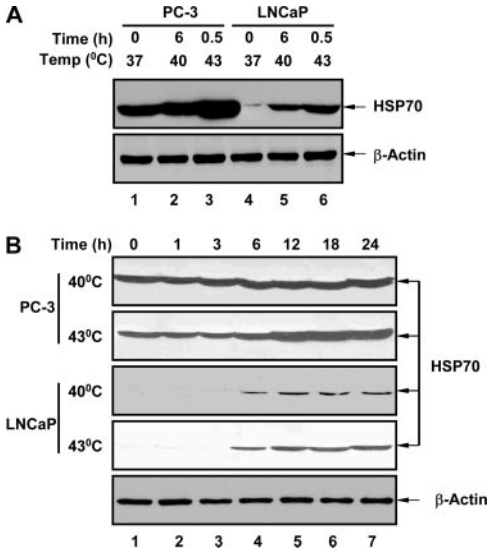

FIGURE 1. *A*, Effects of heat shock on intracellular levels of HSP70 at various temperatures. A total of 1×10^6 cells/ml PC-3 and LNCaP cells were heat treated at 40°C for 6 h (*lanes 2* and *5*) and at 43°C for 30 min (*lanes 3* and *6*) followed by recovery at 37°C overnight. Control samples (*lanes 1* and *4*) were incubated at 37°C overnight. Following cell lysis, proteins were analyzed by 10% SDS-PAGE and probed for HSP70 by Western blot. Experiments were performed reproducibly in duplicate. *B*, Kinetics of intracellular levels of HSP70 following hyperthermia treatment. Cells were incubated at 40°C for 6 h and at 43°C for 30 min in tissue culture dishes containing 1×10^6 cells. At the times indicated, cell lysates were prepared and probed for the presence of HSP70 by Western blot. Each experiment was performed twice with similar results.

and deposit LAMP1 on the cell surface, whereas HSP70 is released into the external milieu. The factors required for HSP70 release further resemble those involved in IL-1β secretion and glibenclamide and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), potent inhibitors of ABC family transporter activity, inhibit HSP70 secretion.

We also examined signals that may be involved in HSP70 release and our experiments suggest that HSP70 release requires extracellular ATP. HSP70 secretion therefore appears to share mechanisms with other leaderless cell proteins and may play a part in cell regulation by interaction with receptors on the cell surface.

Materials and Methods

Cell culture

The prostate carcinoma cell lines PC-3 and LNCaP were cultured respectively in complete Ham's F-12 and RPMI 1640 medium supplemented with 10% FCS (Mediatech), glutamine (Invitrogen Life Technologies), penicillin, and streptomycin (Invitrogen Life Technologies).

Hyperthermia treatments and HSP70 release

Five million cells at a concentration of 1×10^6 cells/ml (PC-3 or LNCaP) were cultured at 37°C overnight and fed with fresh medium before treatment at 37°C and 43°C for 30 min or 37°C and 40°C for 6 h representing the untreated control group and the heat shock group, respectively. Heat shock was conducted in a circulating water bath and temperature monitored to a resolution of 0.1°C using a high-resolution mercury-in-glass thermometer. Aliquots of medium were harvested at the indicated times in experiments and cleared by spinning at $900 \times g$ for 15 min. These aliquots of medium were replaced by replicate volumes of fresh medium to preserve a constant extracellular volume. HSP70 release was corrected for the resultant dilution.

Detection of extracellular HSP70 by ELISA

Sandwich ELISA was used for detection of HSP70 in supernatants described previously (23). Briefly, ultra-high-binding 96-well microtiter ELISA plates (ThermoLabsystems) were coated overnight with HSP70 mAb (StressGen Biotechnologies) in carbonate buffer followed by three washes in PBS plus 0.1% Tween 20 (PBST; Sigma-Aldrich). The plates were blocked with 1% BSA (Sigma-Aldrich) in PBST followed by three washes in PBST. Samples and recombinant human HSP70 protein (StressGen Biotechnologies) were added to the wells and incubated for 1 h followed by three washes in PBST. Rabbit polyclonal anti-HSP70 (StressGen Biotechnologies) was added to each well and incubated for 1 h followed by three washes with PBST. Anti-rabbit IgG alkaline phosphatase-conjugated mAb (σ) was added to the 96-well plates followed by three washes with PBST. Finally, the substrate *p*-nitrophenyl phosphate (Sigma-Aldrich) was added, and

