HSP72 INTERACTS WITH PAXILLIN AND FACILITATES THE REASSEMBLY OF FOCAL ADHESIONS DURING RECOVERY FROM ATP DEPLETION

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Running Title: Hsp72-paxillin interaction
Index words: Ischemia, vinculin, talin, cell attachment, HSP70, acute renal failure

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SUMMARY

The cytoprotective effect of heat stress proteins on epithelial cell detachment, an important cause of acute, ischemic renal failure, was examined after ATP depletion by evaluating focal adhesion complex (FAC) integrity. The intracellular distribution of FAC proteins (paxillin, talin and vinculin) was assessed by immunohistochemistry before, during and after exposure of renal epithelial cells to metabolic inhibitors. The resulting ATP depletion caused reversible re-distribution of all three proteins from focal adhesions to the cytosol. Paxillin, a key adaptor protein, was selected as a surrogate marker for FAC integrity in subsequent studies. Prior heat stress increased hsp72, a molecular chaperone, in both the triton X-100 (TX-100) soluble and insoluble protein fractions. Compared to ATP deplete control, heat stress significantly decreased paxillin and hsp72 shift from the TX-100 soluble to the insoluble protein fraction (an established marker of denaturation and aggregation); increased paxillin-hsp72 interaction detected by co-immunoprecipitation; enhanced paxillin extractability from TX-100 insoluble precipitates; increased the reformation of focal adhesions and improved cell attachment (P < 0.05). To determine whether hsp72 mediates protection afforded by heat stress, cells were infected with adenovirus containing human hsp72 or empty vector. Hsp72 over-expression increased its interaction with paxillin and improved focal adhesion reformation during recovery, mimicking the protective effects of heat stress. These data suggest that hsp72 facilitates the reassembly of focal adhesions and improves cell attachment by reducing paxillin denaturation and by increasing its re-solubilization after ATP depletion.
INTRODUCTION

Acute ischemic renal failure is characterized by detachment of proximal tubule epithelial cells from the substratum, permitting backleak of glomerular filtrate and intratubular obstruction (1-3). ATP depletion, an *in vitro* model of ischemia, disrupts the cytoskeleton, causing filamentous actin to be replaced by macromolecular aggregates of short actin polymers (4-6). In intact organisms (3,7) and in culture (3), viable cells that originate from the proximal tubule have been harvested after an ischemic insult, suggesting that cell death is not a prerequisite for detachment.

The tri-partite structure composed of integrins, the cytoskeleton and cell-cell contact sites permit epithelial cells to adhere to the extracellular matrix. Attachment is directly mediated by the interaction between $\alpha$ and $\beta$ integrins, transmembrane proteins that reside on the basolateral surface, and arginine-glycine-aspartic acid (RGD) residues of extracellular matrix proteins (8). Adhesion is stabilized by the actin cytoskeleton by anchoring to the cytosolic domain of integrins (9) as well as to cell–cell contact sites (10). This cytoskeleton-integrin connection is formed and regulated by the focal adhesion complex, which is located at the basolateral region of the epithelial cell (11,12). This complex, comprised of talin, vinculin, paxillin, focal adhesion kinase, src and other proteins, is crucial for regulating the cell attachment. Stress-induced alterations in the activity and/or intracellular distribution of focal adhesion complex proteins cause detachment (13).

During ischemia *in vivo* or ATP depletion *in vitro*, many cytoskeletal-associated component proteins are either denatured or form large macromolecular aggregates (5,14-18). Following exposure to solvents, toxins, heat or ATP depletion, the increased burden of detergent insoluble proteins contributes to cell dysfunction and
impairs recovery (16,19-21). Perturbations in protein conformation correlate with their solubility in non-selective detergents such as triton X-100 (14,17,20). Intracellular proteins that denature or aggregate during ATP depletion are likely targets for rescue by molecular chaperones (20,22,23). Structural proteins that comprise the cytoskeleton or cell adhesion sites are likely to interact with molecular chaperones since: (1) disruption of these cellular sites is one of the earliest morphologic features of ischemia (24-27) and ATP depletion (5,28); (2) ischemia increases the content of detergent-insoluble cytoskeletal-associated proteins (14,16,17,20,29) and (3) ischemia promotes the formation of large protein aggregates (5,16,26). In contrast to the pathologic changes in the actin cytoskeleton and cell-cell contact sites that accompany ATP depletion, alterations in focal adhesion complex proteins are poorly characterized.

