Journal of Cell Science & Therapy

Pradeep et al., J Cell Sci Ther 2016, 7:5
DOI: 10.4172/2157-7013.1000254

Research Article Open Access

Nucleolin Transports Hsp72 to the Plasma Membrane Preparatory to its Release into the Microenvironment

Appukuttan NR Pradeep[1], Alexzander Asea[2] and Punit Kaur[3,4]*

[1]Divisions of Investigative Pathology, Scott and White Memorial Hospital and Clinic, Texas A&M Health Science Center College of Medicine, Texas, USA
[2]Department of Neuroscience Research and Deanship for Scientific Research, University of Dammam, Dammam Khobar Coastal Road, Dammam, 33441 Saudi Arabia
[3]Department of Microbiology, Biochemistry and Immunology, Morehouse School of Medicine, 720 Westview Drive SW, Atlanta, GA 30310, USA
[4]Department of Experimental Radiation Oncology, University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030, USA

Abstract

Purpose: Our previous studies demonstrated that thermal stress induces the release of Hsp72 from cells by a mechanism independent of the classical protein transport pathway. However, the exact mechanism by which Hsp72, a leaderless protein, gains access to the extracellular milieu remains unknown.

Materials and methods: This study is designed to determine the mechanism by which intracellular Hsp72 trafficking and release occurs. The data presented in this study suggests intracellular Hsp72 trafficking using Western blotting and Flow Cytometry. Also, unknown membrane bound proteins were identified doing in-gel digestion and LC-MS/MS.

Results: We demonstrate that within 60 minutes after first exposure of cells to heat shock treatment, plasma membrane bound Hsp72 is shed and redistributed into cytosolic compartments. Inhibition of active cell transport by pre-treatment of cells with Cytochalasin B completely abrogated Hsp72 redistribution from the plasma membrane into the cytosol. Cross-linking of plasma membrane bound proteins with Hsp72 followed by Western blot analysis and LC-MS/MS analysis revealed at least seven interacting partners with Hsp72, including nucleolin, Hsp90, gp96, CAP2, TLR2, 4 and 7. Transfection of cells with nucleolin-siRNA completely inhibited baseline and heat shock-induced Hsp72 release.

Conclusions: Taken together, this study for the first time demonstrates that the plasma membrane acts as a reservoir for Hsp72 and suggests that nucleolin plays an important role in Hsp72 trafficking and release.

Keywords: Cell trafficking; Hsp72; Heat shock; LC-MS/MS; Membrane proteins; Nucleolin; Protein transport; siRNA

Abbreviations: CAP2: Adenylyl Cyclase Associated Protein 2; ER: Endoplasmic Reticulum; gp96: Glucose Regulated Protein 96; HSF: Heat Shock Factor; HSP: Heat Shock Protein family; *hsp*: Heat Shock Protein gene; Hsp72: Seventy-two kilo-Dalton Heat Shock Protein; MHC: Major Histocompatibility Complex; NK: Natural Killer; TLR: Toll-like Receptors.

Introduction

The seventy-two kilo-Dalton heat shock protein (Hsp72) belongs to a family of highly conserved group of proteins involved in protein folding, stabilization and cytoplasmic transportation. Hsp72 expression is induced by various stressors, including heat shock, radiation, heavy metals and infection by disease causing microorganisms [1]. The Hsp72 is not only expressed within the cytoplasm, but also in the plasma membrane of cells, from where it is actively released into the extracellular milieu to bind to and be internalized by professional antigen presenting cells [2,3]. The extracellular release of Hsp72 was initially reported in neuronal cells by Hightower and Guidon [4]. Recently, the scavenger receptor, LOX-1 [5], Toll-like receptors (TLR) [6-9], CD14 [10,11], CD91 [12] and CD40 [13] have been demonstrated to effectively bind extracellular Hsp72. Internalization of extracellular Hsp72 has been suggested to require endocytosis through binding with lipid components present in plasma membrane [9]. Although the chaperokine activity of extracellular Hsp72 is now well accepted [14], the exact mechanism by which Hsp72 passes through the plasma membrane and thus gains access to the extracellular milieu is less well understood. Recently it has been shown that plasma membrane Hsp72 enhances the plasma membrane density of CD56/CD94 and initiates the cytolytic activity of NK cells [15]. In addition, plasma membrane Hsp72 has been shown to bind with nucleotide binding and substrate binding domains at distinct sites that are differentially localized on the membrane of macrophage cell lines [16]. In this study, we demonstrate that heat shock induces the shedding and subsequent subcellular redistribution of Hsp72 from the plasma membrane into cytosolic compartments. We also demonstrate that the plasma membrane acts as a reservoir for Hsp72 and that heat shock stimulates complex formation between Hsp72, Hsp90, nucleolin, gp96, CAP2, TLR2, 4 and 7.

Materials and Methods

Cell culture conditions

THP-1 cells were purchased from American Type Cell Culture (ATCC; **Manassas, VA**) were maintained at 37°C with 5% CO_2, in complete medium RPMI 1640 containing 10% FBS and antibiotics

***Corresponding author:** Kaur P, Department of Microbiology, Biochemistry and Immunology, Morehouse School of Medicine, 720 Westview Drive SW, Atlanta, GA 30310 and Department of Experimental Radiation Oncology, University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030, USA, Tel: +1(404)756-5795; Fax: +1(404)7521179; E-mail: pkaur@msm.edu, pkaur@mdanderson.org

Received: September 19, 2016; **Accepted:** October 26, 2016; **Published:** October 28, 2016

Citation: Pradeep ANR, Asea A, Kaur P (2016) Nucleolin Transports Hsp72 to the Plasma Membrane Preparatory to its Release into the Microenvironment. J Cell Sci Ther 7: 254. doi: 10.4172/2157-7013.1000254

(Penstrep, Gibco). Survival rate of the culture was assessed by Trypan blue exclusion method. Cells were cultured to 1×10^6 per mL and used for experiments. The insect cell line Sf9 (ATCC) was cultured at 27°C, without CO_2, in insect cell culture medium (Novagen) containing 5% FBS (Invitrogen) and 0.1% Pluronic F68 Prill (BASF). Sf9 was used for over expression of Hsp72 under aseptic conditions.

Flow cytometry

THP-1 cells (10^6) were maintained at 37°C in complete medium with 10% FBS. Before analysis of plasma membrane expression of Hsp72, normal THP-1 cells were kept in FBS-free medium at 4°C for 2 h and washed with ice-cold PBS twice. The cells were incubated with the human Hsp72 monoclonal antibody (C92F3A-5) (ADI-SPA-810AP-D, Enzo Life Sciences) for 30 minutes on ice in 1/100 dilution. The cells were washed with PBS twice and incubated with FITC-conjugated goat anti mouse IgG (AP124F, Sigma-Aldrich) in 1/100 dilution for 30 minutes on ice. After washing with PBS twice, the cells were resuspended in 500 µL of PBS and filtered to FACS tube to estimate the plasma membrane expression. For the analysis of total Hsp72 expression, the cells were washed with 1% PBS-BSA and treated with 500 µl of a fixation and permeabilization reagent, Permiflow™ (Invirion, Oak Brook, IL) that made the cell wall porous. All washings were done using 1% PBS-BSA and other procedures remain same as described above. Analysis was done using a FACS Area II Flow Cytometer (BD Biosciences). Individual cells were gated based on forward (FSC) and orthogonal scatter (SSC). The photomultiplier (PMT) for FITC (FL1-height) was set on a logarithmic scale. Cell debris was excluded by raising the FSC-height PMT threshold. The flow rate was adjusted to <200 cells/second and at least 30,000 cells were analyzed for each sample.

Sucrose gradient ultracentrifugation

A stock of 60% sucrose was prepared using ultrapure sucrose (SigmaUltra) in 10 mM HEPES buffer (pH 7.4). From the stock, gradient of 20%, 30%, 40% and 50% sucrose was prepared in 10 mM HEPES buffer and syringe-filtered. Layering of the sucrose was done above the 60% sucrose bottom layer and in a gradient fashion in the decreasing order with 20% sucrose as the top layer. Protein sample (400 µL) of control and heat shocked cells after normalization for 0 h, 5 h, 11 h and 23 h in 10% 10 mM HEPES-sucrose were layered on the sucrose gradient. The gradient was ultracentrifuged at 28000 RPM for 16 h at 4°C in a swinging-bucket MLS-50 rotor using Beckman Optima-Max bench top ultracentrifuge. Eight fractions of 15 drops (or 500 µl) each were collected from each tube and diluted with equal quantity of ice cold 10 mM HEPES buffer. The diluted samples were ultracentrifuged at 40,000 rpm for 2 h at 4°C. The pellet was collected and diluted in 2X Sample loading buffer [17] without β-mercaptoethanol and bromophenol blue. Protein content was quantified by Biorad-BSA assay and 50 µg protein was resolved on 10% SDS-PAGE and used for Western blotting.