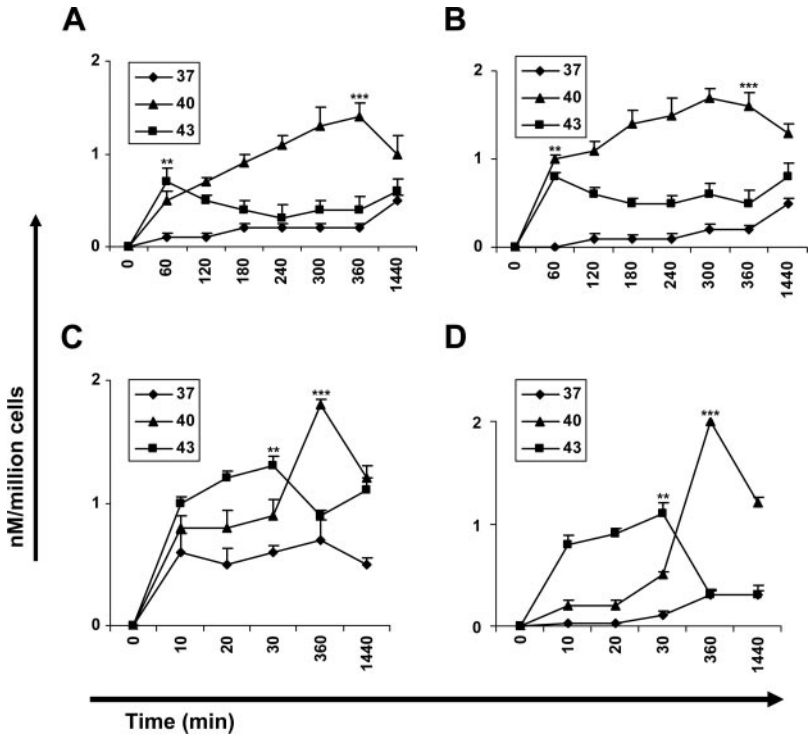

FIGURE 2. Kinetics of HSP70 release following heat shock treatment. A total of 1×10^6 cells/ml PC-3 (*A* and *C*) and LNCaP (*B* and *D*) cells were incubated at 40°C for 6 h (▲), 43°C for 30 min (■), and 37°C control group (♦) or nonheat shocked cells in tissue culture dishes containing 5×10^6 cells. Following heat treatments, the dishes were incubated at 37°C for recovery. Supernatants were taken at 60-min intervals (*A* and *B*) and 10-min intervals (*C* and *D*) and analyzed for the presence of HSP70 by ELISA. Data represent the mean ± SD of three independent experiments. Statistical analyses compared 0 time point and 60 in *A* and *B* and 0 min vs 30 min in *C* and *D*. **, $p < 0.01$; comparing 0 vs 360 min in *A* and *B* and 0 vs 360 in *C* and *D* (***, $p < 0.001$).

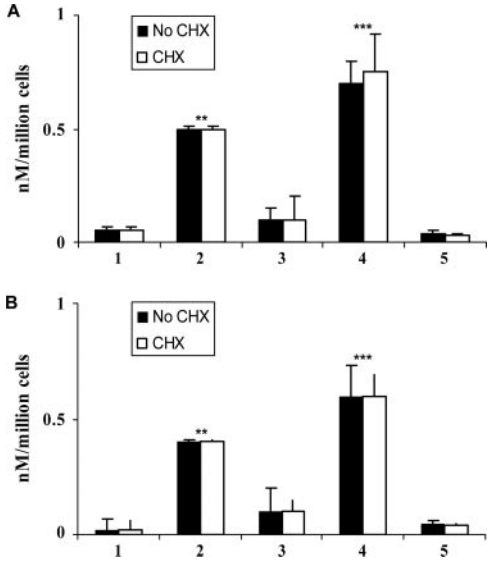

FIGURE 3. HSP70 release does not require mRNA translation. A total of 1×10^6 cells/ml PC-3 (*A*) and LNCaP (*B*) were pretreated for 30 min with 15 μg/ml cyclohexamide (CHX; □) and control group without cyclohexamide treatment (CHX; ■) followed various heat treatments, followed by the heat treatments at 37°C and 43°C for 30 min represented by 1 and 2, 37°C and 40°C for 6 h represented by 3 and 4, respectively, whereas 5 represents 4°C. Supernatants were harvested and tested for the presence of HSP70 by ELISA. Data represent the mean ± SD of three experiments done in triplicate. Statistical analyses compared conditions 2 vs 1 (**, $p < 0.01$) and 4 vs 3 (***, $p < 0.001$).

absorbance was read by spectrophotometer (Bio-Rad) at absorbances of 405 and 650 nm (reference wavelength).

Western analysis of intracellular HSP70 expression

Aliquots of 1×10^6 cells were washed three times in ice-cold PBS, solubilized in reducing sample buffer, and resolved by 10% SDS-PAGE under reducing conditions. Proteins were then electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences) and blocked for 1 h with 10% nonfat dry milk (Bio-Rad) in PBST buffer (Boston BioProducts). Filters were hybridized sequentially with the following: HSP70 mAb (StressGen Biotechnologies), anti-mouse IgG hp-conjugate (Amersham Biosciences) and Ab-Ag complexes developed by ECL-plus luminescence-based detection agent (Amersham Biosciences) according to the manufacturer's instructions.

Western analysis of ABCA-1 expression

A total of 1×10^6 cells was grown until growth was confluent, and cells were harvested and washed three times in PBS. Total cell lysates were prepared by solubilizing cells in reducing sample buffer, and samples were run on 6% SDS-PAGE under reducing conditions. Transfer of proteins was conducted as described above, and detection was confirm by using ABCA-1 Abs (Novus Biologicals). Actin controls were run on each lysate.

Isolation of cell lysosomal fraction

PC3 and LNCaP cells were grown until there was packed cell volume of 3 ml, which was resuspended in an equal volume of packed cells with hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT) and allowed to swell for 15 min on ice. Cells were lysed by rapidly pushing cells through a 1 ml syringe with a 25-gauge needle five times while keeping the cells on ice. Crude nuclear pellet was pelleted by centrifugation for 30 s at $12{,}000 \times g$. Supernatants were stored and lysosomal fraction isolated as described by Andrei et al. (12). Briefly, supernatants were treated with proteinase K for 30 min on ice and inhibited with protease inhibitors. The supernatant was diluted 10-fold in homogenizing buffer and centrifuged at $50{,}000 \times g$ for 20 min. The pellet was washed once in homogenizing buffer and solubilized in reducing sample buffer, and HSP70 was detected by Western blots as described previously. Cathepsin D was detected as the control for the lysosomal compartment by Western blot.

Immunofluorescence microscopy

Cells were plated onto fibronectin-coated 8-well chamber glass slides with cover (Nalge Nunc International) at 10^5 cells per well. Twenty-four hours later, the culture medium was removed and replaced with fresh medium before the cells were hyperthermia treated as described above. The cells were immediately fixed with 3.7% paraformaldehyde. After fixation for 15 min, the cells were washed once with PBS and blocked with blocking buffer (PBS containing 3% BSA) for 30 min. For intracellular staining, the cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 15 min at room temperature after the fixing step. The cells were next washed once with PBS, incubated for 1 h anti-HSP70 (StressGen Biotechnologies) or LAMP1 (CD107a; BD Pharmingen) mAbs followed by five washes in wash buffer (PBS containing 0.05% Tween 20). The cells were incubated with fluorescein-conjugated anti-mouse/rabbit IgG Ab (Alexa 594 and Alexa 488; Invitrogen Life Technologies), respectively. Slides were then washed and mounted in aqueous mounting solution using cover glass (Fisher Scientific) before fluorescent microscopy. Images were acquired using a Nikon Eclipse E600 microscope fitted with a RT SPOT digital camera and processed using SPOT software (Diagnostic Instruments).

Statistical analysis

Data are shown as mean ± SD. Statistical significance of differences between experimental groups was analyzed by using a Student's *t* test.

