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Extracellular heat shock protein 70 has novel functional effects on sea urchin eggs and coelomocytes

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Summary

Numerous reports document that the 70 kDa heat shock proteins are not only intracellular proteins but are also present in blood and other extracellular compartments. How they affect cell function from the extracellular space remains unclear. Using two well-characterized cell types from the sea urchin, we show that extracellular mixtures of the constitutive and inducible forms of the 70 kDa heat shock proteins (Hsc70 and Hsp70, respectively) have dramatic effects on initiation of cell division in fertilized eggs and on the clotting reaction of hypotonically stressed coelomocytes. In suspensions of fertilized eggs to which Hsc70 or a 2:3 mixture of Hsc and Hsp70 was added, progression to the first mitotic division was accelerated. Evidence is provided that the extracellular Hsc70 passes into the egg cells in an unconventional manner, being distributed through the cytoplasm, and that it may alter the intracellular signaling cascade initiated by sperm penetration. In coelomocytes that were stimulated by hypotonic shock to mimic injury, the spreading reaction of the clotting response was significantly inhibited when either Hsp70 or Hsc70 was in the medium. These results suggest that the presence of Hsc and/or Hsp70 in the extracellular fluid may promote mitosis of dividing cells and suppress the reactivity of immune system cells.

Key words: cell division, egg, extracellular, fertilization, Hsp70, Hsc70, hypotonic shock, mitosis, nucleus, nuclear envelope breakdown, immune response, *Lytechinus variegatus*, sea urchin, uptake, wound response.

Introduction

All cells respond to heat and other physiological stresses by the production of heat shock proteins (Hsps). Hsps act as chaperone molecules, binding to and assisting the folding of other proteins, they aid in transport across cell membranes and they modulate immune and inflammatory responses (Hartl et al., 1996; Latchman, 1990; Jacquier-Sarlin et al., 1994; Malhotra and Wong, 2002). Physical stresses induce activation of the heat shock transcription factor (HSF-1), which binds to the heat shock element (HSE), leading to transcription of Hsp genes (Morimoto, 1998). In addition to their inducible gene expression in response to stress, other members of the Hsp family are constitutively expressed in non-stressed cells (Kochevar et al., 1991; Kregel, 2002; Welch, 1992).

The Hsps are divided into several families, distinguishable by molecular mass and function (Tytell and Hooper, 2001). The highly conserved Hsp70 family includes Hsc70 (heat shock cognate protein), which is synthesized constitutively and is only moderately induced by stress, and Hsp70, which is present at low levels in many cells and is highly induced by stress, regardless of the stage of the cell cycle (Hang and Fox, 1996). Both Hsc70 and Hsp70 participate in the folding of nascent proteins, refolding of mature proteins (Hartl, 1996; Rassow et al., 1997) and the translocation of proteins from one compartment to another in the cell (Glick, 1995). However, they also influence the activities of intracellular signaling molecules involved in mitosis and inflammation. In dividing cells, members of the Hsp70 family were found to associate with important cell cycle regulatory proteins and may serve as scaffold-promoting agents in complexes of mitogen-activated signaling proteins (Helmbrecht et al., 2000). During the inflammatory response, Hsp70 contributes to antigen processing and presentation (Bachelet et al., 1999; Stephanou and Latchman, 1999; Malhotra and Wong, 2002; van Eden et al., 2005) on the one hand, whereas on the other hand, it ameliorates some aspects of inflammation, like leukocyte diapedesis and autoimmune responses (House et al., 2001; Jacquier-Sarlin et al., 1994; van Eden et al., 2005).

Additionally, Hsp70 promotes cell survival by inhibiting apoptosis (Beere and Green, 2001; Garrido et al., 2001; Guzhova et al., 2001; Jäättelä, 1999; Mosser et al., 2000; Park et al., 2002).

Although Hsps are known primarily for their intracellular functions, an increasing number of reports shows that Hsp70 also functions extracellularly, interacting with the plasmalemma and being present in blood. The first evidence for this unexpected function of Hsp70 was suggested in the 1980s, when it was shown to be transferred from glial cells to axons and to be released from cultured cells (Hightower and Guidon, 1989; Tytell et al., 1986). More recently, Hsp70 and artificially produced NF-κB–Hsp70 fusion proteins have been shown to be taken up into the cytoplasm and nuclei of a number of different types of cultured cells (Fujihara and Nadler, 1999) and are released *via* exosomes from dendritic cells. Especially intriguing are the observations that Hsp70 is a normal constituent of blood and that its level changes with physiological stress and disease (Lancaster and Febbraio, 2005; Pockley et al., 1998; Pockley, 2002; Walsh et al., 2001). Both Hsp70 and Hsc70 have been shown to interact with lipids, inserting into liposomes and forming a cation channel in a calcium- and ATP-dependent manner (Arispe et al., 2002; Arispe and De Maio, 2000). At the same time that the existence of extracellular Hsp70 was becoming known, other work revealed that exogenously applied Hsc70 or Hsc/Hsp70 interacts with a number of different cell types and influences cell function. An Hsc/Hsp70 mixture bound to the surfaces of cultured smooth muscle cells (Johnson and Tytell, 1993) and U937 cells (Guzhova et al., 1998) and was internalized in the latter. It also inhibited apoptosis of dorsal root ganglion neurons and motor neurons in the mouse *in vivo* and in cultured chick motor neurons (Houenou et al., 1996; Robinson et al., 2005; Tidwell et al., 2004). These effects may be a consequence of the protein's unexpected ability to interact directly with membranes. For example, Hsp70 induced calcein leakage from liposomes and formed ion-conducting channels in artificial phospholipid bilayers (Alder et al., 1990; Arispe and De Maio, 2000). In patch-clamp experiments with a human premonocyte cell line (U9367), exposure of membrane patches to a mixture of Hsc70 and Hsp70 increased membrane K^+ channel conductance (Negulyaev et al., 1996), and in *Aplysia* bag cell neurons Hsc70 elevated Ca^{2+} efflux (Smith, P. J. S. et al., 1995). The potential of exogenously applied Hsc70 and Hsp70 to alter cellular homeostasis of calcium has implications for the many cellular processes that are dependent on that ion and may, in fact, be the mechanism underlying many of the above effects on cell function.

The implications of the above reports that extracellular Hsc70 and Hsp70 can alter cell function through effects on cation homeostasis and intracellular signaling cascades prompted us to test its effects on two sea urchin cell types: the fertilized egg and the coelomocyte. These cells have been useful models for studying many calcium-dependent processes and signaling cascade-dependent responses to physiological stressors. For both cell types, the advantages are that large numbers of cells can be easily isolated and they can all be triggered simultaneously to go through well-characterized structural transformations, i.e. mitosis in the case of the fertilized egg and the spreading/clotting response of stressed coelomocytes. The sea urchin egg is a large cell (~100 μm in diameter) that has been used extensively to study the processes of fertilization and mitosis (Doree and Hunt, 2002; Whitaker and Larman, 2001), and the coelomocyte is a motile, phagocytic, immune-type cell in the coelomic fluid of sea urchins and many other lower invertebrates that plays a role in protection against physical trauma and bacterial invasion (Edds, 1977; Henson and Schatten, 1983; Johnson, 1969). Additionally, numerous studies have documented the presence of both constitutive and stress-inducible forms of the 70 kDa Hsps in sea urchin embryos (reviewed in Giudice et al., 1999), although the latter is not present under normal conditions, only after metabolic stress (Geraci et al., 2004). We report that in *Lytechinus variegatus*, certain preparations of Hsc/Hsp70 can accelerate the cell cycle time of fertilized eggs and inhibit the spreading reaction of coelomocytes stressed by hypotonic shock.