Western blot analysis

Fifty microgram protein was resolved on 10% SDS-PAGE gel and blotted on PVDF membrane by Western transfer. The blot was blocked with 5% BSA in TBS with Tween-20 (TBST) and probed with primary antibodies against human Hsp72 (C92F3A-5) (ADI-SPA-810AP-D, Enzo Life Sciences), human Hsp90 (16F1) (ADI-SPA-835-D, Enzo Life Sciences), CAP2 (ALX-804-655-C100, Enzo Life Sciences), Nucleolin (sc-8031 Santa Cruz Biotechnology, CA), human gp96 (Zymed Labs, Invitrogen), or human β-integrin antibody (sc-8978, Santa Cruz Biotechnology) and countered stained with secondary antibody (anti-mouse HRP (1:15000) (BD Pharmingen). Signals were detected on X-ray film after treatment with chemiluminiscent substrate (Pierce).

Expression and purification of endotoxin-free recombinant Hsp72

Endotoxin-free recombinant Hsp72 was produced using the baculovirus expression vector system (BEVS) and expressed in insect cells as described in detail previously [18]. Briefly, recombinant baculovirus transfer plasmids containing individual inserts (*hsp70*) were transfected along with the linearized wild-type baculovirus (*Autographa californica* nuclear polyhedrosis virus, AcMNPV) DNA (home-made) into Sf9 cells. The transfection procedure was performed as described previously [19]. Sf9 cells were seeded into T25 flasks at a density of 2.0×10^6 cells per flask. After 1 hour, media was removed and replaced with 0.75 ml Grace's insect medium (Sigma Aldrich, G9771) containing 10% FBS. 0.75 ml transfection buffer (25 mM HEPES, pH 7.1, 140 mM NaCl, 125 mM $CaCl_2$) was added to a 1.5 ml polypropylene tube which contains 1 µg of linearized AcMNPV DNA and 2.5 µg baculovirus transfer vector. The cells were incubated at 27°C for 4 hours following adding DNA solution dropwise to the Grace's insect medium in the flask. Medium was removed and replaced with 5ml BacVector Insect Cell Medium. After 6 days, post-infection, medium was collected and recombinant virus was identified and purified by plaque assays. Recombinant viruses were plaque-purified three times to eliminate contamination by wild-type baculovirus. To confirm the recombinant virus containing corresponding *hsp72* gene, recombinant viral DNAs were isolated and examined for the correct gene insert by PCR. The two primers used for PCR verification of recombinant virus production were EcoRV-For (5′-CCATTGTAATGAGACGCAC-3′) and DOWN1629-Rev (5′-CTGTAAATCAACAACGCACAG-3′). Sf9 cells were infected with purified recombinant virus from each purified plaque at a multiplicity of infection (MOI) of 10. After 72 h post infection at 27°C, Sf9 cell culture was collected and added 0.05 culture volume of Insect PopCulture Reagent (Novagen, San Diego, CA), followed by 4U Benzonase Nuclease per 1 ml of the original culture volume. The mixture was inverted gently and incubated at room temperature for 15 min. The cell pellets were removed by centrifugation for 20 min at 15,000 rpm (4°C). For purification of His-tagged proteins, the supernatants were subjected to metal-chelation column chromatography using His-Bind resin (Novagen) equilibrated with column buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 1 mM imidazole, pH 8.0). The column was washed twice with 10 ml of wash buffer (300 mM NaCl, 50 mM sodium phosphate buffer, pH 8.0) containing 5 mM imidazole. The bound proteins were eluted with elute buffer (300 mM NaCl, 50 mM sodium phosphate buffer, pH 8.0) containing 250 mM imidazole. Fractions containing Hsp72 protein was further desalted and identified by Western Blot and Mass Spectrometry. Purified proteins were analyzed for endotoxin content using the *Limulus* amebocyte lysate assay (Cambrex). Protein concentration was detected by *RC DC* protein assay (Bio-Rad).

Plasma membrane cross-linking assay

To identify the interacting proteins of Hsp72 on cell plasma membrane, an experimental protocol has been followed, combining biotin labeling, UV cross-linking as well as DTT reduction, induced biotin transfer to interacting molecules by label transfer experiments. Biotinylated complexes were identified from the reduced and non-reduced fractions that had been resolved on 10% SDS-PAGE gel. Hsp72 was labeled with 3M Sulfo-SBED dissolved in DMSO (dimethyl sulfoxide, cell culture grade; Gibco) using Pro Found label transfer kit (Pierce) following manufactures' instructions and dialyzed to label transfer buffer (Pierce) for 24 h at 4°C under dark. Briefly, Sulfo-SBED contains tri-functional arms [20], an amine reactive sulfo-NHS ester on

one arm which has a cleavable disulfide bond which permits transfer of biotin, a photoreactive phenyl azide group on the other side and a biotin handle that will be transferred to the interacting partner. On UV cross-linking, interacting molecule (prey protein) will be coupled with bait protein (SulfoSBED labeled Hsp72 in this case) through covalent cross-link. On treatment with a reducing agent such as DTT (dithiothreitol), biotinylated arm will be transferred to the interacting molecule which can be detected by streptavidin. Ten-micrograms of Hsp72 was labeled with sulfoSBED and kept in dark at -80°C and used within 24 h for the experiments. THP-1 cells were maintained at 1×10^6 cells per mL per well of 24 good plate (BD Sciences) at 37°C in FBS-free medium for 24 h and used for the labeling experiments. Ten-micrograms of the Hsp72 labeled with SulfoSBED was added to the well and incubated for 1 h at 4°C, in ice, under dark. Immediately after the incubation, the cells kept on ice were exposed to UV (Stratalinker) for 5 minutes at a distance of 5 cm at 12,000 microjoules for cross-linking. The cross-linked cells were centrifuged at 10,000 rpm for 5 min at 4°C and pelleted. After washing with PBS, the pellet was lysed with cell lysis buffer (Cell Signaling) containing 10X protease inhibitor and phosphatase inhibitor and centrifuged at 13,000 rpm for 15 minutes at 4°C. Protein content of the supernatant was assessed by BSA standard-Biorad assay. A sample of the cell lysate with 50 micrograms of the protein was reduced with 100 mM DTT and another sample was used without reduction. Both reduced and non-reduced samples of cell lysate of normal THP-1 cells, only UV linked cells, only labeled cells and labeled plus UV linked cells were analyzed. Each sample was mixed with Lamelli's buffer containing bromophenol blue and denatured at 95°C for 5 minutes, loaded and resolved on 10% SDS-PAGE gel. Simultaneously resolved gels were either used for Western blotting or Coomassie (Biosafe, Biorad) staining. The blot was probed with streptavidin (Pierce) to identify the biotinylated fragments resolved on the gel. Simultaneously, from the gel stained with Coomassie, the biotinylated fragments were identified using mass spectrometry.

Laser scanning confocal microscopy

Plasma membrane localization and internalization of Hsp72 was observed in control and heat shocked (43°C for 1 h) THP-1 cells under FBS-free conditions. The cells were incubated at 37°C for 0 h and 5 h after the heat shock, washed with 25 mM HEPES solution, blocked with 5% BSA for 20 minutes at 4°C and incubated with antihuman Hsp72 antibody for 1h at 4°C and fixed in 2% paraformaldehyde and further incubated with FITC-conjugated IgG for 30 minutes at 4°C in dark. All washings were done using PBST. After washing with PBST, cells were mounted in DAPI (Vector) medium for nuclear staining. The cells were observed under fluorescent microscope. To recognize the co-localization of Hsp72, Gp96, and nucleolin proteins on the cell plasma membrane, THP-1 cells were blocked with 5% BSA, simultaneously labeled with primary monoclonal antibodies for 1 h at 4°C, fixed in 2% paraformaldehyde in PBS and incubated with secondary antibodies conjugated with different fluorescent stains, FITC, Phycoerythrin and Alexa 405 for 30 minutes at 4°C under dark. Three washes with PBST were allowed after incubation with the primary and secondary antibodies. THP-1 cells were heat shocked and the co-localization studies were made after 0 h and 23 h of normalization at 37°C. Microscopic observations were made using a FV1200 Laser Scanning Confocal Microscope (Olympus) with sequential scanning feature turned on to eliminate the possibility of cross-excitation. All the experiments were performed at least twice and representative figures are given.

