hsp72 Inhibits Focal Adhesion Kinase Degradation in ATP-depleted Renal Epithelial Cells*

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Prior heat stress (HS) or the selective overexpression of hsp72 prevents apoptosis caused by exposure to metabolic inhibitors by protecting the mitochondrial membrane and partially reducing caspase-3 activation. Focal adhesion kinase (FAK), a tyrosine kinase, exhibits anti-apoptotic properties and is a potential target for degradation by caspase-3. This study tested the hypothesis that hsp72 interacts with FAK, preventing caspase-3-mediated degradation during ATP depletion. ATP depletion (5 mM NaCN and 5 mM 2-deoxy-D-glucose in the absence of medium glucose) caused FAK degradation within 15 min. FAK degradation was completely prevented by a caspase-3-specific inhibitor. HS induced the accumulation of hsp72, increased the interaction between hsp72 and FAK, and significantly inhibited FAK degradation during ATP depletion. Selective overexpression of wild-type hsp72 (but not hsp72ΔEEVD) reproduced the protective effects of HS on FAK cleavage. Purified hsp72 prevented the degradation of FAK by caspase-3 in vitro in a dose-dependent manner without affecting caspase-3 activity. Interaction between hsp72 and FAK is critical because both exogenous ATP and deletion of the substrate-binding site decreased protection of FAK by hsp72. These data indicate that FAK is an early target of injury in cells exposed to metabolic inhibitors and demonstrate that hsp72 reduces caspase-3-mediated proteolysis of FAK, an anti-apoptotic protein.

Transient ischemia in vivo (1) and ATP depletion in vitro (2–4) induce apoptosis in renal epithelial cells. Apoptosis in renal cells (like many eukaryotic cells) is characterized by mitochondrial injury and the subsequent activation of caspase-3 (5). In addition to mitochondrial membrane injury, ischemia and ATP depletion perturb cell-cell contact sites (6, 7) and cause cell detachment (7, 8). These untoward events predispose cells to apoptosis and contribute to the acute deterioration of renal function (9–12). Several studies suggest that hsp72 exerts anti-apoptotic effects in a variety of cell types subjected to diverse stresses (5, 13–18). In renal epithelial cells, prior heat stress protects cells contact sites (6) and inhibits apoptosis caused by ATP depletion (3). Recent studies in renal epithelia show that heat exposure and the selective overexpression of hsp72 decrease, but do not abolish, mitochondrial membrane injury and the subsequent activation of caspase-3 (5, 17). These observations suggest that hsp72 could provide additional cytoprotection by inhibiting caspase-3 and/or by preventing caspase-3 from degrading anti-apoptotic proteins.

Focal adhesion kinase (FAK)1 is a multifunctional non-receptor protein-tyrosine kinase that promotes the formation of cell-substrate contact sites in response to integrin-mediated contact with the extracellular matrix (19, 20). Integrin-associated focal adhesion complexes localize to these contact sites and provide the primary “adhesive link” between the actin cytoskeleton and extracellular matrix proteins (21). FAK regulates the turnover of the focal adhesion complex (22) that is required for migration (23), differentiation (24), and cell growth (21, 25). FAK itself is regulated by its state of tyrosine phosphorylation, its intracellular distribution, and protease-mediated cleavage of its kinase and focal adhesion-targeting domains (26–28).

Recent evidence suggests that FAK suppresses apoptosis (26–33). The selective overexpression of FAK protects against apoptosis induced by a variety of noxious insults (29, 32, 34). Conversely, FAK proteolysis (26, 28, 35) or the overexpression of mutant FAK proteins (33) promotes apoptosis. During cell stress, cysteinyl aspartate-specific proteases (caspases) cause the sequential cleavage of intact FAK into several smaller protein fragments (26, 27). Of the intracellular proteases, caspase-3 has been shown to exert a major role in the degradation of FAK into pro-apoptotic fragments (20, 27, 36).

