Identification and Characterization of a Novel Protein from Sertoli Cells, PASS1, That Associates with Mammalian Small Stress Protein hsp27

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hsp27 is involved in development of tolerance to stress, possibly by its involvement in molecular chaperoning, maintenance of glutathione status, and/or modulation of microfilament structure and function. We hypothesize that hsp27 function depends on specific association with other proteins. To discover proteins that associate with hsp27, we made a differentiated rat Sertoli cell cDNA expression library and screened it using the yeast two-hybrid system. We obtained a cDNA coding for a novel protein of 428 amino acids that we have named PASS1 (protein associated with small stress proteins 1). BLAST searches did not reveal major similarity of PASS1 to any known protein, but the cDNA sequence matched several mouse EST clones and shares 34% homology with a Caenorhabditis elegans genomic sequence. In vitro, bacterially expressed glutathione S-transferase-PASS1 fusion protein bound to hsp27, and hsp27 was co-immunoprecipitated with c-Myc-tagged PASS1 overexpressed in several cell lines. The region of PASS1 responsible for association with hsp27 was identified as existing predominantly between amino acids 108 and 208 of PASS1. Northern hybridization and Western blot analysis demonstrated that PASS1 is expressed in several tissues, with the highest expression occurring in testis, primarily in Sertoli cells. The presence of a 1.4-kilobase PASS1 mRNA in kidney as well as the 1.8-kilobase mRNA seen in other tissues suggests that alternate splicing may occur in this organ. Ectopic expression of PASS1 in two cultured cell lines was observed to inhibit the ability of hsp27 to protect cells against heat shock, indicating that PASS1 does interact with hsp27 in the live cell.

The small heat shock or stress proteins of vertebrates, including hsp27 (also known as hsp25) and α-crystallin, are believed to play a significant role in the cellular stress response (1–6) and have been suggested to be involved in a broad range of other physiological activities (7, 8). In response to toxic conditions, or stress, cells increase the synthesis of heat shock proteins, including hsp27, and coincidentally become more stress-resistant. Elevated levels of hsp27 have been shown to be coincident with elevated resistance to many types of stress, including elevated temperature, toxic metals, drugs, and oxidants (9–14). Three primary hypotheses have been offered to explain how hsp27 might protect cells against stress. These proposed hypotheses are that hsp27 has chaperone-like activity (15–17), that hsp27 stabilizes microfilaments (see 8), and that expression of hsp27 enhances cellular glutathione levels (18, 19).

hsp27 is believed to exist in cells primarily as oligomers of as many as 8–40 hsp27 protein monomers. It is the large oligomers that permit denatured proteins to regain some of their enzymatic activity in vitro (17), suggesting that large oligomers of hsp27 have a chaperone-like activity by serving as a site where unfolding proteins may bind (by doing so, the proteins will not irreversibly aggregate) until ATP and hsp70-dependent refolding can occur (17). αB-crystallin has also been shown to have a chaperone-like activity in vitro (20).

It has also been suggested that hsp27 regulates microfilament organization, in a manner dependent on its phosphorylation and oligomeric status. Hsp27 inhibits actin polymerization in vitro (21). This ability of hsp27 to inhibit actin polymerization appears to be regulated by its phosphorylation status, because only the nonphosphorylated lower molecular weight forms of hsp27 were determined to bind actin barbed ends and inhibit polymerization (22). Several in vivo studies suggest an interaction between expression and phosphorylation of hsp27 and the organization of the actin cytoskeleton. In cell lines transfected to overexpress hsp27, cortical actin arrays were increased, as was pinocytotic activity (23). Microfilaments in cells transfected to overexpress hsp27 are more stable to heat shock, oxidative stress, or treatment with cytochalasin D than are microfilaments in parental cells (11, 23, 24). Cells transfected with an hsp27 antisense construct were seen to have a drastic reduction in microfilament arrays (25). Increased microfilament stability was not observed in cell lines transfected with mutant hsp27 incapable of being phosphorylated (serine residues had been replaced with glycine residues) (24). Migration was reduced in endothelial cells that had been transfected with this mutant form of hsp27, while cells transfected with wild type hsp27 migration was enhanced (26). Interestingly, this mutant form of hsp27 is not only incapable of being phosphorylated, but it also does not form high molecular weight oligomers (19).

The third mechanism by which hsp27 may protect cells is by enhancing cellular glutathione levels. Elevated glutathione...
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levels have been measured in cells overexpressing hsp27, while cells underexpressing hsp27 contained less glutathione (19). When cells overexpressing hsp27 were treated with buthionine-S-sulfoximine (to deplete cellular glutathione stores), the protective effect of hsp27 was abolished, so that hsp27 can apparently protect cells only when glutathione is present. Transfection of cells with wild-type and mutant forms of hsp27 in which the serine phosphorylation sites were mutated to alanines (3A), glycines (3G), or aspartates (3D) demonstrated that the effect of hsp27 on cellular glutathione levels depended on the oligomerization of hsp27, with only the large oligomeric forms of hsp27 being able to protect cells by enhancing glutathione levels (19).

