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## **Hsp70 Translocates into the Plasma Membrane after Stress and Is Released into the Extracellular Environment in a Membrane-Associated Form that Activates Macrophages<sup>1</sup>**

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# Hsp70 Translocates into the Plasma Membrane after Stress and Is Released into the Extracellular Environment in a Membrane-Associated Form that Activates Macrophages<sup>1</sup>

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and Antonio De Maio<sup>2\*†</sup>

Heat shock proteins (hsps) are intracellular chaperones that play a key role in the recovery from stress. Hsp70, the major stress-induced hsp, has been found in the extracellular medium and is capable of activating immune cells. The mechanism involved in Hsp70 release is controversial because this protein does not present a consensual secretory signal. In this study, we have shown that Hsp70 integrates into artificial lipid bilayer openings of ion conductance pathways. In addition, this protein was found inserted into the plasma membrane of cells after stress. Hsp70 was released into the extracellular environment in a membrane-associated form, sharing the characteristics of this protein in the plasma membrane. Extracellular membranes containing Hsp70 were at least 260-fold more effective than free recombinant protein in inducing TNF- $\alpha$  production as an indicator of macrophage activation. These observations suggest that Hsp70 translocates into the plasma membrane after stress and is released within membranous structures from intact cells, which could act as a danger signal to activate the immune system. *The Journal of Immunology*, 2008, 180: 4299–4307.

Expression of heat shock proteins (hsps)<sup>3</sup> is markedly increased as part of the response to an array of stressors. These proteins participate in the refolding of denatured polypeptides that become damaged as a consequence of an insult. During nonstress conditions, hsps participate in the folding of nascent polypeptides and the stabilization of receptors and signal transduction molecules (1). Although hsps are localized intracellularly, they have also been found outside the cell, particularly after various pathological conditions (2–4). Extracellular hsps, specifically Hsp70, have been reported to activate macrophages, dendritic cells, and NK cells by a receptor-mediated process (5–10). An important question that remains unanswered is how does Hsp70, which does not have a consensual secretory signal, reach

the extracellular environment? Initially, it was thought that Hsp70 was released from necrotic cells after injury (11). However, secretion of Hsp70 also occurs in the absence of cell death (12). Consequently, an active, nonclassical secretory pathway may be involved. We have previously shown that both Hsp70 and heat shock cognate 70 (Hsc70) interact with artificial membranes (13). Moreover, these proteins displayed a particular specificity for phosphatidylserine (PS), a phospholipid that is normally present in the cytosolic side of cellular membranes (14). In the present study, we investigate whether the interaction of Hsp70 with membranes acts as a platform for its release into the extracellular environment.

## Materials and Methods

### Materials

Cell lines were obtained from American Type Culture Collection. Alexa Fluor 532-conjugated cholera toxin (CTX) subunit B, Alexa Fluor 594-conjugated transferrin, and Amplex Red cholesterol assay kits were purchased from Invitrogen Life Technologies. Ab against the C-terminal region of human Hsp70, cmHsp70.1, was obtained from Multimmune. Abs against the N-terminal region of Hsp70 (SPA-810), Hsc70 (SPA-815), Hsp40 (SPA-450), Hsp27 (SPA-800), and Hsp90 (SPA-830) were purchased from StressGen Bioreagents. HRP-conjugated streptavidin was purchased from Invitrogen Life Technologies. HRP-conjugated CTX, methyl- $\beta$ -cyclodextrin, and mouse monoclonal anti-actin Ab were purchased from Sigma-Aldrich. EZ-link *N*-hydroxysulfosuccinimidobiotin (sulfo-NHS-biotin) reagent was purchased from Pierce. Palmitoyl-oleoyl-phosphatidylserine and palmitoyl-oleoyl-phosphatidylcholine were obtained from Avanti Polar Lipids. TNF- $\alpha$  ELISA was obtained from BioSource International. FuGENE transfection reagent was purchased from Roche Applied Science.

### Immunostaining

Cells ( $1 \times 10^6$ ) were grown on a sterile glass cover slide. Nonspecific binding was blocked by incubation with serum (20%) from the host of secondary Ab and 0.2% Tween 20 in PBS (0.5 h at 25°C). Cells were incubated with primary Abs (1/200 dilution) for 1 h at 4°C, extensively washed with PBS, and incubated with secondary Abs (1/1000 dilution) for 0.5 h at 4°C. When indicated, cells were fixed with 4% paraformaldehyde (PFA) (10 min at 25°C) and permeabilized with cold acetone (15 s). In

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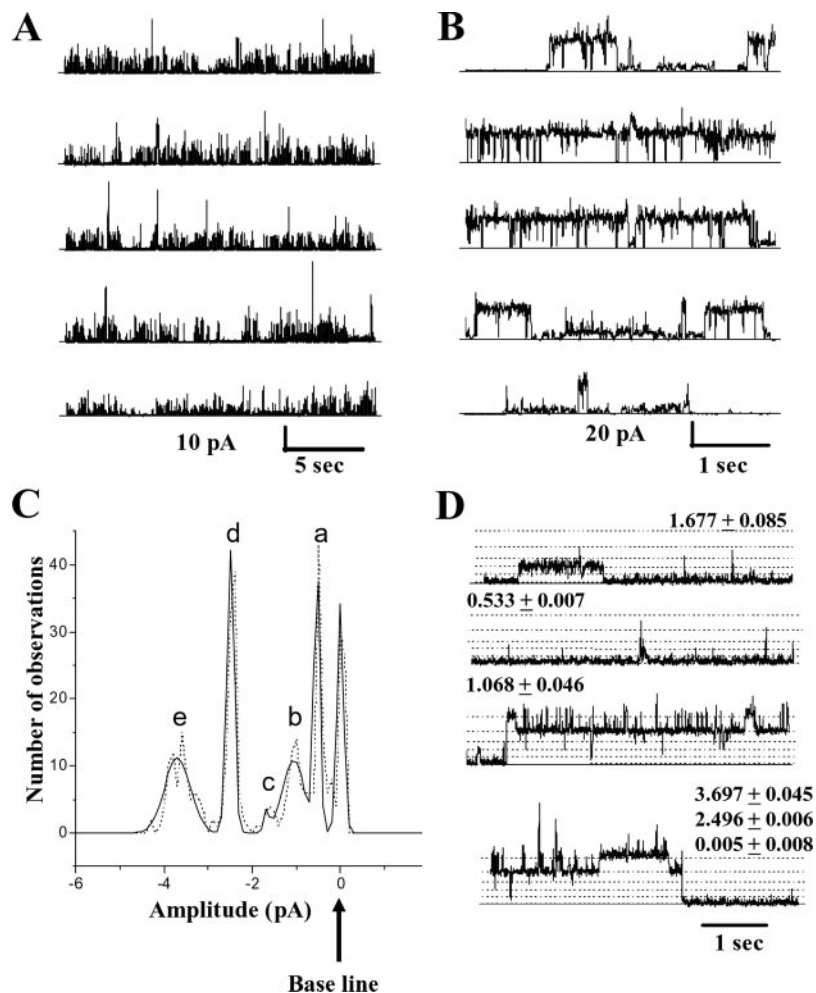
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<sup>3</sup>Abbreviations used in this paper: hsp, heat shock protein; CTX, cholera toxin; DAPI, 4',6'-diamidino-2-phenylindole; DRM, detergent-resistant membrane; ECM, extracellular membrane; GA, geldanamycin; GM1, monosialoganglioside; HS, heat shock; Hsc70, heat shock cognate 70; M $\beta$ CD, methyl- $\beta$ -cyclodextrin; PFA, paraformaldehyde; POPE, 1-palmitoyl-2-oleoyl-phosphatidylethanolamine; POPS, 1-palmitoyl-2-oleoyl-phosphatidylserine; PS, phosphatidylserine; SF, serum free; sulfo-NHS-biotin, *N*-hydroxysulfosuccinimidobiotin; WT, wild type; YFP, yellow fluorescent protein.