In-gel digestion and LC-MS

The biotinylated fragments were processed by in-gel digestion with trypsin. After reduction and alkylation, the gel pieces were digested overnight with sequencing grade trypsin (Promega) at 37°C. The peptide samples were zip-tip cleaned with trifluroacetic acid (TFA) and acetonitrile mixture and dried in Speedvac. The samples were re-dissolved in 0.1% TFA, fractionated and mass spectra was obtained by automated LC-MS/MS analysis (Agilent). CID data was searched against the SwissProt all species database, using the Agilent Spectrum Mill Server software (Rev A.03.03.) installed on a HP Intel® Xeon (TM) dual processor server. Peak lists were created with the Spectrum Mill Data Extractor program with the following attributed: scans with the same precursor ± 1.4 m/z were merged within a time frame of ± 15 seconds. Precursor ions needed to have a minimum signal to noise value of 25. Charges up to a maximum of 7 were assigned to the precursor ion and the 12°C peak was determined by the data extractor. The SwissProt database was searched for tryptic peptides with a mass tolerance of ± 2.5 Da for the precursor ions and a tolerance of ± 0.7 Da for the fragment ions. Two missed cleavages were allowed. A Spectrum Mill autovalidation was performed first in the protein details followed by peptide mode using default values (Minimum scores, minimum scored peak intensity (SPI), forward minus reversed score threshold, and rank 1 minus rank 2 score threshold). All protein hits found in a distinct database search by Spectrum Mill are non-redundant. The LC-MS/MS data are representation of three independent experiments.

Statistical analysis

Data was analyzed using a two-tailed *t*-test after applying ANOVA to the data. Differences were considered significant when $p<0.05$.

Results

Heat shock induces the shedding and redistribution of plasma membrane bound Hsp72

Initial studies were performed to determine the effect of heat shock on plasma membrane bound Hsp72. Flow cytometric analysis of plasma membrane Hsp72 on THP-1 human monocytic cells demonstrated a slight albeit significant decrease in plasma membrane bound Hsp72 after heat shock (43°C, 60 min) treatment as compared to controls maintained at 37°C for 60 minutes (Figure 1). Before the FACS analysis, cells were maintained for 24 h in FBS-free medium to avoid the influence of FBS on internalization as previously demonstrated [8,21]. Plasma bound Hsp72 was detected on >90% THP-1 cells under control conditions, 37°C, 60 min (Figure 1A). Exposure of THP-1 cells to heat shock (43°C, 60 min) and immediate measurement of Hsp72 resulted in the complete loss of plasma membrane bound Hsp72, when measured 2 h post exposure, with <5% THP-1 cells expressing Hsp72 on the cell surface (Figure 1E).

To determine whether the plasma membrane Hsp72 was shed to the extracellular milieu or redistributed into the cell. THP-1 cells were grown on microscope slides, heat shocked, permeabilized and counter stained with Hsp72-FITC. Confocal microscopy images revealed that heat shock (43°C, 60 min) induced the redistribution of Hsp72 primarily from the plasma membrane under control conditions (37°C, 60 min; Figure 1B-1D), to the cytoplasm (Figure 1F-H).

To determine the kinetics of heat shock-induced redistribution of Hsp72, THP-1 cells were heat shocked at 43°C for 60 min and incubated at 37°C for a further 0 h, 5 h, 11 h and 23 h. Homogenized cells were layered over a sucrose gradient and the recovered fractions were subjected to Western blot analysis. Subcellular fractions 1-4 were demonstrated to be plasma membrane fractions due to the expression of β-integrin (loading control for control and heat shock treatment) in these fractions (Figure

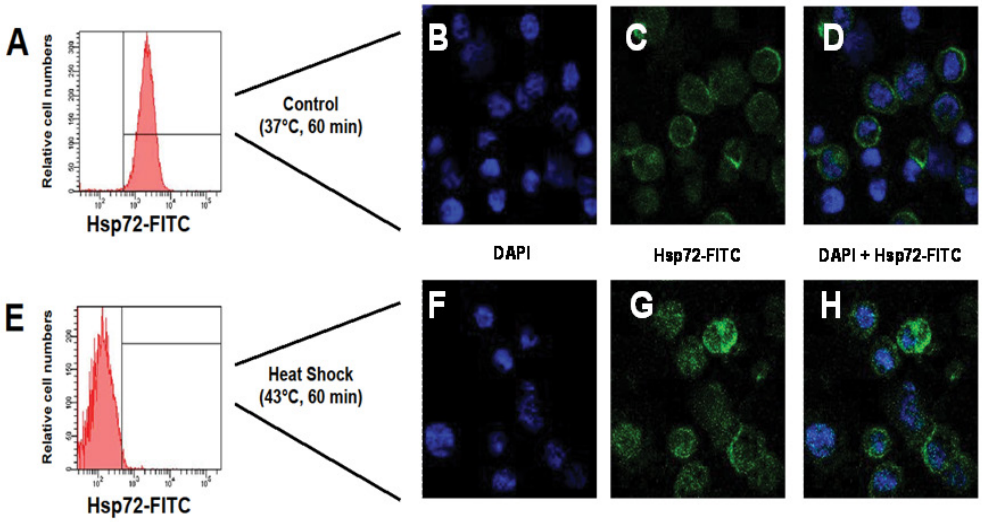

Figure 1: Plasma membrane bound Hsp72 is shed and redistributed into the cytoplasm immediately following thermal stress. **A** THP-1 cells (10^6) were maintained at 37°C for 60 minutes or **E** exposed to heat shock (43°C for 60 minutes) and immediately incubated with mouse anti-human Hsp72 antibody or isotype control, and the relative expression of Hsp72 was measured by flow cytometry as described in detail in the Experimental Procedures section. Histograms represent the relative number of THP-1 cells expressing Hsp72 from the gated population of cells and is a representative experiment from six independently performed experiments with similar results. THP-1 cells (10^4) were grown on Falcon Culture Slides (BD Labware, Franklin Lakes, NJ) overnight and either maintained at 37°C for 60 min (top panels) or exposed to heat shock (43°C for 60 min; bottom panels). Cells were later washed twice with ice-cold PBS and simultaneously fixed and permeabilized using Cytofix/Cytoperm™ kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions and counter stained with anti-Hsp72-FITC. One drop of mounting media containing DAPI stain to visualize nuclear structures (Oncogene, Boston, MA) was placed onto the glass slide before the cover slip was sealed with nail polish. Results show fluorescence microscope pictograms using laser confocal microscopy; **B** and **F** DAPI stain that reveal the cells nuclear morphology (blue); **C** and **G** FITC stained cells to reveal the intensity of total Hsp72 expression (green); **D** and **H** merged image of DAPI overlaid with Hsp72-FITC. Results are a representative experiment from four independently performed experiments with similar results.

2). Reprobing the gels with anti-Hsp72 revealed that at 0 h post heat shock treatment, Hsp72 could be expressed in all the subcellular fractions 1-8 (Figure 2). From 5 h to 23 h post heat shock treatment, we demonstrate a redistribution of Hsp72 from the plasma membrane fraction to the fractions representing the cytosolic fractions (Figure 2). To confirm that the redistribution of Hsp72 from the cell plasma membrane to the cytosol is an active process requiring various components of the cell transport system, THP-1 cells were pre-treated with Cytochalasin B (50 μM/ml) prior to heat shock treatment. This concentration of Cytochalasin B was chosen since it does not induce significant cell death but disrupts the lipid raft membrane (data not shown). We demonstrate that Cytochalasin B changed the redistribution of Hsp72 in the plasma membrane and cytosolic compartments (Figure 2).

Identification of protein partners complexed with Hsp72 in the plasma membrane

To determine the identity of proteins which are complexed to Hsp72 in the plasma membrane, we produced functional endotoxin-free recombinant human Hsp72 using a baculovirus expression vector system (BEVS) and expression the protein in Sf9 insect cells [18]. The purity of the Hsp72 protein from the eluate was determined both by Western blot analysis and shown to be free of other contaminating proteins by Coommassie blue staining (data not shown). To identify proteins that interact with Hsp72 within the plasma membrane, sulfoSBED labeled-Hsp72 was added as a bait protein to THP-1 cells and UV light was used to cross-link neighboring interacting proteins (prey protein) with Hsp72 on the plasma membrane. The cross-linked cells were lysed and the protein was resolved on 10% SDS-PAGE under non-reducing and reducing (with DTT) conditions and processed for Western blot analysis. Principally, DTT reduction transfers the biotin arm from the SulfoSBED-labeled Hsp72 (bait) to the interacting proteins (prey) and separates the interacting cross-linked proteins from the labeled Hsp72. Biotinylation of these fragments was detected on Western blot labeled with HRP-conjugated streptavidin (Fig. 3A). Low molecular weight, biotinylated fragment (~70 kDa) was resolved from samples reduced with DTT along with high molecular weight biotinylated fragments (110 kDa, 95 kDa, 72 kDa and 70 kDa) from reduced samples (Figure 3A).