Expression of hsp27 also correlates with growth and differentiation in the developmental processes (reviewed in Ref. 8). For example, the accumulation of hsp27 in muscle tissues and certain neuronal cells during murine development has been observed (27, 28). Interestingly, hsp27 seemed to be associated with reduced growth or proliferation and increased differentiation state in mammalian cells, such as murine Ehrlich ascites tumor cells (29, 30), embryonal carcinoma, embryonic stem cell lines (31), NIH/3T3 cells (32), and other cell types (33–36).

Unfortunately, no precise mechanism has been proposed, much less demonstrated, to explain how hsp27 may participate in all of the activities suggested. We have hypothesized that hsp27 may participate in differing cellular functions as a consequence of association with different specific binding protein partners. For example, the localization of hsp27 with microfilaments in some cell types but not others might be a consequence of the expression in some cells, e.g. muscle and Sertoli cells, of as yet unknown proteins that specifically bind to both hsp27 and microfilaments or a microfilament-associated protein. More generally, we further hypothesized that the various functions suggested for hsp27 and the other members of the small stress protein family may depend on the existence of different proteins that interact with the small stress proteins and mediate different events. Thus, by identifying proteins associating with small stress proteins, functions of hsp27 may be elucidated, and mechanisms may be understood.

Previously, hsp27 has been reported to associate with the mammalian transglutaminase, platelet factor XIII (37) and to a Drosophila nuclear ubiquitin-conjugating enzyme (38). Here we report the molecular cloning of a novel binding protein we have termed PASS1 (protein associated with small stress proteins 1). PASS1 was isolated from a rat Sertoli cell cDNA library using the yeast two-hybrid system and biochemically shown to interact specifically with mammalian hsp27. Additionally, we demonstrate that PASS1 expression affects the transcription of genes 1). PASS1 was isolated from a rat Sertoli cell cDNA library using the yeast two-hybrid system and biochemically shown to interact specifically with mammalian hsp27. For example, the localization of hsp27 with microfilaments in some cell types but not others might be a consequence of the expression in some cells, e.g. muscle and Sertoli cells, of as yet unknown proteins that specifically bind to both hsp27 and microfilaments or a microfilament-associated protein. More generally, we further hypothesized that the various functions suggested for hsp27 and the other members of the small stress protein family may depend on the existence of different proteins that interact with the small stress proteins and mediate different events. Thus, by identifying proteins associating with small stress proteins, functions of hsp27 may be elucidated, and mechanisms may be understood.

Experimental Procedures

Construction of Sertoli Cell cDNA Library—Sertoli cells were isolated from testes of 27-day-old Harlan Sprague-Dawley rats and were cultured for 3 days as described previously (39). Sertoli cells were isolated at 37 °C with 5% CO2. Culture medium and serum were purchased from Life Technologies, Inc.

Northern Hybridization—For Northern blotting, a rat multiple tissue Northern blot (CLONTECH) was used and fused in the BamHI–HindIII site, with the coding sequence of PASS1 derived from pGal4AD-PASS1. The fusion-expression vector was designated prkPASS1. Re- moving the 3′-end sequence of PASS1 cDNA to the BgII, SmaI, EcoRI, and XhoI sites, respectively, generated four serial C-terminal truncated versions of prkPASS1. The corresponding plasmids prkPASS1ΔC1, prkPASS1ΔC2, prkPASS1ΔC3, and prkPASS1ΔC4, each containing PASS1 inserts of 1.155, 0.843, 0.624, and 0.324 kb, respectively, were obtained. Two N-terminal truncated PASS1 constructs were made as follows. Cloning a 1.020-kb PCR fragment from PASS1 into prk5Myc gave the 5′-Myc-tagged PASS1 expression plasmid, pcDNA-myc, was subcloned into a complete rat β-crystallin cDNA sequence into pcDNA3.1 (Invitrogen) at EcoRI-XhoI sites. For expression of PASS1 as a glutathione S-transferase (GST) fusion protein, a 1.5-kb PASS1 cDNA fragment containing the complete coding region of PASS1 protein was cut with BamHI–XhoI from the cloning construct pGal4AD-PASS1. The fragment was then subcloned into pGex-SX-1 (Amersham Pharmacia Biotech), and the resulting construct was designated pGEX-PASS1. For constructing a c-Myc-tagged PASS1 expression plasmid, a modified prk5 vector designated prkMyc, containing coding sequence for c-Myc peptide (EQKLISEEDL), was used and fused in the BamHI–HindIII site, with the coding sequence of PASS1 derived from pGal4AD-PASS1. The fusion-expression vector was designated prkPASS1. Removing the 3′-end sequence of PASS1 cDNA to the BgII, SmaI, EcoRI, and XhoI sites, respectively, generated four serial C-terminal truncated versions of prkPASS1. The corresponding plasmids prkPASS1ΔC1, prkPASS1ΔC2, prkPASS1ΔC3, and prkPASS1ΔC4, each containing PASS1 inserts of 1.155, 0.843, 0.624, and 0.324 kb, respectively, were obtained. Two N-terminal truncated PASS1 constructs were made as follows.