**FIGURE 1.** Incorporation of Hsp70 into a planar lipid bilayer opens a stable ion conductance pathway. Proteoliposomes made of purified human recombinant Hsp70 and POPS were added to one of the sides of the experimental chamber, and the current activity at the planar lipid bilayer was measured. The planar lipid bilayer, made of a suspension of POPS/POPE, was separating ionic solutions of asymmetrical concentrations of KCl (200 mM *cis* and 50 mM *trans*). *A*, At low time resolutions, the Hsp70 channel activity shows stable trains of brief current spikes, indicating brief multiple channel conductance changes. *B*, At a more expanded time scale, the current traces show the long duration channel openings, indicating channel stabilization into one preferred conductance state. *C*, The channel activity made of discrete events shows frequent switching between well-defined, long-lasting current levels. The dotted lines on the traces indicate the best-defined current levels and their values, which were obtained from the current amplitude histogram (*D*). The current amplitude histogram shows the frequency of unitary opening events lasting >2 milliseconds. Marquardt least square fitting of this histogram indicates that the current values distributed mainly into five Gaussians. The values corresponding to each current peak in the histogram are: *a*,  $0.533 \pm 0.007$ ; *b*,  $1.068 \pm 0.046$ ; *c*,  $1.677 \pm 0.085$ ; *d*,  $2.496 \pm 0.006$ ; and *e*,  $3.697 \pm 0.045$ . Data are from a representative experiment of  $\geq 42$  determinations.



some cases, immunostained cells were incubated with 1% Triton X-100 in PBS for 20 min on ice. Nuclei were stained with 4',6'-diamido-2-phenylindole (DAPI) hydrochloride (15 s at 25°C), and cells were visualized using a fluorescent microscope.

#### Recording of Hsp70 ion channel activity using a planar lipid bilayer

The experimental chamber consisted of two compartments separated by a thin Teflon film with a hole of ~100- to 120- $\mu\text{m}$  in diameter. Planar lipid bilayers were formed by applying a suspension of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS) (1:1, 50 mg/ml, each in *n*-decane) to the hole in the Teflon film. The ionic solutions contained asymmetrical concentrations of KCl (200 mM *cis* and 50 mM *trans*), and 0.5 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , and 5 mM potassium-HEPES (pH 7). Hsp70 was incorporated into the lipid bilayer from POPS liposomes bearing the peptide and added to the *cis* compartment in small aliquots. To prepare Hsp70 liposomes, POPS dissolved in  $\text{CHCl}_3$  (10 mg/ml) was air dried and resuspended in 1 M potassium aspartate to a final concentration of 5 mg POPS/ml. The resulting mixture was bath sonicated for 5 min. Aliquots of the stock solution of Hsp70 were added to the liposome suspension, and the combination was sonicated for an additional period of 2 min. Recombinant Hsp70 used in this study was a special order from StressGen Bioreagents that was dialyzed extensively to eliminate ATP and salts. Incorporation of Hsp70 occurred directly from the solution by spontaneous fusion of the proteoliposomes with the lipid bilayer. Channel currents associated with the incorporation of Hsp70 were recorded using a patch-clamp amplifier and stored on computer disk memory. Off-line analysis of the recorded Hsp70 channel activity was conducted using the software package pClamp.

#### Detergent-resistant membrane (DRM) isolation

HepG2 cells (90% confluent, 10-cm culture dish) were harvested in 1 ml of cold TNE buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA,

4 mg/ml trypsin inhibitor, 1 mg/ml benzamide, 5  $\mu\text{mol/ml}$  leupeptin, 200  $\mu\text{M}$  sodium vanadate, 100 nM okadaic acid, and 1 mg/ml PMSF) containing 1% Triton X-100. Cell lysates were incubated at 4°C for 0.5 h and an equal volume of 85% sucrose in TNE was added. The mixture was placed at the bottom of a centrifuge tube and overlaid with 4 ml of 35% sucrose and 1 ml of 5% sucrose followed by 4.5 ml of TNE buffer and centrifuged at  $200,000 \times g$  for 18 h at 4°C (15). After centrifugation, 3.5 ml were removed from the top of the tube and 13 fractions (615  $\mu\text{l}$  each) were collected from the top of the gradient. Each fraction (535  $\mu\text{l}$ ) was precipitated with 10% TCA (2 h at 4°C) and centrifuged at  $15,600 \times g$  (10 min at 4°C). Samples were washed twice with cold acetone, resuspended in 1 $\times$  loading buffer, boiled, and separated by SDS-PAGE. The rest of each fraction (80  $\mu\text{l}$ ) was used for the detection of monosialoganglioside (GM1) slot blots using HRP-conjugated cholera toxin.

#### Extracellular membrane (ECM) isolation

HepG2 cells, (90% confluent, 150-cm culture dish) were heat shock (HS) or not in serum-free (SF) medium and allowed to recover for different lengths of time at 37°C. The ECM was collected and centrifuged at  $1,500 \times g$  for 10 min. The supernatant was centrifuged at  $10,000 \times g$  (0.5 h at 4°C) followed by ultracentrifugation at  $100,000 \times g$  (1 h at 4°C). The pellet was resuspended in 1 ml of supernatants and ultracentrifuged again at  $100,000 \times g$  (1 h at 4°C). The resulting pellet (ECM) was resuspended in loading buffer 1 $\times$  or PBS. This protocol has been previously used to isolate exosomes from cells (16, 17).

#### Cell surface and ECM biotinylation

HepG2 cells (90% confluent) were washed twice with cold PBS and incubated with sulfo-NHS-biotin in PBS (0.2 mg/ml) for 0.5 h at 4°C (18). Free reagent was removed by two washes with cold PBS and cells were lysed. Isolated ECM were incubated with sulfo-NHS-biotin in PBS (1.6 mg/ml for 2 h at 4°C) and centrifuged at  $100,000 \times g$  (60 min at 4°C). The detection of biotinylated proteins was performed using HRP-conjugated

streptavidin (1/5000 at 3 h) after SDS-PAGE and transferring onto nylon membranes.