Intracellular proteins that are denatured or aggregate during ATP depletion are likely targets for rescue by molecular chaperones (20,22,23). Hsp72, a known cytoprotectant protein, is induced by renal ischemia in vivo (30) and acts as a molecular chaperone, binding and repairing non-native proteins (20,23,31). Although the chaperone function of hsp72 is well characterized, its role in protecting proteins involved in cell attachment has not been previously described. The present study evaluated the hypothesis that hsp72 protects cell attachment, at least in part, by interacting with proteins that comprise the focal adhesion complex. ATP depletion caused hsp72 and paxillin (an adaptor protein that localizes to the focal adhesion plaque) to shift from the triton X-100 soluble protein fraction (containing primarily cytosolic proteins) to the triton X-100 insoluble pool (containing cytoskeletal and other structural proteins). In addition, ATP depletion caused the reversible re-distribution of talin, vinculin, and paxillin from focal adhesion plaques into the cytosol. Loss of focal adhesion staining was associated with cell detachment. Prior heat stress prevented the loss of paxillin from the detergent-soluble protein pool, increased paxillin extractability from the detergent-insoluble pool, enhanced paxillin
staining in focal adhesion plaques during recovery from ATP depletion and increased cell attachment (P < 0.05). Both heat stress and ATP depletion increased the interaction between paxillin and hsp72, suggesting that protein repair facilitates the re-entry of paxillin into the focal adhesion plaque during recovery from ATP depletion. Selective over-expression of hsp72 also increased its interaction with paxillin and reproduced the protective effect of prior heat stress on the reformation of focal adhesion plaques. These studies suggest that hsp72 prevents focal adhesion protein denaturation and increases the release of focal adhesion components from detergent-insoluble protein aggregates. By preventing primary protein denaturation and secondarily, by increasing the content of functional proteins available for re-assembly of focal adhesions, hsp72 exerts cytoprotective effects on cell attachment.
EXPERIMENTAL PROCEDURES

Materials: All reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Cell Culture: Opossum Kidney (OK) cells were obtained from the American Type Culture Collection (ATCC #CRL-1840) and were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS). Cells were used within 72 hr. of achieving confluence as assessed by visual inspection with a phase-contrast microscope.

ATP Depletion and hsp72 Induction: In OK cells, ATP content was reduced by exposure to 1 hr of glucose-free medium (cat #5030) containing sodium cyanide (5mM) and 2-deoxy-d-glucose (5mM). This maneuver causes equivalent reductions in ATP content to < 10% of the baseline value within 10 minutes in both control and previously heated renal epithelial cells (32). DMEM containing 10 mM glucose without metabolic inhibitors was added to initiate recovery. Parallel medium changes were made in controls using glucose-containing DMEM. To induce hsp72, OK cells were heated to 43±0.5°C for 45-60 minutes in a temperature-regulated incubator followed by incubation at 37°C for 12-16 hours (32).

Selective over-expression of hsp72: Cells were co-infected with adenoviruses containing wild-type human hsp72 and green fluorescent protein (AdTR5/hsp70-GFP) expressed on separate cistrons and a tetracycline-regulated promoter (AdCMV/tTA) kindly provided by Dick Mosser (University of Guelph, Ontario Canada) as recently described (33). This maneuver increase hsp72 content to a level compared to that of heat stress (33). Control cells were co-infected for 24 hr. at 37°C with 3 x 10^7 PFU/35 mm² petri dishAdTR5/GFP and AdCMV/tTA. Infection efficiency for both viruses was
>95% as estimated by direct visualization of GFP. After 24 hr exposure to either adenovirus, cells were subjected to ATP depletion as described above.

**Preparation of cell fractions:**

**Whole cell lysate:** Harvested cells were re-suspended in cell lysis buffer (described below) containing a protease cocktail (10 units/ml). Cells were sonicated and then centrifuged at 10,000xg for 5 minutes at 4°C. The supernatant was designated as the whole cell lysate.

**Triton X-100 extraction:** Monolayers of cells in individual, 60 mm² culture dishes were incubated for 30 minutes on a rocker at 4°C with 0.4 ml Triton X-containing CSK extraction buffer containing (in mM): NaCl, 50; sucrose, 300; PIPES, 10; MgCl₂, 3; and triton X-100 (0.5% v/v) and protease inhibitor cocktail (10 units/ml) at pH 7.40. After centrifugation (13,000 rpm x 15 min at 4°C), the supernatant (the Triton-X soluble protein fraction) was harvested. The pellet (the Triton-X insoluble protein fraction) was sonicated at 4°C in 0.1 ml SDS IP buffer containing (in mM): Tris HCl, 10; EDTA, 0.5; DTT, 0.5; with DNAase, 0.1 mg/ml; RNAase, 0.1 mg/ml), a protease inhibitor cocktail (10 units/ml) and SDS (1%).

**Immunologic Analyses:** Protein components of the focal adhesion plaque including paxillin (cat# 05-417), talin, (cat# 05-385) vinculin (cat# 05-386) were examined using commercially available antibodies (Upstate Biotechnologies, Lake Placid, NY). Hsp72 (cat #SPA-810, StressGen Biotechnologies, Victoria, BC Canada) and hsp27 (Santa Cruz #SC-1049) were detected with mouse monoclonal antibodies specific for these proteins. Immunoreactive proteins were detected with a horseradish peroxidase-based enzyme-linked chemiluminescence system as previously described (33). Immunodetected protein bands were quantified using NIH-Image Quant Software after scanning the blot with a densitometer (Hewlett-Packard, Desk Scan II). Species specific Cy3-linked secondary antibodies (Jackson Immunoresearch, West Grove, PA) were
used for all immunohistochemical studies in cells fixed with 2% paraformaldehyde after permeabilization with 1% sodium dodecyl sulfate as previously reported (5).