Cell Culture and Transfection—Monkey COS cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum. Human 293T cells were cultured in the DMEM supplemented with nonessential amino acids. NIH/3T3 cells were cultured in DMEM supplemented with 5% calf serum. Cells were maintained at 37 °C in 5% CO2. Culture medium and serum were purchased from Life Technologies, Inc.

Transient transfections were performed using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s protocol. Briefly, 24 h prior to transfection, 5 × 105 cells were plated onto each well of six-well plates, with 1 µg of each construct used per well. Total DNA was adjusted to the same concentration for all treatments. Cells were harvested or processed for further treatments 48 h after transfection.

Northern Hybridization—Total RNA was prepared from whole testis or primary culture of rat Sertoli cells (39) with TRIZOL (Life Technologies, Inc.), and poly(A)−RNA was prepared from the total RNA (40). For Northern blotting, a rat multiple tissue Northern blot (CLONTECH) was used, or 2 µg of poly(A)−RNA was run on a 1% agarose gel electrophoresis. The purified cDNA (100 ng) was ligated into 1 µg of the gel-purified vector PACT II arms (41) at 12 °C for 2 days. The ligation mixture was packaged using the Gigapack II Gold packaging extract (Stratagene). After cre-lox conversion, plasmid preparation was performed using Mega Plasmid Kit (QIAGEN).
and transferred onto a nylon membrane. The probe, a gel-purified 1-kb *BamHI–HindIII fragment from plasmid PAD-PASS1, was labeled with [32P]dCTP in a random primer reaction (kit from Roche Molecular Biochemicals).

Northern blots were prehybridized for 2 h at 42 °C in a solution containing 5% SDS, 400 mM sodium phosphate buffer (pH 7.2), 1 mM EDTA, 1 mg/ml bovine serum albumin, and 50% formamide. The labeled probe was added to the mixture, and hybridization was allowed to proceed for 18 h. After hybridization, blots were washed in 1× SSC, 0.1% SDS two times for 30 min at 42 °C. These washes were followed by three 20-min high stringency washes in 0.2× SSC, 0.1% SDS at 65 °C, after which the blot was covered in plastic wrap and exposed to x-ray film (Eastman Kodak Co.) using a Kodak Lanex enhancer screen.

**GST Pull-down Assay**—Bacterial strain BL21 (Stratagene) was used to produce GST fusion proteins. Briefly, an overnight culture was diluted 10-fold and allowed to continue growth until the optical density at a wavelength of 600 nm reached 1.0 before the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.2 mM for induction of the fusion protein. Cells were harvested after another 4 h of incubation. After lysis of bacterial cells by French press, cell supernatants were batch affinity-purified using glutathione-agarose beads (Sigma). Fusion proteins were eluted from the beads using a buffer containing reduced glutathione and were subsequently analyzed by SDS-PAGE. The pull-down assay was performed as follows. Total protein extracts from rat kidneys prepared in Nonidet P-40 buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40) were incubated for 2 h with bead-immobilized GST alone or GST-PASS1 at 4 °C. The beads were washed extensively with PBS to remove any proteins that would not bind to GST or PASS1. The column was then washed with PBS containing reduced glutathione (10 mM) to elute proteins binding to the column. Eluted proteins were subsequently analyzed by SDS-PAGE and Western blotting.

**Extraction of Proteins from Rat Tissues for Western Blotting**—An adult male laboratory rat (about 200 g) was sacrificed, and the organs to be studied were immediately removed and processed for protein extraction. About 200 mg each of the more dense tissues (brain, skeletal muscle) were ground under liquid nitrogen using a precooled mortar and pestle. After evaporation of liquid nitrogen, the remaining frozen tissue powder was transferred into a Dounce tissue grinder and homogenized with 300 μl of extraction solution (3% SDS, 68.5 mM Tris-HCl, pH 6.8, 5% glycerol, 10 mM dithiothreitol, 1 pill/20 ml of protease inhibitor mixture (Roche Molecular Biochemicals)). The tissue mixtures were chilled on ice, and the resulting homogenates were supplemented with protease inhibitors (1 mM phenylmethysulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and centrifuged at 14,000 g for 10 min at 4 °C. The supernatant fluids were incubated with the indicated antibodies on ice for 2 h. Protein A-agarose beads (25 μl; Sigma) were then added to the mixture for another hour at 4 °C. The beads were washed three times with lysis buffer before being resuspended in sample loading buffer and analyzed by SDS-PAGE.