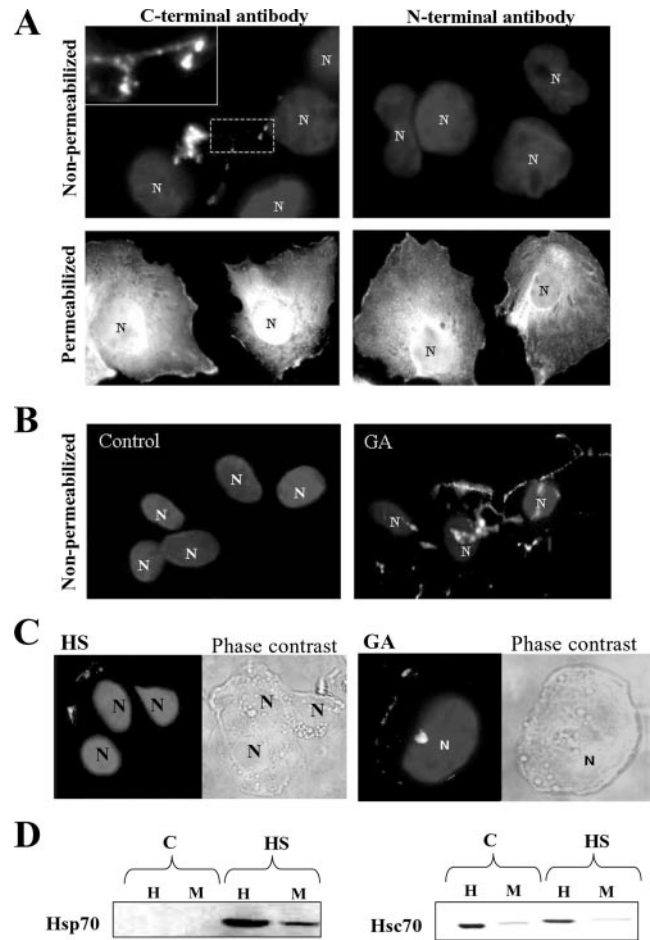
## Results

### *Hsp70 forms stable multiconductance ion channels in planar lipid bilayers*

We investigated the interaction of Hsp70 with membranes using planar lipid bilayers. Proteoliposomes made of PS and human recombinant Hsp70 were added to ionic solutions that were separated by the artificial lipid bilayer in an experimental chamber. The incorporation of Hsp70 into the lipid bilayer occurred by spontaneous fusion of the proteoliposome with the artificial membrane. Soon after the addition of Hsp70 liposomes, an electrical current activity was observed across the voltage-clamped bilayer, indicating a flow of charges across a newly formed ion pathway. This current activity was observed in all cases where the incorporation of Hsp70 into the lipid bilayer was attempted ( $n = 42$ ) and showed high stability. The direction of the current recorded under a transbilayer chemical gradient, at zero transbilayer potential, indicated that Hsp70 preferentially permits the flow of cations. Representative recordings illustrate two different patterns of electrical activity within the same channel performance: a stable train of swift current spikes that indicate brief, multiple channel conductance changes (Fig. 1A) and an extended duration of opening activity that indicates channel stabilization into one preferred conductance state (Fig. 1B). At a long, expanded time interval and a constant membrane potential, Hsp70 channel activity showed frequent changes between well-defined and long-lasting current levels (Fig. 1, C and D). Analysis of the current amplitudes at different membrane potentials showed that Hsp70 channels displayed a range of measurable, stable conductance between 3 and 60 pS. Recombinant Hsp70 used in this study was extensively dialyzed to eliminate any possible low m.w. contaminants. In addition, the recombinant protein displayed a single band after SDS-PAGE. Neither Hsp90 nor LPS added to the lipid bilayer displayed any channel activity, which is consistent with a prior report (19).

### *Hsp70 is present on the plasma membrane after stress*

The preceding observations suggest that Hsp70 is capable of interacting with lipids within membranes. We further investigated whether Hsp70 is present in cellular membranes. HepG2 cells, which were subjected to a 43°C HS and recovered for 7 h at 37°C, were incubated with Abs that recognize different Hsp70 epitopes. One of these Abs (cmHsp70.1) is directed against a peptide localized in the C terminus of Hsp70, whereas the other Ab (SPA-810) recognizes the N terminus of the molecule. The presence of Hsp70 on the plasma membrane was observed after staining with FITC-conjugated cmHsp70.1 Ab at 4°C in nonpermeabilized, nonfixed cells (Fig. 2, A and C). Under the same conditions, SPA-810 Ab failed to reveal the presence of Hsp70 (Fig. 2A). In contrast, both Abs showed a similar staining pattern in acetone-permeabilized cells (Fig. 2A). These observations suggest that Hsp70 is inserted into the plasma membrane with the C terminus exposed outside of the cell. Treatment of HepG2 cells with geldanamycin (GA), an inhibitor of Hsp90 that triggers the induction of Hsp70 (20, 21), and destabilizes membranes, also resulted in cell surface detection of Hsp70 by cmHsp70.1 Ab (Fig. 2, B and C), suggesting that the translocation of Hsp70 to the membrane is not an exclusive effect of HS. Hsc70, the constitutive form of the Hsp70 family, was not detected on the cell surface of nonstressed or stressed cells (data not shown). The presence of Hsp70 on the cell surface, which was detected by FITC-conjugated cmHsp70.1 Ab (C terminus of the molecule), was not affected by pretreatment of the cells (10 min) with low pH (5.0) or high salt buffer, suggesting that the protein is



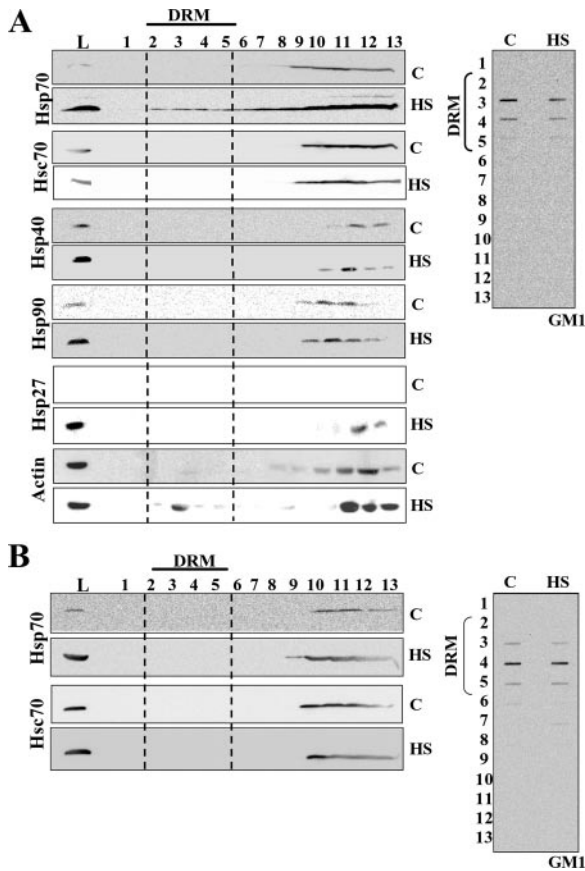
**FIGURE 2.** Hsp70 is present on the surface of stressed cells. *A*, HepG2 cells were HS (43°C for 1.5 h), recovered for 7 h at 37°C, and incubated with Abs against Hsp70 C-terminal (cmHsp70.1) or N-terminal (SPA-810) Ab at 4°C. Nonbound Ab was washed off and cells were fixed with PFA (*top panel*). The *inset* shows the detection of Hsp70 on the cell surface. Heat shocked cells were fixed with PFA, permeabilized with cold acetone, and stained for Hsp70 using either C- or N-terminal Ab (*bottom panel*). Nuclei (N) were stained with DAPI. *B*, Cells were also treated with GA (1 µg/ml) for 8 h or not (control) and incubated with FITC-conjugated cmHsp70.1 (C-terminal) Ab at 4°C as described above. *C*, A comparison between surface staining using cmHsp70.1 Ab at 4°C and phase contrast for cells after HS or GA treatment. *D*, Detection of Hsp70 or Hsc70 by Western blotting on total cell homogenates (H) or membrane fractions (M) obtained from unstressed (C) or thermally stressed (HS) cells.