**Immunoprecipitation and Co-immunoprecipitation**  As previously reported (33,34), aliquots of samples obtained from the whole cell lysates or triton x-100 soluble and insoluble protein extracts were subjected to co-immunoprecipitation (IP). Samples were diluted to 1 mg/ml with IP buffer (in mM): NaCl, 150; TRIS-HCl, 10; EDTA, 5; EGTA, 1 at pH 7.40; to which triton X-100 (0.1%) and a protease inhibitor cocktail (10 units/ml; cat#539131; Merck Biosciences AG, Germany) were added. Apyrase and EDTA prevent ATP and Mg-mediated release of potential binding partners from hsp72 (33,34). After centrifugation (14,000 x g x 5 min. at 4°C), 250-500 µg of total protein of supernatant was "pre-cleared" for 1 hour with non-immune serum (10 µl/mg protein) obtained from the same host species as the primary antibody. Supernatant was incubated overnight at 4°C with either anti-paxillin (Santa Cruz, #SC-5574), anti-hsp27 (Stressgen, cat#SPA-803) or anti-hsp72 (Stressgen, cat#SPA-810) antibody titrated to permit equivalent yields of paxillin under each experimental condition (8 µg/mg protein/ml IP buffer). Immobilized protein G agarose was added to the solution during the final 2 hours of incubation. An agarose pellet was obtained by centrifugation and then washed for 5 min. with high stringency buffer (0.1% sodium dodecyl sulfate, 1% deoxycholic acid, 0.5% Triton X-100, 20 mM TRIS-HCl, 120 mM NaCl, 25 mM KCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM DTT ["HS-B"]) with 1 M sucrose, pH 7.5. Samples were then washed in a high salt buffer (HS-B + 1 M NaCl) followed by a final wash in low salt buffer (2 mM EDTA, 0.5 mM DTT, 10 mM TRIS-HCl, pH 7.5). Samples were mixed with 2X-sample buffer in preparation for SDS-PAGE. Blots were then probed for paxillin, hsp27 and hsp72 as described above.
**Protein Assay:** Protein concentrations were determined from a colorometric dye-binding assay (BCA Assay, Pierce, Rockford, IL) and expressed in milligrams protein per milliliter.

**Quantifying focal adhesion plaques:** The mean number ± standard error of cells containing at least 10 focal adhesion plaques was assessed in 4-7 random fields of ATP depleted in two separate experiments by an observer blinded to the experimental conditions.

**Cell Detachment:** The mean number of detached cells was determined in at least three randomly selected fields of subconfluent monolayers (85-90% confluent) 30 min after 1 or 1.5 hr ATP depletion by an observer blinded to the experimental conditions. Minimal apoptosis (34) or necrosis (32) have been observed at this time point in renal epithelial cells subjected to transient ATP depletion.

**Statistical Analysis:** Data are expressed as the mean ± SE. Experimental groups were compared using an unpaired, two-tailed Student's t-test. A P value of < 0.05 was considered significant. Analysis was performed with standard statistical software (Excel, Microsoft Corporation, Santa Monica, CA).
RESULTS

Effect of ATP depletion and prior heat stress on cell attachment. After transient ATP depletion followed by 30 min recovery, numerous detached cells were visible by routine contrast microscopy. After 1 or 1.5 hr ATP depletion, approximately 25% and 41% of cells respectively, were detached (fig 1). Prior heat stress significantly reduced the number of detached cells to 9 and 13%, respectively after both periods of injury (P < 0.05 vs. control at each time point). In the absence of ATP depletion, only 4-5% of cells were detached in control or heat stressed cells (data not shown; P > 0.05).

Effect of ATP depletion and prior heat stress on focal adhesion integrity. Several components of focal adhesion complex were examined by immunohistochemical analyses. ATP depletion caused a marked re-distribution of vinculin, talin, and paxillin from the adhesion plaque into the cytosol. In control, focal vinculin (fig 2A) and talin (fig 2B) staining in adhesion plaques were visualized. Immediately after ATP depletion however, virtually no intact adhesion plaques (i.e., vinculin or talin staining) could be observed. After 30 min recovery, many adhesion plaques were again visualized. Similar results were obtained in control cells stained with antibody directed against paxillin, another protein that localizes to the focal adhesion (fig 3A). At baseline, both control and previously heat stressed cells exhibited an identical pattern of paxillin staining in focal adhesions (left upper panel vs. left lower panels). As described for both vinculin and talin, no focal adhesions were seen immediately after 1 or 1.5 hr of ATP depletion in either control or heated cells (images not shown). After 30 min recovery from transient ATP depletion, paxillin staining was more abundant in heat stressed cells (right lower vs. right upper panels). To quantify this difference, the number of cells with at least 10 visible focal adhesion plaques was counted before, during and after ATP depletion (fig 3B). At baseline, >10 focal adhesion plaques were observed in virtually all control and previously heated cells. Immediately after 1 hr ATP
depletion, no adhesion plaques were visible in either experimental group. After 15 or 30 min recovery however, less than 5% of control cells exhibited ≥10 focal adhesion plaques. In contrast, >10 focal adhesions were visible in nearly 30% of previously heated cells (P < 0.05 vs. control at both time points). Since all three focal adhesion proteins exhibited a similar pattern of re-distribution following ATP depletion, paxillin was selected as a marker of focal adhesion integrity in subsequent studies.