In this study, we hypothesized that hsp72, an inducible cytoprotective protein, prevents FAK cleavage by caspase-3 during ATP depletion. Our study shows that transient exposure to metabolic inhibitors results in the rapid proteolysis of FAK. Heat stress with recovery induced hsp72 and significantly inhibited FAK proteolysis during ATP depletion. Acute heat stress without recovery did not increase hsp72 content and failed to prevent FAK degradation in ATP-depleted cells. In intact cells, caspase-3 inhibition or the selective overexpression of hsp72 reproduced the protective effect of heat stress on FAK proteolysis. Wild-type hsp72 co-immunoprecipitated with FAK. Prior heat stress and ATP depletion markedly increased the interaction between hsp72 and FAK. In an in vitro assay, purified human caspase-3 produced virtually complete fragmentation of intact human FAK. Purified human hsp72 inhib-

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1 The abbreviations used are: FAK, focal adhesion kinase; OK, opossum kidney; Ad, adenovirus; GFP, green fluorescent protein; CMV, cytomegalovirus; tTA, tetracycline-controlled transactivator; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.
ited FAK cleavage by caspase-3 in a dose-dependent manner. Preservation of FAK was more striking in the absence of ATP and was not observed when the hsp72EEVD deletion mutant was overexpressed, suggesting that interaction with hsp72 mediates protection of FAK. This study supports the hypothesis that hsp72 inhibits apoptosis, at least in part, by preventing caspase-3-mediated proteolysis of FAK.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents were obtained from Sigma unless otherwise indicated.

**Cell Culture**—Renal epithelial cells (CRL-1840) derived from opossum kidney (OK) were purchased from American Type Culture Collection. Previously characterized human kidney cells (37) were generously provided by Dr. Richard Zager (University of Washington, Seattle, Washington). Cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum. Human kidney cells required the addition of 50% Dulbecco’s modified Eagle’s medium and 50% Ham’s F-12 medium in the presence of 10% fetal calf serum. Cells were used within 72 h of achieving confluence.

**ATP Depletion**—To induce ATP depletion, cells were incubated for 15–60 min at 37 °C in glucose-free medium (Dulbecco’s modified Eagle’s medium) containing sodium cyanide and 2-deoxy-D-glucose (5 mM each) as previously described (38). In control cells, parallel medium changes were performed using 10 mM glucose-containing Dulbecco’s modified Eagle’s medium.

**Induction of Wild-type hsp72 and hsp72EEVD**—Wild-type hsp72 content was increased either by transient heat stress (42.5 ± 0.5 °C for 45 min) in a temperature-regulated incubator followed by incubation at 37 °C for 16–18 h (38) or by co-infection of OK cells with adenoviruses containing human wild-type hsp72 and green fluorescent protein (AdTR5/hsp70-GFP) expressed on separate cistrons and a tetracycline-regulated promoter (AdCMV/tTA) as previously described (18). Control cells were co-infected with AdTR5/GFP and AdCMV/tTA. To increase hsp72 content, cells were infected for 24 h at 37 °C with 3 × 10^7 plaque-forming units/35-mm^2 Petri dish. hsp72EEVD was transduced in cells lacking the C-terminal EEVD sequence that is essential for peptide binding, was also overexpressed using adenovirus as previously described (18). After removing the virus, cells were incubated for an additional 24 h. Infection efficiency was >95% as estimated by direct visualization of GFP. Preliminary experiments were performed to determine the amount of adenovirus and the incubation conditions required to approximate hsp72 content in heat-stressed cells as measured by immunoblot analysis (described below).