Table I. *HSP70 release from PC-3 cells (nM/million cells) after indicated treatment*[a]

	30 min		6 h	
PC-3	37°C	43°C	37°C	40°C
Continuous media	1.19 ± 0.18	3.29 ± 0.30	1.5 ± 0.5	2.86 ± 0.28
+ Media[b]	0.83 ± 0.08	3.43 ± 0.15	1.47 ± 0.40	3.57 ± 0.30
Ionomycin	1.41 ± 0.50*	3.56 ± 0.82*	1.73 ± 0.20*	3.21 ± 0.29*
Ca^{2+} 0	Nd	Nd	Nd	Nd
Ca^{2+} 40	Nd	Nd	Nd	Nd
Ca^{2+} 400	Nd	Nd	Nd	Nd
1% FBS	0.21 ± 0.02	0.63 ± 0.03	Nd	Nd
10% FBS	0.20 ± 0.06	0.61 ± 0.06	Nd	0.25 ± 0.05
BAPTA-am	0.43 ± 0.03	3.86 ± 0.86	1.29 ± 0.51	4.72 ± 0.43
EGTA	0.14 ± 0.04	2.86 ± 0.94	1.28 ± 0.47	2.57 ± 0.56

[a] PC-3 cells were pretreated with 1 μM ionomycin for 30 min, serum starved in PBS without calcium magnesium, and incubated with 0, 40, 400 μM calcium and 1% and 10% FBS in the presence of 0.2 mM glucose, in complete media containing 25 μM BAPTA and 250 mM EGTA, for 2 h before heat treatment as described previously. Supernatants were harvested and analyzed for the presence of HSP70 by ELISA.

[b] Cells were serum starved in PBS without calcium magnesium in the presence of 0.2 mM glucose and incubated for 2 h and 10 min before the heat treatment complete medium was added to the cell cultures. Data represent the mean ± SD of three experiments done in triplicate. Statistical analyses compared ionomycin vs continuous medium control at each condition (*, $p < 0.05$). Nd, Not Detected.

Table II. *HSP70 release from LNCaP cells (nM/million cells) after indicated treatment*[a]

LNCaP	30 min		6 h	
	37°C	43°C	37°C	40°C
Continuous media	0.41 ± 010	1.84 ± 0.28	0.62 ± 0.10	3.91 ± 0.32
+ Media[b]	0.21 ± 0.11	1.93 ± 0.27	0.57 ± 0.12	3.32 ± 0.35
Ionomycin	0.8 ± 0.23*	2.43 ± 0.71*	1.23 ± 0.46*	4.30 ± 0.94*
Ca^{2+} 0	Nd	Nd	Nd	0.14 ± 0.04
Ca^{2+} 40	Nd	Nd	Nd	0.11 ± 0.02
Ca^{2+} 400	0.04 ± 0.01	0.04 ± 0.01	0.09 ± 0.03	0.17 ± 0.05
1% FBS	Nd	Nd	Nd	0.17 ± 0.03
10% FBS	Nd	0.25 ± 0.01	0.19 ± 0.02	0.27 ± 0.06
BAPTA-am	0.46	3.0 ± 0.57	0.49 ± 0.03	4.02 ± 0.56
EGTA	0.35	2.86 ± 0.10	0.54 ± 0.06	3.28 ± 0.21

[a] LNCaP cells were pretreated with 1 μM ionomycin for 30 min, serum starved in PBS without calcium magnesium, and incubated with 0, 40, 400 μM calcium and 1% and 10% FBS in the presence of 0.2 mM glucose, in complete medium containing 25 μM BAPTA and 250 mM EGTA, for 2 h before heat treatment as described previously. Supernatants were harvested and analyzed for the presence of HSP70 by ELISA.

[b] Cells were serum starved in PBS without calcium magnesium in the presence of 0.2 mM glucose and incubated for 2 h and 10 min before the heat treatment complete medium was added to the cell cultures. Data represent the mean ± SD of three experiments done in triplicate. Statistical analyses compared ionomycin vs continuous medium control at each condition (*, $p < 0.05$). Nd, Not detected.

Statistical significance was defined as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Results

Rate of HSP70 release from prostate carcinoma cells after heat shock

We first investigated whether the rate of HSP70 release is related to the overall intracellular levels of the protein using PC-3 and LNCaP cells. These cell lines were chosen for their contrasting levels of intracellular HSP70. We have shown previously that although LNCaP express minimal levels of intracellular HSP70, PC-3 express HSP70 abundantly even at nonstress temperatures (24). As a stimulus for HSP70 release, we used heat shock which has previously been shown to cause release of a number of leaderless proteins including FGF-1 and IL-1α (25). We used two heat shock conditions for the experiments, including 40°C, which is in the fever range and minimally toxic in tissue culture and stimulates tumor immunity in mice (26, 27) and 43°C, which strongly induces transcriptional activation of *hsp70* genes (28). As mentioned, PC-3 cells have high endogenous HSP70 levels which increase significantly after heat shock at 43°C although with comparatively minor increases at 40°C. Similar trends were observed in LNCaP cells that have almost undetectable levels of HSP70 in unstressed cells but undergo significant HSP70 induction at 40°C and 43°C (Fig. 1, *A* and *B*). These heat shock treatments resulted in minimal (<1%) levels of necrosis and apoptosis at the 40°C or 43°C condition in both cell lines at the period investigated (data not shown). This is in line with many previous studies of the kinetics of heat-induced cell death that show minimal effects on cell survival of 40°C heating or heating at 43°C up to 30 min reviewed by Hahn and Li (28). Low, but significant levels of necrosis and apoptosis (5–10%) did develop in the prostate cancer cells at later times (24–48 h) (29, 30). Our recent studies indicate that increasing temperatures above 43°C when necrosis increases actually decreases HSP70 secretion, presumably by denaturing components in the secretion pathway (30). HSP70 release at the time period examined in this study is apparently due to active secretion (30).