Materials and methods

Heat shock protein preparations

Human recombinant Hsp70 and brain recombinant Hsc70 were obtained from StressGen Biotechnologies (Victoria, BC, Canada; now Assay Designs, Ann Arbor, MI, USA). A mixture of Hsc/Hsp70, purified from bovine skeletal muscle (~40% constitutive and ~60% stress-inducible forms, 97% purity), was a gift from Boris Margulis (Russian Academy of Sciences, St Petersburg, Russia) (see Guzhova et al., 1998) for purification procedure and western blot analysis. The endotoxin activity of a 5 μg ml^{-1} solution of the recombinant Hsp70 solution was 500 U ml^{-1}, as determined by Norman Wainwright of the Marine Biological Laboratory, Woods Hole, MA, USA using the *Limulus* ameobocyte lysate assay. Actin, β-lactoglobulin, bovine serum albumin (BSA), casein, bacterial endotoxin and goat antimouse-FITC were obtained from Sigma (St Louis, MO, USA).

Hsp refolding activity was measured using a luciferase assay system (Promega, Madison, WI, USA) according to the method of Schumacher et al. (Schumacher et al., 1996). Bovine brain Hsc70, bovine skeletal muscle Hsc/Hsp70 and fluorescein-labeled Hsc/Hsp70 were tested. Luciferase (Sigma) was combined with each Hsp preparation to a final concentration of 2 μg ml^{-1}, heated for 5 min at 40°C and then allowed to incubate at room temperature for 1 h. The heated luciferase was combined with 100 μl of luciferin reagent, and luminescence was measured on a Turner TD-20e luminometer. The luminometer readings were converted to percent renaturation by dividing them by the values measured for unheated control luciferase–luciferin reaction.

Egg collection, treatments and analyses

Lytechinus variegatus (Lamarck) (Duke Marine Laboratories) were maintained at room temperature in running

seawater. Gametes were obtained by intracoelomic injection of 1 ml of 1 mol l^{-1} KCl. Sperm were collected dry and diluted with seawater just before use by adding 10 μl of sperm to 100 ml of seawater. Eggs that had been washed several times in artificial seawater (ASW–Marine Biological Laboratory formulation) were fertilized by the addition of 10 ml of diluted sperm suspension to 90 ml of egg suspension. After fertilization, the eggs were again washed in ASW to remove unattached sperm. Fertilization membranes were removed by filtration of the eggs through a 100 μm nylon mesh filter. Fertilized eggs were then incubated in 0.1–20 μg Hsp preparations or in artificial sea water (ASW). Hsp70, Hsc70 and Hsc/Hsp70 solutions were prepared in ASW, pH 8.0.

To determine the time of nuclear envelope breakdown and cleavage, samples of the egg suspension were taken at regular intervals, placed on a slide and examined with phase-contrast optics at 250× magnification using a Zeiss standard microscope. This analysis was continued through completion of second cleavage. A minimum of 25 cells was counted per slide at each interval. The percentage of eggs showing nuclear envelope breakdown as the cell cycle progressed was noted. Each experiment was repeated a minimum of eight times. Care was taken to minimize polyspermy, and eggs that were obviously polyspermic were not scored. Only those populations of fertilized eggs that demonstrated synchronous progression through the cell cycle, defined as at least 90% of the cells undergoing cleavage within a 10 min window of time, were scored.

The Hsp70 and Hsc70 received from Stressgen, but not the Hsc/Hsp70 preparation, were shipped in 1 mmol l^{-1} dithiothreitol (DTT) and 0.1 mmol l^{-1} phenyl methyl sulphonyl fluoride (PMSF). Incubation of fertilized eggs in concentrations of DTT and PMSF comparable to those to which Hsp-exposed eggs were exposed had no effect on the time to nuclear envelope breakdown or cleavage.

To evaluate the uptake of extracellular Hsc/Hsp70 in unfertilized eggs, various preparations of labeled protein were used. For some experiments, Hsc70 or Hsc/Hsp70 was labeled with succinimidyl esters of fluorescein or biotin according to the manufacturer's instructions (Invitrogen/Molecular Probes, Eugene, OR, USA; cat. # F-6129 or Sigma Aldrich BTAG microbiotinylation kit, respectively). In other experiments, a preparation of human recombinant Hsc70 tagged at the amino terminus with six histidines was used (kindly provided by M. B. Robinson and C. E. Milligan, Dept of Neurobiology and Anatomy, Wake Forest University School of Medicine, Winston-Salem, NC, USA). Additionally, the uptake of control proteins, ovalbumin and bovine skeletal muscle actin labeled with fluorescein or biotin, was also examined. The eggs were incubated in 5, 10 or 20 μg ml^{-1} of labeled protein in small volumes of ASW either in suspension or after attachment to poly-L lysine-coated slides for up to 120 min. They were then washed twice by application of ASW to the slides or, in the case of the egg suspension, by centrifugation at about 300 ***g*** and resuspension in about 1 ml of ASW. The slide-mounted eggs were permeabilized and fixed by treatment with methanol at 4°C for 4 min followed by 4% paraformaldehyde. The egg suspensions were fixed by resuspension in 3.7% formalin in ASW for 10 min followed by centrifugation and resuspended in 400 μl ASW. They were then permeabilized by incubation in 0.5% Triton X-100 for 2 min, then washed twice, 5 min each, in phosphate-buffered saline (PBS). To detect biotinylated proteins, the slide-mounted eggs were incubated with fluorescein-conjugated avidin. The N-His–Hsc70 was detected by resuspending the eggs in either 2 or 4 μg ml^{-1} of an anti-histidine mouse monoclonal antibody (anti-His tag; Upstate Cell Signaling Solutions Co./Millipore, Chicago, IL, USA; cat. # 05-53) prepared in PBS, pH 7.2 with 2% normal donkey serum (NDS) for 1 h at room temperature. Eggs were washed twice in PBS–2% NDS, centrifuging at 500 ***g*** each time, after which they were incubated in a 1:50 dilution (280 μg ml^{-1}) of donkey anti-mouse, Cy3-labeled, secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; cat. #715-165-150) for 45 min at room temperature and rinsed twice in PBS. About 30 μl of each egg suspension was placed on a Fisher ProbeOn Plus slide, coverslipped with ProLong Gold mounting medium (Molecular Probes/Invitrogen), and examined with a Zeiss LSM 510 confocal microscope under both DIC (differential interference contrast) and epifluorescence illumination using either of the two following 40× objectives: a 1.3 NA Plan-Neofluar oil immersion or a 1.2 NA C-Apochromat water immersion. Optical sections of 0.46, 0.48 or 0.72 μm were collected from 25 positively labeled eggs and from 13 negative controls. The negative controls were used to adjust the baseline sensitivity of the microscope so that only positive N-His–Hsc70 immunofluorescence was detected.

