Interaction of Human HSP22 (HSPB8) with Other Small Heat Shock Proteins*

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Mammalian small heat shock proteins (sHSP) are abundant in muscles and are implicated in both muscle function and myopathies. Recently a new sHSP, HSP22 (HSPB8, H11), was identified in the human heart by its interaction with HSP27 (HSPB1). Using phylogenetic analysis we show that HSP22 is a true member of the sHSP superfamily. sHSPs interact with each other and form homo- and hetero-oligomeric complexes. The function of these complexes is poorly understood. Using gel filtration HPLC, the yeast two-hybrid method, immunoprecipitation, cross-linking, and fluorescence resonance energy transfer microscopy, we report that (i) HSP22 forms high molecular mass complexes in the heart, (ii) HSP22 interacts with itself, cvHSP (HSPB7), MKBP (HSPB2) and HSP27, and (iii) HSP22 has two binding domains (N- and C-terminal) that are specific for different binding partners. HSP22 homo-dimers are formed through N-N and N-C interactions, and HSP22-cvHSP hetero-dimers through C-C interaction. HSP22-MKBP and HSP22-HSP27 hetero-dimers involve the N and C termini of HSP22 and HSP27, respectively, but appear to require full-length protein as a binding partner.

In a previous publication we identified mammalian HSP22 (other names: HSPB8, H11, E21G1), a protein similar to known small heat shock proteins (sHSP),¹ by a two-hybrid (TH) screen using HSP27 (HSPB1, HSPB5), the classic heat-inducible sHSP, as “bait,” and a heart cDNA library (1). While in this and other reports HSP22 was characterized as an sHSP (2, 3), in some reports the newly described protein was classified as a protein kinase with similarity to the Herpes simplex protein kinase ICP10 (4–6). HSP22 occurs preferentially in striated muscle cells. In addition to HSP22, also abundant in muscles are other sHSPs including HSP27, myotonic dystrophy kinase-binding protein (MKBP, HSPB2), HSPB3, αB-crystallin (αB-Cry, HSPB5), HSPB20 (HSPB6), and cvHSP (HSPB7) (7, 8). It is now widely accepted that sHSPs play a major role in muscles, although their precise role is not understood. A point mutation in the αB-Cry gene causes desmin-related cardiomyopathy in humans (9), and MKBP binds to and activates the myotonic dystrophy protein kinase, an enzyme that when absent results in myotonic dystrophy (10). Overexpression of HSP22 in heart was recently shown to be associated with hypertrophy (6). Also, the specific location of sHSPs in the sarcomeres (11) and the protection of myocytes by sHSPs from ischemic stress (12) suggest an important role for these proteins in muscles. Phosphorylation of HSP27 has been implicated in the contraction (13), and phosphorylation of HSP20 in the relaxation (14) of smooth muscle cells.

sHSPs have been shown to form two types of hetero-oligomeric complexes in striated muscles: type I complex consisting of HSP27, αB-Cry, and HSP20, and type II complex consisting of MKBP and HSPB3 (8). sHSP complexes are heterogeneous in size and composition. The molecular mass of cellular sHSP complexes varies over a wide range (~50–1000 kDa) and changes under stress conditions leading usually to smaller complexes (15). The crucial role of these complexes for cell survival under stress conditions has been demonstrated (16). Based on these findings it is believed that the formation of homo- and hetero-oligomeric complexes of sHSPs is essential for their function.

Most available data concerning formation and structure of sHSP complexes are based on studies of α-crystallin, αB-Cry, and HSP27. These three sHSPs form dynamic homo- and hetero-oligomeric complexes which may have a micellar structure and rapidly exchange subunits (17–19). HSP27 dimerizes, and two such dimers form tetramers, which are in equilibrium with larger complexes (20). Human HSP27 is phosphorylated at three sites (Ser15, Ser78, Ser82) by the protein kinase MAPKAP-K2 resulting in the disassembly of large oligomeric structures predominantly into tetramers (15, 21). Interactions within HSP27 complexes involve at least two sites, one site within the C-terminal α-crystallin domain and the other site at the far N terminus (22). The site in the α-crystallin domain has been proposed to be involved in dimer formation (23), and these dimers are thought to further multimerize into larger complexes using the N-terminal dimerization site.

If HSP22 belongs to this group of proteins, we hypothesized that it interacts with itself and with other sHSPs which may result in the formation of homo- and hetero-oligomeric complexes, similarly to other sHSPs. In an initial effort to characterize HSP22, we have studied its phylogenetic relationship among Bilateria proteins and confirmed that it is a member of

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¹ The abbreviations used are: sHSP, small mammalian heat shock protein; HSP22 (HSPB8, H11, E21G1), heat shock protein 22; HSPB1, heat shock protein 27; MKBP (HSPB2), myotonic dystrophy kinase-binding protein; CL, cross-linking; co-IP, co-immunoprecipitation; FRET, fluorescence resonance energy transfer; TH, two hybrid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography.