**Effect of transient ATP depletion on triton-X 100 soluble and insoluble protein:** In cells subjected to 1 hr. ATP depletion followed by 30 min recovery, a progressive, reciprocal shift in the relative amounts of triton X-100 soluble and insoluble total cell protein was observed (fig 4). At baseline, approximately 28% of total cell protein was insoluble. Insoluble protein increased to 35% after 1.5 hr. ATP depletion and represented almost half (48%) of total cell protein following 30 min recovery. These data demonstrate that the absence of ATP alters protein conformation and precipitates aggregate formation.

**Effect of prior heat stress on the triton X-100 solubility of paxillin:** Compared to control, the relatively rapid recovery of paxillin staining suggested that heat stress exerted a cytoprotective effect on the re-assembly of focal adhesions. To evaluate this hypothesis, the detergent solubility of paxillin was examined. In control, ATP depletion resulted in the progressive shift of paxillin from the detergent-soluble to the-insoluble protein fraction (fig 5A). This shift was significantly reduced by heat stress (fig 5B; P < 0.05), suggesting that heat inducible proteins protect paxillin from ATP depletion-mediated denaturation.

In addition to preventing paxillin denaturation, prior heat stress could promote focal adhesion reassembly by enhancing paxillin release from the detergent-insoluble protein pool. To evaluate this hypothesis, the content of immunoreactive paxillin was serially assessed in triton X-100 insoluble protein fractions obtained from control and previously...
heated cells subjected to ATP depletion as outlined (fig 6A). In this in vitro assay, various maneuvers were used to extract paxillin from the triton X-100 insoluble protein fraction. The content of immunodetectable paxillin was used to estimate the amount of paxillin available to reassemble focal adhesions. In the absence of ATP (+ ayprase) or the presence of ATP and magnesium (“ATP + Mg”), virtually no paxillin could be detected at any time point before, during or after ATP depletion in the detergent insoluble protein fraction (fig 6B; upper portions of the top and middle panels). The combination of ATP, magnesium, high temperature (100°C) and a reducing agent (DTT) resulted in modest paxillin release during recovery from ATP depletion (upper portion of panel C). The addition of prior heat stress to each of these maneuvers markedly increased paxillin release under all experimental conditions (lower portion of panels A-C). Heat stress was most effective for extracting paxillin from the detergent insoluble protein fraction.

**Effect of ATP depletion on the triton-X solubility of hsp72:** Since hsp72 has been reported to bind and repair non-native proteins, the detergent solubility of hsp72 was serially examined. At baseline, hsp72 was detected in both the triton X-100 soluble and insoluble fractions (fig 7A). Prior heat stress markedly increased hsp72 content in both the detergent-soluble and -insoluble protein fractions. ATP depletion (1 hr) and recovery (30 min) resulted in a marked, reciprocal shift in immunoreactive hsp72 from the detergent-soluble to the detergent-insoluble protein fraction (fig 7B). During ATP depletion and recovery, prior heat stress was associated with as much as a 40% reduction in the shift of hsp72 to the detergent insoluble fraction (fig 7C, P< 0.05).

Since ATP depletion and recovery resulted in a parallel shift of hsp72 and paxillin into the triton X-100 insoluble protein pool and hsp72 preferentially binds non-native proteins, potential interaction between these two proteins was assessed by co-immunoprecipitation. Minimal interaction between hsp72 and paxillin was observed at
baseline (lane 1, upper panel, fig 8A). ATP depletion and recovery modestly increased their interaction (lanes 2,3). In contrast, prior heat stress markedly increased the interaction between hsp72 and paxillin under all experimental conditions. Attempts to detect paxillin in hsp72 immunoprecipitates was complicated by interference of the overlying heavy chain band with paxillin (data not shown). Selective over-expression of human hsp72 (“+Hsp72”) also increased the interaction between these two proteins when compared to empty vector that contained GFP alone (“-Hsp72”; middle panel). The increase in hsp72-paxillin interaction associated with heat stress cannot be attributable to changes in the amount of paxillin contained in the immunoprecipitates, since paxillin content was comparable in each sample (lower panel).