To further identify the interacting proteins, the biotinylated bands from the gel were cut using a spot cutter, in-gel digested and the peptides were purified and analyzed by LC-MS/MS. A total of 143 peptides of proteins interacting with Hsp72 were identified from the reduced lysate samples (Table 1). Peptides of Hsp90α, TLR2, TLR4, TLR7 (Toll-like receptors), gp96 (tumor rejection antigen), cyclic AMP associated protein-2 (CAP2), nucleolin and calnexin precursor were identified from the different biotinylated interacting protein conjugates (Table 1). Additional proteins identified include cytoskeletal proteins such as actin, β-integrin and tubulin. Importantly, 84 peptides of Hsp90β were identified from different fragments, whereas only one peptide of Hsp90α was identified from the ~95 kDa fragment. We validated these results using Western blot analysis with antibodies directed against Hsp90α, CAP2, gp96, TLR2, TLR4, TLR7 and nucleolin determined that all five proteins co-localized in the ~110 kDa fragment resolved from the reduced samples (Figure 3B). Calnexin precursor was absent in this fragment and hence further studies on this protein were not conducted (data not shown). Confocal microscopy was used to visually confirm the co-localization of Hsp72 with nucleolin and gp96. Under control conditions (37°C, 60 min), there was weak co-localization of all three proteins, as indicated by the arrow (Figure 4; left column, arrow). However, exposure of THP-1 cells to heat shock (43°C, 60 min) treatment, resulted in significant co-localization of both nucleolin and gp96 with Hsp72 (Figure 4; right column).

To demonstrate the relative contribution of nucleolin in the cytosol

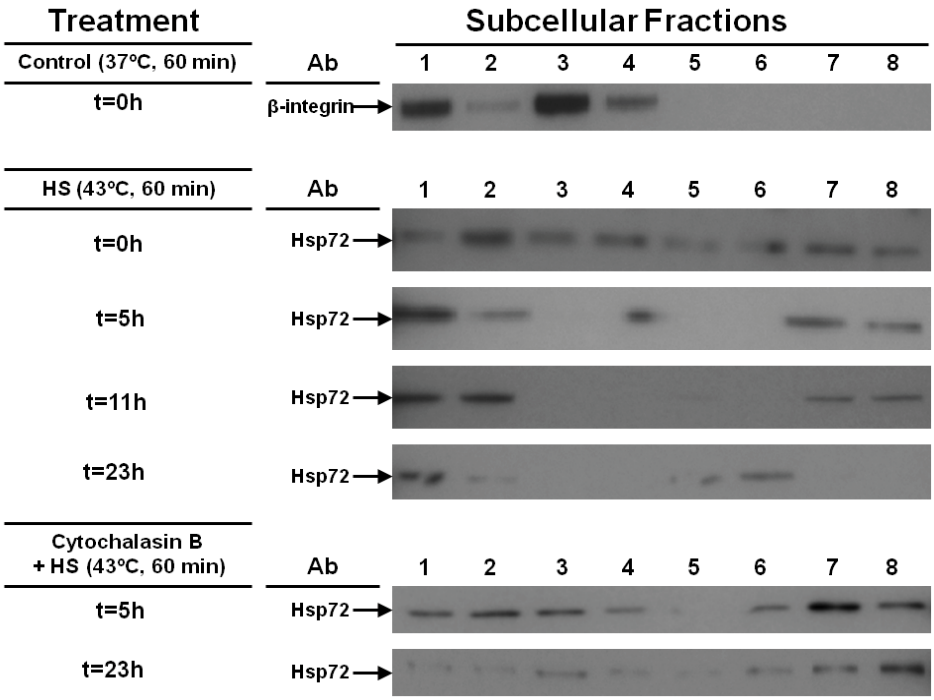

Figure 2: Heat shock-induced redistribution of Hsp72 from the plasma membrane to the cytoplasm is abrogated by pre-treatment with transport inhibitor. THP-1 cells (10^6) were maintained at 37°C (top panel) or exposed to heat shock (43°C for 60 min; middle panels) or pre-treated with Cytochalasin B (50 μM/ml) and exposed to heat shocked (43°C for 60 min; bottom panel). At 0 h, 5 h, 11 h and 23 h post heat shock treatment cells were lysed and homogenized in 10% HEPES-sucrose buffer, overlaid onto a sucrose gradient and ultracentrifuged at 28,000 rpm for 16 h. Eight subcellular fractions (1-8) were collected for each time point and ultracentrifugation at 40,000 rpm for 2 h. The precipitated protein was resolved onto a 10% SDS-PAGE and Western blotted with anti-human β-integrin or anti-human Hsp72. Data is a representative experiment from seven independently performed experiments with similar results.

in heat shock-mediated Hsp72 trafficking, the expression of nucleolin in THP-1 cells was silenced using specific nucleolin-siRNA prior to heat shock treatment. Initial experiments demonstrated that nucleolin-siRNA (Nu-siRNA) effectively suppressed baseline (Ctrl; 37°C, 60 min) and heat shock (43°C, 60 min) induced nucleolin expression (Figure 5A) and Hsp72 expression (Figure 5B) in THP-1 cells, as judged by Western blot analysis. We consistently obtained the knockdown efficiency of 900% on the expression of both nucleolin and Hsp72 respectively in HT vs. Nu-siRNA+HT. Pre-treatment of THP-1 cells with Cytochalasin B strongly inhibited baseline and heat shock-induced nucleolin (Figure 5C). Although similar treatment did not significantly alter the baseline expression of Hsp72, it drastically suppressed heat shock-induced Hsp72 expression (Figure 5D). Scrambled siRNA was used as a control (data not shown). To determine the role of the proteins shown to co-localize with Hsp72 on its release, THP-1 cells were transfected with a specific siRNA prior to heat shock treatment and Hsp72 release measured using the classical sandwich Hsp72 ELISA. We demonstrated that silencing nucleolin expression using nucleolin-siRNA strongly suppressed baseline and heat shock-induced Hsp72 release (Figure 5E). However, similar suppression of Hsp72 release was not observed using Hsp90-siRNA (Figure 5F), gp96-siRNA (Figure 5G), CAP2-siRNA (Figure 5H), TLR2-siRNA (Figure 5I), or TLR4-siRNA (Figure 5J) or TLR7-siRNA (Figure 5K).

Discussion

This study was undertaken to determine the effect of heat shock on plasma membrane bound Hsp72. The THP-1 human monocytic cell is a good model system for studying Hsp72 trafficking since Hsp72 is highly expressed on the cell plasma membrane under control conditions. The biological significance of plasma membrane bound Hsp72 is demonstrated in its ability to act as a target structure for natural killer (NK) cell-mediated cytotoxicity [22] and elicit anti-tumor immunity [23]. Increased expression of Hsp72 on the plasma membrane is consistent with our earlier findings [6] and reports from various tumor cell line [24]. Different classes of HSP on tumor cells differentially activate monocytes and granulocytes to generate pro-inflammatory cytokines [10,25], transfer antigen to APC to stimulate tumor specific T-cells [26,27], enhance the ability of tumors to process and present MHC-class I antigens directly to T-cells [28] or induce tumor cell lysis mediated by non-MHC restricted NK cells [29,30]. However, the overexpression of plasma membrane bound Hsp72 induced negative feedback to prevent activation of heat shock factor (HSF) and subsequent Hsp72 production [31]. The significant decrease in plasma membrane expression of Hsp72 after the heat shock of THP-1 cells (Figure 1) suggests a similar attenuated expression of HSP observed under stress conditions such as aging and heat shock induced in fibroblasts, T lymphocytes, Jurkat cells and CCRF-CEM leukemia cell lines [32]. The selective depletion of Hsp72 was found to enhance malignant cell immunogenicity in rat colon cells [33], and its internalization was facilitated by its binding with lipid raft present in the cell membrane [9,34,35].