**Immunoblot Analysis and Co-immunoprecipitation**—Harvested cells were resuspended in lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1 mM EGTA, 1% Triton-X-100) and a mixture of protease inhibitors (5 μM 4-(2-aminoethyl)benzenesulfonyl fluoride HCl, 10 mM leupeptin, 1.5 mM aprotinin, 10 mM E-64, and 5 mM EDTA; pH 7.40; Calbiochem-Novabiochem). The cells were sonicated and then centrifuged at 10,000 × g for 10 min at 4 °C. hsp72 (SPA-810, Stressgen Biotech Corp., Victoria, British Columbia, Canada), hsp72EEVD (SPA-811, Stressgen Biotech Corp.), and FAK (BD Biosciences) were detected in the supernatant by immunoblotting using commercially available monoclonal antibodies. Specific protein bands were detected with an anti-IgG antibody coupled to a horseradish peroxidase-based enzyme-linked chemiluminescent detection kit (Lumiglow, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). After digitizing the image of each immunoblot (Desk Scan II, Hewlett-Packard Co.), band intensities were quantified using NIH ImageQuant software. Immunoprecipitation of FAK using a specific monoclonal antibody (2 μg of protein/ml of immunoprecipitation buffer; Santa Cruz Biotechnology, Santa Cruz, CA) was performed as described recently by us for cytochrome c (5). Appyrase (10 units/ml), a compound that causes ATP hydrolysis, was used to prevent the ATP-mediated release of bound ligands from hsp72 (5). Co-immunoprecipitation was assessed by probing the membranes with an antibody directed against hsp72.

**Caspase-3 Activity**—Caspase-3 enzyme activity was measured as previously described by us (5) using a fluorometric assay (ApoAlert caspase-3 fluorescence assay, Clontech) according to the manufacturer’s protocol. To assess caspase-3-specific activity, assays were also performed either without substrate or in the presence of a caspase-3-specific inhibitor (1 μM of DEVD-CHO-aldehyde [Upstate Biotechnology, Inc.]).

**FAK Proteolysis in Vivo**—In intact cells, FAK proteolysis was assessed by comparing the quantity of cleaved FAK by immunoblot analysis of homogenates obtained from control and ATP-depleted OK cells in the presence and absence of caspase-3 inhibition. Immunoblot analysis was performed with an antibody that detects both intact FAK (125 kDa) and its cleavage products (70–85 kDa). FAK cleavage products were included in the analysis. *p < 0.05 (n = 5). C, effect of a caspase-3 inhibitor on FAK cleavage. OK cells were subjected to 30 min of ATP depletion in the presence and absence of a cell-permeable caspase-3 inhibitor (100 μM DEVD-CHO). Data are representative of three separate experiments. Each lane contains 20 μg of total protein. Both intact and degraded FAks are indicated by arrows.

**FIG. 1. A and B, effect of ATP depletion on FAK fragmentation. A, FAK proteolysis was examined in control and previously heated OK cells immediately after 15–90 min of ATP depletion using an antibody that detects both intact FAK (125 kDa) and its cleavage products (70–85 kDa). B, densitometric analysis was used to assess the protective effect of prior heat stress on FAK degradation after 15, 30, and 60 min of ATP depletion in control cells (white bars) and previously heat-stressed cells (black bars). All FAK cleavage products were included in the analysis. †, *p < 0.05 (n = 5). C, effect of a caspase-3 inhibitor on FAK cleavage. OK cells were subjected to 30 min of ATP depletion in the presence and absence of a cell-permeable caspase-3 inhibitor (100 μM DEVD-CHO). Data are representative of three separate experiments. Each lane contains 20 μg of total protein. Both intact and degraded FAks are indicated by arrows.**
Protein Assay—Protein concentrations were determined with a colorimetric dye binding assay (BCA assay, Pierce). Results are expressed in mg of protein/ml.

Statistical Analysis—Data are expressed as means ± S.E. Comparison of two groups was performed using one- or two-tailed Student’s t test. Bonferroni’s adjustment was applied to correct the p value when multiple tests were performed using a single control group. A result was considered significant if p < 0.05.

RESULTS

Exposure to metabolic inhibitors caused the de novo appearance of several FAK cleavage products with apparent molecular sizes of 70–90 kDa (Fig. 1A). Although virtually no FAK fragments were detected in control cells maintained at 37 °C (first lane), FAK proteolysis was evident after only 15 min of exposure to metabolic inhibitors (second lane). The magnitude of FAK degradation was greatest after 30 min. More prolonged ATP depletion (60–90 min) was associated with less immunoreactive FAK cleavage products (fourth and fifth lanes). Prior heat stress inhibited FAK degradation at all time points of ATP depletion (sixth through tenth lanes). Prior heat stress significantly decreased FAK degradation after 15, 30, and 60 min of ATP depletion (p < 0.05 versus control) (Fig. 1B). To confirm that in situ FAK proteolysis was mediated by caspase-3, a cell-permeable caspase-3-specific inhibitor was added prior to exposure to metabolic inhibitors. DEVD-CHO completely prevented the degradation of FAK after 30 min of ATP depletion (Fig. 1C). In contrast to FAK, ATP depletion minimally increased the proteolysis of paxillin, another regulatory focal adhesion protein that is susceptible to degradation by caspase-3 (39). Unlike FAK, prior heat stress did not inhibit ATP depletion-mediated degradation of paxillin (Fig. 2).