**Western Blotting**—After SDS-PAGE, proteins were blotted onto polyvinylidene difluoride membranes (Millipore Corp.) by a semidy filter method (42). Blots were blocked with 5% dry milk in PBS (PBS containing 0.1% Tween 20), treated with appropriate primary antibody followed by horseradish peroxidase-conjugated secondary antibody. Protein detection was performed using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Sometimes membranes were striped and reprobed, following protocols also recommended by Amersham Pharmacia Biotech. Developed blots were scanned into files using a GS 200 Imaging densitometer (Bio-Rad) and analyzed by the accompanying software, Molecular Analyst (Bio-Rad).

**Antibodies**—A peptide sequence (SRISGPFPKDYHSSKFWA) derived from PASS1 was selected as an antigen based on its antigenic index and hydropathy properties as determined by MacVector (Oxford Molecular Group). The peptide was synthesized with a T cell epitope from botulinum toxin (RAHYIVNTF) (43) coupled to multiple antigenic peptide by the University of Michigan Protein and Carbohydrate Structure Facility. Antigen was injected into sheep for raising an anti-PASS1 antisera designated MJW22. For affinity chromatography purification of MJW22 serum, a column of the PASS1-derived peptide was immobilized on Affi-Gel beads (Bio-Rad) and provided by the manufacturer. The resulting antibody recognized PASS1 as judged from the pronounced band at the apparent molecular mass of approximately 64 kDa obtained after transfection of COS cells with PASS1-pcDNA3.1 (cf.Fig. 4C). In COS cells, this antibody did not cross-react with any other protein, indicating a sufficient specificity. Hsnt-1 protein had a certain degree of non-specific cross-reactivity with other proteins with lower apparent molecular masses when rat tissues were analyzed (not shown). The identity of the labeled proteins is not known.

Monoclonal antibody against hspt7 (anti-hspt7) was described previously (44, 45). Rabbit antisera against human and murine/rat hspt7 were purchased from StressGen. The specific antibody against αβ-crystallin (anti-αβ-crystallin) was also purchased from StressGen. Myc monoclonal 9E10 (anti-Myc) and horseradish peroxidase-conjugated 9E10 antibody were purchased from Roche Molecular Biochemicals. Anti-mouse horseradish peroxidase-conjugated secondary antibody preabsorbed with Fc and anti-rabbit horseradish-conjugated peroxidase secondary antibody were purchased from Jackson ImmunoResearch Laboratories.

**Protein Detection**—Western blot was probed with the indicated antibody, and following Washes in PBS, the membrane was exposed to x-ray film (Eastman Kodak Co.) using a Kodak Lanex enhancer screen. Protein detection was performed using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Blots were incubated with the indicated antibodies on ice for 2 h. Protein A-agarose beads (25 μl; Sigma) were then added to the mixture for another hour at 4 °C. The beads were washed three times with lysis buffer before being resuspended in sample loading buffer and analyzed by SDS-PAGE.

**RESULTS**

**Identification of PASS1 and Cloning of Its Full-length cDNA**—In studies designed to identify hspt7-binding proteins using the yeast two-hybrid system, hspt7 cDNA was fused with Gal4-binding domain as “bait.” After transformation of yeast, the expression of this fusion protein was verified by Western blotting using anti-hspt7 monoclonal antibody (not shown). After a second transformation of the yeast with the library plasmid representing the cDNA of a primary culture of Sertoli cells isolated from 27-day-old rats, a total of approximately 5 million yeast colonies were screened. Seventeen positive clones were recovered containing potential hspt7-binding proteins. Recovery and sequencing of the cDNA derived from the 17 positive clones identified hspt7 itself (9 times), αβ-crystallin (2 times), a novel ATP-binding protein with slight homology to thioredoxin (2 times), a mitochondrial NADH dehydrogenase (ubiquinone) (2 times), ubiquitin (1 time), a rat homologue of the yeast transcription enhancer mpr1 (1 time), and the novel protein (1 time) described in this paper. We have named this protein...
The sequencing was conducted by automated sequencing at the University of Michigan Biomedical Core Facility, and the accuracy was confirmed by sequencing both strands. The start codon and stop codon are marked in boldface type. Putative Kozak sequence is underlined, and polyadenylation signals are double underlined.

PASS1 (protein associated with small stress proteins 1). Its cloned cDNA (1.6 kb) has an open reading frame coding for a novel protein of 428 amino acids. By the RACE-PCR method, using rat kidney cDNA, it was confirmed that the open reading frame of PASS1 represents a full-length coding sequence. The complete nucleotide sequence and inferred amino acid sequence of PASS1 is shown in Fig. 1. PASS1 has a calculated molecular mass of 49 kDa and a pI of 6.47. The BLAST search against the GenBank database did not reveal any known homologous proteins. However, several EST sequences were identified having 34% homology with C. elegans having 34% homology with C. elegans. Putative Kozak sequence is indicated in Fig. 2A, showing that both hsp27 and PASS1 are expressed in cells expressing both proteins.