To explore the possibility that exogenous Hsc/Hsp70 altered signal transduction events are associated with the first mitosis after fertilization, samples of fertilized eggs with or without treatment with N-His–Hsc70 were processed for phospho-cdc2 analysis by western blotting in the following manner. Batches of eggs collected in ASW from two sea urchins were fertilized as described above and then 1 ml samples of each were dispensed into the wells of a 12-well culture plate. Additionally, two 1 ml portions of unfertilized eggs from each batch were dispensed into wells of the culture plate. All but two wells of fertilized eggs received N-His–Hsc70 to give a final concentration of 5 μg ml^{-1}. At 25 and 50 min after fertilization, 100 μl of the Hsc70-treated eggs were taken, mixed with 0.5 ml electrophoresis sample buffer, and heated for 5 min at 95°C to dissolve and denature the proteins. For comparison, similarly treated samples of fertilized eggs without Hsc70 treatment were collected at 35 min and 50 min. The 10 min difference between the first collection of Hsc70-treated and untreated eggs meant that both samples of eggs should have been at similar points in their progress through first mitosis, given the acceleration of mitosis in the presence of Hsc70. In addition, samples of unfertilized, Hsc70-treated eggs were collected at zero and 45 min after Hsc70 to monitor for fertilization-independent changes in phosphor-cdc2. The protein concentration of each

sample was measured using the BioRad RC DC Protein Assay (Hercules, CA, USA; cat. #500-0119). 5 μg of protein from each sample were analyzed by SDS-polyacrylamide gel electrophoresis using Pierce 10% Precise™ 10-well precast gels (Rockford, IL, USA; cat. #25201) according to the manufacturer's specifications, followed by western blotting onto 0.2 μm pore-sized Immobilon membranes (Millipore cat. #ISEQ10100). To detect phosphor-cdc2, the dried membranes were re-wetted in 100% methanol, then incubated for 1 h in 50 mmol l^{-1} Tris-buffered saline, pH 7.0, with 0.1% Tween and 1% BSA (TBST-BSA). The blots were then probed with a rabbit polyclonal anti-phospho-cdc2 (Stressgen, now Assay Designs; cat# KAP-CC015), 1:3000 for 1 h while shaking. They were then rinsed four times for 5 min per rinse in TBST, followed by incubation in 1:10 000 donkey anti-rabbit horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch Laboratories; cat. #711-035-152) in TBST-BSA for 1 h. Following four washes in TBST as before, the bound antibody was detected using Millipore chemiluminescent HRP substrate (cat. #WKBLS005) according to the manufacturer's directions and by exposing the blot to X-ray film for a few seconds. The film was developed using a standard automated x-ray film processor. Digital images of the film were made using an Agfa Arcus II scanner and processed using for reproduction using Adobe Photoshop 7.0. For densitometry, an additional digital image of the film was made using a BioRad Gel Doc 2000 system and analyzed using 'The Discovery Series™ Quantity One®' 1-D analysis software that BioRad provides.

Coelomocyte collection, testing and analyses

Several different types of coelomocytes have been described, including phagocytic amoebocytes, flagellated vibratile cells, and colorless and red spherule cells, which can be separated by density gradient centrifugation in sucrose (Edds, 1977; Gerardi et al., 1990; Otto et al., 1979). Coelomocytes were collected from the coelomic cavity of *Lytechinus variegatus* as described previously (Edds, 1977). 5 ml of coelomic fluid was drawn into a syringe containing an equal volume of anticoagulant solution (30 mmol l^{-1} EGTA, 0.5 mol l^{-1} NaCl and 20 mmol l^{-1} Hepes, pH 7.4). The coelomocyte–anticoagulant mixture was then layered onto an equal volume of 0.75 mol l^{-1} sucrose and centrifuged in a Beckman JS 13.1 rotor for 10 min at 8000 ***g***. The cells found at the interface between the sucrose and anticoagulant medium were predominantly phagocytic amoebocytes. The isolated cells were kept on ice and used within 2 h of centrifugation.

To test the effect of Hsp70 protein on coelomocyte spreading, coelomocytes prepared as described above were incubated at room temperature on uncoated glass cover slips in 250 μl of the appropriate medium for 20 min. Hsp70, Hsc70 and control proteins were dissolved in either 0.5 mol l^{-1} NaCl (control) or 0.3 mol l^{-1} NaCl (hypotonic medium) containing 0.1 mol l^{-1} Tris-HCl, and 0.001 mol l^{-1} EGTA, pH 7.4. For each experiment, at least 100 cells were measured or characterized for each experimental treatment. Each experiment was conducted with cells from different animals and repeated a minimum of six times.

Isolated coelomocytes were heat-shocked by placing the cells in isotonic medium in microcentrifuge tubes in a water bath at 31°C for 30 min. Control cells remained at room temperature. After incubation, the cells were transferred to hypotonic medium, allowed to attach to and spread on uncoated glass cover slips for 15 min. Cells were fixed onto glass cover slips in 4% paraformaldehyde for 10 min. Cover slips were rinsed in coelomocyte buffer (100 mmol l^{-1} NaCl, 16.9 mmol l^{-1} K_2HPO_4, 10 mmol l^{-1} EGTA, 3.1 mmol l^{-1} KH_2PO_4 and 2 mmol l^{-1} $MgCl_2$) and incubated in 1% BSA, 0.1% Triton X-100 in isolation medium for 15–20 min. For detection of tubulin, cover slips were then incubated in anti-tubulin antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) in coelomocyte buffer containing 1% BSA and 0.1% Triton X-100, for 2–3 h at room temperature. The cells were washed and then incubated for 4–5 h in FITC-labeled goat anti-mouse antibody (Sigma). For detection of actin, cover slips were incubated for 4 h with Texas Red–phalloidin (Molecular Probes) in coelomocyte buffer.

Slides were viewed using a Zeiss LSM510 confocal microscope with multitrack fluorescence and 100× oil-immersion objective or a Zeiss Axiophot epifluorescence microscope using a 63× water-immersion objective. Cells were measured across the longest diameter and categorized according to shape.

Statistics

For statistical analysis, percentages were transformed to obtain a normal distribution. Analysis was done by single-factor or two-factor analysis of variance (ANOVA), followed by comparison of means test using 95% confidence limit intervals. In all graphs, error bars represent standard error of the mean (s.e.m.).

Results

Hsp effects on fertilized eggs

Fertilized sea urchin eggs were incubated in varying concentrations of bovine Hsc/Hsp70. This 2:3 mixture of the constitutive and inducible forms is similar to what is found in many cell types under physiological conditions (e.g. Brown et al., 1993; Gutierrez and Guerriero, 1995). The concentrations of Hsc/Hsp70 ranged from 0.1 to 10 μg ml^{-1}. 1 μg ml^{-1} of BSA or ovalbumin was used to control for non-specific protein effects. Samples of the fertilized egg suspensions were examined microscopically at 5 min intervals to detect nuclear envelope breakdown (NEB).

There were no significant differences in the time to reach NEB or cleavage among ovalbumin-treated, BSA-treated or untreated eggs. However, eggs incubated in Hsc/Hsp70 underwent NEB significantly earlier than controls (Fig. 1). Although the time to NEB was accelerated in the presence of Hsc/Hsp70, the time between NEB and mitosis was unchanged.

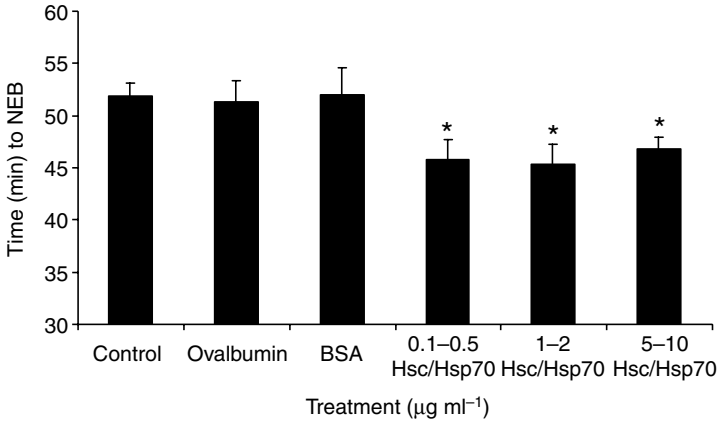

Fig. 1. The effect of Hsp/Hsc70 on nuclear envelope breakdown (NEB) in fertilized sea urchin eggs. Fertilized sea urchin eggs were incubated in varying concentrations of Hsc70 and Hsc/Hsp70 ranging from 0.1–10 μg ml^{-1}, in artificial sea water (ASW) (control) or in 1 μg ml^{-1} of bovine serum albumin (BSA) or ovalbumin, and the times to NEB and cleavage were observed. Eggs exposed to Hsp/Hsc70 reached NEB earlier than eggs incubated in ASW, ovalbumin or BSA. There were no significant differences in the time to NEB between untreated control eggs and BSA- and ovalbumin-treated eggs. The difference between Hsc/Hsp70-treated and control, BSA and ovalbumin-treated eggs was statistically significant at all concentrations, as indicated by the asterisks (P<0.05). Values are means ± s.e.m. N=6 replicate experiments in which at least 100 eggs were observed.