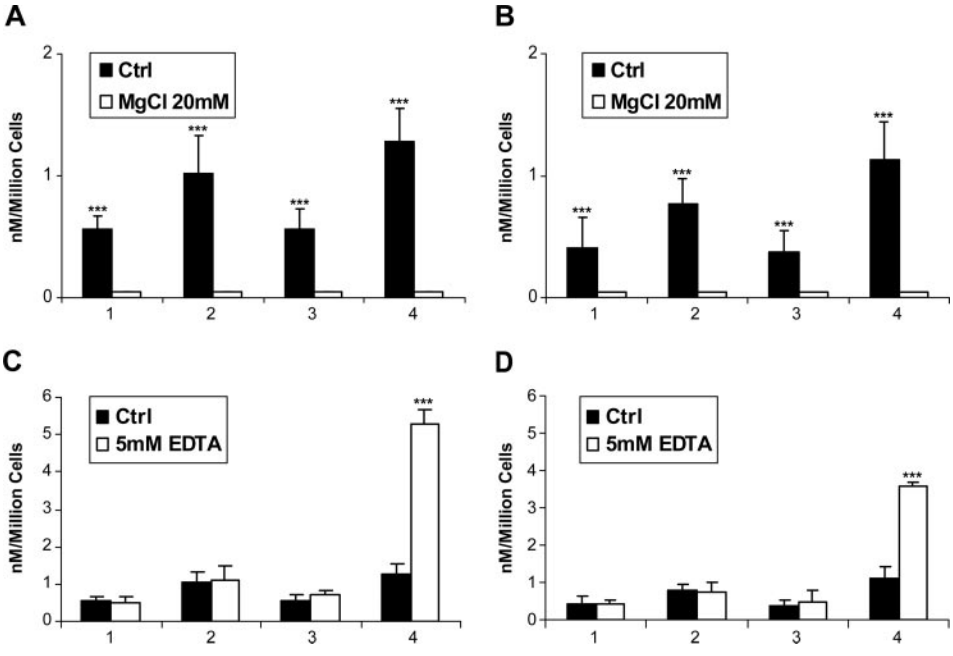

FIGURE 4. Magnesium, an ATP chelator, inhibits HSP70 release, whereas EDTA enhances HSP70 release. PC-3 (*A*) and LNCaP (*B*) cells were pretreated for 2 h with 20 mM magnesium chloride ($MgCl_2$; □) compared with control group of just medium (■) PC-3 (*C*) and LNCaP (*D*) and 5 mM EDTA (□) compared with control untreated cells (■) before hyperthermia treatments previously described. Supernatants were harvested and tested for the presence of HSP70 using ELISA methods described above. The 37°C, 43°C for 30-min data are represented by 1 and 2, whereas 37°C and 40°C for 6 h is represented by 3 and 4, respectively. Data represent the mean ± SD of three experiments done in triplicate. Statistical analyses compared $MgCl_2$ vs control at each condition and EDTA vs control at condition 4 (***, $p < 0.001$).

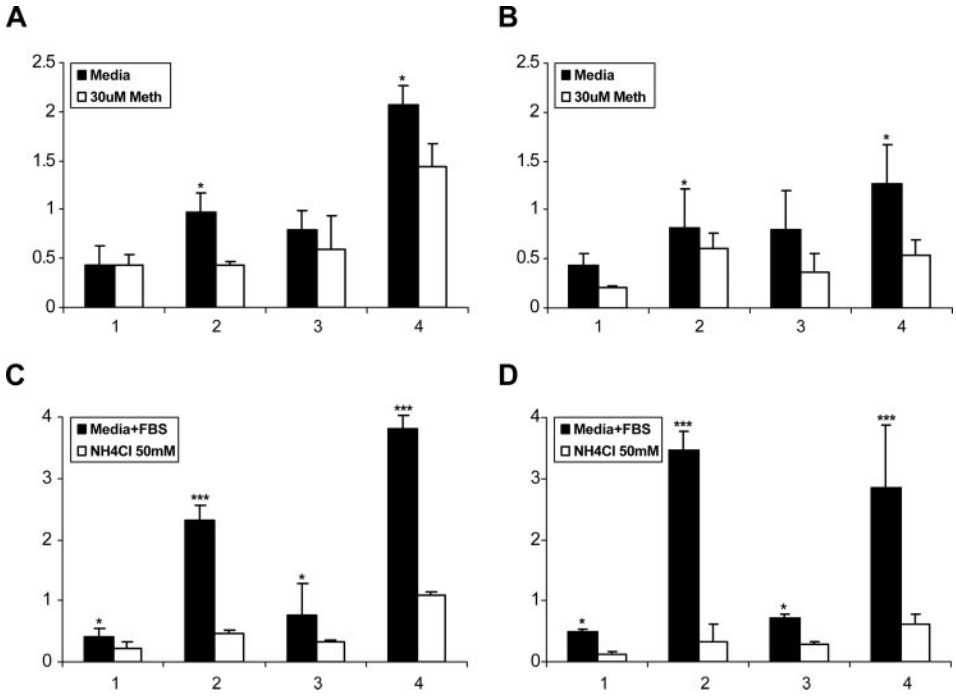

FIGURE 5. The effects of lysomotrophic agents that lead to increased endoluminal pH inhibits vesicular accumulation and secretion of HSP70. A total of 1×10^6 cells/ml PC-3 (*A*) and LNCaP (*B*) were pretreated for 2 h with 30 μM methylamine (Meth; □) and 50 mM ammonium chloride (NH_4Cl; □) compared with control (■) untreated cells PC-3 (*C*) and LNCaP (*D*) followed by the heat treatments at 37°C and 43°C for 30 min represented by 1 and 2 and 37°C and 40°C for 6 h represented by 3 and 4, respectively. Supernatants were harvested and tested for the presence of HSP70 by ELISA. Data represent the mean ± SD of three experiments done in triplicate. Statistical analyses compared Meth vs control conditions 2 and 4 (*, $p < 0.05$) and NH_4Cl vs control at conditions 2 and 4 (***, $p < 0.001$).