PASS1 binds specifically to both hsp27 and αB-crystallin—In experiments designed to test if the interaction between hsp27 and PASS1 is specific, we reasoned that if PASS1 binding with hsp27 were specific, we would be able to demonstrate a decrease in interaction between the two proteins when a specific domain of PASS1 was deleted from the protein’s sequence. This would represent the binding region of the protein. In order to define such a region of PASS1, we constructed a series of truncated protein constructs tagged with c-Myc. To test the interaction between each truncated PASS1 and hsp27, each truncation construct was co-transfected with the rat hsp27 expressing construct pcDNA-hsp27 (lane 5). Cell lysates were immunoprecipitated with monoclonal anti-hsp27 antibody. Both monkey (endogenous) and rat (ectopically expressed) hsp27 were co-immunoprecipitated with the anti-Myc 9E10 antibody (Fig. 2A, lane 5). This indicates that PASS1 and hsp27 interact with each other in solution.

To further confirm the association between hsp27 and PASS1, we constructed a c-Myc-tagged PASS1, expressed it in mammalian COS cells, and immunoprecipitated the Myc-tagged PASS1 from cell extracts using the anti-Myc 9E10 monoclonal antibody. The immunoprecipitate was tested for the presence of hsp27 by Western blotting. As shown in Fig. 2B, immunoprecipitation of c-Myc-tagged PASS1 expressed in COS cells also co-immunoprecipitated the endogenous hsp27, as detected by Western blotting analysis (Fig. 2B, lane 4). Hsp27 was not co-immunoprecipitated from COS cells not expressing Myc-PASS1 (Fig. 2B, lane 3). Furthermore, when COS cells were co-transfected with the rat hsp27 expressing construct pcDNA-hsp27, the expressed rat hsp27 was also co-immunoprecipitated with PASS1, resulting in a double band as indicated in Fig. 2B, lane 5. The additional lower band was identified by rodent-specific antibodies as rat hsp27, while the upper band is monkey hsp27 (data not shown). Similar results were observed when 293T or NIH/3T3 cells were used instead of COS cells (data not shown), thus confirming the results shown for COS cells. αB-crystallin was also co-immunoprecipitated with PASS1, as detected by probing the Western blots for hsp27. As shown in Fig. 2B, co-immunoprecipitation of c-Myc-tagged PASS1 expressed in COS cells also co-immunoprecipitated the endogenous hsp27, as detected by Western blotting analysis (Fig. 2B, lane 4). Hsp27 was not co-immunoprecipitated from COS cells not expressing Myc-PASS1 (Fig. 2B, lane 3). Furthermore, when COS cells were co-transfected with the rat hsp27 expressing construct pcDNA-hsp27, the expressed rat hsp27 was also co-immunoprecipitated with PASS1, resulting in a double band as indicated in Fig. 2B, lane 5. The additional lower band was identified by rodent-specific antibodies as rat hsp27, while the upper band is monkey hsp27 (data not shown). Similar results were observed when 293T or NIH/3T3 cells were used instead of COS cells (data not shown), thus confirming the results shown for COS cells. αB-crystallin was also co-immunoprecipitated with PASS1, as detected by probing the Western blots for hsp27.
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**Fig. 3. Co-immunoprecipitation of a series of truncated PASS1 proteins and hsp27.** A, diagram showing the series of deletions of PASS1. cDNAs coding for four fragments of PASS1 truncated from the carboxyl-terminal end (Ct1-Ct4) and two PASS1 fragments truncated from the amino-terminal end (Nt1 and Nt2) were generated. The numbers indicate the numbers of amino acids in each fragment. The cDNAs coding for PASS1 fragments were subcloned into prk5Myc. Human 293T cells were co-transfected with each truncated PASS1-expressing construct and with pcDNA-hsp27. Myc-tagged PASS1 fragments expressed in 293T cells were immunoprecipitated using anti-Myc 9E10 antibody. B, truncated PASS1 proteins detected by anti-Myc 9E10 antibody. Lane 1 is of nontransfected 293T cells showing the presence of endogenous 64-kDa c-Myc protein. Full-length PASS1 ran at the same apparent molecular weight (lane 2). The other expression constructs were as follows (lanes 3–8, respectively): prkPASS1Dct1, prkPASS1Dct2, prkPASS1Dact3, prkPASS1Dact4, prkPASS1Dnt1, and prkPASS1Dnt2. Molecular weight markers are indicated on the left. This blot was first probed for hsp27, as shown in C. It was then stripped and reprobed with horseradish peroxidase-conjugated anti-Myc 9E10 to give the blot seen in B. C, detection of hsp27 co-immunoprecipitated with the series of truncated PASS1 protein fragments. In the absence of expression of any PASS1, no hsp27 was precipitated by the 9E10 anti-Myc antibody (lane 1).

Co-immunoprecipitating with truncated PASS1 proteins was similar until amino acids 109–208 were deleted from the protein. The amount of hsp27 co-immunoprecipitated with PASS1 decreased by more than 80% when amino acids 108–208 were deleted from the C terminus (Fig. 3C, lane 6). Additionally, when amino acids 88–208 were deleted, co-immunoprecipitated hsp27 decreased by more than 60% (Fig. 3C, lane 8).