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the superfamily of sHSPs. By gel filtration high performance liquid chromatography (HPLC) we show that HSP22 forms high molecular mass complexes in the heart, similarly to other sHSPs. By yeast TH experiments, co-immunoprecipitation (co-IP), cross-linking (CL), and fluorescence resonance energy transfer (FRET) microscopy we show that HSP22 interacts with itself and with the other tested sHSPs. We show also that HSP22 has two binding domains that are specific for individual sHSPs.

EXPERIMENTAL PROCEDURES

Phylogeny of sHSPs—A profile hidden Markov model in HMMR 2.2 g was employed to characterize, search for and align diverse sHSP superfamily members. The non-redundant protein data base (GenBankTM CDS translations + PDB + SwissProt + PIR) was queried with sHSP-HMM for significant matches (protein with scores of 4 or greater and expectation values of 0.05 or less were retained). The resulting sequences were aligned with the sHSP-HMM. To estimate phylogeny and to approximate the posterior probabilities of the trees, a Bayesian inference approach with Metropolis-coupled Markov chain Monte Carlo, or MC, was used. More details are given elsewhere (24).

Origin of sHSP cDNAs and Plasmid Constructs—All relevant data on origin of sHSP cDNAs, plasmid constructs, used cloning methods and PCR primers are given in Table I. The PCR cycle conditions for cHSP cDNA amplification from a human heart cDNA library (Clontech) were 50 s 95 °C, 50 s 63 °C, and 60 s 72 °C. For all TH experiments, the vectors pAS2–1 (abbreviated: pAS) and pACT2 (abbreviated: pACT) (Clontech) were used. For eukaryotic expression of Myc- and FLAG-tagged sHSPs the vectors pDNAs, 1-myc (In vitrogen) and pFlag-CMV2 (Stratagene) were used. For eukaryotic expression of YFP- and CFP-sHSP fusion proteins the vectors pEFYFP and pECFP (Clontech) were used. All plasmid constructs were verified by sequencing. sHSP cDNAs were separated into N- and C-terminal fragments in the central region or beginning of the α-crystallin domain as indicated in Fig. 1A.

Two Hybrid Experiments—Small scale quantitative transformation of the yeast strain Y190 was performed as described in the manufacturer's instructions (Clontech). Interactions between full-length (F), N-terminal (N), and C-terminal halves (C) of sHSPs were analyzed. Briefly, yeast was first transformed with the constructs 2, 5, or 6 and grown on -Leu medium, or with construct 1 and grown on -Trp medium. In the second step, the yeast was transformed with vectors as specified in the Figs., and plated on -Trp, -Leu, -His selective medium. Y190 has two reporter genes (his +, growth of colonies; gal +, blue colonies), and two proteins were considered as interacting partners only if both reporter genes were activated. In order to reduce false positive results, every used vector with an insert was tested with an empty partner vector. In some of these controls the his + reporter gene was moderately activated resulting in between 10 and 100 colonies/10 cm Petri dish; however, the gal + reporter gene was never activated (not shown).

Cell Culture, Transfection, Co-immunoprecipitation, and Cross-linking—293T and COS-7 cells (ATCC) were maintained at 37 °C in 5% CO2 humidified atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. To prevent detachment of 293T cells, tissue culture plates were treated with poly-L-lysine before seeding the cells. For transient transfection FuGENE 6 (Roche Applied Science) was used according to the manufacturer's protocol. 48 h later, cells were harvested and processed for co-IP, CL, or FRET analysis.

For co-IP, 2 μg of a rabbit anti-FLAG polyclonal antibody (Sigma) or mouse anti-Myc monoclonal antibody (Roche Applied Science) were bound to 50 μl of protein G-Sepharose (20% slurry, Sigma) by incubating for 2 h at 4 °C in 500 μl of buffer A (50 mM Tris-HCl, pH 8.0; 1% Triton X-100; 5 mM EDTA; 1 mM EGTA; 1× protease inhibitor mix, Roche Applied Science). Transfected and non-transfected COS-7 cells were rinsed with ice-cold PBS and lysed in buffer A on ice for 10 min. The lysate was centrifuged at 14,000 × g for 10 min at 4 °C before it was incubated with the antibody-coated beads overnight at 4 °C on a rotating platform. Then the beads were collected by centrifugation and washed three times with buffer A. Bound immunocomplexes were released from the beads by a 3-min boiling in 100 μl of buffer B (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 1% β-mercaptoethanol; 0.01% protease inhibitor mix). Thereafter, samples were analyzed using SDS-PAGE followed by Western blotting. Negative controls were run with rabbit and mouse preimmune sera instead of anti-FLAG or anti-Myc antibodies (not shown).