not bound to the plasma membrane via another surface protein (data not shown). Hsp70 was detected by Western blotting in isolated total cellular membrane fractions from stressed cells, confirming the interaction of Hsp70 with membranes. In contrast, Hsc70 was not detected in the membrane fraction, suggesting that the presence of Hsp70 is not due to cytosolic contamination (Fig. 2D).

### *Hsp70 on membranes is present in Triton X-100 in soluble form*

Prior studies have shown that Hsp70 is present within DRM fractions. Consequently, we investigated whether Hsp70 on membranes could be present in this fraction. DRM were isolated after Triton X-100 treatment of HepG2 cells following HS by sucrose gradient centrifugation. Hsp70 was detected in DRM fractions, which were identified by the presence of GM1, as early as 4 h into the recovery time (37°C) following HS. Other hsp, such as

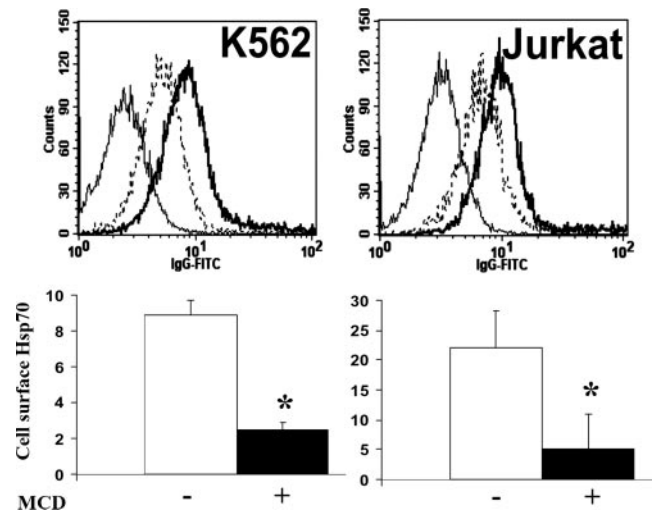




**FIGURE 3.** Hsp70 is present in DRM fractions depending on the presence of cholesterol. *A*, HepG2 cells were maintained at 37°C (C) or HS (43°C for 1.5 h), recovered (37°C for 4 h), and lysed in TNE buffer containing 1% Triton X-100. DRM fractions were isolated as described in *Materials and Methods*. The presence of Hsp70, Hsc70, Hsp90, Hsp27, and actin within DRM fraction was detected by Western blotting (*left panel*), whereas the levels of GM1 were measured by slot blotting using HRP-conjugated CTX (*right panel*). *B*, Cells, controls or after HS and recovery, were treated with M $\beta$ CD (10 mM for 30 min at 37°C) and DRM were analyzed as described above for the presence of Hsp70 or Hsc70. These are representative experiments of at least three independent determinations.

Hsc70, Hsp90, Hsp27, and Hsp40, were not detected in DRM fractions. Actin was only observed within DRM fractions after 4 h of recovery from HS (Fig. 3A). Treatment of cells after HS with methyl- $\beta$ -cyclodextrin (M $\beta$ CD), which removes cellular cholesterol without compromising cellular viability, disrupted the interaction of Hsp70 with DRM fractions (Fig. 3B). Under these conditions, the presence of GM1 within DRM fractions was not affected. The previous observations were extended to other cell types. The presence of surface Hsp70 was also revealed on K562 and Jurkat cells analyzed by fluorocytometry using FITC-conjugated cmHsp70.1 Ab. Treatment of these cells with M $\beta$ CD reduced considerably the detection of Hsp70 on the cell surface (Fig. 4). These observations altogether suggest that the interaction of Hsp70 with membranes is enhanced by the presence of cholesterol.

To further investigate the presence of Hsp70 on the cell surface, HepG2 cells after HS and recovery were incubated with FITC-conjugated cmHsp70.1 Ab, Alexa Fluor 532-conjugated CTX, or Alexa Fluor 592-conjugated transferrin at 4°C, fixed with PFA (4%) after ligand binding and treated or not with Triton X-100 (1% for 20 min at 4°C). The signal for Hsp70 was still preserved on

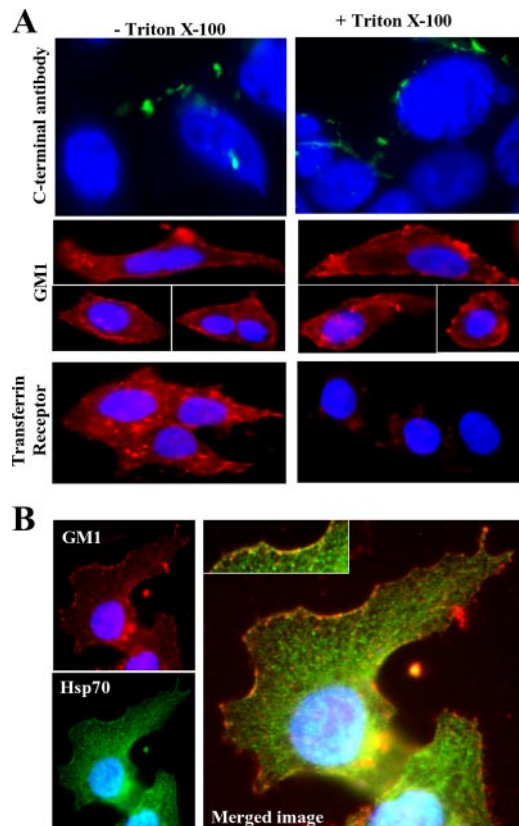


**FIGURE 4.** Cell surface presence of Hsp70 is affected by M $\beta$ CD treatment. K562 or Jurkat cells ( $0.5 \times 10^6$  cells/ml) were treated or not with M $\beta$ CD (1 mM for 6 h at 37°C), washed with PBS, and stained with FITC-conjugated cmHsp70.1 Ab (Multimmune). Labeled cells were analyzed using a FACSCalibur apparatus. *Top panel*, representative histograms of mean fluorescence intensity, cmHsp70.1 (thick line), and isotype-matched control Ab (light line); dashed lines correspond to M $\beta$ CD treatment; *Bottom panel*, Signal intensity quantification for cell surface Hsp70 ( $n = 4$ ). \*,  $p < 0.05$  vs untreated cells.