We next examined the rate of extracellular HSP70 release in both cell lines after heat shock at 40°C and 43°C (Fig. 2). HSP70 release was assayed in samples of medium by ELISA. Because some reports in the literature indicate that HSP70 family members may be released from cells in membrane-bounded vesicles, we investigated whether treating samples with detergents such as Nonidet P-40 or Lubrol affects recovery of extracellular HSP70 by causing release of HSP70 from vesicles. However, we did not observe significant changes when medium from PC-3 or LNCaP cells was treated with detergents, suggesting that most HSP70 is released from these cells is in free solution (S. S. Mambula and S. K. Calderwood, unpublished data). The rate of HSP70 release was therefore assayed as free extracellular HSP70 released into cell medium. The rate of accumulation of extracellular HSP70 in PC-3 and LNCaP cells measured at hourly intervals is shown in Fig. 2, *A* and *B*. Fig. 2, *C* and *D*, show more detailed kinetic analyses of the initial heat shock period with extracellular HSP70 measured at 10-min intervals. In each cell line, we observed basal HSP70 release that was significantly increased after heat shock (Fig. 2). The rate of HSP70 accumulation was at least as great at 40°C as at the higher temperature (43°C). In addition, HSP70 release occurred during exposure to heat shock and was not markedly increased in the recovery period at 37°C (Fig. 2, *A–D*). A role for de novo HSP70 synthesis in heat-induced release seems unlikely because the bulk of the HSP70 release occurs before we observe increased intracellular levels of the protein (compare Fig. 1*B* with 2, *C* and *D*); HSP70 expression was not increased by 30 min at 43°C or by 6 h at 40°C in the PC-3 cell line, whereas LNCaP only then shows HSP70 expression (Fig. 1*B*) when HSP70 release is maximal at either temperature (Fig. 2, *A–C*). The effects of heat shock thus appear to be largely due to a direct stimulation of secretion of endogenous HSP70, and increased HSP70 expression due to heat shock appears to be an independent aspect of the heat shock response.

Heat shock-induced HSP70 release does not require HSP70 mRNA translation

The experiments in Fig. 2 suggested that HSP70 release is independent of de novo synthesis. We have directly examined the requirement for translation using protein synthesis-inhibitor cycloheximide (Fig. 3, *A* and *B*). Cycloheximide did not inhibit the rate of HSP70 release in either cell line further indicating that HSP70 translation is not required for release (Fig. 3, *A* and *B*). In addition, we found that the rate of HSP70 release was temperature dependent and minimal release was seen at 4°C, suggesting a requirement for metabolic activity in HSP70 secretion (Fig. 3, *A* and *B*). There were significant differences observed in HSP70 release at both 43°C and 40°C compared with 37°C controls. These experiments thus suggest an active process of HSP70 release that can be stimulated by heat shock. Interestingly, we observed that HSP70 release was stimulated only in full medium and was not observed in cells incubated in balanced salt solutions (Tables I and II). This effect did not appear to involve energy depletion or the absence of the stimulatory effects of serum, as HSP70 release was not observed in PBS supplemented with either 5 mM glucose or 10% FCS (Tables I and II). As the active secretion of some intracellular proteins may require Ca^{2+}, we therefore tested the potential role of extracellular Ca^{2+} in HSP70 release (Tables I and II). In cells incubated at various concentrations of extracellular Ca^{2+} (40–400 mM) in PBS (Tables I and II), we did not see restoration of stress-induced HSP70 release. In addition, we did not observe differences in HSP70 release when cells in full medium were pretreated with the intracellular Ca^{2+} chelator BAPTA-AM or the extracellular Ca^{2+} chelator EGTA before stress (Table I and II). We did, however, observe a significantly higher rate of HSP70 release in cells treated with the calcium ionophore ionomycin alone compared with nontreated cells, whereas there was a significant additive increase when the cells were treated with ionomycin and heat compared with heat treatment alone (Table I and II).

We next examined the effects of modulating extracellular Mg^{2+} on HSP70 secretion because the concentration of extracellular Mg^{2+} can mediate the release of other leaderless proteins though its effects on the state of extracellular ATP (12). Modulation of extracellular Mg^{2+} has a profound effect on the release of IL-1β; addition of Mg^{2+} decreases secretion due ATP sequestration, whereas addition of Mg^{2+} chelator EDTA can increase secretion though its chelation of Mg^{2+} and release of ATP in unbound form (12). We incubated the prostate carcinoma cells in growth medium

supplemented with 20 mM $MgCl_2$ before heat shock. Indeed, the addition of $MgCl_2$ had a profound significant effect on the rate of HSP70 release and almost completely inhibited both the basal release and heat-induced HSP70 release in both cell lines (Fig. 4, *A* and *B*). This suggests that extracellular ATP is present in a free form and that addition of $MgCl_2$ sequesters free ATP and blocks HSP70 release. EDTA significantly enhanced HSP70 release but only under the 6 h at 40°C condition. The rationale behind these findings is not clear but may be related to the longer incubation time at 40°C (Fig. 4, *C* and *D*) (21). However, addition of ATP alone does not cause release of HSP70 in the absence of heat shock, suggesting that it is necessary but not sufficient for HSP70 release (data not shown). Similarly, findings are observed with IL-1β in which extracellular ATP is required for secretion, but not sufficient in itself (21).

Potential role of endosomal lysosomes in stress-induced secretion of HSP70

The ability of HSP70 to exit cells under nontoxic conditions suggests a pathway for traversing cell membranes and active secretion. Therefore, we next examined the pathway of HSP70 release, exploring the hypothesis that HSP70 release may follow mechanisms comparable to IL-1β secretion. We first explored a role for secretory lysosomes in HSP70 release and investigated whether increasing pH in these compartments using lysosomotropic agents inhibits HSP70 secretion. Both methylamine and ammonium chloride, which increase intralysosomal pH, decreased the rate of HSP70 release in PC-3 and LNCaP cells (Fig. 5). Incubation with methylamine significantly caused a >50% reduction in HSP70 release in both cell lines exposed to either heat shock condition (Fig. 5, *A* and *B*). The effects of NH_4Cl were significantly more marked, with abolition of both basal and heat shock-induced HSP70 release (Fig. 5, *C* and *D*). Thus, lysomotropic drugs inhibit HSP70 secretion, and these experiments indicate a key role for the endolysosme pathway in HSP70 release.

We next examined whether the heat shock conditions that cause HSP70 release lead to the transport of secretory lysosomes to the cell surface using the intralysosmal protein LAMP1 as a marker (Fig. 6). LAMP1 was detected by indirect immunofluorescence in small patches on the cell surface of both cell types at 37°C. However, conditions that lead to HSP70 secretion including 30 min at 43°C and 6 h at 40°C lead to a major increase in cell surface LAMP1, which becomes concentrated in large caps on the surface of PC-3 and LNCaP cells (Fig. 6, *A* and *B*, respectively). These data are consistent with a hypothesis involving stress-induced transport of secretory lysosomes, containing HSP70 to the cell surface, fusion with the plasma membrane, and display of LAMP1 on the extracellular face of the plasma membrane accompanied by HSP70 secretion. We further confirmed this hypothesis by isolating the lysosomal fraction from LNCaP and PC3 cells and detecting the presence of HSP70 in this compartment as shown in Fig. 6*C*. HSP70 levels and cathepsin D controls were minimal in the lysosomal fraction before heating but increased after both the heat shock conditions (Fig. 6*C*).