To track the redistribution of Hsp72 within the subcellular components, sucrose gradient ultracentrifugation combined with Western blotting was performed and revealed the redistribution of Hsp72 in all the subcellular fractions immediately after the heat shock (Figure 2). The redistribution of Hsp72 was limited to 5 subcellular fractions after 5 h of normalization and to 4 subcellular fragments after 11 h of normalization, indicating inter-organellar transport of Hsp72

Fragment size (kDa)	Protein ID	Accession number	Number of peptides	Molecular weight (kDa) of the peptides (range)
110	Hsp70[1]	16507237	2	944.523-1838.013
	Hsp70[2]	24234686	6	
	Hsp70[3]	13676857	3	
	Hsp70[4]	62896815	1	
	Hsp70[5]	5729877	1	
	Hsp90[6]	20149594	9	1141.56-1847.797
	CAP[7]	5453595	10	1151.515-2073.035
	Nucleolin	55956788	7	937.499-1561.68
	Gp96[8]	4507677	30	961.458-2260.063
	TLR2	88878	6	911.325-2116.258
	TLR4	88880	10	945.658-2287.264
~95	Hsp70[1]	16507237	22	1074.554-2175.993
	Hsp70[2]	24234686	1	
	Hsp90[6]	20149594	28	951.464-2176.945
	Hsp90[9]	83699649	1	
	CAP[7]	5453595	6	1426.315-2073.035
	Calnexin precursor	10716563	12	902.462-2283.052
	TLR2	88878	8	899.458-2125.125
	TLR4	88880	11	920.894-2221.956
~75	Hsp70[1]	16507237	21	1074.554-2175.993
	Hsp90[6]	20149594	20	951.964-1847.797
	CAP[7]	5453595	9	1003.567-2073.035
	Calnexin precursor	10716563	10	902.462-2462.128
~70	Hsp70[1]	16507237	23	1074.554-2175.993
	Hsp70[3]	13676857	2	
	CAP[7]	5453595	11	1084.589-2073.035

[a]THP-1 cells were incubated with SulfoSBED-labeled Hsp70 and cross-linked using UV light. Proteins obtained from subcellular fractions recovered after cell lysis and sucrose gradient ultracentrifugation were resolved from the reduced lysates, *in-gel* trypsinized, purified and the protein mass spectra were determined by LC-MS/MS as described in detail in the Methods section. Data are a representative experiment from four independently performed experiments with similar results.
[1] Hsp70 kDa protein-5; [2] Hsp70 kDa protein8 isoform-2; [3] Hsp70 kDa protein-2; [4] Hsp70 kDa protein 8 isoform-2 variant; [5] Hsp70 kDa protein 8 isoform-1; [6] Hsp90 kDa protein-1 beta; [7] Adenylyl cyclase-associated protein; [8] Tumor rejection antigen (Gp96); [9] Hsp90 kDa protein 1 alpha.

Table 1: Identification of candidate proteins interacting with Hsp72 within the plasma membrane[a].

during the initial period of normalization. The absence of Hsp72 in most of the fractions and its strong presence on plasma membrane fractions by 23 h of normalization suggested a utility-based transport of Hsp72 after the heat shock, from plasma membrane to the cytosol, inter-organellar transport and subcellular compartmentalization (Figure 2). These results were confirmed by the pre-treatment of THP-1 cells with the cell transport blocker, Cytochalasin B. The detection of Hsp72 signals in all subcellular fragments indicate that pretreatment of THP-1 cells with Cytochalasin B before heat shock did not prevent internalization of Hsp72 (Figure 2). Our data suggests that heat shock influences the membrane transport of Hsp72 [36,37]. The internalization of Hsp72 appears to be an early event preparatory to signaling to elicit intracellular Ca^{2+} flux, activates NF-κB nuclear translocation and augments expression and release of inflammatory cytokines [10,35,38,39]. The continued presence of Hsp72 in all the Cytochalasin-treated subcellular fractions after 5 h of normalization demonstrates inhibition of transport of Hsp72 among the subcellular components. The absence of Hsp72 in plasma membrane fractions (fractions 1 and 2) after 23 h normalization further demonstrate the inhibition of transport of Hsp72 from cytosolic fractions to the cell membrane (Figure 2). These data suggest that Hsp72 is redistributed on the plasma membrane within 23 h of normalization after the heat shock. We hypothesize that the cell membrane acts as a reservoir for Hsp72, which will be transported into the cytosol in response to stress to induce its chaperokine activity [6] and redistribute to the plasma membrane without retaining in the cytosolic compartments after normalization.

In the present study, we demonstrated that 6 proteins including, Hsp90, CAP2, gp96, TLR2, TLR4 and nucleolin co-localize with Hsp72 on the plasma membrane. Of the Hsp72 interacting partners, we [6] and others [8,40] have previously demonstrated that TLR2 and TLR4 are surface receptors for exogenous Hsp72. Hsp90, CAP2 and gp96 are well known partners of Hsp72 [41]. However, nucleolin has not previously demonstrated to co-localize with plasma membrane Hsp72 (Table 1 and Figure 3B). To serve as an effective chaperone, Hsp72 must interact with specific binding molecules that effect membrane transport as well as antigen cross presentation. Herein, we observed that heat shock induced the transport of plasma membrane Hsp72 to cytoplasmic compartments. The co-localization of gp96 and nucleolin with Hsp72 was confirmed by immunoblotting (Figure 3B) and confocal microscopy (Figure 4H). Under control conditions (37°C, 60 min), Hsp72, nucleolin and gp96 on the plasma membrane expression was found in only a few areas. However, exposure of cells to heat shock conditions (43°C, 60 min) augmented the co-localization of Hsp72, nucleolin and gp96 throughout the cell surface (Figure 4H). We further demonstrate that nucleolin plays a major role in Hsp72 release, as demonstrated by the significant decreases in Hsp72 release in response to transfection with nucleolin-siRNA (Figure 5C), but not gp96-siRNA (Figure 5E). This is interesting because nucleolin was originally proposed to be exclusively a nuclear and cytoplasmic protein that performs functions in rRNA maturation and ribosome assembly [42]. However, recently plasma membrane expression of nucleolin was established in different cell lines, including THP-1 cells [43,44]. Several studies establish nucleolin's involvement in protein shuttling between cytoplasm and nucleus [45,46] and between the plasma membrane and nucleus [43,47-49]. We hypothesize that nucleolin acts in a similar fashion by shuttling Hsp72. Plasma membrane nucleolin is a signaling receptor for P-selectin, in human colon carcinoma [50]. Co-localization of nucleolin with Hsp72 after normalization suggests a nucleolin-mediated transport of Hsp72 from cellular compartments to the plasma membrane for the re-localization of Hsp72 after recovery from heat shock (Figure 1 and 4). Our studies are supported by earlier observations on the simultaneous increase in Hsp72 and nucleolin mRNAs in rat hepatocytes [51] and binding of plasma membrane nucleolin with the anti-HIV cytokine, midkine [43].

It has previously been suggested by us [6] and others [9] that Hsp72 recruits pattern recognition molecules such as TLR to move across the membrane over lipid rafts. These studies are strengthened by the finding that TLR2 and TLR4 are interacting partners of Hsp72 (Table 1 and Figure 3B). TLR-mediated Hsp72 action requires intermediate molecules which have yet to be identified. Suggestively, nucleolin may be capable of direct interaction with TLR which may be a "missing link adapter" that exists in the nucleolin-TLR-Hsp72 association. Our data does not support a role for TLR2, 4 or 7 in the release of Hsp72 (Figure 5G-5I).

Co-localization of gp96 with plasma membrane-bound Hsp72 and nucleolin indicated the association of gp96 with plasma membrane Hsp72. The strong expression of gp96 after heat shock indicates a stress-induced upregulation of gp96 on THP1 plasma membrane (Figure 4A-4H). Gp96 is a glycoprotein of the endoplasmic reticulum (ER)