No significant relationship was detected between the Hsp concentration and the degree to which NEB and cleavage were accelerated. These results were compared with those of fertilized eggs incubated in recombinant bovine Hsc70 (rbHsc70). This protein produced a dose-related trend towards decreases in the time to NEB relative to control that achieved significance at the highest concentration, 5 μg ml^{-1} (Fig. 2). Thus, in contrast to Hsc/Hsp70, rbHsc70 seemed less effective.

In order to determine whether the differing effects of skeletal muscle Hsc/Hsp70 and recombinant Hsc70 on the fertilization to NEB interval might be related to differences in the chaperone function of the two forms, the refolding activity of these proteins was assayed by measuring their abilities to renature firefly luciferase that had been inactivated by heat shock at 40°C. Renaturation activity of Hsc/Hsp70, Hsc70 and control preparations was measured by restoration of luminescence activity of heat-denatured luciferase in the presence of substrate. Both Hsp preparations showed comparable, significant ability to protect against denaturation, with the skeletal muscle Hsp/Hsc70 preparation being more effective (Fig. 3).

The fact that exogenous Hsc/Hsp70 accelerated a basic function like time to NEB in fertilized eggs made us wonder if it might also make them resistant to heat stress, an effect for which Hsp70 is well-known. Sea urchin embryos are incapable of upregulating Hsps in response to temperature or other physical stresses until after hatching. Thus, prior to that time, they cannot be conditioned to survive an elevated temperature challenge (reviewed in Giudice et al., 1999). To test whether exogenously applied Hsp70 might compensate for this deficit and offer protection for early embryos against heat shock, unfertilized eggs were exposed to 2 μg ml^{-1} Hsc/Hsp70 for one

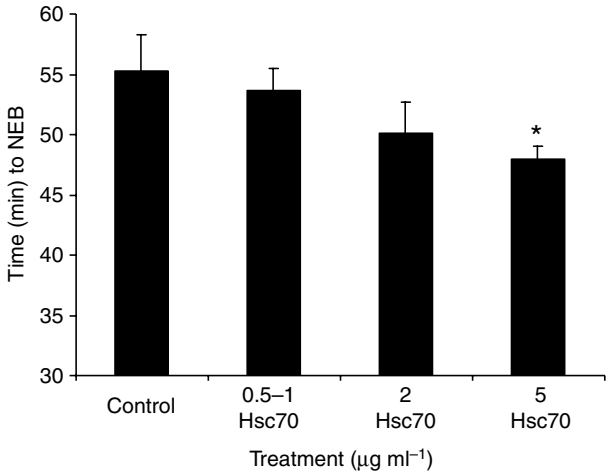

Fig. 2. The effect of rbHsc70 on nuclear envelope breakdown (NEB) in fertilized sea urchin eggs. Fertilized sea urchin eggs were incubated in varying concentrations of Hsc70, and the times to NEB and cleavage were observed. Exogenous Hsc70 reduced the time to NEB at all concentrations tested. The difference between Hsc-treated and control eggs was statistically significant at 5 μg ml^{-1} Hsc, as indicated by the asterisk (P<0.05). Values are means ± s.e.m. N=6 replicate experiments in which at least 100 eggs were observed.

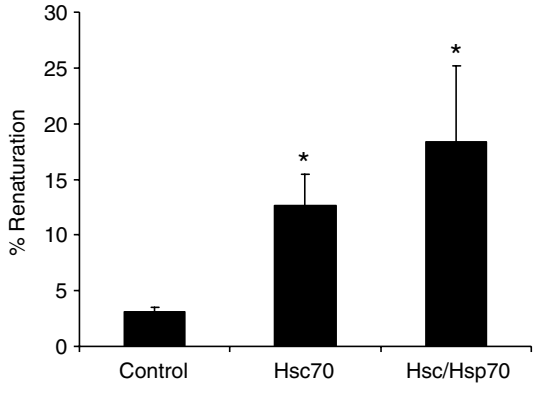

Fig. 3. Renaturation of luciferase in the presence of Hsc/Hsp70 or Hsc70 as a measure of their refolding activities. 2 μg ml^{-1} of Hsp70 and of Hsc/Hsp70 was heated in the presence of luciferase to determine the ability of the Hsp preparations to prevent luciferase denaturation. The heated luciferase–Hsp mixture was combined with luciferin reagent and luminescence recorded as a percentage of unheated luciferase. The difference in phosphorescence between luciferase heated in the absence of Hsp and in the presence of Hsp was statistically significant as indicated by the asterisk (P<0.05). There was no difference in effectiveness between the two Hsp preparations. Values are means ± s.e.m. N=6 replicate experiments.

hour, fertilized and then subjected to 35°C heat shock for 20 or 40 min. This temperature is about 13°C higher than that at which *Lytechinus variegatus* are normally maintained. These eggs did not proceed through cleavage. Control eggs incubated in Hsc/Hsp70 prior to fertilization but then incubated at 22°C after fertilization, cleaved normally (not shown).

The ability of exogenously applied Hsp70 to alter the cell cycle of fertilized eggs suggested binding at the cell surface and/or internalization. To determine whether one or both of these events occurred, unfertilized eggs were incubated in 5, 10 or 20 μg ml^{-1} of N-His–recombinant human Hsc70 (N-His–rhHsc70) for 30 or 120 min, followed by fixation and Hsc70 immunolocalization with a mouse monoclonal anti-His antibody and Cy3-labeled secondary antibody. The cells were examined by confocal microscopy. Control eggs incubated in ASW lacking N-His–Hsc70 showed low levels of nonspecific fluorescence (not shown). A diffuse, heterogeneous distribution of fluorescence was observed in all eggs exposed to N-His–Hsc70 that did not change in relation to the amount of N-His–Hsc70 or the duration of incubation. In nearly one-third of the eggs (eight out of 25), the N-His–Hsc70 immunofluorescence (Hsc70-IF) was excluded from the nucleus. Fig. 4A,B shows two examples. This distribution of Hsc70-IF suggested that it was mainly in the cytoplasm; there was no indication of vesicular localization of fluorescence inside the cell as would be expected if the protein were endocytosed. In fact, as shown in Fig. 4C, when the DIC and fluorescence images of identical regions of an egg were compared, vesicles and Hsc70-IF hot spots did not co-localize.