To determine whether hsp72 per se inhibits FAK cleavage in heat-stressed cells subjected to ATP depletion, hsp72 content was selectively increased by transiently infecting renal epithelial cells with adenovirus encoding human hsp72. Exposure to an hsp72-expressing adenovirus increased hsp72 to a level similiar to that observed after heat stress (Fig. 3A). Both heat stress and adenoviral infection resulted in a significant 5-fold increase in steady-state hsp72 content (Fig. 3B). Approximately 95–99% of the adenovirus-infected cells were positive for GFP (data not shown). Compared with control cells (infected with GFP- and tTA-encoding adenoviruses), increased expression of hsp72 inhibited FAK proteolysis after 15, 30, and 60 min of ATP depletion (Fig. 3C). The decrease in FAK degradation associated with selective hsp72 overexpression was significant at all three periods of ATP depletion (Fig. 3D). Of note, differences in the conditions of gel electrophoresis (10 versus 12%) accounted for the apparent alteration in the sizes of FAK fragments of hsp72 inhibited FAK cleavage during ATP depletion. A, hsp72 content was examined in control (C), previously heat-stressed (HS), and adenovirus-infected (AdV) OK cells. Each lane contains 10 μg of total protein. The effect of selective overexpression of hsp72 on ATP depletion-mediated FAK cleavage was examined in cells infected with adenovirus containing human hsp72. B, densitometric analysis was used to assess the hsp72 content after heat stress or adenoviral infection. Data are expressed as means ± S.E. *, p < 0.05 (n = 3). C, cells were subjected to transient ATP depletion for 15–60 min. Cells were infected with AdCMV/tTA and either AdTR5/GFP (− HSP72) or AdTR5/hsp70-GFP (+ HSP72). Each lane contains 20 μg of total protein. D, densitometric analysis of FAK degradation after 15–60 min of ATP depletion in control cells (white bars) or in cells infected with adenovirus (black bars). All FAK cleavage products were included in the analysis. Data are expressed as means ± S.E. *, p < 0.05 (n = 3).
ysis in each of the samples in A exposure to 42.5 through first through ATP depletion. A or after 15 and 30 min of ATP depletion in cells as follows: control (separate experiments. Each lane in A and B contains 20 μg of total protein. These immunoblots are representative of three separate experiments.

ments observed in Fig. 3C compared with Fig. 1A. The observation that hsp72 per se inhibited FAK proteolysis during ATP depletion suggests that hsp72 and FAK might interact with one another.

Acute heat stress per se (42.5 °C for either 40 or 60 min) did not cause FAK degradation (Fig. 4, first lanes versus seventh and tenth lanes). In contrast to heat stress with recovery, acute heat exposure without recovery did not prevent FAK degradation after 15 or 30 min of ATP depletion (Fig. 4A). In addition, acute heat stress without recovery did not increase steady-state hsp72 content (Fig. 4B).

In control cells, interaction between hsp72 and FAK could not be detected by co-immunoprecipitation prior to ATP depletion (Fig. 5A, upper panel), and only minimal interaction was observed after 15–60 min of ATP depletion (second through fourth lanes). In contrast, prior heat stress sufficient to increase hsp72 content increased the interaction between these two proteins at all time points (fifth through eighth lanes). Maximal interaction between hsp72 and FAK occurred after 30 min of ATP depletion (seventh lane). Increased interaction between hsp72 and FAK could not be attributed to differences in the amount of immunoprecipitable protein given that FAK content was virtually identical in each sample (Fig. 5A, lower panel). Because the content of intact FAK differed in control and heat-stressed cells subjected to ATP depletion, the amount of antibody used to immunoprecipitate FAK was calibrated to permit an equivalent yield of protein from both experimental groups. hsp72 content was similarly increased in all lysates obtained from cells subjected to heat stress (Fig. 5B).