Triton X-100-treated cells (Fig. 5A). Similarly, the signal corresponding to Alexa Fluor 532-conjugated CTX, which binds to GM1, was resistant to Triton X-100 treatment (Fig. 5A). In contrast, the presence of transferrin receptor was significantly decreased after incubation with Triton X-100 (Fig. 5A). To determine whether Hsp70 and GM1 are located on the same membrane domain, cells after HS and recovery were incubated with Alexa Fluor 532-conjugated CTX at 4°C, fixed with PFA (4%), permeabilized with cold acetone, and incubated with FITC-conjugated cmHsp70.1 Ab. Cells were then treated with Triton X-100 (1% for 20 min at 4°C). Under these conditions, colocalization of Hsp70 and GM1 could be easily demonstrated (Fig. 5B).

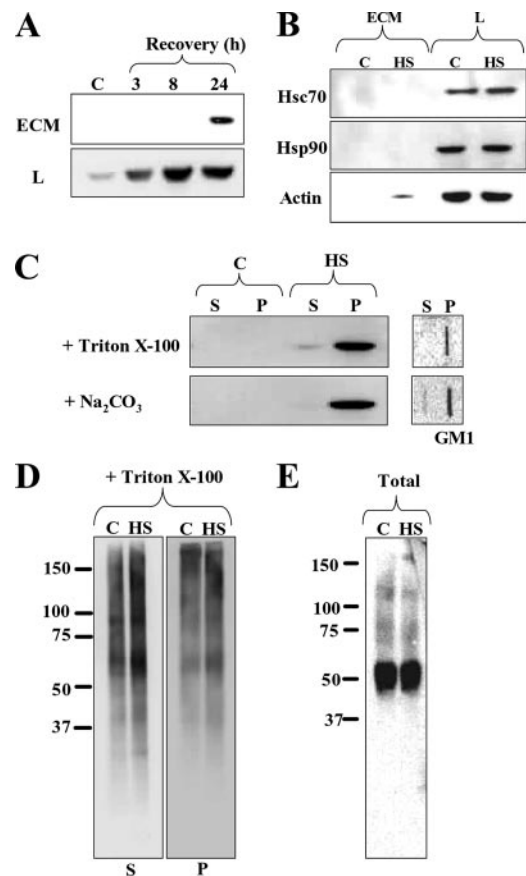
#### Hsp70 can be detected in extracellular membranes

We investigated whether Hsp70 could be present in the extracellular environment in a membrane-bound form. HepG2 cells were subjected to HS (43°C, for 1.5 h) in SF medium followed by recovery at 37°C for different lengths of time. The ECM was collected and subjected to differential centrifugation. Hsp70 was only detected in the high-speed centrifugation pellet fraction after 24 h of recovery following HS by Western blotting. Hsp70 was not observed in similar high-speed centrifugation fractions obtained from nonstressed (control) cells (Fig. 6A). This high-speed centrifugation fraction, which we have named extracellular membranes or ECM, has been referred to as exosomes in other studies (17, 22). ECM derived from both control and HS cells were positive for acetyl cholinesterase activity and contained Rab4. Actin was detected only in fractions derived from cells subjected to HS followed by 24 h of recovery (Fig. 6B). In contrast, other hsp, such as Hsc70, Hsp90, or Hsp27, were not detected in ECM (Fig. 6B). ECM contain cholesterol and GM1 and can be stained by FM4-64 reagent, which interacts with phospholipids. Hsp70 within ECM was resistant to Triton X-100 solubilization and Na<sub>2</sub>CO<sub>3</sub> extraction suggesting that Hsp70 is inserted into the membrane (Fig. 6C). To demonstrate that solubilization by Triton



**FIGURE 5.** Hsp70 on the cell surface is resistant to Triton X-100 treatment. *A*, HepG2 cells were heat shocked (43°C for 1.5 h), recovered for 7 h at 37°C, and incubated with FITC-conjugated cmHsp70.1 Ab, Alexa Fluor 532-conjugated CTX, or Alexa Fluor 592-conjugated transferrin at 4°C. Cells were fixed with PFA and treated or not with Triton X-100 (1% for 20 min at 4°C). *B*, HepG2 cells after HS and recovery were incubated with Alexa Fluor 532-conjugated CTX at 4°C for 30 min, fixed with PFA, permeabilized with cold acetone, incubated with FITC-conjugated cmHsp70.1 Ab at 4°C, and treated with Triton X-100 (1% for 20 min at 4°C). Nuclei were stained with DAPI. The images corresponding to Hsp70 and GM1 were merged to show colocalization between these two molecules. The inset shows the colocalization of Hsp70 and CTX on the membrane.

X-100 indeed removes some proteins from ECM, they were labeled with sulfo-NHS-biotin after isolation. Treatment of these labeled membranes with Triton X-100 (1% for 30 min at 4°C) resulted in the solubilization of the majority of the proteins (Fig. 6D), whereas treatment with Na<sub>2</sub>CO<sub>3</sub> resulted in the release of some proteins (not shown). The presence of Hsp70 in ECM was resistant to hyaluronidase treatment, suggesting that the protein is not associated with the extracellular matrix. The total protein pattern of ECM was similar between samples isolated from control or HS cells (Fig. 6E), which suggests that a significant difference between ECM derived from stressed and nonstressed cells is the presence of Hsp70. To investigate whether these ECM were derived from the plasma membrane, control or HS cells were surface labeled using sulfo-NHS-biotin, extensively washed to remove the reagent excess, and incubated in SF conditions for 24 h. The ECM fraction was isolated by high-speed centrifugation and found labeled with biotin, suggesting that they were derived from the plasma membrane of both control and HS cells. ECM were unlikely to be derived from apoptotic cells because PS was not detected on the surface of cells after HS and recovery. Moreover, we did not observe any change in cellular viability during HS/