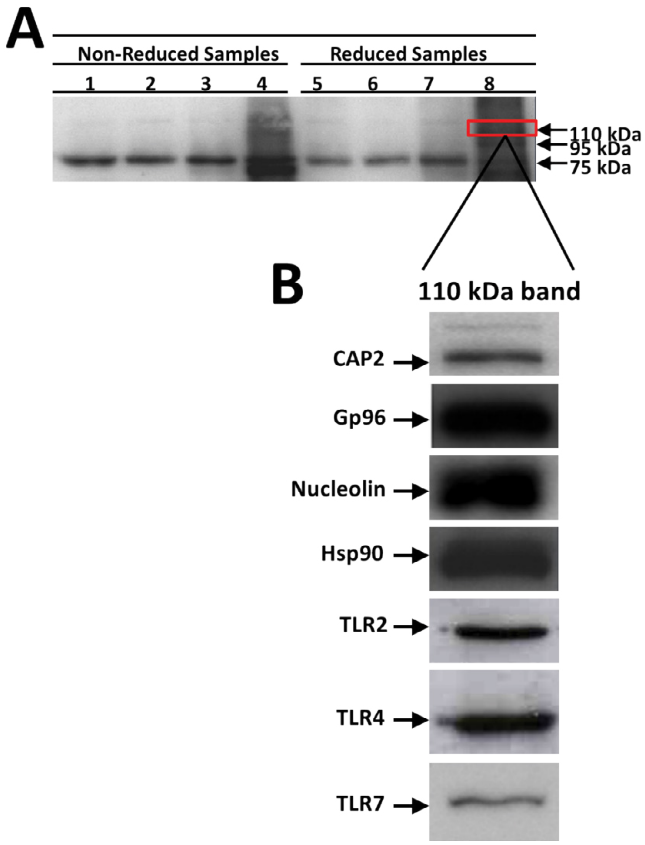

Figure 3: Hsp90, CAP2, nucleolin, TLR2, TLR4, TLR7 as possible interacting proteins with plasma membrane bound Hsp72**.** THP-1 cells (10^6) were stained with Hsp72-labeled with Sulfo-SBED that contains tri-functional arms with a biotin handle that will be transferred to the interacting partner on reduction with a reducing agent as described in detail in the Experimental Procedures section. Cells were later exposed to UV light and lysed with either DTT (reduced samples) or control (non-reduced samples). **A** The cell lysate was later denatured at 95°C for 5 min and resolved on 10% SDS-PAGE and stained with Coomassie (Biosafe, Biorad). Lane 1, non-reduced cell lysate (control); lane 2, non-reduced cell lysate from UV-linked cells; lane 3, non-reduced cell lysate from SulfoSBED-labeled cells; lane 4, non-reduced cell lysate from SulfoSBED-labeled and UV-linked cell; lane 5, reduced cell lysate (control); lane 6, reduced cell lysate from UV-linked cells; lane 7, reduced cell lysate from SulfoSBED-labeled cells; lane 8, reduced SulfoSBED-labeled and UV-linked cells lysate. Data is a representative experiment from four independently performed experiments with similar results. **B** The 110 kDa band (red rectangle) was cut, denatured at 95°C for 5 min and resolved on 10% SDS-PAGE and Western blotted with antibodies against human Hsp90 or CAP2 or nucleolin, or gp96 or TLR2 or TLR4 or TLR7. Data is a representative experiment from four independently performed experiments with similar results.

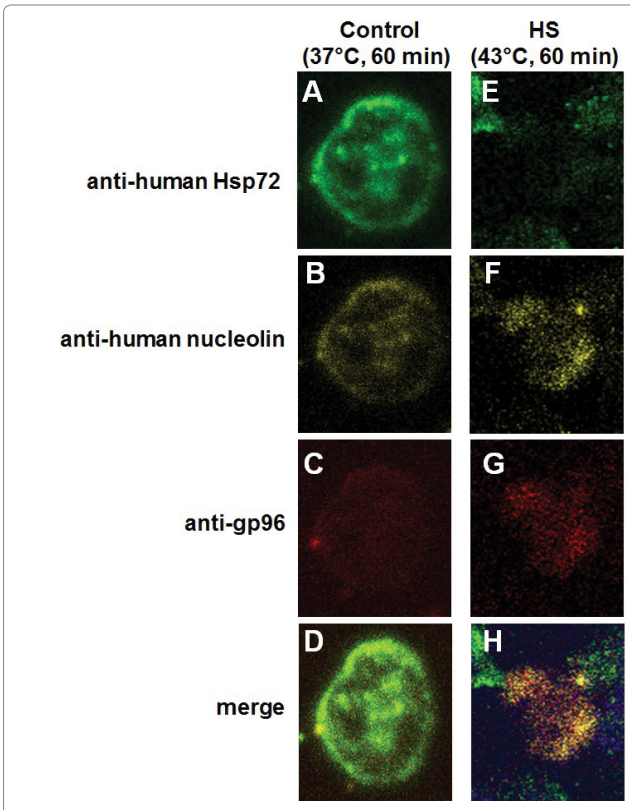

Figure 4: Heat shock augments the co-localization of Hsp72 with gp96 and nucleolin. THP-1 cells (10^4) were grown on Falcon CultreSlides (BD Labware, Franklin Lakes, NJ) overnight and either maintained at 37°C for 60 min (left column) or exposed to heat shock (43°C for 60 min; right column). Cells were later washed twice with ice-cold PBS and simultaneously fixed and permeabilized using Cytofix/Cytoperm™ kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions and counter stained with anti-Hsp72-FITC **A** and **E**, anti-nucleolin-Alexa 405 **B** and **F**, and anti-gp96-phycoerythrin **C** and **G**. One drop of mounting media containing DAPI (Oncogene, Boston, MA) to visualize nuclear structures was placed onto the glass slide before the cover slip was sealed with nail polish. Results show fluorescence microscope pictograms using laser confocal microscopy. Merged images represent an overlay of anti-Hsp72-FITC, anti-nucleolin-Alexa 405, and anti-gp96-phycoerythrin **D** and **H**. Arrow indicates single area in which co-localization can be found under control conditions **D**. Data is a representative experiment from three independently performed experiments with similar results.

and an ER paralog of Hsp90 that is involved in antigen processing as an intermediate peptide carrier. Gp96 has previously been identified as tumor specific antigen that regulates antigen presenting cells [52]. Interaction of gp96 with Hsp72 on the plasma membrane reflects the peptide-binding activity of gp96 to serve as cross-priming antigen to initiate T lymphocyte responses [27,53]. Binding of gp96 with associated proteins induces immune responses [23,54,55] and protecting cells from complete degradation-peptides [56]. However, gp96 does not appear to play a major role in Hsp72 release (Figure 5E).

Cytosolic Hsp90 regulates numerous important cellular activities and trafficking of many signal transducing proteins [57,58]. Hsp90 function is intimately coupled to a cycle of ATP binding and hydrolysis [59,60]. Mass spectrometry identified 57 peptides of Hsp90β and one peptide of Hsp90α showed preference of Hsp90β to Hsp90α to form complexes with the co-chaperone Hsp72 (Table 1). The finding of a differential interaction of the two isoforms of Hsp90 has recently been reported with another chaperone GCUNC45 [61], which has been suggested to be due to the larger presence of monomeric form of Hsp90β in the cell lysates [62]. Adenylyl cyclase associated protein-2 (CAP2) is a multifunctional protein in which the N-terminal region with amino acids 1-168 is required for heat shock sensitivity [63]. Adenylyl cyclase forms a complex with CAP [64] which was associated with $RAS2^{val-19}$-dependent heat shock sensitivity [65,66]. CAP may not be directly involved in physical association with Ras protein instead acts through alternated confirmation of adenylyl cyclase [67]. The identification of CAP2 as an interacting protein of Hsp72 (Table 1) provides evidence for indirect involvement of CAP2 in the heat shock response, probably by using the chaperone effect of Hsp72. Interestingly, silencing CAP2 expression reduced the release of Hsp72, albeit, not significantly (Figure 5F).

The role of plasma membrane Hsp72 in induction of immune system is obviously more complex than our results suggest. Our