Role of ABC-binding cassette membrane transporter in HSP70 secretion

Because previous studies have implicated a role for ABC family transporters in the secretion of IL-1β, we examined whether this mechanism might also relate to HSP70 secretion (12). Cells were pretreated with the ABC inhibitor glibenclamide before examination of basal and heat shock-induced HSP70 secretion. Glibenclamide significantly inhibited heat-induced HSP70 release in LNCaP and PC-3 cells although it had a less-pronounced effect on basal release, suggesting a role for ABC family transporters in HSP70 secretion (Fig. 7, *A* and *B*). Previous studies have implicated the ABC family member ABCA-1 in IL-1β secretion (17). The role for ABCA-1 can be examined using another chemical, inhibitor DIDS. DIDS inhibits ABCA-1 by blocking the chloride current necessary for its function (21). DIDS proved to significantly inhibit HSP70 secretion at both basal and heat shock-stimulated PC-3 and LNCaP cells (Fig. 7, *C* and *D*). Fig. 7*E* indicates that ABCA-1 is expressed in both cell lines both under nonheat shock and heat shock conditions. ABCA-1 may be involved in the multidrug resistance phenotype exhibited by both of the tumor cells. These experiments therefore indicate that HSP70 secretion requires the ABCA-1 isoform ABC transporters.

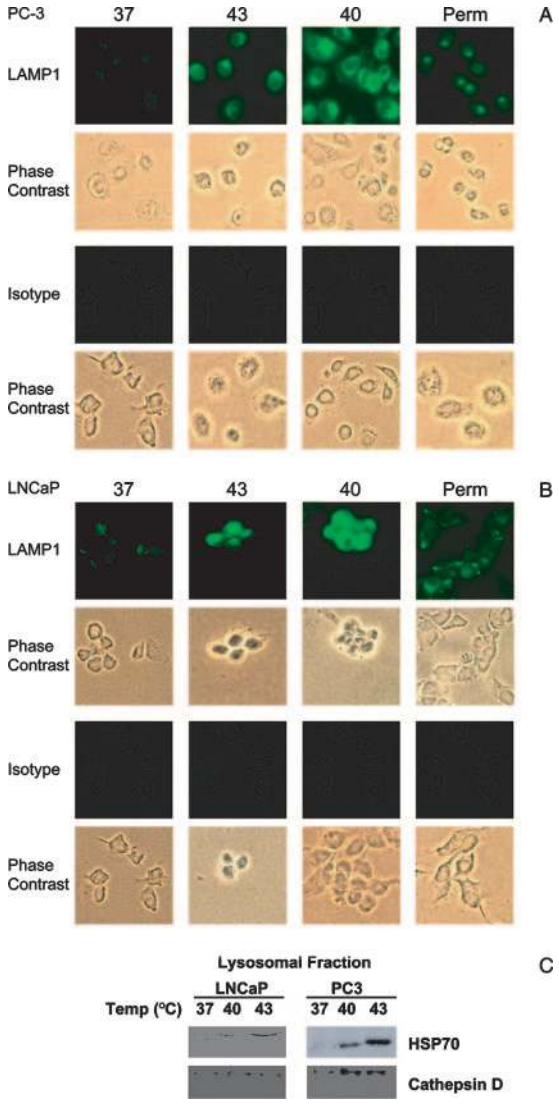

FIGURE 6. Immunostaining for surface LAMP1. Following no heat treatment at 37°C and heat shock at 43°C for 30 min and 40°C for 6 h, PC-3 (*A*) and LNCaP (*B*) were fixed and stained with mAbs to human LAMP-1 (Isotype Labeling, mouse IgG1κ) and mouse IgG1κ T-2 mycotoxin (Isotype Labeling, matched controls) followed by Alexa-488-conjugated secondary Ab (green). Perm represents intracellular staining for LAMP1. Images were acquired using fluorescent microscopy at a magnification of ×400. This experiment was repeated in triplicate with reproducible results. Presence of HSP70 was determined using Western blots of the lysosomal fraction (*C*) in both PC3 and LNCaP cells.