The cytoplasmic distribution of the added Hsc70 suggested that the promotion of NEB reflected its alteration of one or

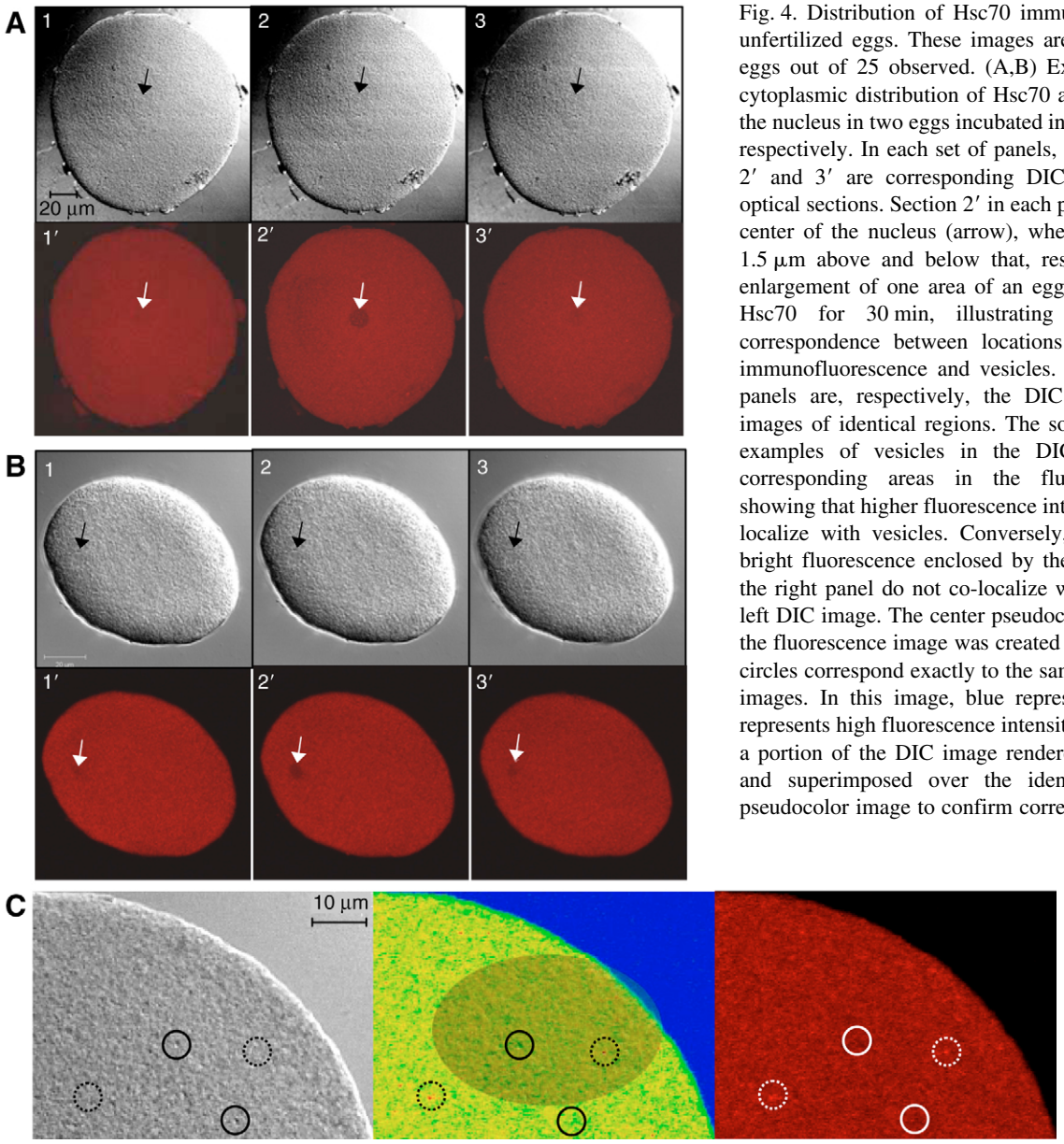

Fig. 4. Distribution of Hsc70 immunofluorescence in unfertilized eggs. These images are examples of two eggs out of 25 observed. (A,B) Examples of diffuse cytoplasmic distribution of Hsc70 and exclusion from the nucleus in two eggs incubated in 20 or 5 μg Hsc70, respectively. In each set of panels, 1, 2 and 3, and 1′, 2′ and 3′ are corresponding DIC and fluorescence optical sections. Section 2′ in each panel is through the center of the nucleus (arrow), whereas 1′ and 3′ are 1.5 μm above and below that, respectively. (C) An enlargement of one area of an egg treated with 5 μg Hsc70 for 30 min, illustrating the absence of correspondence between locations of higher Hsc70 immunofluorescence and vesicles. The left and right panels are, respectively, the DIC and fluorescence images of identical regions. The solid circles enclose examples of vesicles in the DIC image and the corresponding areas in the fluorescence image, showing that higher fluorescence intensity does not co-localize with vesicles. Conversely, two locations of bright fluorescence enclosed by the broken circles in the right panel do not co-localize with vesicles in the left DIC image. The center pseudocolored rendition of the fluorescence image was created to confirm that the circles correspond exactly to the same areas in the two images. In this image, blue represents low and red represents high fluorescence intensity. The gray oval is a portion of the DIC image rendered semitransparent and superimposed over the identical area in the pseudocolor image to confirm correspondence.

more signaling cascades that occur after fertilization. One candidate for this effect is the cell division cycle kinase, cdc2, because of its purported role in phosphorylation of nuclear lamins (Peter et al., 1991), which is required for NEB (Burke, 1990). In the resting egg, cdc2 is highly phosphorylated and inactive and is dephosphorylated during the initiation of mitosis (Edgecombe et al., 1991). Therefore, we examined the levels of phosphorylated cdc2 in unfertilized and fertilized eggs with or without treatment with Hsc70. Fig. 5 illustrates that Hsc70-treated eggs had a higher level of phospho-cdc2 within the 25–35 min period after fertilization compared with those without Hsc70 treatment (an average of 2.5 times greater by densitometry), but that 50 min after fertilization, both groups had similar levels of phospho-cdc2. Thus, it seems that increased Hsc70 and Hsp70 enhanced or helped to maintain phospho-cdc2, but how that relates to its promotion of NEB remains a question (see Discussion).

Hsp effects on coelomocytes

The relatively large diameter, spherical shape of a sea urchin egg is not easily amenable to spatial analysis of the cytoskeleton. However, that is not the situation with the coelomocytes. To determine the effect of exogenous Hsp on spreading of coelomocytes, cells were isolated into hypotonic buffer, with or without 5 μg ml^{-1} of rhHsp70 or rbHsc70, and plated onto glass slides. After 20 min, the cells were washed, fixed, stained with fluorescent probes for actin and tubulin and visualized with confocal microscopy. To quantify the degree of spreading, the longest axis of the cells was measured. Cells with diameters of less than 25 μm were considered 'petaloid' cells, those between 26 and 39 μm were defined as partially spread, those between 40 and 69 μm were said to have a fibroblast-like or small filopodial morphology, and those that measured above 70 μm were called filopodial. Cells exposed to hypotonic medium alone showed few petaloid cells, with the majority of the cells showing an intermediate degree of spreading – the fibroblast morphology. About 20% of the cells could be characterized as well-spread or filopodial.

The spreading of coelomoctyes in hypotonic medium in the presence of Hsp was significantly inhibited (Fig. 6). Only 1% of the cells exposed to Hsp70 and 3% of those exposed to Hsc70 could be characterized as well-spread, having the fibroblast-like or filopodial appearance. Overall, the coelomocytes responded differently to Hsc70 and Hsp70. Hsp70 inhibited the spread of cells to a greater degree, with over 60% of the cells having the petaloid morphology. Hsc70 produced a more even distribution of cell shapes and sizes than did Hsp70, but the cells were still skewed dramatically towards smaller sizes as compared with cells in hypotonic medium alone.

Because the Hsp preparations used in the coelomocyte study were recombinant proteins, we were concerned about potential contamination with small amounts of bacterial endotoxin. By itself, endotoxin may induce the expression of endogenous Hsp70 in antigen-sensitive cells (Smith, L. C. et al., 1995) and has been reported to activate coelomocytes and stimulate cell spreading through stimulation of profilin transcription (Smith et al., 1992). In order to determine whether the effects of the recombinant rhHsp70 and rbHsc70 preparations on spreading might be due to endotoxin contamination, the effect of a comparable concentration of endotoxin on hypotonically shocked coelomocytes was tested. Coelomocytes in hyptotonic medium containing up to 500 U ml^{-1} of bacterial endotoxin in the absence of Hsp had no effect on the spreading response (Fig. 7), indicating that the inhibition observed with Hsp70 and Hsc70 could not be attributed to endotoxin.