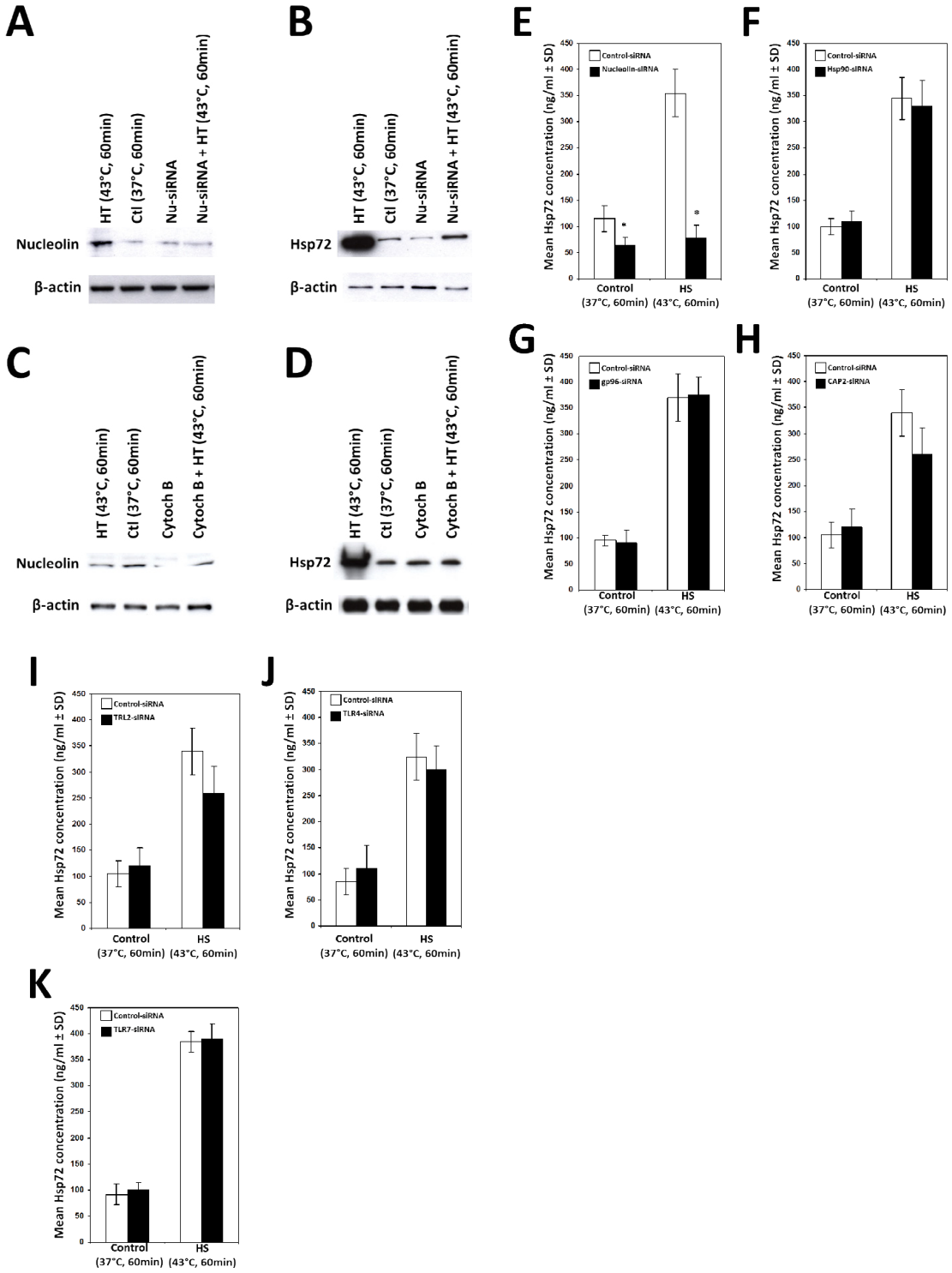

Figure 5: Silencing nucleolin prior to heat shock abrogates Hsp72 release from THP-1 cells. THP-1 cells (10^6) were pre-treated with 25 nM nucleolin-siRNA or Cytochalasin B (50 μM/ml) then exposed to either heat shock (HT; 43°C for 60 min) or maintained at 37°C (Ctl; 37°C, 60min). Twenty-four hours later cells were lysed and proteins measured using the classical Bradford method and equal amounts of protein were resolved onto a 10% SDS-PAGE and Western blotted with **A** and **C** anti-human nucleolin or **B** and **D** anti-human Hsp72. β-actin was used as a loading control in all experiments. Data is a representative experiment from seven independently performed experiments with similar results. THP-1 cells (10^6) were transfected for 48 hours with **E** 25 nM nucleolin-siRNA, **F** 20 nM Hsp90-siRNA, **G** 15 nM gp96-siRNA, **H** 25 nM CAP2-siRNA, **i** 20nM TLR2-siRNA, **J** 25 nM TLR4-siRNA, **K** 25 nM TLR7-siRNA or 25 nM control-siRNA using siPORT NeoFX (Ambion). The transfected cells were either maintained at control conditions (37°C, 60 min) or exposed to heat shock (43°C, 60 min). Five hours later, supernatant was recovered, centrifuged to remove floating dead cells and cellular debris, treated 1% Lubrol for 10 min at 4°C with gentle rocking and the concentration of Hsp72 measured using the classical sandwich ELISA as described in detail in the Methods section. Data represent the mean Hsp72 concentration (pg/ml ± SD) and is the sum of three independent experiments performed in quadruplicates. *, $p<0.05$ vs control-siRNA (Student's *t*-test).

working hypothesis is that the plasma membrane of cells functions as a reservoir for Hsp72 and thermal stress induces the redistribution of plasma membrane bound Hsp72 into subcellular cytosolic components, from where; nucleolin-mediated transport system carries the Hsp72 to the plasma membrane for its re-localization and final release into the extracellular milieu. In this fashion, the plasma membrane also acts as a reservoir for Hsp72.

Acknowledgements

This work was funded in part by Department of Biotechnology - Overseas associateship 2007 grant of Government of India (to A.N.R.P.), institutional support from Morehouse School of Medicine and U54 CA118638 (to P.K.) and the US National Institutes of Health grant RO1CA91889, Texas A&M Health Science Center College of Medicine, the Central Texas Veterans Health Administration, an Endowment from the Cain Foundation and the University of Dammam (to A.A.A.A.). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Author Contributions

Conceived and designed the experiments: AA PK. Performed the experiments: ANRP PK. Analyzed the data: ANRP AA PK. Contributed reagents/materials/analysis tools: PK AA. Wrote the paper: ANRP AA PK.

References

1. Noonan EJ, Place RF, Giardina C, Hightower LE (2007) Hsp70B' regulation and function. Cell Stress Chaperones 12: 393-402.
2. Mambula SS, Calderwood SK (2006) Heat shock protein 70 is secreted from tumor cells by a nonclassical pathway involving lysosomal endosomes. J Immunol 177: 7849-7857.
3. Tytell M, Greenberg SG, Lasek RJ (1986) Heat shock-like protein is transferred from glia to axon. Brain Res 363: 161-164.
4. Hightower LE, Guidon PT Jr. (1989) Selective release from cultured mammalian cells of heat-shock (stress) proteins that resemble glia-axon transfer proteins. Journal of Cellular Physiology 138: 257-266.
5. Theriault JR, Mambula SS, Sawamura T, Stevenson MA, Calderwood SK (2005) Extracellular HSP70 binding to surface receptors present on antigen presenting cells and endothelial/epithelial cells. FEBS Lett. 579: 1951-1960.
6. Asea A, Rehli M, Kabingu E, Boch JA, Bare O, et al. (2002) Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem 277: 15028-15034.
7. Takeda K, Kaisho T, Akira S (2003) Toll-like receptors. Annu Rev Immunol 21: 335-376.
8. Vabulas RM, Ahmad-Nejad P, Ghose S, Kirschning CJ, Issels RD, et al. (2002) HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. J Biol Chem 277: 15107-15112.
9. Wang R, Town T, Gokarn V, Flavell RA, Chandawarkar RY (2006) HSP70 enhances macrophage phagocytosis by interaction with lipid raft-associated TLR-7 and upregulating p38 MAPK and PI3K pathways. J Surg Res 136: 58-69.
10. Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, et al. (2000) HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nat Med 6: 435-442.
11. Kol A, Bourcier T, Lichtman AH, Libby P (1999) Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. J Clin Invest 103: 571-577.
12. Basu S, Binder RJ, Ramalingam T, Srivastava PK (2001) CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. Immunity 14: 303-313.
13. Wang Y, Kelly CG, Karttunen JT, Whittall T, Lehner PJ, et al. (2001) CD40 is a cellular receptor mediating mycobacterial heat shock protein 70 stimulation of CC-chemokines. Immunity 15: 971-983.
14. Asea A (2008) Hsp70: a chaperokine. Novartis Found Symp 291: 173-179.
15. Gross C, Holler E, Stangl S, Dickinson A, Pockley AG, et al. (2008) An Hsp70 peptide initiates NK cell killing of leukemic blasts after stem cell transplantation. Leuk Res 32: 527-534.
16. Zitzler S, Hellwig A, Hartl FU, Wieland F, Diestelkotter-Bachert P (2008) Distinct binding sites for the ATPase and substrate-binding domain of human Hsp70 on the cell surface of antigen presenting cells. Mol Immunol 45: 3974-3983.
17. Sambrook J, Fritsch E, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor, NewYork.
18. Zheng HY, Nagaraja GM, Kaur P, Asea EE, Asea A (2010) Chaperokine Function of Recombinant Hsp72 Produced in Insect Cells Using a Baculovirus Expression System Is Retained. Journal of Biological Chemistry 285: 349-356.
19. Smith GE, Summers MD, Fraser MJ (1983) Production of human beta interferon in insect cells infected with a baculovirus expression vector. Mol Cell Biol 3: 2156-2165.
20. Geselowitz DA, Neumann RD (1995) Quantitation of triple-helix formation using a photo-cross-linkable aryl azide/biotin/oligonucleotide conjugate. Bioconjug Chem 6: 502-506.
21. Vabulas RM, Ahmad-Nejad P, da Costa C, Miethke T, Kirschning CJ, et al. (2001) Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. J Biol Chem 276: 31332-31339.
22. Multhoff G, Botzler C, Jennen L, Schmidt J, Ellwart J, et al. (1997) Heat shock protein 72 on tumor cells: a recognition structure for natural killer cells. J Immunol 158: 4341-4350.
23. Chen X, Tao Q, Yu H, Zhang L, Cao X (2002) Tumor cell membrane-bound heat shock protein 70 elicits antitumor immunity. Immunol Lett 84: 81-87.
24. Multhoff G (2007) Heat shock protein 70 (Hsp70): membrane location, export and immunological relevance. Methods 43: 229-237.
25. Negulyaev YA, Vedernikova EA, Kinev AV, Voronin AP (1996) Exogenous heat shock protein hsp70 activates potassium channels in U937 cells. Biochim Biophys Acta 1282: 156-162.
26. Albert ML, Pearce SF, Francisco LM, Sauter B, Roy P, et al. (1998) Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. Journal of Experimental Medicine 188: 1359-1368.
27. Srivastava PK, Menoret A, Basu S, Binder RJ, McQuade KL (1998) Heat shock proteins come of age: primitive functions acquire new roles in an adaptive world. Immunity 8: 657-665.
28. Wells AD, Rai SK, Salvato MS, Band H, Malkovsky M (1998) Hsp72-mediated augmentation of MHC class I surface expression and endogenous antigen presentation. Int Immunol 10: 609-617.
29. Gross C, Hansch D, Gastpar R, Multhoff G (2003) Interaction of heat shock protein 70 peptide with NK cells involves the NK receptor CD94. Biol Chem 384: 267-279.
30. Mosser DD, Caron AW, Bourget L, Denis-Larose C, Massie B (1997) Role of the human heat shock protein hsp70 in protection against stress-induced apoptosis. Mol Cell Biol 17: 5317-5327.
31. Balakrishnan K, De Maio A (2006) Heat shock protein 70 binds its own messenger ribonucleic acid as part of a gene expression self-limiting mechanism. Cell Stress Chaperones 11: 44-50.
32. Hromadnikova I, Sedlackova L (2008) Analysis of cell surface and relative gene expression of heat shock protein 70 in human leukemia cell lines. Leuk Lymphoma 49: 570-576.
33. Gurbuxani S, Bruey JM, Fromentin A, Larmonier N, Parcellier A, et al. (2001) Selective depletion of inducible HSP70 enhances immunogenicity of rat colon cancer cells. Oncogene 20: 7478-7485.
34. Arispe N, Doh M, De Maio A (2002) Lipid interaction differentiates the constitutive and stress-induced heat shock proteins Hsc70 and Hsp70. Cell Stress Chaperones 7: 330-338.
35. Bausero MA, Gastpar R, Multhoff G, Asea A (2005) Alternative mechanism by which IFN-gamma enhances tumor recognition: active release of heat shock protein 72. J Immunol 175: 2900-2912.
36. Barreto A, Gonzalez JM, Kabingu E, Asea A, Fiorentino S (2003) Stress-induced release of HSC70 from human tumors. Cell Immunol 222: 97-104.
37. Guzhova I, Kislyakova K, Moskaliova O, Fridlanskaya I, Tytell M, et al. (2001) In vitro studies show that Hsp70 can be released by glia and that exogenous Hsp70 can enhance neuronal stress tolerance. Brain Res 914: 66-73.