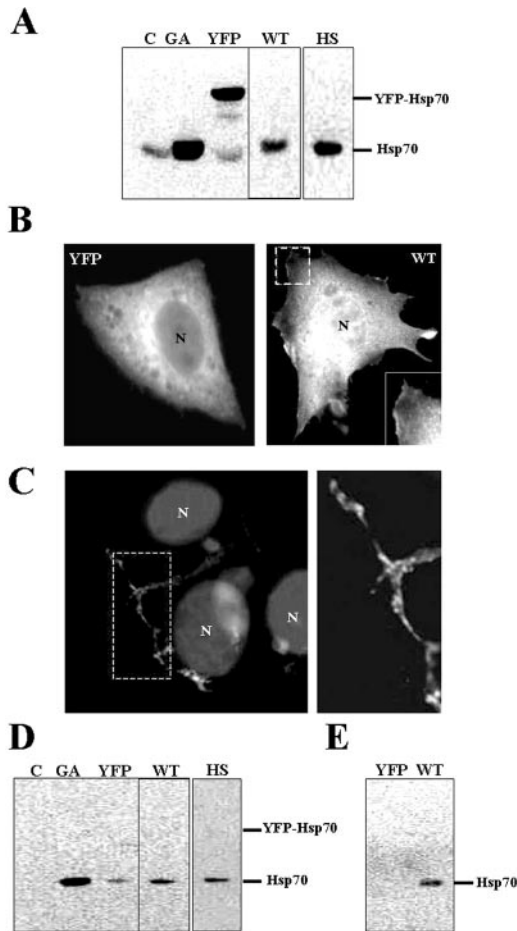


**FIGURE 6.** ECM derived from stressed cells contain Hsp70. HepG2 cells were maintained at 37°C (C) or heat shocked (43°C for 1.5 h), and recovered (37°C) in SF medium. ECM were isolated from culture medium by high-speed centrifugation as described in *Materials and Methods*. *A*, Detection of Hsp70 by Western blotting in total cell lysates (L) or in isolated ECM derived from cells after HS and recovery for 3, 8, and 24 h. *B*, Detection of Hsc70, Hsp90, and actin by Western blotting in ECM derived from HS (24-h recovery at 37°C) or control (C) cells. *C*, Isolated ECM from HS or control cells were incubated with Triton X-100 (1% for 30 min at 4°C) or Na<sub>2</sub>CO<sub>3</sub> (0.1 M at pH 11.5) and 2 mM EDTA (30 min at 4°C), centrifuged at 100,000 × *g* for 1 h, and the pellet and supernatant were analyzed for the presence of Hsp70 or GM1. *D*, Isolated ECM from HS or control cells were labeled using sulfo-NHS-biotin and treated with Triton X-100 (1% for 30 min at 4°C), centrifuged at 100,000 × *g* for 1 h, and the pellet (P) and supernatant (S) were collected. *E*, Protein pattern of ECM labeled with sulfo-NHS-biotin. The protein pattern in each fraction was visualized after SDS-PAGE and transferred onto nylon membranes using HRP-conjugated streptavidin.

recovery in comparison with control cells as assessed by the MTT method.

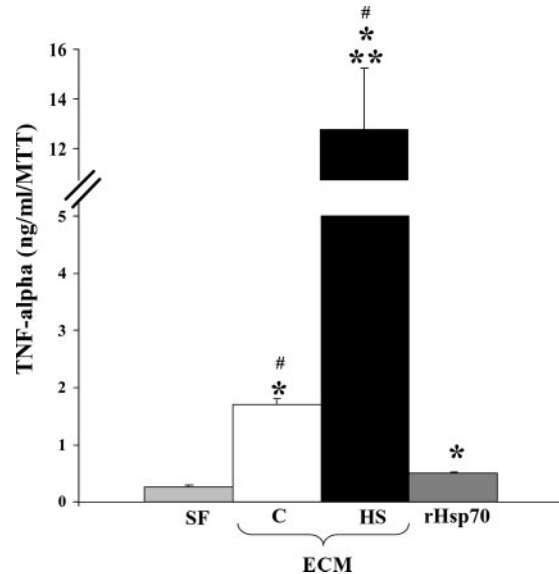
*The addition of yellow fluorescent protein (YFP) to the Hsp70 C terminus abolished the insertion of Hsp70 into the plasma membrane and its release into the extracellular medium*

Our results suggest that Hsp70 is inserted into the plasma membrane with the C terminus outside the cell. A plasmid containing a quimeric Hsp70 with YFP added to the C-terminal of the molecule (provided by Dr. H. Kampinga at the University of Groningen, The Netherlands) was transfected into HepG2 cells. Cells were also transfected with wild-type (WT) Hsp70. As positive control, cells were treated with GA or subjected to thermal stress and recovery (HS). Western blotting of lysates obtained from transfected cells demonstrated the expression



**FIGURE 7.** The interaction of Hsp70 with membranes is affected by addition of YFP at the C-terminal of the hsp. HepG2 cells (30% confluency) were transfected using FuGENE with either pYFP-N1-Hsp70 (YFP) encoding a YFP-Hsp70 quimera (provided by H. Kampinga, University of Groningen, The Netherlands) or pSG5-12c, a WT Hsp70 plasmid. After transfection (72 h), cells were lysed or total membrane fraction was isolated. Lysates or total membranes were obtained from nonstressed cells (C) as negative control or cells treated with GA or stressed (HS) as positive controls. *A*, Detection of Hsp70 by Western blotting from cell lysates (notice that Hsp70-YFP displayed a higher m.w.). *B*, Detection of YFP-Hsp70 or WT transgene expression in permeabilized cells by fluorescent microscopy. *Inset* marked by dotted line is magnified in *lower right corner inset*. *C*, Presence of Hsp70 on the cell surface after transfection with WT Hsp70 visualized by the Ab against the C-terminal of the molecule (cells were incubated with the Ab at 4°C). *Inset* on the left panel is displayed at high magnification on the *right panel*, demonstrating the presence of WT Hsp70 on the cell surface. *D*, Detection of Hsp70 by Western blotting in isolated total membrane preparations. *E*, Detection of Hsp70 in ECM obtained from cells transfected with Hsp70-YFP or Hsp70 WT constructs (72 h).

levels for YFP-Hsp70 and WT Hsp70 in comparison to Hsp70 induced by HS or GA treatment. Hsp70-YFP displayed a higher m.w. than WT Hsp70 (Fig. 7A). In addition, the presence of Hsp70-YFP was visualized by fluorescent microscopy (Fig. 7B). Although a robust expression level was observed for the quimeric protein, Hsp70-YFP was not observed on the plasma membrane (Fig. 7B). In contrast, cells transfected with WT Hsp70 showed the presence of Hsp70 in the cytosol as well as on the plasma membrane (Fig. 7B; see *inset*). The presence of WT Hsp70 on the plasma membrane of transfected cells was confirmed by the detection of this protein using FITC-conjugated cmHsp70.1 Ab at 4°C (Fig. 7C). Furthermore, Western