**PASS1 Expression in Rat Tissues and Sertoli Cells—**We also examined the expression pattern of PASS1 mRNA by Northern blot hybridization in several rat tissues including heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis (Fig. 4A). Additionally, Sertoli cells isolated from rat testis (27 days postpartum) were examined. PASS1 mRNA, with a size of about 1.8 kb, could be detected in all tissues analyzed and in the Sertoli cells. Among the tissues analyzed, the greatest abundance of PASS1 mRNA was seen in kidney and testis. A moderate abundance was seen in brain, spleen, and liver, while it was least abundant in heart and skeletal muscle. In kidney, in addition to the 1.8-kb signal, a 1.4-kb signal was also observed. A direct comparison of the signal obtained from equivalent quantities of RNA isolated from testes and Sertoli cells revealed that there was at least a 10-fold higher signal for PASS1 mRNA in Sertoli cells than in the whole testis (Fig. 4B).

PASS1 expression was also examined at the protein level in the same rat tissues and in rat Sertoli cells using the Western blotting technique. To define the position of PASS1 on the membrane, COS cells before and after transient transfection with PASS1 were also analyzed. The anti-PASS1 antibody labeled a protein at an apparent molecular mass of 64 kDa in transfected COS cells, while no signal was obtained in control COS cells (Fig. 4C, lanes 10 and 11, respectively). In Sertoli cells (27 days postpartum, from which the cDNA library was made) a pronounced signal was also observed (lane 9), while in testes the intensity of the signal was reduced (lane 8). In this respect, the data obtained from both Western and Northern blot analysis are consistent. In most tissues with a moderate or low abundance of PASS1 mRNA, no detectable protein was observed by Western blot analysis, which may be due to the low sensitivity of the antibody used. Surprisingly, in kidney, which had a high PASS1 mRNA level, no protein was observed, while in brain, which demonstrated a moderate expression of mRNA, a pronounced protein band was observed by Western analysis. These data suggest that the expression of PASS1 may be regulated post-transcriptionally in some tissues. Since intracellular localization of HSP27 in Sertoli cells changes during the maturation of the testis of rats (45), we also compared the PASS1 level in Sertoli cells isolated from testes of two different ages (18 and 27 days postpartum). As is seen on the Western blot in Fig. 4D, Sertoli cells isolated from testes 18 days (lane 1) or 27 days postpartum (lane 2) contain similar amounts of PASS1.
Concerning its intracellular location, PASS1 was diffusely distributed in the cytoplasm of cultured COS, 293T, and NIH/3T3 cells when it was overexpressed as a green fluorescent protein fusion protein, as detected by fluorescence microscopy (results not shown). The intracellular distribution of PASS1 was similar to that of hsp27, which is also distributed diffusely throughout the cytoplasm of the three cell lines examined.

PASS1 Affects the Ability of Hsp27 to Protect Cells from Heat Shock—If PASS1 truly interacts with hsp27 in live cells, we reasoned that expression of PASS1 should affect the function of hsp27 in cells, e.g. its ability to protect cells against sublethal heat shock. To test this possibility, NIH/3T3 cells were transfected with vector pCDNA3.1 without additional insert (control), with pCDNA3.1-hsp27 alone, or with both pCDNA3.1-hsp27 and prkPASS1. Two days after transfection, when expression of foreign proteins reached a maximum, the cells were heat-shocked at 45 °C for increasing lengths of time in a water bath. Cell survival was determined after 24 h of recovery of the cells at 37 °C. Experiments were conducted with eight replicates for each heat treatment, and means and S.E. values were calculated and plotted. An asterisk indicates the significant difference at p < 0.05 level by t test.

As expected, increased expression of hsp27 was able to significantly increase the tolerance of the cells to elevated temperature (Fig. 5A). After heat treatment for up to 60 min, the protective effect of hsp27 on cell survival was significant. However, when cells were co-transfected with expression constructs for both PASS1 and hsp27, the ability of hsp27 to confer heat resistance to cells was eliminated. In fact, the co-expression of PASS1 and hsp27 in 3T3 cells made the cells more vulnerable to heat than control cells. This is most obvious after 10 and 20 min of heat treatment (Fig. 5A). Moreover, the difference of cell survival between cells expressing hsp27 alone and cells co-expressing hsp27 plus PASS1 was significant at every tested time point, i.e. from 10 to 60 min of heat shock.