To examine the role of substrate binding in preventing FAK proteolysis, hsp72ΔEEVD, a mutant hsp72 protein that exhibits defective substrate binding (13, 18), was selectively overexpressed in OK cells (Fig. 6). In contrast to wild-type hsp72 (ninth through twelfth lanes), hsp72ΔEEVD (fifth through eighth lanes) failed to prevent FAK proteolysis in cells subjected to ATP depletion (Fig. 6A) despite a marked increase in mutant hsp72 content (Fig. 6B). Overexpression of hsp72ΔEEVD did not affect the steady-state content of wild-type hsp72 (Fig. 6C).

If hsp72 prevented FAK proteolysis by caspase-3, then similar protection might be observed in vitro. To test this hypothesis, FAK was obtained from whole cell lysates of control and previously heated cells with an antibody directed against FAK (upper panel). Samples were obtained prior to ATP depletion (first and fifth lanes) and after ATP depletion for 15 min (second and sixth lanes), 30 min (third and seventh lanes), and 60 min (fourth and eighth lanes). To assess the amount of FAK in each sample, immunoprecipitates were probed with an antibody directed against FAK (lower panel). Immunoprecipitation was performed on 1 mg of whole cell lysate, and the resulting immunoprecipitates were separated by SDS-PAGE. B, hsp72 content in whole cell lysates used for the immunoprecipitation in A.

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FIG. 7. Effect of purified hsp72 on FAK cleavage by caspase-3 in vitro. A, FAK obtained from whole cell lysates (lane 1). FAK incubated with 5 µg of purified hsp72 (lane 2), FAK incubated with 200 ng of caspase-3 (lane 3), FAK preincubated with 3 µg of hsp72 prior to the addition of 200 ng of caspase-3 (lane 4), and hsp72 (3 µg) incubated with 200 ng of caspase-3 prior to the addition of FAK (lane 5). All incubations were performed at 37 °C for 60 min. The arrows indicate the locations of intact FAK and a major degradation product. B, intact FAK immunoprecipitated from human kidney cells (lane 1) and subjected to degradation by 200 ng of caspase-3 (lane 2) in the presence of 1 µg (lane 3) or 3 µg (lane 4) of purified hsp72. FAK cleavage was examined in the presence of exogenous ATP (10 mM; lane 5) or in the absence of ATP (10 units/ml apyrase; lane 6). The results are representative of three separate experiments.

FIG. 8. Dose-response effect of purified hsp72 on caspase-3-mediated FAK fragmentation in vitro. FAK obtained from whole cell lysates was incubated with human caspase-3 (200 ng) in the presence of purified hsp72 (0.01–3 µg) for 60 min at 37 °C as described under “Experimental Procedures.” The arrows indicate the locations of intact FAK and its degradation products. Each lane contains 50 µg of total protein. The results are representative of two separate studies.

purified human hsp72 to FAK obtained from whole cell lysates did not alter the migration or content of FAK (Fig. 7A, lane 2). Exposure of FAK to purified human caspase-3 resulted in the formation of at least one major cleavage product (at ~85 kDa) (lane 3). In contrast, co-incubation with human hsp72 reduced FAK degradation (lane 4). If hsp72 was preincubated with caspase-3 prior to the addition of FAK, FAK cleavage was not inhibited despite equivalent contact time between FAK and caspase-3 (lane 5). Similar results were obtained with FAK obtained by immunoprecipitation (Fig. 7B, lanes 2–4). Exogenous ATP (10 mM) resulted in a relative increase in FAK degradation by caspase-3 (lane 5). In contrast, ATP hydrolysis with apyrase (10 units/ml) (3) increased the inhibitory effect of hsp72 on caspase-3-mediated FAK proteolysis (lane 6).