To further evaluate protein interaction, triton x-100 soluble and insoluble immunoprecipitates were assessed. Triton x-100 insoluble protein extracts were solubilized by exposure to 1% SDS without disrupting paxillin-hsp 72 binding. Prior heat stress markedly increased the interaction between hsp72 and paxillin, particularly during ATP depletion in both the triton X-100 soluble (upper panel) and insoluble (lower panel) protein fractions (fig 8B). Unlike hsp72, minimal interaction between hsp27 (a small hsp known to interact with cytoskeletal-related proteins (35) and paxillin could be detected when either hsp27 or paxillin were used for the initial immunoprecipitation. Furthermore, ATP depletion and recovery were associated with only modest changes in the degree of interaction between paxillin and hsp27 in either triton X-100 soluble (upper panel) or insoluble (lower panel) protein extracts (fig 8C).

To test the hypothesis that hsp72 is rate-limiting in restoring focal adhesion complexes in injured cells, human, wild type hsp72 was selectively over-expressed prior to ATP depletion. Exposure to adenovirus containing hsp72, but not empty vector, increased the steady state content of hsp72 to a level comparable to that of heat stress (data not shown). Selective increase of hsp72 (“+Hsp72”) did not affect paxillin staining in focal
adhesion plaques compared cells containing the empty vector (“-Hsp72”; Fig 9, upper panels) or to non-transfected control cells at baseline (fig 3). Immediately after ATP depletion, paxillin was virtually absent from focal adhesions in both groups (middle panels). In contrast, over-expression of hsp72 increased the number of visible focal adhesions after 30 min recovery (lower panels).
DISCUSSION

ATP depletion is associated with the detachment of viable renal epithelial cells from the substratum (3). In the present study (fig 1), detachment is unlikely to be due to renal cell death, since minimal necrosis (manifested as loss of lactate dehydrogenase or trypan blue uptake; ref (32)) or apoptosis (34) are observed in ATP depleted cells at this time point. How does ATP depletion cause detachment independent of cell death? The present study proposes that alterations in the distribution and function of focal adhesion proteins compromise attachment. A central thesis of this hypothesis is that cell stressors, including ATP depletion, disrupt native protein conformation and expose hydrophobic regions thereby increasing their propensity to form detergent insoluble aggregates (20,21,36,37). These aggregates are primarily comprised of cytoskeletal-related proteins (28,38,39).

The present study focuses on the effect of ATP depletion on the intracellular distribution and detergent solubility of focal adhesion proteins that regulate cell attachment. Not only does the focal adhesion complex regulate the interaction between the cytoskeleton and integrins, it serves as a focal point for the reassembly of actin filaments during recovery from ATP depletion (27). In proximal tubule cells in vitro, exposure to metabolic inhibitors induced a marked shift in the detergent solubility of total cell protein (fig 4). These changes are similar to those reported in non-renal cells subjected to ATP depletion (16,20,40,41) suggesting that disruption of protein conformation is a generalized response to this form of stress (16,21).

In renal cells, transient ATP depletion causes marked changes in the distribution and detergent solubility of focal adhesion component proteins. Rapid, reversible redistribution of three components of the focal adhesion complex: paxillin, talin and vinculin
(figs 2-3) was detectable within 15 min of ATP depletion (data not shown). Of these proteins, paxillin is relatively well characterized. Paxillin localizes to the focal adhesion, where it acts as an adaptor molecule or “platform”, linking the cytoskeleton with β–integrins (42,43). Paxillin also co-ordinates cell adhesion, motility and spreading by recruiting multiple signaling molecules to the focal adhesion complex (35,39,43). Since paxillin is central to focal adhesion assembly and turnover, alterations in its detergent solubility were examined. In response to ATP depletion and in parallel with total cell protein, paxillin progressively shifted from the detergent soluble to insoluble protein fraction (fig 5) after ATP depletion, suggesting that paxillin is sensitive to denaturation and aggregation.

Inducible molecular chaperones prevent protein denaturation and facilitate their repair (44). Increasing the content of cytoprotective chaperone proteins prior to stress enhances cellular defense by improving their stoichiometry with the burden of denatured proteins (19,20,38). The hypothesis that stress protein content is rate-limiting in protecting paxillin is supported by the observation that prior heat stress significantly prevented the translocation of paxillin into the detergent insoluble fraction during ATP depletion and recovery (fig 5). By preventing aggregation, heat stress proteins increase the amount of native paxillin available to reconstitute the focal adhesion plaque during recovery. Protection of paxillin by HSP70 is not unique to paxillin or to ATP depletion, as HSP70 has been shown to improve the function of several enzymes following exposure to alcohol, hyperthermia and other noxious stimuli that alter protein conformation (20,31,33).