The transfection of 3T3 cells with hsp27 or PASS1 cDNA as performed for the growth assay in Fig. 5A may affect the induction of endogenous hsp27 in response to the applied heat shock with possible consequences for cell survival. We performed an experiment to monitor the induction of endogenous human hsp27 in control cells and in rat hsp27- and PASS1-transfected cells using human 293T cells. This approach allows the immunological distinction between endogenous human hsp27 and foreign rat hsp27 (cf. “Experimental Procedures”). 293T cells exhibited a similar survival pattern as 3T3 cells (results not shown). For the experiment, 293T cells were either transfected with control vectors (pcDNA3.1, prk5Myc), with pcDNA3.1-hsp27 alone, with prkPASS1 alone, or with both pcDNA3.1-hsp27 and prkPASS1. 48 h after transfection, cells were heat-shocked for 45 °C for 20, 30, or 60 min and allowed to recover for 6 h to permit the synthesis of sufficient amounts of endogenous hsps.

As shown in Fig. 5B, a 48 h after transfection, control cells (kept at 37 °C) expressing either none of the foreign genes, PASS1 alone, rat hsp27 alone, or rat hsp27 and PASS1 had a low level of expression of endogenous hsp27, which was close to the detection limit of the applied method. When the cells were heat-shocked 48 h after transfection for 20 or 30 min and allowed to recover, endogenous hsp27 was induced to a similar extent in cells expressing either none of the foreign genes, rat hsp27 alone, PASS1 alone, or rat hsp27 and PASS1 together. Thus, none of the performed transfections interfered with the endogenous heat shock response as judged by the induction of endogenous hsp27. When the cells were heat-shocked 48 h after transfection for 60 min at 45 °C and allowed to recover for 6 h, no induction of endogenous hsp27 was detected in any of the control or transfected cells. This indicates that the heat shock at 45 °C for 60 min was severe enough to prevent the cells from recovering within the 6 h. However, as is shown in the viability assay in Fig. 5A, cells expressing foreign hsp27 alone do tolerate the severe heat shock treatment to a certain extent better than the other cells used.

DISCUSSION

Observations suggest that hsp27 plays a role in Sertoli cell differentiation and function. Like many cell types, Sertoli cells of the testis respond to physiologically relevant stimuli with phosphorylation of endogenous hsp27. Sertoli cell hsp27 phosphorylation is rapidly stimulated by a factor from germ cells (47). Expression of hsp27 mRNA in seminiferous tubules also varies during the cycle of the seminiferous epithelium (45), suggesting that hsp27 gene expression in Sertoli cells is closely regulated by local signals. The subcellular localization of hsp27 in Sertoli cells is also distinct, with the protein being diffuse throughout the cell cytoplasm in Sertoli cells of young rats but being closely associated with microfilaments in older, more differentiated Sertoli cells (45). The fact that hsp27 localization in Sertoli cells changes as the cells differentiate led us to hypothesize that the change in distribution in Sertoli cells might be mediated by expression of an hsp27-binding protein during Sertoli cell differentiation. To test this hypothesis, we constructed a cDNA library from differentiated Sertoli cells and used the yeast two-hybrid system to screen this library for proteins associating with small stress proteins, or PASS.
hsp27 (51, 52). We found no evidence from the two-hybrid assay that actin would bind hsp27. This was unexpected, because in vitro results have shown that hsp27 can bind actin (21). Moreover, in some cell types such as Sertoli cells (45) and muscle (53), hsp27 co-localizes with microfilaments.

There are many reasons why the yeast two-hybrid assay might not have identified actin as a protein to which hsp27 could bind. It is possible that hsp27 interacts only with polymerized actin or that, although unlikely (22), only oligomers of hsp27 may bind actin. Since the fusion proteins of the two-hybrid assay cannot form polymers of either actin or hsp27, the assay is inadequate for the detection of this type of interaction. Alternatively, hsp27 may not directly bind to actin in vivo as it does in vitro. Hsp27 interaction with microfilaments may be mediated by another protein that co-purifies with actin when it is isolated for in vitro polymerization studies, and it is with this protein that hsp27 would interact in order to affect actin polymerization in vivo or to co-localize with microfilaments in some cell types. Another possibility is that the form of hsp27 that is functional in the yeast two-hybrid assay is different from the form that interacts with actin. For example, the hsp27 expressed in yeast is probably not phosphorylated, because yeast appears to have no MAPK kinase-23 family gene homologs as revealed by BLAST searches of the yeast genome. This kinase family is responsible for phosphorylation of hsp27 in mammalian cells. Although unlikely (22), if only phosphorylated hsp27 could interact with actin in vivo, then the yeast two-hybrid assay would be expected to be negative for showing an interaction between hsp27 and actin. Indeed, because the hsp27 in this assay was not phosphorylated, our results may not give a full account of the possible binding partners of hsp27.

Therefore, in another set of yeast two-hybrid experiments, we also screened expression libraries for proteins binding to the 3D mutant form of human hsp27 (serines 15, 78, and 82 substituted by aspartate to mimic phosphorylation). Also in these experiments, we still have not identified actin to bind to hsp27 (results not shown).