Exposure of FAK to caspase-3 resulted in a 93% decrease in the content of intact FAK (Fig. 8, second lane). Wild-type hsp72 inhibited FAK degradation by caspase-3 in a dose-dependent manner at hsp72 concentrations between 2.3 and 700 nM (third through sixth lanes). The correlation coefficient for the relationship between the dose of hsp72 and the degree of protection of intact FAK was 0.979 as assessed by densitometry of the 125-kDa bands in Fig. 8.

To examine the possibility that hsp72 interfered with caspase-3, in vitro enzyme activity was compared in the presence and absence of purified hsp72. The addition of either 1 or 3 µg of hsp72 (150 and 400 nM) did not alter the activity of purified caspase-3 (Fig. 9). These results are in agreement with a previous report showing that purified hsp70 has no effect on the activity of caspase-3 (40). To determine whether or not hsp72 might compete with FAK as a substrate for caspase-3, purified hsp72 was incubated with 100 ng of caspase-3 (Fig. 10). Caspase-3 failed to degrade intact hsp72 at doses between 1 and 10 ng of the latter protein. These data suggest that hsp72-FAK interaction, rather than inhibition of caspase-3 activity or competition between hsp72 and FAK for proteolysis by caspase-3, is required to protect FAK.

**DISCUSSION**

This study demonstrates that focal adhesion kinase is a target for injury caused by exposure to metabolic inhibitors. In renal epithelial cells, metabolic inhibitors reduce ATP content to <10% of the basal level within 10 min (38). Concomitant with the reduction in ATP content, caspase-3 is activated by 2.5-fold (5). Activated caspase-3 participates in the proteolysis of several substrates, including FAK (26–28, 30). FAK is preferentially cleaved by caspase-3 after the DQTD sequence that terminates at residue 772 (27). Sequential degradation by caspase-3 yields multiple fragments ranging in size from 17 to

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90 kDa (27). Some of these fragments are intrinsically unstable (28, 30), whereas a 45-kDa N-terminal fragment translocates to the nucleus (41). Either observation could explain the apparent disappearance of FAK fragments during prolonged ATP depletion (60–90 min) (Fig. 2). Although caspase-6 and various calpains have also been reported to cleave FAK (20, 27), caspase-3 is the primary enzyme responsible for FAK degradation (27, 28). In this study, a cell-permeable caspase-3-specific inhibitor completely prevented FAK proteolysis in intact cells (Fig. 1C). These results were reproduced in an in vitro system (Figs. 7 and 8), further supporting the notion that caspase-3 is responsible for the proteolysis of FAK in renal epithelial cells.

This study tested the hypothesis that heat stress and hsp72 exert anti-apoptotic effects, at least in part, by decreasing the proteolysis of FAK. Cell stress has been associated with FAK degradation in a variety of cell lines (26, 30, 31, 35). FAK cleavage not only separates the kinase from the focal adhesion-targeting domain, but generates “FAK-related non-kinase,” a peptide fragment that interferes with the kinase function of intact FAK (27). FAK degradation could precipitate apoptosis by acting at one or more sites in the cell death cascade (26–32, 35, 36). FAK regulates the Akt pathway (29) and stimulates protein inhibitors of apoptosis (32), and intact FAK prevents anoikis, a form of apoptosis that occurs as a consequence of the loss of cell adherence (28).

Stewart et al. (41) recently demonstrated that an N-terminal fragment of FAK derived from the focal adhesion-targeting domain migrates into the nuclei of epithelial cells in a manner that is independent of its tyrosine phosphorylation or the presence of the C terminus. Within the nucleus, FAK may directly regulate survival (42, 43). The mechanism by which the N-terminal fragment mediates cell survival is not presently known (41). It is conceivable that FAK degradation represents a redundant signaling pathway that ensures death by apoptosis following caspase-3 activation. Similar redundant pro-apoptotic functions have been attributed to apoptosis-inducing factor, a cause of caspase-independent cell death (44).