In addition to preventing changes in protein conformation, HSPs also facilitate protein re-solubilization and repair (44). By repairing non-native proteins, HSPs increase the
mass of functional proteins available during recovery. In vitro experiments show that prior heat stress also accelerates focal adhesion recovery by increasing the amount of paxillin available to re-constitute the focal adhesion complex. Compared to the presence of ATP, magnesium, or exposure to both 100°C and a reducing agent (common maneuvers for re-solubilizing aggregated proteins), heat stress was more potent in extracting immunoreactive paxillin from the detergent-insoluble protein aggregates (fig 6B). In the absence of heat stress, only magnesium-ATP released detectable amounts of paxillin from these extracts (upper portion of panel c). ATP dependent release of substrate proteins from members of the HSP70 family suggests that their interaction is specific (23,45). In addition to ameliorating protein aggregation and repair, inducible HSP70 members mediate the delivery of proteins to their appropriate intracellular compartment (46,47). Hsp72 has been reported to facilitate the delivery of Na⁺,K⁺-ATPase to the basolateral compartment, a requirement for re-establishing cell polarity, during recovery from renal ischemia (14,48,49). In the present study, hsp72 could stabilize cell attachment by re-targeting paxillin to the focal adhesion plaque.

To identify the specific protein responsible for cytoprotection, interaction between paxillin and hsp72, the major inducible HSP70 member in mammalian cells, was examined. Hsp72 is an ideal candidate chaperone, since prior heat stress increases its steady state content in both the detergent soluble and insoluble protein fractions (fig 7A) and significantly decreases the shift in hsp72 from the detergent soluble to the insoluble fraction during ATP depletion and recovery (fig 7C). In the present study, selective over-expression of hsp72 mimicked the effect of heat stress on focal adhesion plaque formation during recovery from ATP depletion (fig 9). Taken together, these observations suggest that hsp72 protects paxillin and mediates the improvement in cell attachment associated with prior heat stress. A role for hsp72 in
protecting paxillin is supported by the observation that both heat stress and the selective over-expression of hsp72 increased interaction between these two proteins in whole cell lysates (fig 8A). Furthermore, ATP depletion enhanced hsp72-paxillin interaction in both the detergent soluble and insoluble protein fractions, especially in cells that over-express hsp72 (fig 8B). This suggests that hsp72 not only ameliorates paxillin denaturation, but also facilitates its repair.

To assess the specificity of paxillin-hsp72 interaction, the degree of co-immunoprecipitation between paxillin with hsp27 was examined. Hsp27 associates with cytoskeletal proteins (18,51) and has been reported to protect myocardial cells against ischemia both in vitro (52) and in vivo (53). Unlike hsp72, minimal interaction between paxillin and hsp27 were detected in renal epithelial cells and ATP depletion exerted almost no effect on hsp27-paxillin interaction (fig 8C). In addition, microinjection of hsp27 in non-renal cells actually inhibited focal adhesion formation after thermal stress (54).

Heat stress proteins such as hsp72 are likely to promote cell attachment by protecting multiple intracellular sites in addition to the focal adhesion. Prior heat stress significantly preserved the actin cytoskeleton and improved the functional integrity of the tight junction in ATP depleted renal epithelial cells (5). Heat stress also inhibited the activation of src, a key regulatory tyrosine kinase that mediates both cell attachment and cell-cell contact, and minimized alterations in the phosphotyrosine content of key src substrate proteins including paxillin, in ATP depleted renal epithelial cells. Hsp72 also binds to Na⁺,K⁺-ATPase, a cytoskeletal tethering protein(14,56). Finally, during ATP depletion in renal cells, hsp72 interacts with focal adhesion kinase (FAK), a regulatory component of the focal adhesion complex. The interaction between hsp72 and FAK is required to prevent the degradation of FAK by caspase 3 (33). By protecting the actin
cytoskeleton, preserving cell-cell contact sites and inhibiting events that promote cell shrinkage (thereby decreasing the surface area available for attachment), prior heat stress could promote attachment despite the fact that talin, vinculin and paxillin redistribute from the focal adhesion plaque during ATP depletion even in previously heat stressed cells (fig 2).

Preservation of renal epithelial cell attachment is likely to improve organ function after an ischemic insult. Attached, viable cells contribute to vectoral solute transport, prevent glomerular backleak and minimize the formation of casts that cause intra-tubular obstruction, important contributors to the pathogenesis of acute ischemic renal failure (1,57,58). The contribution of detached cells to acute renal failure has been emphasized by Noiri and colleagues, who showed that RGD peptides improved organ function, presumably by preventing intra-tubular obstruction in rats subjected to transient ischemia (2). Maintenance of cell-matrix interactions could also promote cell survival by preventing anoikis, a form of apoptosis precipitated by the loss of attachment (28,59). Since hsp72 has a half-life measured in days (30,32), maneuvers that increase hsp72 content may ameliorate renal epithelial cell injury and improve organ function after an ischemic insult.
ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health Grant DK-47994 (SCB) and DK-5298 (JHS).
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FIGURE LEGENDS

Figure 1: Effect of ATP depletion and prior heat stress on renal cell attachment. The number of detached cells 30 min after 1 or 1.5 hr. ATP depletion was quantified in control (open bars) or previously heated (solid bars) cells. At least three random fields were counted in six separate experiments. Data shown are mean ± SE. * P < 0.05 vs. control.