38. Basu S, Binder RJ, Suto R, Anderson KM, Srivastava PK (2000) Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. Int Immunol 12: 1539-1546.

39. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 3: 991-998.

40. Vabulas RM, Wagner H, Schild H (2002) Heat shock proteins as ligands of toll-like receptors. Curr Top Microbiol Immunol 270: 169-184.

41. Kim SR, Imoto S, Nakajima T, Ando K, Mita K, et al. (2009) Well-differentiated hepatocellular carcinoma smaller than 15 mm in diameter totally eradicated with percutaneous ethanol injection instead of radiofrequency ablation. Hepatol Int 3: 411-415.

42. Srivastava M, Pollard HB (1999) Molecular dissection of nucleolin's role in growth and cell proliferation: new insights. FASEB J 13: 1911-1922.

43. Said EA, Krust B, Nisole S, Svab J, Briand JP, et al. (2002) The anti-HIV cytokine midkine binds the cell surface-expressed nucleolin as a low affinity receptor. J Biol Chem 277: 37492-37502.

44. Sinclair JF, O'Brien AD (2002) Cell surface-localized nucleolin is a eukaryotic receptor for the adhesin intimin-gamma of enterohemorrhagic Escherichia coli O157:H7. J Biol Chem 277: 2876-2885.

45. Borer RA, Lehner CF, Eppenberger HM, Nigg EA (1989) Major nucleolar proteins shuttle between nucleus and cytoplasm. Cell 56: 379-390.

46. Yu D, Schwartz MZ, Petryshyn R (1998) Effect of laminin on the nuclear localization of nucleolin in rat intestinal epithelial IEC-6 cells. Biochem Biophys Res Commun 247: 186-192.

47. Christian S, Pilch J, Akerman ME, Porkka K, Laakkonen P, et al. (2003) Nucleolin expressed at the cell surface is a marker of endothelial cells in angiogenic blood vessels. J Cell Biol 163: 871-878.

48. Schmidt-Zachmann MS, Nigg EA (1993) Protein localization to the nucleolus: a search for targeting domains in nucleolin. J Cell Sci 105: 799-806.

49. Shibata Y, Muramatsu T, Hirai M, Inui T, Kimura T, et al. (2002) Nuclear targeting by the growth factor midkine. Mol Cell Biol 22: 6788-6796.

50. Reyes-Reyes EM, Akiyama SK (2008) Cell-surface nucleolin is a signal transducing P-selectin binding protein for human colon carcinoma cells. Exp Cell Res 314: 2212-2223.

51. Ohmori H, Murakami T, Furutani A, Higashi K, Hirano H, et al. Simultaneous activation of heat shock protein (hsp 70) and nucleolin genes during in vivo and in vitro prereplicative stages of rat hepatocytes. Exp Cell Res 189: 227-232.

52. Schild H, Rammensee HG (2000) gp96: The immune system's Swiss army knife. Nat Immunol 1: 100-101.

53. Srivastava PK (1993) Peptide-binding heat shock proteins in the endoplasmic reticulum: Role in immune response to cancer and in antigen presentation. Adv Cancer Res 62: 153-177.

54. Srivastava PK, DeLeo AB, Old LJ (1986) Tumor rejection antigens of chemically induced sarcomas of inbred mice. Proc Natl Acad Sci U S A 83: 3407-3411.

55. Zheng H, Dai J, Stoilova D, Li Z (2001) Cell surface targeting of heat shock protein gp96 induces dendritic cell maturation and antitumor immunity. J Immunol 167: 6731-6735.

56. Demine R, Walden P (2005) Testing the role of gp96 as peptide chaperone in antigen processing. J Biol Chem 280: 17573-17578.

57. Pratt WB, Toft DO (2003) Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med (Maywood) 228: 111-133.

58. Richter K, Buchner J (2001) Hsp90: chaperoning signal transduction. J Cell Physiol 188: 281-290.

59. Grenert JP, Sullivan WP, Fadden P, Haystead TAJ, Clark J, et al. (1997) The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. J Biol Chem 272: 23843-23850.

60. Huai Q, Wang H, Liu Y, Kim HY, Toft D, et al. (2005) Structures of the N-terminal and middle domains of E. coli Hsp90 and conformation changes upon ADP binding. Structure 13: 579-590.

61. Chadli A, Felts SJ, Toft DO (2008) GCUNC45 is the first Hsp90 co-chaperone to show alpha/beta isoform specificity. J Biol Chem 283: 9509-9512.

62. Minami Y, Kawasaki H, Miyata Y, Suzuki K, Yahara I (1991) Analysis of native forms and isoform compositions of the mouse 90-kDa heat shock protein, HSP90. J Biol Chem 266: 10099-10103.

63. Shima F, Yamawaki-Kataoka Y, Yanagihara C, Tamada M, Okada T, et al. (1997) Effect of association with adenylyl cyclase-associated protein on the interaction of yeast adenylyl cyclase with Ras protein. Mol Cell Biol 17: 1057-1064.

64. Field J, Vojtek A, Ballester R, Bolger G, Colicelli J, et al. (1990) Cloning and characterization of CAP, the S. cerevisiae gene encoding the 70 kd adenylyl cyclase-associated protein. Cell 61: 319-327.

65. Gerst JE, Ferguson K, Vojtek A, Wigler M, Field J (1991) CAP is a bifunctional component of the Saccharomyces cerevisiae adenylyl cyclase complex. Mol Cell Biol 11: 1248-1257.

66. Wang J, Suzuki N, Nishida Y, Kataoka T (1993) Analysis of the function of the 70-kilodalton cyclase-associated protein (CAP) by using mutants of yeast adenylyl cyclase defective in CAP binding. Mol Cell Biol 13: 4087-4097.

67. Osipiuk J, Walsh MA, Freeman BC, Morimoto RI, Joachimiak A (1999) Structure of a new crystal form of human Hsp70 ATPase domain. Acta Crystallogr D Biol Crystallogr 55: 1105-1107.