Several lines of evidence support the contention that hsp72 prevents the degradation of FAK. First, the selective overexpression of hsp72 (Fig. 3) reproduced the inhibitory effects of prior heat stress (Fig. 1) on the proteolysis of FAK during ATP depletion. In contrast, acute heat stress did not increase hsp72 content and failed to prevent FAK degradation during ATP depletion (Fig. 4). Second, hsp72 interacted with FAK (Fig. 5). This interaction was markedly increased in cells subjected to prior heat stress and was greatest during the time period when FAK degradation occurred. The increased interaction was due in part to the up-regulation of hsp72 content as well as to alterations in their mutual affinity. Third, hsp72 inhibited FAK cleavage by caspase-3 in an in vitro system (Figs. 7 and 8). The ability of hsp72 to prevent FAK degradation is dose-dependent across a range of hsp72 concentrations reported to have physiologic effects (15, 26). These observations may explain, in part, the fact that the selective overexpression of hsp72 inhibits apoptosis in ATP-depleted renal epithelial cells (17).

Several mechanisms could explain the ability of hsp72 to inhibit FAK degradation by caspase-3. For instance, hsp72 could interfere with caspase-3. This does not appear to be likely because hsp70 has been shown to act upstream (16) or down-stream (45) of caspase-3 without directly affecting enzyme activity in vitro (40). In addition, we have shown that purified hsp72 did not interfere with caspase-3 activity in whole cell lysates obtained from ATP-depleted renal cells (Fig. 9) (5). Also, preincubation of hsp72 with caspase-3 prior to the addition of FAK actually reduced the protective effect of hsp72 on FAK degradation (Fig. 7A). Finally, purified hsp72 did not alter caspase-3 activity (Fig. 9). As an alternative mechanism, hsp72 could compete with FAK for degradation by caspase-3. However, this hypothesis is not supported by the observation that caspase-3 failed to degrade purified hsp72 in vitro (Fig. 10). This may be due to the fact that human hsp72 (NCBI BLAST Protein Database accession number XP_175177) does not have a DQTD cleavage sequence targeted by caspase-3 (27).

Taken together, these data support the hypothesis that cytoprotection of FAK requires interaction with hsp72. hsp72, like other members of the hsp70 family, releases non-native proteins in an ATP-dependent manner (46–49). The observation that exogenous ATP decreased protection of FAK by hsp72 (and removing ATP increased protection) (Fig. 7B) implies that hsp72-FAK binding is required to prevent caspase-3-mediated FAK proteolysis. This hypothesis is strengthened by the observation that the hsp72 ΔEVD mutant failed to inhibit FAK degradation during ATP depletion (Fig. 6A). Deletion of the substrate-binding domain also sensitizes cells to apoptosis caused by thermal stress (13, 18, 50), suggesting that this domain mediates cytoprotection. The ability of ATP to affect protection of FAK by hsp72 could be the result of changes in either hsp72 or FAK. In addition to directly altering the binding affinity of hsp72 for FAK, exogenous ATP could also change the conformation of FAK by affecting its degree of phosphorylation (51). Either mechanism (or both) could explain the enhanced interaction between FAK and hsp72 observed during ATP depletion in the absence of changes in hsp72 content (Fig. 7).

This study shows that caspase-3-mediated FAK degradation occurs early in the course of ATP depletion and clearly precedes morphologic evidence of apoptosis (3, 17). It is therefore possible that pharmacologic intervention could inhibit this deleterious event. hsp72 is poised to be a potent anti-apoptotic protein because it acts at multiple sites in the cell death cascade. hsp72 suppresses the activation of stress kinases that participate in the caspase-3 pathway (13), prevents the release of cytochrome c from mitochondria and the subsequent activation of caspase-3 (5, 14, 18), and reduces caspase-independent cell death by apoptosis-inducing factor (15). Both heat stress and selective hsp72 overexpression have recently been shown to decrease primary mitochondrial membrane injury and to partially inhibit caspase-3 activation in ATP-depleted renal epithelial cells (5, 17). Interaction between FAK and hsp72 prevents FAK degradation by activated caspase-3 and may represent a secondary cytoprotective pathway. These two points of intervention ensure that FAK cleavage is prevented. This study identifies a new mechanism by which hsp72 could prevent cell death by apoptosis.

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