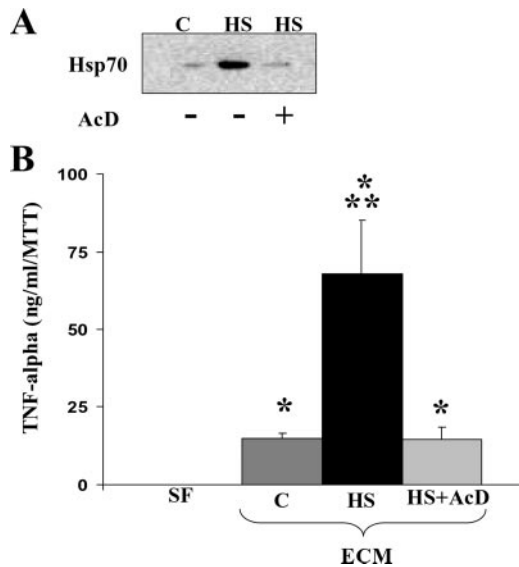
**FIGURE 8.** ECM derived from HS cells activates the production of TNF- $\alpha$  by macrophages. Macrophages (J774;  $2.5 \times 10^5$  per well) were incubated with ECM (50  $\mu$ l) derived from control or heat-shocked HepG2 cells in SF medium for 3 h at 37°C. At the end of the incubation period, extracellular medium was collected and stored at -20°C. Levels of TNF- $\alpha$  in the extracellular medium were measured by ELISA and normalized by the number of viable cells in each well measured by the MTT assay. Results are presented as mean  $\pm$  SEM ( $n = 10$ ). Statistical analysis was performed by one-way ANOVA, followed by Newman-Keuls test. \*,  $p < 0.05$  vs cells maintained in SF medium without additions; \*\*,  $p < 0.05$  vs cells incubated with ECM derived from control (C) or rHsp70; #,  $p < 0.05$  vs cells incubated with rHsp70.

blot analysis of isolated total membrane fraction from transfected cells revealed the presence of WT Hsp70, whereas Hsp70-YFP (higher m.w.) was not observed (Fig. 7D). Isolated ECM from transfected cells revealed the presence of WT Hsp70, but not YFP-Hsp70 (Fig. 7E). These observations suggest that insertion of Hsp70 into the plasma membrane requires an intact C terminus of the molecule. In addition, insertion in the plasma membrane may be a requirement for its release within ECM.

#### ECM containing Hsp70 are capable of activating macrophages

To investigate the potential biological role of Hsp70-positive ECM, macrophages (J744.A1) were incubated for 3 h with ECM derived from either control or HS (24 h of recovery from the stress) cells in SF medium. At the end of the incubation period, the extracellular culture medium was collected for the detection of TNF- $\alpha$ . Macrophages exposed to ECM containing Hsp70 produced higher levels of TNF- $\alpha$  (7.5-fold higher) in comparison with ECM derived from control cells. TNF- $\alpha$  production was also induced by the addition of recombinant human Hsp70 (50 ng) under similar conditions (Fig. 8). However, TNF- $\alpha$  production by Hsp70-positive ECM was 25.9-fold higher than the cytokine levels after incubation with recombinant Hsp70. The amount of Hsp70 within HS-derived ECM was estimated to be  $\sim$ 5 ng by Western blotting using recombinant protein as standards. Thus, the specific difference in TNF- $\alpha$  production between Hsp70 within ECM and recombinant protein is  $\sim$ 260-fold. The fact that macrophages were incubated with ECM in SF medium reduces the possibility that the activation of macrophages was due to contamination with endotoxin. This assumption was further supported by the observation that boiling ECM for 30 min resulted in an  $\sim$ 84% reduction of





**FIGURE 9.** ECM derived from HS cells in the presence of actinomycin D did not induce the production of TNF- $\alpha$  by macrophages. **A**, HepG2 cells were heat shocked (HS for 1.5 h) in presence or absence of actinomycin D (AcD; 1  $\mu$ g/ml). Cells were allowed to recover (37°C) for 3 h in the presence or absence of actinomycin D and then the drug was removed and the cells were allowed to recover for up to 24 h. Nonstressed cells were used as a control (C). **B**, ECM were isolated from control or HS cells treated or not with actinomycin D as described above. ECM were incubated with macrophages (J774;  $2.5 \times 10^5$  per well) in SF medium for 3 h at 37°C. At the end of the incubation period, extracellular medium was collected and stored at  $-20^\circ\text{C}$ . Levels of TNF- $\alpha$  in the extracellular medium were measured by ELISA and normalized by the number of viable cells in each well measured by the MTT assay. Results are presented as mean  $\pm$  SEM ( $n = 8$ ). Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls test. \*,  $p < 0.05$  vs cells maintained in SF medium without additions; \*\*\*,  $p < 0.05$  vs cells incubated with ECM derived from control (C).

TNF- $\alpha$  production by J744 cells. To demonstrate that the biological effect of ECM containing Hsp70 is due to the presence of this protein, cells were HS in the presence of actinomycin D, which was washed off after 3 h of recovery from the stress. This treatment did not result in any loss of cellular viability. However, expression of Hsp70 was dramatically reduced in cell lysates from actinomycin D-treated cells in comparison with cells in absence of the drug (Fig. 9A). ECM were isolated from HS cells treated or not with actinomycin D or from nonstressed cells. ECM isolated from actinomycin D-treated HS cells showed the same level of TNF- $\alpha$  production as controls cells, which was considerably lower than incubation with ECM derived from HS cells in absence of actinomycin D treatment (Fig. 9B).

## Discussion

Hsp70 is an intracellular chaperone, which is mainly involved in protein folding. A great body of evidence has demonstrated that this protein could also be detected outside cells where it has the capacity to activate the immune system (2, 5). Early assumptions indicate that Hsp70 was released into the extracellular medium from necrotic cells (11). Although this possibility cannot be discarded completely, it has been demonstrated that extracellular hsp70s are present in the absence of cell death (12). Because Hsp70 does not have a consensual secretory signal, the mechanism for translocation of this protein across membranes is intriguing. The earliest observation regarding the release of Hsp70 from viable cells by a nonclassical mechanism was reported by

Hightower and Guidon (23). In their study, extracellular Hsp70 was found noncovalently associated with fatty acids. More recently, it has been shown that Hsp70 could be secreted from intact cells via exosomes (16, 17, 22, 24) or by an endo-lysosomal-dependent pathway (25). In the present study, we provide evidence that Hsp70 is inserted into the plasma membrane before release into the extracellular environment in membrane-associated structures from intact stressed cells. This membrane-bound Hsp70 is capable of activating macrophages.