Figure 2 (A,B) Effect of ATP depletion on focal adhesion protein distribution. 
(A) Vinculin distribution in normal cells (“baseline”; upper panel), after ATP depletion (“ATP Deplete”; middle panel) and following 30 min recovery (“REC30”; lower panel); 
(B) Talin distribution in normal cells (“baseline”; upper panel), after ATP depletion (“ATP Deplete”; middle panel) and following 30 min recovery (“REC30”; lower panel).

Figure 3 (A,B): Effect of prior heat stress on paxillin in focal adhesions following ATP depletion. 
(A) Control (upper panels) and previously heated (lower panels) cells were examined at baseline and after 1 hr ATP depletion followed by 30 min recovery. Focal adhesions were visualized using antibody directed against paxillin; 
(B) The number of control (open bars) and previously heated (solid bars) cells with visible focal adhesion plaques was determined as described in Methods. Open bars Data represent the mean± SE. Some SE bars are too small to be seen. * P < 0.05 vs. control.

Figure 4: ATP depletion induces a shift in detergent soluble total cell protein. Whole cell lysates was divided into to triton X-100 soluble (open bars) and insoluble (solid bars) protein fractions as described in Methods. Detergent solubility was
examined at baseline, immediately after 1.5 hr ATP depletion and following 30 min recovery. One of two representative studies is shown.

**Figure 5 (A,B): Effect of prior heat stress on the triton-X 100 solubility of paxillin following ATP depletion.**

**(A)** Immunodetectable paxillin was examined in triton X-100 soluble (*upper panels*) and insoluble (*lower panels*) in control and previously heat stressed cells before, during and after ATP depletion. A representative immunoblot is shown;

**(B)** Quantitative analysis of the change in detergent soluble (*left panel*) and insoluble (*right panel*) paxillin was determined in control (*open squares*) and previously heated (*solid triangles*) cells. *P* < 0.05 vs. control; *n* = 3. Data are mean ± SE.

**Fig 6 (A,B) Effect of heat stress on paxillin release from triton X-100 insoluble extracts.**

**(A)** Schematic diagram for quantifying the re-solubilization of immunoreactive paxillin in triton X-100 insoluble protein extracts;

**(B)** Immunoreactive paxillin released from detergent insoluble extracts in the presence or absence of Mg-ATP (“ATP/Mg” or “Apyrase”, respectively); after exposure to 100°C + dithiothreitol (“100°C-DTT”); or following transient heat stress (“HS”) was measured under control conditions (“C”) after ATP depletion (“A”) and after 5, 15 and 30 min recovery (“R_5, R_{15}, R_{30}”). Boxes are shown to highlight the additive effect of heat stress on paxillin re-solubilization compared to each of the other maneuvers. Compared to each maneuver alone, prior heat stress markedly increased paxillin re-solubilization from detergent insoluble extracts.
Figure 7 (A-C): Effect of ATP depletion on the triton X-100 solubility of hsp72.

(A) Hsp72 content in the triton X-100 soluble and insoluble protein fractions of control (left panel) and previously heat stressed (right panel) cells. Each lane contains 10 µg total protein;

(B) Hsp72 content in triton X–100 soluble (upper panel) and insoluble (lower panel) fractions harvested from control or previously heat stressed cells under normal conditions (“Base”), immediately after 1 hr ATP depletion (“ATP deplete”) and following 30 min recovery (“REC_{30}”). Differences in the amount of protein loaded in the lanes of each panel account for the apparent lack of induction of hsp72 by heat stress;

(C) Densitometric analysis of immunoreactive bands obtained from three separate studies as described in panel B. Date obtained from control (open circles) and previously heated (solid triangles) cells are presented as mean ± SE. Some SE bars are too small to be seen; * P < 0.05 vs. control.

Figure 8 (A-C): Hsp 72 interacts with paxillin. The degree of co-immunoprecipitation of hsp72 with paxillin was examined in samples using an anti-paxillin antibody. Immunoprecipitates were separated by SDS-PAGE and then exposed to anti-hsp72, hsp27 or paxillin antibodies.

(A) Whole cell lysates containing 250 µg or 500 µg were obtained from control (“Control”), previously heated (“Heat stress”) and cells selectively over-expressing human hsp72 (“+Hsp72”). Empty vector containing GFP only (“-Hsp72”) was used as the control. Increased expression of hsp72 increased its interaction with paxillin at baseline (“Base”), immediately after 1 hr ATP depletion (“ATP deplete”) and following recovery (“REC_{30}”); After IP, virtually no residual paxillin was detected in the supernatants (data not shown).

(B) Interaction between paxillin and hsp72 in triton X-100 soluble and insoluble protein fractions harvested from control and heat stress cells in immunoprecipitates as described
in A. Exposure times in each panel were selected to optimize hsp72 and resulted in apparent differences in the amount of immunoglobulin heavy chain; 

(C) Interaction between paxillin and hsp27 triton X-100 soluble and insoluble samples harvested from control and heat stress cells as described in A. After IP, virtually no residual hsp27 was detected in the supernatants (data not shown).