To eliminate the possibility that the inhibition of spreading observed upon the addition of Hsp was due to a non-specific effect of exogenous protein, the effects of several control proteins were tested. Exposure of coelomocytes to hypotonic medium containing 5 μg ml^{-1} of lactoglobulin, casein or actin had no effects on spreading (Fig. 8).

The coelomocytes of sea urchins exposed to heat stress have

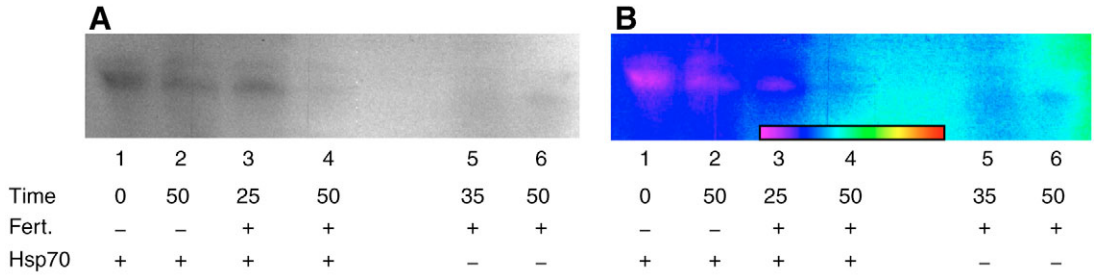

Fig. 5. Effect of Hsc70 on immunoreactive phospho-cdc2 levels following fertilization. (A) Gray-scale image of one blot of immunoreactive phospho-cdc2. (B) The same image in pseudocolor to emphasize the relative differences in each lane. The color spectrum bar shows the grayscale-to-color correspondence, with violet representing black, and red representing white. Variation in background is what accounts for the blue color in the left half of the blot and the blue-green on the right. Lanes 1 and 2 are unfertilized eggs collected at 0 and 50 min after 5 μg ml^{-1} Hsc70. Lanes 3 and 4 are fertilized eggs in 5 μg ml^{-1} Hsc70 at 25 and 50 min after fertilization, respectively. Lanes 5 and 6 are fertilized eggs in the absence of Hsc70 at 35 and 50 min after fertilization, respectively. Phospho-cdc2 remained at a higher level in Hsc70-treated eggs for the first 25–35 min after fertilization (compare lanes 3 and 5), but was about the same in the two groups by 50 min after fertilization. This blot is one of two, each showing the same trends.

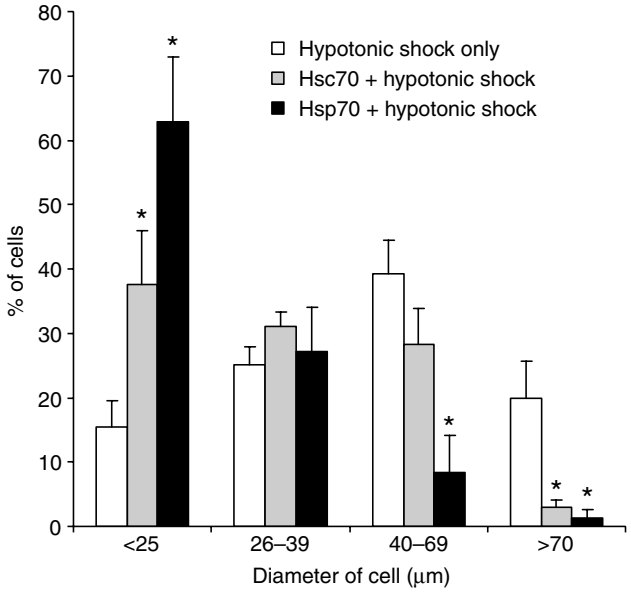

Fig. 6. Inhibition by Hsp of the spreading of hypotonically shocked coelomocytes. Phagocytic coelomocytes were exposed to hypotonic shock in the presence or absence of 5 μg ml^{-1} recombinant Hsp70 or 5 μg ml^{-1} bovine Hsc70. Both Hsps dramatically inhibited hypotonic medium-induced spreading in coelomocytes, increasing the numbers of smaller diameter coelomocytes and decreasing the numbers of larger diameter ones. Significant differences (P<0.05) between Hsc70- or Hsp70-treated cells and controls are indicated by asterisks (determined by a two-factor ANOVA). Values are means ± s.e.m. N=5 replicate experiments in which at least 100 cells were observed.

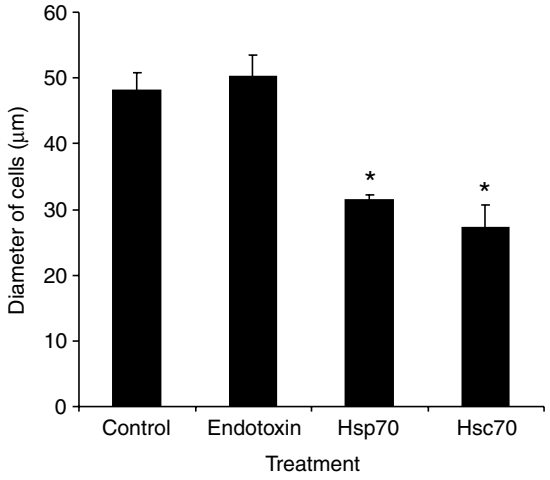

Fig. 7. The effect of endotoxin on cell spreading in hypotonically shocked coelomocytes. Recombinant proteins may be contaminated with small amounts of bacterial endotoxin. To confirm that inhibition of coelomocyte spreading in the presence of recombinant Hsps was not a result of endotoxin, hypotonically shocked coelomocytes were exposed to endotoxin in concentrations similar to those measured in the recombinant Hsps. The endotoxin-treated cells were compared to cells hypotonically shocked (control) and hypotonically shocked in the presence of Hsp70 or Hsc70. Endotoxin-treated cells were not significantly different from control cells that had received only a hypotonic shock. The Hsp70- and Hsc70-treated cells were significantly different from both the endotoxin-treated and control cells, as indicated by the asterisk (P<0.05). Values are means ± s.e.m. N=5 replicate experiments in which at least 100 cells were observed.

been shown to have increased expression of Hsp70 and to include a higher proportion of red sperula cells (Matranga et al., 2000). In order to determine whether heat shock itself could inhibit spreading of coelomocytes exposed to hypotonic medium, isolated coelomocytes were exposed to 31°C for 30 min and then transferred to hypotonic medium (Fig. 9). Heat shock prior to hypotonic shock inhibited coelomocyte spreading similar to the way exogenously applied Hsp did. While the majority of the cells in hypotonic medium are usually well-spread and have numerous thin cytoplasmic projections, coelomocytes that were heat shocked prior to hypotonic shock displayed a more rounded, petaloid morphology, with few cytoplasmic projections (not shown). As with Hsp-treated cells, heat-shock-treated cells also showed less cytoplasmic spreading than unshocked cells in isotonic medium (not shown).