Previous studies from our laboratory have shown that Hsp70 and Hsc70 interact with lipid membranes (13), displaying a high degree of specificity for the presence of PS (14). The interaction of Hsp70 and Hsc70 with lipids is a unique characteristic of these two proteins because other hsp70s, including Hsp90 and Hsp60, do not associate with artificial membranes (A. De Maio and N. Arispe, unpublished observations). In agreement with these observations, prior investigations have detected Hsp70 in close proximity to cellular membranes (26, 27). Moreover, the presence of Hsp70 on the surface of transformed cells has been well documented (17, 28). We observed that, indeed, Hsp70 could be detected on the surface of nonpermeabilized cells after heat shock, which is consistent with prior observations in tumor cells (28). Our results indicate that Hsp70 is embedded within the plasma membrane as demonstrated by selective Ab binding. These observations suggest that Hsp70 may span the plasma membrane, leaving a small region of the C terminus exposed outside of the cell while the N terminus is located on the cytosolic side. This assumption is further supported by the ability of Hsp70 to integrate into artificial lipid bilayers and open ion conductance pathways. A similar channel activity has been previously reported for Hsc70 (19). The C-terminal of Hsp70 (peptide binding domain) is long enough to span the typical 50-Å width of the plasma membrane. In addition, this region presents  $\beta$ -sheet structures, which may act as centers for Hsp70 oligomerization and insertion into the lipid membrane. Other molecules containing similar  $\beta$ -sheet structures have also been found to form channels, including annexin,  $\beta$ -amyloid, and amylin (29–31). The mechanism that has been proposed to explain the selective insertion of Hsp70 into membranes assumes that Hsp70 is capable of assembling into low-order oligomers when the protein is in excess of polypeptide targets, such as when proteins become unfolded after stress (14). Indeed, Hsp70 has been reported to oligomerize in the absence of peptide targets or denatured proteins in an ATP-dependent manner (32, 33). Higher-order oligomerization of proteins has been proposed to target them to the plasma membrane before exosome budding (34). Because Hsp70 displayed a high degree of specificity for PS (14), which is a component of the cytosolic side of cellular membranes, we speculate that Hsp70 could translocate spontaneously from the cytosol into the plasma membrane after oligomerization and binding to PS. Thus, Hsp70 could initially associate with PS on the cytosolic phase of the plasma membrane and translocate within the lipid bilayer by the spontaneous flipping of this lipid into the outside of the cell. This process may be followed by the rapid ATP-dependent return of PS to the inner side of the plasma membrane, leaving Hsp70 inserted into the membrane. Elevation of surface PS is a characteristic of apoptotic cells due to inhibition of the ATP-dependent flippase that returns the phospholipid inside the plasma membrane. It is unlikely that the insertion of Hsp70 into the plasma membrane is related to the apoptotic process. In fact, we did not detect surface PS on HepG2 cells after HS and recovery. Cell viability was not compromised under HS and recovery conditions either. Moreover, there are no reports, to the best of our knowledge, indicating that apoptotic cells contain a large amount of Hsp70 on the plasma membrane.



Our data also showed that Hsp70 could be colocalized within DRM depending on the presence of cholesterol, because this protein was no longer detected in these fractions after treatment with M $\beta$ CD. Similar observations were obtained with cells expressing Hsp70 on the surface. Hsp70 was observed to colocalize with GM1 on the plasma membrane of cells after treatment with Triton X-100. Other studies have also reported the presence of Hsp70 in DRM (12, 35–38). However, these studies did not present evidence of Hsp70 insertion into the membrane. We could not detect other hsp on DRM, which is in apparent contradiction with other studies. The presence of Hsp90 within DRM as reported by other investigators is very weak, and it could be due to cytosolic contamination of this extremely abundant protein (36). Other studies used mouse brain samples (37), which are likely to have a different lipid membrane composition than the cell line used in this study. Extensive studies from our laboratory using different artificial lipid membrane models have failed to detect the interaction of other hsp with membranes.

ECM derived from control or HS cells were rich in cholesterol, GM1, and acetyl cholinesterase. Hsp70 within ECM could not be released by incubation with Na<sub>2</sub>CO<sub>3</sub>, suggesting that the protein is not loosely bound to the membrane. In addition, treatment of ECM with Triton X-100 did not solubilize Hsp70, suggesting that this protein is embedded within structures that may resemble DRM. Previous reports have shown the presence of Hsp70 on exosomes. For example, exosomes derived from tumor cells that bear Hsp70 on the surface contain this protein, whereas exosomes isolated from Hsp70 surface negative cells do not display this hsp. These exosomes were assumed to be derived from the inward budding of endosomes and also to contain tubulin, Hsc70, and Rab4 (39). Other studies have assumed the presence of Hsp70 in the lumen of exosomes (16, 24). Our results clearly showed the presence of Hsp70 in the membrane of these released membrane structures, which were apparently derived from the plasma membrane. A quimeric protein between Hsp70 and YFP in which the former was added to the C terminus of Hsp70 could not be detected in membranes isolated from transfected cells with this construct. In addition, ECM derived from cells transfected with the YFP-Hsp70 construct were negative for the presence of the quimeric Hsp70. These observations suggest that the appearance of Hsp70 in the plasma membrane precedes the release of this protein into ECM.

Purified Hsp70, as well as recombinant protein, has been shown to activate cells of the immune system, in particular macrophages (5). We found that ECM containing Hsp70 isolated from HS HepG2 cells were capable of mounting a robust activation of macrophages, at least at the level of TNF- $\alpha$  production. The production of TNF- $\alpha$  after incubation with Hsp70-positive ECM was 260-fold higher than that observed with recombinant Hsp70. The protein pattern of ECM derived from HS or control cells was overall very similar, with the exception of Hsp70. Thus, the difference in macrophage activation between these vesicles is likely due to the presence of Hsp70. In fact, inhibition of Hsp70 expression during stress by blockers of transcription resulted in ECM that did not display the robust activation of macrophages observed in Hsp70-bearing ECM. This assumption is supported by the fact that Hsp70 is the only hsp detected in ECM. Several possibilities could be assumed to explain the high degree of macrophage activation displayed by membrane-bound Hsp70. On one hand, it is possible that the membrane environment makes Hsp70 a better target for macrophages that could recognize and engulf these membranous structures via phagocytosis. On the other hand, it is likely that Hsp70 within membranes is in a multimeric form, which may be more active in triggering a response by macrophages. Similarly, previous observations have shown that exosomes containing Hsp70

stimulated the cytosolic capacity of NK cells (17). Hsp70 has been found in serum obtained from patients suffering from an array of conditions, such as cancer (40, 41), diabetes (42), coronary artery disease (43, 44), myocardial infarction (45), and trauma (46). The presence of Hsp70 in circulation within membranes or in a free form still needs to be established. Finally, we cannot discard the possibility that the release of Hsp70 could be part of a mechanism to limit the presence of Hsp70 in cells. There is evidence that Hsp70, despite being protective in the short term, could be cytotoxic in the long term (14, 47). Indeed, the expression of Hsp70 is tightly regulated at the level of transcription (48) and mRNA stability (49). It is possible that the release of Hsp70 upon interaction with lipid rafts is an additional mechanism for avoiding a secondary negative effect of this protein.

In summary, we have shown that Hsp70 is present on the cell surface. We propose that Hsp70 translocates into the plasma membrane before the release of this protein in a membrane-associated form, which may be derived by inverse evagination, exocytosis, or membrane shedding. Hsp70 within these membranes could act as an immune modulator, serving as a danger signal. Thus, it could be speculated that ECM-bound Hsp70 is involved in the activation of the immune system to generate a systemic response to a localized insult to avoid the propagation of the initial stress.

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## Disclosures

The authors have no financial conflict of interest.

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