Figure 9: Effect of selective hsp72 over-expression on the recovery of focal adhesion plaques after ATP depletion. Focal adhesion plaques were examined in control (“-Hsp72”) and hsp72 (“+Hsp72”) over-expressing cells (as described in the fig 8 legend) using an antibody directed against paxillin prior to ATP depletion (“baseline”), immediately after ATP depletion (“ATP depletion”) and after 30 min recovery (“Recovery”). Over-expression of hsp72 increased the number of focal adhesions detected during recovery from ATP depletion.
Fig 1

![Graph showing percentage of detached cells over duration of ATP depletion.](http://www.jbc.org/)

- % detached cells
- Duration ATP depletion (hr)
- Bars with error bars and asterisks indicating statistical significance.
Fig 2A

Baseline

ATP deplete

REC₃₀
Fig 2B

Baseline

ATP Deplete

REC_{30}
Fig 3
Fig 3B

![Bar graph showing % focal adhesion + cells for different conditions: Base, ATP deplete, Rec_{15}, Rec_{30}. Significant differences are indicated by asterisks (*)]
Fig 4

![Bar chart showing % total protein content for Baseline, ATP Depletion, and REC30.](image)
**Fig 5A**

The figure shows Western blots for Paxillin in different conditions:

- **TX Soluble**
  - **CONTROL**
  - **HEAT STRESS**

- **TX Insoluble**
  - **CONTROL**
  - **HEAT STRESS**

Legend:

- **Base**
- **ATP deplete**
- **REC<sub>30</sub>**

Paxillin expression levels are compared between control and heat stress conditions for both soluble and insoluble fractions.
Fig 5B
Fig 6A

Control  Heat Stress

ATP depletion

Harvest whole cell lysate

Triton X-100

Soluble  Insoluble

ATP depletion

ATP -ATP  ATP + Mg  100°C + DTT

Standard sample buffer

Immunoblot for paxillin
### Fig 6B

<table>
<thead>
<tr>
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<th>C</th>
<th>A</th>
<th>R₅</th>
<th>R₁₅</th>
<th>R₃₀</th>
<th>Apyrase</th>
<th>ATP +Mg</th>
<th>Boil</th>
<th>HS +DTT</th>
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Fig 7A

**Triton X-100 solubility**

<table>
<thead>
<tr>
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<th>Soluble</th>
<th>Insoluble</th>
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<tr>
<td>CONTROL</td>
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<td>![CONTROL Image]</td>
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<tr>
<td>HEAT STRESS</td>
<td>![HEAT STRESS Image]</td>
<td>![HEAT STRESS Image]</td>
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</tbody>
</table>

**Hsp72**
**Fig 7B**

Triton X-Soluble

**CONTROL**
(10 µg/lane)

**HEAT STRESS**
(2.5 µg/lane)
Fig 7C

TX-100 Soluble Fx

hsp72 content (vs. % baseline)

Baseline  ATP deplete  REC_{30}

TX-100 Insoluble Fx

hsp72 content (vs. % baseline)

Baseline  ATP deplete  REC_{30}
**Fig 8A**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Western Blot</th>
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<tr>
<td>Base deplete</td>
<td>Hsp72</td>
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<tr>
<td>ATP Rec&lt;sub&gt;30&lt;/sub&gt;</td>
<td>250 µg IP</td>
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<tr>
<td></td>
<td>Control</td>
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<tr>
<td>ATP deplete</td>
<td>Hsp72</td>
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<td>Heat stress</td>
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<tr>
<td>- Hsp72</td>
<td>Paxillin</td>
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<tr>
<td></td>
<td>250 µg IP</td>
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<td>+ Hsp72</td>
<td>Control</td>
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Fig 8B

<table>
<thead>
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<th>TX-100 Soluble</th>
<th>TX-100 Insoluble</th>
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<tbody>
<tr>
<td>Control</td>
<td>Control</td>
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<tr>
<td>Heat stress</td>
<td>Heat stress</td>
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</table>

hsp72
Fig 8C

<table>
<thead>
<tr>
<th>Cell lysate</th>
<th>Base deplete</th>
<th>ATP</th>
<th>Rec$_{30}$</th>
<th>Base deplete</th>
<th>ATP</th>
<th>Rec$_{30}$</th>
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</table>

TX-100
Soluble

TX-100
Insoluble

Control → Heat stress
Fig 9

Baseline

ATP depletion

Recovery

- Hsp72  + Hsp72

5 µm
HSP72 interacts with paxillin and facilitates the reassembly of focal adhesions during recovery from ATP depletion
Haiping Mao, Yihan Wang, Zhijian Li, Kathleen L. Ruchalski, Xueqing Yu, John H. Schwartz and Steven C. Borkan

J. Biol. Chem. published online January 12, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313484200

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