The actin cytoskeleton in Hsp-treated cells was visualized by staining with Texas Red phalloidin. In both Hsp70-treated and untreated cells, the degree of actin polymerization was positively correlated with the extent of cell spreading. Petaloid cells showed actin aggregated in the center of the cell, and individual filaments were not visible regardless of whether or not they had been treated with Hsp70 or Hsc70. The same was found to be true in well-spread cells; the actin filament network appeared as a radial array, with filaments extending

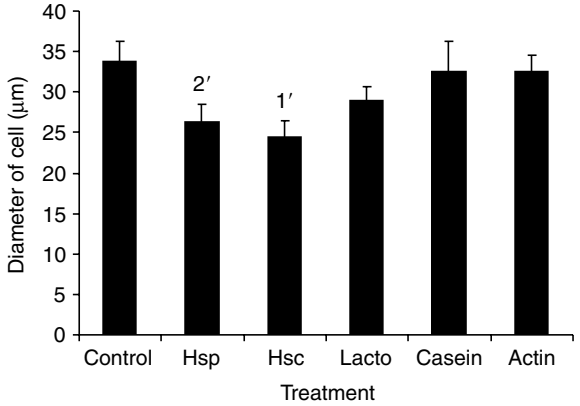

Fig. 8. The inhibition of coelomocyte spreading in response to hypotonic shock is specific to Hsc70 and Hsp70. Coelomocytes exposed to 5 μg ml^{-1} of other proteins (lactoglobulin, casein and actin) during hypotonic shock spread normally, while those exposed to Hsp during hypotonic shock were inhibited. This result confirms that inhibition of spreading is Hsp-specific and not a result of the interaction of any protein with the cells during hypotonic shock. Values are means ± s.e.m. N=3 replicate experiments in which at least 100 cells were observed.

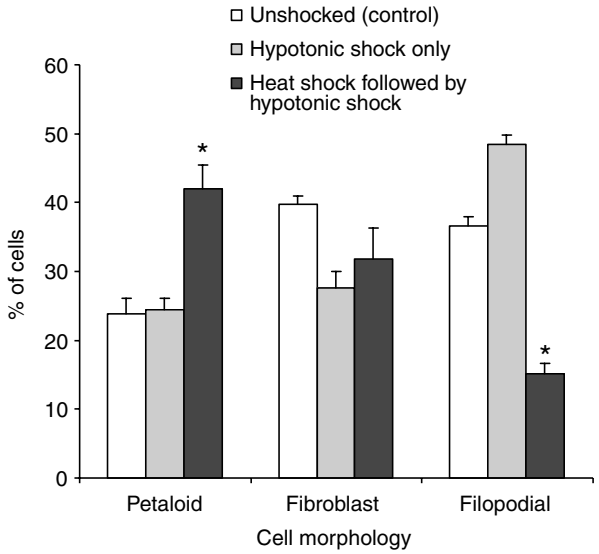

Fig. 9. The effect of heat shock on coelomocyte spreading. Isolated coelomocytes were heat-shocked in isotonic medium at 31°C for 30 min. Controls remained at room temperature. The percentages of cells on each slide that had petaloid, fibroblastic, or filopodial morphology were determined. Heat-shocked cells showed significantly more petaloid forms and fewer filopodial forms than unshocked or hypotonically shocked cells (asterisks, $P<0.05$). Thus, heat shock prior to hypotonic shock inhibited coelomocyte spreading to a similar extent as exogenous Hsp70 and Hsc70. Values are means ± s.e.m.

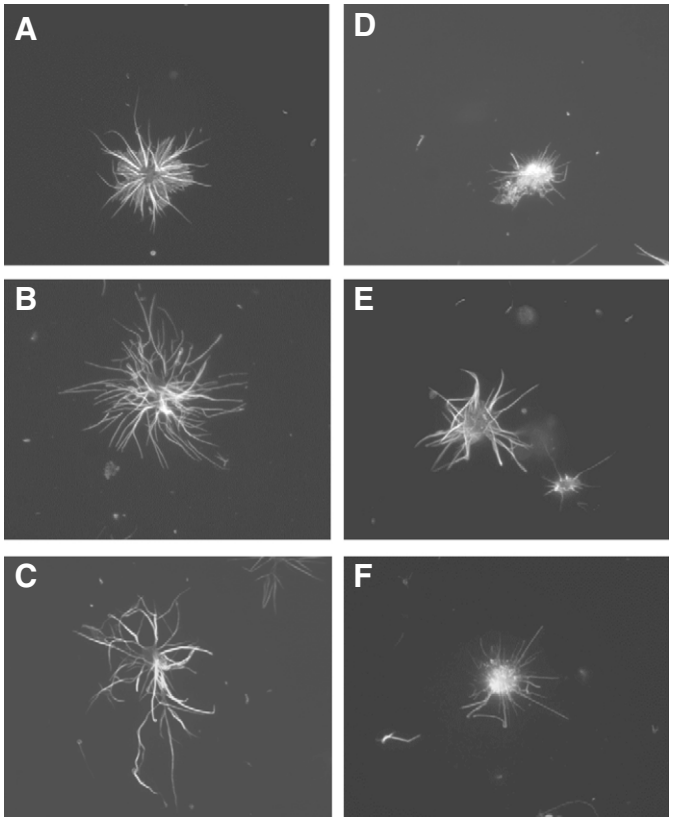

Fig. 10. The appearance of the actin cytoskeleton in Hsp70-treated coelomocytes. Coelomocytes exposed to hypotonic shock in the presence or absence of Hsp70 were fixed and stained with Texas Red phalloidin to visualize the actin cytoskeleton. The actin in coelomocytes incubated in hypotonic medium polymerized into long filaments that extend to the edges of the large, flattened cells (A–C). Hsp70-treated cells failed to spread or spread only partially and the actin appears as unpolymerized pools in the center of the rounded cells (D–F).

to the periphery of the cell, or as parallel bundles that extended the length of the filopodia (Fig. 10). The Hsp-treated coelomocytes that were in the rounded, petaloid shape and had failed to extend cytoplasmic projections showed intense staining for unpolymerized cytoplasmic actin (Fig. 10D,F), while those that did extend projections (Fig. 10E) showed actin morphology indistinguishable from that of untreated cells (Fig. 10A–C).

In additional groups of coelomocytes, we examined the microtubule network using indirect immunofluorescence. The arrangement of microtubules did not correlate with the degree to which the cells were spread. The microtubules were often clustered in circular bundles around the nucleus in both Hsp-treated and untreated cells exposed to hypotonic medium.

Discussion

Egg cell Hsp uptake and effects on mitosis

The original concept of the 70 kDa Hsps being strictly cytoplasmic in their distributions and functions clearly is no longer accurate (Fleshner and Johnson, 2005) (for a review, see Tytell, 2005). However, there is little information about the functional impact of extracellular Hsc70 and/or Hsp70. Our work shows the utility of sea urchin eggs and coelomocytes as models in which one can explore the effects of extracellular Hsps on two fundamental cell functions, mitosis and the physiological stress response, respectively. Because these two cell types are easy to obtain in large numbers and all can be triggered to go through mitosis in synchrony, it was possible to document that extracellular Hsc70 and Hsp70 significantly accelerate the rate of mitosis in eggs at an early step, the breakdown of the nuclear envelope, and markedly inhibit the wound and inflammatory-like spreading and adhesion response of hypotonically-stressed coelomocytes.