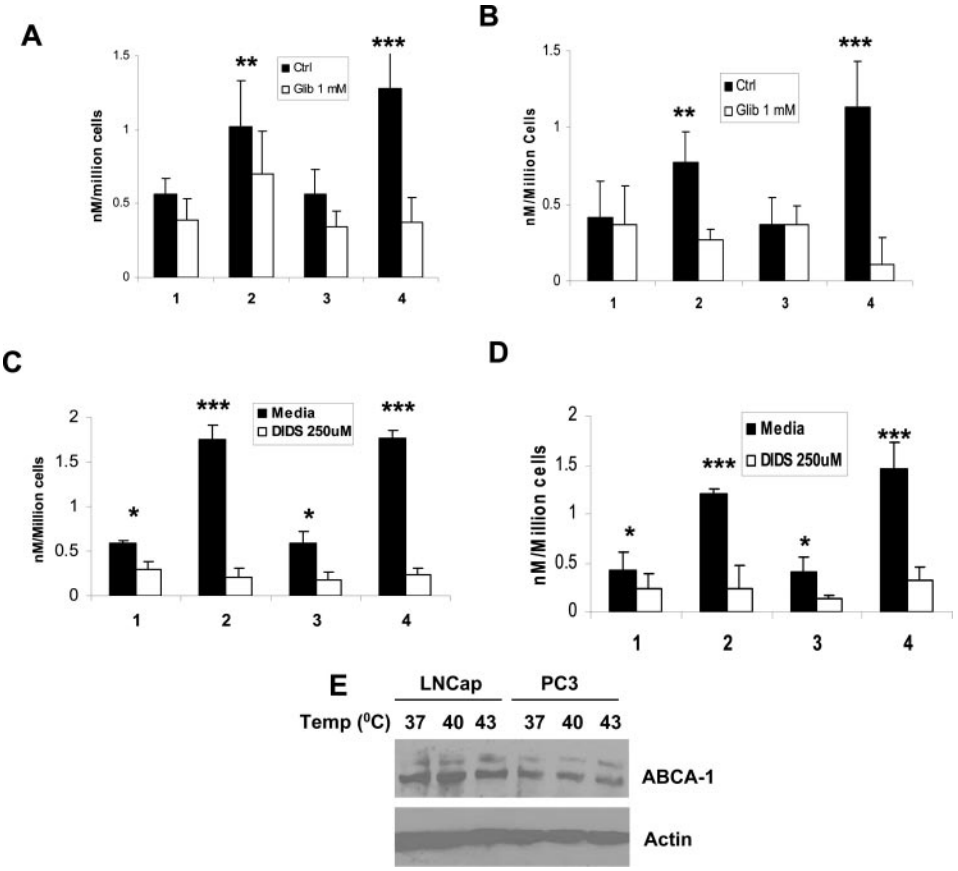

FIGURE 7. HSP70 secretion requires ABC family transporters. PC-3 (*A*) and LNCaP (*B*) cells were pretreated for 2 h with 1 mM glibenclamide (Glib) (□) and 250 μM DIDS (□) compared with controls (■), PC-3 (*C*), and LNCaP (*D*) before hyperthermia treatments previously described. Supernatants were harvested and tested for the presence of HSP70 using ELISA methods described above. The 37°C and 43°C for 30-min data are represented by 1 and 2 and 37°C and 40°C for 6 h is represented by 3 and 4, respectively. ABCA-1 levels in PC3 and LNCaP was determined using Western blots from total lysate (*E*). Data represent the mean ± SD of the experiments done in triplicate. Statistical analyses compared Glib vs control at conditions 2 (**, $p < 0.01$) and 4 (***, $p < 0.001$). DIDS was compared statistically to control at conditions 1 and 3 (*, $p < 0.05$); (*C*), 2 and 4 (***, $p < 0.001$); (*D*) 2 and 4 (**, $p < 0.01$).

HSP70 secreted for prostate carcinoma cells binds to the cell surface prostate cancer cells

We next examine whether a fraction of the HSP70 secreted from tumor cells can bind to the cell surface (Fig. 8). Such a mechanism was suggested by kinetic studies of extracellular HSP70 accumulation as in Fig. 2. In each case, we observed that after initial accumulation, extracellular HSP70 levels declined. This decrease in extracellular HSP70 may be due to binding and internalization of secreted HSP70. Indeed, cell surface HSP70 was minimal in unstressed cells but was readily detectable after 43°C for 30 min or 6 h at 40°C in both cell lines (Fig. 8, *A* and *C*). This increase in surface expression of HSP70 correlates with the increases observed in LAMP1 surface expression (Fig. 6). We also examined the behavior of intracellular HSP70 after heat shock (Fig. 8*B*). HSP70 appears to be evenly distributed within the cell at 37°C but was translocated to the nucleus at 43°C, where it was found in granular structures (31). However, 40°C heating did not appear to markedly alter the intracellular HSP70 distribution (Fig. 8*B*). This experiment therefore indicates a profound and complex redistribution of HSP70 in cells after heat shock. For instance, at 43°C, HSP70 may be translocated to the nucleus (Fig. 8*B*) or associated with the cell surface (Fig. 8*A*). We observed similar findings with the intracellular staining of LNCaP cells (data not shown).

Discussion

Our experiments were conducted with the aim of determining mechanisms of HSP70 release from tumor cells that may be important in understanding its immunological functions. Previous reports suggested that release of HSP70 family proteins may involve physiological mechanisms such as cosecretion in exosomes of HSC70 with transferrin (contains a secretion leader) or discharge from cells undergoing necrosis (32). However, our current studies indicate that HSP70 release from prostate carcinoma cells is not inhibited by inhibitors of endoplasmic reticulum/Golgi function (data not shown) and takes place under conditions in which minimal necrosis is observed (Fig. 1*B* and 2, *A–D*). Thus, HSP70 can be secreted from tumor cells by an active secretion pathway as well as from cells dying from the stress of therapy (10, 32).

The mechanisms of secretion of leaderless proteins such as HSP70 are complex and incompletely understood. However, two main pathways have been deduced. The first (*i*) used by proteins such as FGF-1 and IL-1α, appears to translocate across the plasma membrane on partial denaturation to a molten globule form (15). It has been conjectured that heat shock stimulates this pathway in part by permitting the proteins to partially unfold, a requirement for membrane translocation of many proteins (33, 34). More common (*ii*) appears to be the pathway used by IL-1β, which involves entry of the secreted protein into endolysosomes and release from the cell by a vesiculation mechanism (35). Our largely pharmacological dissection indicates that HSP70 release belongs to this second class of leaderless secretion, and its mechanisms of release further resemble IL-1β in requiring the activity of ABC family transmembrane transporters and the likely participation of purinergic receptors (21). Other secreted proteins that are largely of cytoplasmic/nuclear distribution in addition to HSP70, such as high-mobility protein b1 (HMGB1) and engrailed-2, appear to be secreted along this pathway under specialized conditions that promote secretion (36, 37). Curiously, heat shock has been shown to cause the secretion of two proteins, FGF-1 and IL-1α, through the alternative pathway (*i*) involving heat-induced membrane translocation (15). HSP70 secretion by heat shock may therefore use a hybrid pathway involving mechanisms found in pathways (*i*) and (*ii*). For instance, binding of FGF-1 and IL-1α to phosphatidylserine on the inner leaflet of the plasma membrane before translocation may be involved in a flipping mechanism in which phosphatidylserine