Sequencing of the cDNA for PASS1 indicated that sites for the start and end of translation of the protein were included in the obtained sequence, and this was confirmed by 5’ RACE-PCR using a rat kidney cDNA library. Although BLAST search revealed no similar protein being previously described, the PASS1 cDNA sequence was nearly identical to several mouse EST clones, and the inferred protein sequence exhibited a region of modest similarity to a genomic sequence from C. elegans. The cDNA for PASS1 codes for a protein of 428 amino acids with a calculated pI of 6.47 and a molecular mass of 49 kDa. Unexpectedly, PASS1 runs on SDS-PAGE with an apparent molecular mass of approximately 64 kDa, which is approximately 15 kDa greater than its inferred size. When portions of the protein are deleted from the amino-terminal end, the truncated proteins continue to migrate at higher apparent molecular weights than predicted. However, when the carboxyl-terminal end of PASS1 is deleted from the protein, it migrates in SDS-PAGE at its expected molecular weight (Fig. 3B). Thus, this anomalous migration in SDS-PAGE is a result of some characteristic of the carboxyl-terminal region of PASS1. Possibly, this part of PASS1 confers unusual folding on the final protein, or post-translational modification of the protein is responsible. However, examination of the amino acid sequence of PASS1 indicates no obvious site for carboxyl-terminal modification of the protein. The fact that complete or truncated PASS1 proteins expressed in bacteria migrate similarly to intact or truncated proteins expressed in mammalian cells (data not shown) indicates that glycosylation of the protein is probably not the cause of the anomalous migration.

Several sets of evidence indicated that PASS1 interacts specifically with hsp27. In addition to being selected by the yeast two-hybrid screen for protein-protein interaction, a GST pull-down assay confirmed interaction between hsp27 and GST-PASS1. hsp27 could also be co-immunoprecipitated with Myc-tagged PASS1. Moreover, Myc-tagged PASS1 could be co-immunoprecipitated by anti-hsp27 antibody. Last, when cultured cell lines were transiently transfected with expression vectors for either rat hsp27 alone or for PASS1 together with rat hsp27, increased resistance to heat shock could be measured in cells expressing increased hsp27 alone, but this was not seen in cells expressing hsp27 plus PASS1. This result suggests that PASS1 binds to and sequesters hsp27 in live cells and prevents it from participating in events that would otherwise confer on the cell resistance to heat stress. This result also suggests that PASS1 is not involved in the stress response but that it has another as yet unknown function in cells.

Studies were conducted in an effort to learn what part of the PASS1 sequence might be required for PASS1 to interact with hsp27. Western blots of co-immunoprecipitated proteins indicated that amino acids 108–208 were predominantly responsible for the interaction between PASS1 and hsp27, because the amount of hsp27 co-immunoprecipitated with PASS1 decreased by more than 60–80% when these amino acids were removed from PASS1 (Fig. 3C). Nonetheless, this region of PASS1 may not be the sole area of interaction between hsp27 and PASS1, because there was still detectable hsp27 co-immunoprecipitated with the two proteins having deletions of these amino acids (Fig. 3C). Possibly, a motif exists within this region that can specify the interaction of a protein with hsp27. Further analysis of this sequence or study of other proteins that can bind hsp27 will be needed to verify or refute this possibility.

The expression of PASS1 varies in different tissues, at both transcription and translation levels. Both the mRNA and the protein were relatively highly expressed in testis as compared with other tissues. Moreover, over 10 times as much PASS1 mRNA was observed in isolated Sertoli cells compared with whole testis. Immunofluorescence localization and Western blot had previously shown testicular hsp27 to be expressed mostly in Sertoli cells (45), so this result was anticipated. Sertoli cells isolated from rats of different age (18 and 27 days) that are known to differ in their localization pattern of hsp27 contain approximately similar amounts of PASS1 (Fig. 4D), indicating that PASS1 is probably not involved in relocation of hsp27 during this stage of testes development. PASS1 mRNA was also highly expressed in the kidney and was seen to exist as two different sized transcripts in kidney. This result suggests that PASS1 mRNA may undergo alternate splicing in the kidney.

We investigated the possibility that PASS1 mediates the interaction between hsp27 and actin. When PASS1 was demonstrated to associate with hsp27 by co-immunoprecipitation and Western blot analysis, the same blots were probed with an antisera directed against β-actin. No actin was observed on these blots (data not shown). This result supports the conclusion that the function of PASS1 is not to mediate hsp27 interaction with actin or microfilaments. In the tissues tested other than brain and testis, Western blotting did not reveal any protein expression. This pattern might be due to post-transcriptional regulation. For some unknown reason, the PASS1 protein in those tissues may exist only transiently and/or in a very low concentration. As a new protein, the function of PASS1 could not be determined from sequence information, nor is there sufficient information available at this time about PASS1 to understand the significance of its association with
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hs27. Taken together, our data demonstrating co-immunoprecipitation of PASS1 and hsp27, the association of both proteins revealed by the GST pull-down assay, and the observation that PASS1 inhibits the ability of hsp27 to confer heat resistance indicate that PASS1 does interact with hsp27 within living cells.

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