In the fertilized egg, exogenously applied Hsc/Hsp70 was consistently observed to decrease the time to NEB by more than 10%. There was no significant relationship between the concentration of Hsc/Hsp70 tested and the degree to which NEB and cleavage were accelerated, suggesting that, even at the lowest concentration, the Hsp was present in excess. That concentration equaled about 1.4 nmol l^{-1} Hsc/Hsp70. Being effective at such a low concentration was unexpected and suggested that the protein produced its effects by altering the activity of one or more of the many kinases and phosphatases that play roles in regulating mitosis. We examined the levels of phospho-cdc2 following fertilization as an initial test of this idea. This kinase is known to be activated by being

dephosphorylated rapidly at the onset of mitosis (Edgecombe et al., 1991). Therefore, we expected that the levels of phospho-cdc2 would fall more rapidly in Hsc70-treated eggs compared with controls, so that it could phosphorylate lamin, which is necessary for NEB (Burke, 1990; Peter et al., 1991). Instead, the opposite occurred, with phospho-cdc2 being higher in Hsc70-treated eggs. Thus, it may be that Hsc70 and Hsp70 may modulate cdc2 activity in some other way. In fact, it has been reported to promote the formation of the active cdc2/cyclin B1 complex during meiosis of mouse spermatocytes (Zhu et al., 1997). It is also known that Hsc70 inhibits the activity of P27Kip1, a cyclin-dependent kinase inhibitor, an effect that could accelerate the transition of the cell through G1/S (Nakamura et al., 1999). Furthermore, in other species of sea urchins, Hsc70 was shown to be essential for egg progress through mitosis (Sconzo et al., 1999) and to colocalize with Cdc-2 on the meiotic spindle apparatus (Geraci et al., 2003). Additional work will be required to determine the details of the relationship between the phospho-cdc2 result and the promotion of NEB by exogenous Hsc70 and Hsp70.

There was a difference in the effectiveness of Hsc70 alone to reduce the time to NEB compared with that of the combined Hsc/Hsp70 preparation. Hsc70 did not decrease cell cycle times significantly at lower concentrations, although it showed a trend toward shortening time to NEB at the highest concentration, 5 μg ml^{-1}. This lower effectiveness may be related to differences in the ability of Hsp70 and Hsc70 to interact with membranes, as shown by Arispe et al. (Arispe et al., 2002). Another possible explanation is that the mechanism by which these proteins decrease cell cycle time may be better activated by cooperative interactions of both Hsc70 and Hsp70. This suggestion of a cooperative interaction has a biological basis since both forms of the protein are always present together in the cell, albeit at varying ratios depending on the cell type and the level of metabolic stress to which the cell is subjected. However, we cannot exclude the possibility that the biological activity of the two preparations differed because they were isolated from different tissues, muscle in the case of Hsc/Hsp70 *versus* brain in the case of Hsc70. There exists a variety of accessory proteins that regulate the activity and specificity of interaction of Hsc70 and Hsp90. These include Hip, the Hsc70-interacting protein, Hop, the Hsp70/Hsp90-organizing protein (Frydman and Hohfeld, 1997) and CHIP, the carboxy terminus of Hsp70-interacting protein (Ballinger et al., 1999). The presence of different trace amounts of one or more of these accessory proteins in the two preparations of Hsps may contribute to their different activities and will need to be examined in future studies. Nonetheless, the fact that the Hsc/Hsp70 and Hsc70 preparations used in these experiments had comparable ability to renature luciferase suggests that the observed distinction was not directly related to their refolding activities.

The decrease in time in the initial stages of mitosis in fertilized sea urchin eggs in response to increased levels of Hsp70 has relevance to understanding how endogenous Hsp70 influences cancer cell proliferation. Hsp70 is highly expressed in the cytoplasm of cancer cell lines and has been found to be necessary for their survival (Nylandsted et al., 2000; Rohde et al., 2005). Furthermore, increased expression of Hsp70 has been shown to decrease doubling time in a tumor cell line, perhaps through shortening of the G_0/G_1 and S phases of the cell cycle (Barnes et al., 2001).

The presence in the egg cytoplasm of N-His–rhHsc70 confirmed that extracellular Hsc70 was taken up by the unfertilized egg (Fig. 4). The Hsc70 was excluded from the nucleus in some, but not all, cells. Since Hsc70 has been shown to have a cytoplasmic distribution, moving into the nucleus in response to heat stress (Dingwall and Laskey, 1992; Tsukahara and Maru, 2004; Welch and Mizzen, 1988), the presence or absence of the protein in egg nuclei may reflect varying degrees of metabolic stress in eggs maintained *in vitro*.

The heterogeneous pattern of N-His–Hsc70 immunofluorescence in optical sections of all the eggs suggested that it was enriched and/or reduced in some organelles in the cytoplasm. In fact, Fig. 4C illustrates apparent exclusion from a small vesicle-like structure. Additionally, comparison of the Hsc70 immunofluorescence pattern with the distribution of egg endoplasmic reticulum shown by Henson et al. (Henson et al., 1989) and Teresaki (Teresaki, 2000) suggests that the protein was excluded from that organelle. That interpretation is consistent with the typical distribution of Hsc70 in cells (Dastoor and Dreyer, 2000). There was no indication of any selective binding to the cell surface or evidence of localization to endocytotic vesicles. Therefore, extracellular Hsc70 appears to be able to pass through the plasmalemma and associate with other cytoplasmic constituents by some as yet unknown process. These results are consistent with previous studies (Fujihara and Nadler, 1999; Guzhova et al., 1998; Guzhova et al., 2001) and suggest that Hsc70 uptake is a nonspecific process in eggs. In this regard, it is intriguing that the distribution of endogenous Hsp70 in unfertilized sea urchin eggs appears similar to that seen here after uptake of extracellular Hsc70 (Sconzo et al., 1999) because it suggests that the extracellular 70 kDa Hsps had access to the same intracellular compartments as the endogenous, egg-synthesized Hsp70.

Coelomocyte injury response inhibition by Hsp

In coelomocytes, the inhibition of the injury-related spreading response was unexpected. These cells resemble vertebrate immune system cells, expressing homologues of the vertebrate complement components B and C3, cysteine-rich scavenger receptor genes, toll-like receptors and a C3 receptor (Al-Sharif et al., 1998; Bertheussen, 1982; Pancer, 2000; Smith et al., 1998). Since Hsp70 has been implicated as an extracellular signal in the human immune system, promoting inflammatory responses (Asea et al., 2000), we anticipated it would do the same to coelomocytes. However, other work shows that Hsp70 can be anti-inflammatory, inhibiting leukocyte adhesion to the vascular endothelium and subsequent diapedesis, as well as microglial/monocyte activation in

experimental stroke (Hightower et al., 2000; House et al., 2001). Thus, in the context of osmotic stress of coelomocytes, the anti-inflammatory effects of the 70 kDa Hsps predominated and Hsp70 prevented the spreading response more effectively than Hsc70. This difference in the two isoforms is generally consistent with what we observed in the acceleration of egg NEB, although the preparations of Hsps used to treat them were from different sources, so further experiments will be needed to confirm this point.

Overall conclusions

Using two distinct cell types that are easily isolated from the sea urchin – eggs and coelomocytes – we found that both responded to exogenous Hsc70 and Hsp70 by showing significant alterations in fundamental functions. Thus, the presence of even relatively low concentrations of these Hsps in the extracellular fluid can dramatically alter normal cell processes in ways that are unique to the cell type. The mechanisms of these effects remain to be investigated but are likely to involve interactions of the 70 kDa Hsps with a number of signaling cascades that other studies have shown to be modulated by them. For example, various forms of cellular stress have been shown to activate the p38 stress-activated protein kinase (Martin-Blanco, 2000), and intracellular Hsp70 can interact with and inhibit some members of this and other stress-activated signaling cascades (Gabai et al., 1997). Furthermore, Hsp70 modulates c-Jun kinase by binding directly to it or by altering JNK phosphatase activity (Meriin et al., 1999) and has been implicated in the regulation of the Raf-1/Mek pathway in mammalian tissue culture cells (Song et al., 2001). The data reported here explored and demonstrated the utility of the sea urchin egg and coelomocyte for study of the effects of exogenous Hsp on mitosis and immune cell responsiveness. Future studies can take advantage of these easily obtained cells to reveal the mechanisms by which extracellular Hsc70 and Hsp70 influence these fundamental processes and thereby help to define the necessary studies to be done in mammalian cells, in addition to supporting the phylogenetic conservation of Hsp function.

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