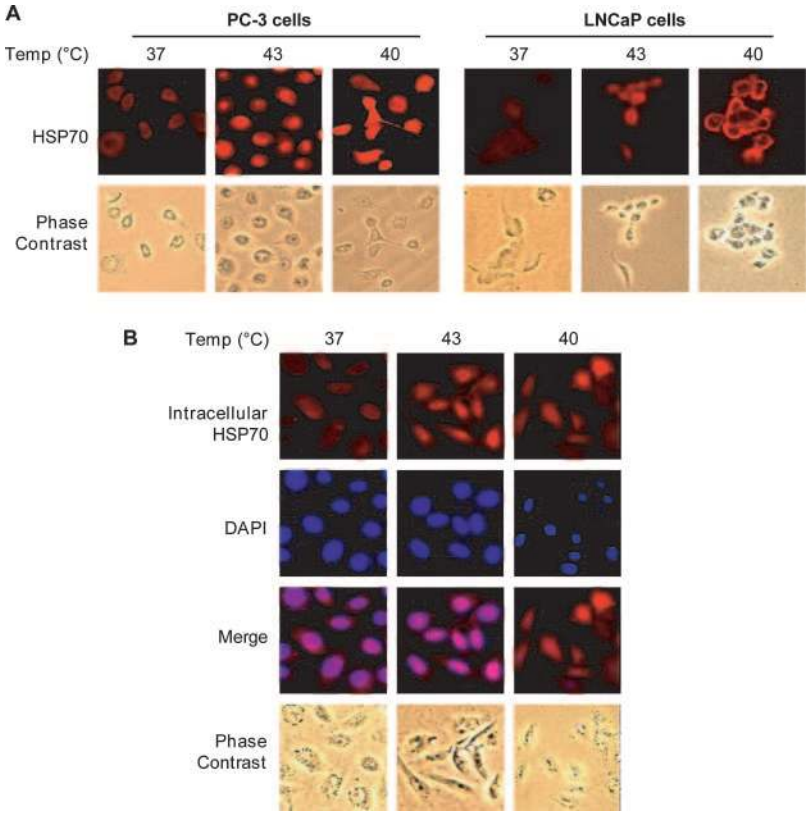

FIGURE 8. Immunostaining for surface and intracellular HSP70. After no heat treatment at 37°C, heat shock at 43°C for 30 min, and 40°C for 6 h PC-3 surface staining (*A*, *left panel*), intracellular staining (*B*), and LNCaP surface staining (*A*, *right panel*) cells were incubated with mAbs to HSP70 (Isotype Labeling, mouse IgG1) followed by Alexa 594-conjugated secondary Ab (red). For intracellular staining (*B*), one drop of mounting medium with DAPI (4,6-diamidino-2-phenylindole) was added to visualize nuclear structure. Row 1, HSP70 intracellular staining is overlaid by row 2, DAPI nuclear stain giving rise to row 3 merged images. Images were acquired using fluorescent microscopy at a magnification of ×400. This experiment was repeated in triplicate with reproducible results.

is externalized to the outer leaflet of the plasma membrane accompanied by associated proteins (38). Intriguingly, HSP70 has been shown to bind phosphatidylserine, although the significance of this interaction for HSP70 release has not been determined (39). There is previous evidence that heat shock stimulates lysosomal exocytosis in vivo in liver cells and peritoneal macrophages (40, 41). Our studies do not indicate the sorting mechanism required for a fraction of the HSP70 to translocate to the lysosomal endosome secretion pathway during heat shock. However, because the majority of intracellular HSP70 is either retained in the cytoplasm or translocates to the nucleus on stress (Fig. 8*B*), it seems likely that a signal such as posttranslational modification may be required for its sorting to the secretion pathway (Fig. 8*A*). HSP70 can be phosphorylated under some circumstances and may be ubiquitinated on association with the ubiquitin ligase CHIP (42, 43). Translocation of a fraction of HMGB1 and engrailed-2 to the secretory pathway involves hyperacetylation and dephosphorylation, respectively (44, 45).

Several other schemes for HSP70 release have been proposed and indeed may be operable under some circumstances. It has been proposed that HSP70 may be released from cells in lipid-bounded endosomes (46). Such a scheme is not supported by the experiments in our study of prostate carcinoma cells in which we could only detect HSP70 in the extracellular medium in free solution. However, it is possible that a small fraction of HSP70 may be released from other cell types by such a mechanism (47, 48). Secretion mechanisms may vary between cell types and, for instance, IL-1β secretion involves quite distinct mechanisms in different cell types (12, 21). In addition, pretreatment of prostate cancer cells with glibenclamide prevented heat-induced HSP70 release but not basal release of HSP70, indicating that heat-induced release is regulated by ABC transporters, whereas basal release likely involves another mechanism (Fig. 7, *C* and *D*). In either case, it seems likely that HSP70 transits though a lipid-bounded vesicular structure along the pathways to secretion, which in the case of this study is a lysosomal compartment, and finally crosses the plasma membrane to be secreted (49).

Our experiments also suggest that HSP70 released from cells exists in free form or may bind to adjacent cells though an autocrine or paracrine mechanism (Fig. 8). Secreted HSP70 may bind to receptors on tumor cells as shown above and mark such cells for destruction by NK cells, or may become associated with APCs such as DC and induce DC maturation and Ag cross-presentation (50, 51). NK cells have been shown to bind directly to HSP70 on the surfaces of tumor cells and lead to direct cell killing (52). The role of HSP70 release in tumor cell killing by T lymphocytes has been examined by Vile and colleagues (7) who showed that HSP70 overexpression in tumors and its induced release can lead to profound immunostimulation and tumor rejection (11). However, it is not clear what the significance of low-level release of HSP70-peptide complexes in untreated tumor cells (Fig. 2) might be. Tumor cell killing by NK cells or alternatively tolerance to tumor Ags could occur. Such tolerance can, however, be broken by increasing intracellular HSP70 levels in tumors and maximizing release (7). The extracellular properties of HSP70 resemble those of HMGB1, another mammalian danger signal, in that it can be released both after cell necrosis or by active secretion mechanisms both in immune and nonimmune cells (53). In addition, stimulation of HSP70 secretion by heat shock may have direct physiological significance and may underlie some of the known effects of febrile heat on the immune response. Febrile heat facilitates some aspects of the innate immunity and causes lymphocyte infiltration and enhanced NK cell-dependent tumor cell killing, some of which changes may involve secreted HSP70 (26). Febrile heat also promotes maturation and migration of APC (27, 54).

Our experiments, therefore suggest that HSP70 under some circumstances may acquire a novel, extracellular role. The modification required in order for HSP70 to be secreted is not known, although the pathway involved has remarkable similarities to

IL-1β secretion in macrophages and glial cells (12, 21). As HSP70 has potent immunological properties, this pathway may play a role in tumor immune response.

Disclosures

The authors have no financial conflict of interest.

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