For CL, transfected 293T cells grown in 6-well plates were washed with cold PBS. Glutaraldehyde (Sigma) was diluted with PBS (0.0001, 0.0002, 0.0005, 0.001, 0.002% final concentrations) immediately before use, and 1 ml of these solutions was added per well. Cells were incubated for 1 h at room temperature, and then cells were cooled on ice and washed with ice-cold PBS. Cells were lysed with 1 ml of buffer B. The lysates were sonicated for 10 s in order to break down DNA, and then boiled for 5 min. These samples were then analyzed by SDS-PAGE and Western blotting.

Size Exclusion HPLC—0.5 g of the left ventricle of a Rhesus monkey heart was homogenized in 2 ml ice-cold buffer C (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM KCl; 1 mM EDTA; 10% glycerol; 1% CHAPS; 1× protease inhibitor mix) using a PowerGen 125 homogenizer. The homogenate was centrifuged at 14,000 × g for 20 min at 4 °C before 1 μl of the supernatant was used for HPLC chromatography. Before loading the sample, the column (Protein PAK 300 sw, 0.75 × 30 cm, Waters) was equilibrated with buffer D (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM KCl; 1 mM EDTA), and separation of the proteins was at a flow rate of 1 ml/min. Fractions were collected as indicated in Fig. 2. Proteins in all fractions were precipitated overnight by 2% of ethanol/1% β-mercaptoethanol at −20 °C, and the precipitates were collected by centrifugation. The air-dried pellets were dissolved in 50 μl of buffer B, briefly sonicated and boiled for 5 min. These samples were then analyzed for the presence of HSP22, HSP27, and MKBP by SDS-PAGE and Western blotting using specific antibodies. The column resolved proteins with molecular masses ranging from <5 to ~670 kDa and was calibrated using the molecular mass markers Dextran blue (>1000 kDa), thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa) (Fig. 2).

Electrophoretic Methods and Western Blotting—SDS-PAGE and Western blotting. Rabbit anti-FLAG antibody (1:5,000) (Sigma), mouse anti-Myc antibody (1:5000) (Roche Applied Science), and sheep anti-HSP22 (1) were used as primary antibodies. As secondary antibodies, goat anti-rabbit IgG (Fc fragment-specific), goat anti-mouse IgG (Fc fragment-specific) and rabbit anti-sheep IgG antibodies conjugated to horseshadish peroxidase were used. Membranes were processed for detection using the ECL plus reagent (Amersham Biosciences).

Fluorescence Resonance Energy Transfer Measurements—COS-7 cells grown on glass coverslips were transfected with various YFP-sHSP and CFP-sHSP constructs as listed in Table I coding for the corresponding fusion proteins. Two days later, cells were fixed with 4% formaldehyde in PBS, pH 7.4, for 30 min at room temperature. The cells were then washed with PBS, and the coverslips were mounted on slides with Prolong antifade mounting medium (Molecular Probes) and used for microscopy. For FRET microscopy, an Axiovert 135TV microscope equipped with a ×40 FLUAR 1.3NA oil immersion objective lens and a CFP/YFP filter set was used. Images were recorded at a temperature of between 10°C and 15°C. Nuclei were stained with 10 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). Images were recorded at room temperature using a cooled integrated CCD camera DAGE RT 3000 (DAGE-MTI Inc.). NIH Image software was used to acquire 8-bit gray scale images from a Scion LG3 video capture board and to quantify the image pixel intensities within user selected regions of each cell. The same region was selected for analysis in each of the images obtained from an individual cell. Individual images were exported in TIFF format and image montages created using Adobe Photoshop. No post-processing of images was conducted after acquisition.

FRET was measured by determining the CFP emission before and after an 8-min photo bleaching step of YFP as described (25). The increase in the bleaching of CFP emission is a direct measure of the FRET due to interaction. Interaction as measured by FRET is expressed as FRET Factor (FF) in Equation 1.

\[
FF = \frac{I_{\text{prebleach}} - I_{\text{postbleach}}}{I_{\text{bleach}}} 
\]

The symbols are defined as: \( I_{\text{prebleach}} \), \( I_{\text{postbleach}} \), intensity of the CFP-signal in the region of interest after and before photo bleaching, respectively; \( I_{\text{bleach}} \), background signals, outside of cell areas, after and before photo bleaching, respectively; CF, correction factor (the ratio of the intensities of the CFP-signals after and before photo bleaching of an image area with cells which were not photo bleached). The CF is used to correct for fluctuations of the CFP signal during the course of the experiment. Ideally, if two partners do not interact, the FF-value would be 0, and any value above 0 would indicate interaction. However, individually expressed CFP and YFP control proteins are known to interact to a certain extent (26). The FF value of this negative control (FF\(_{\text{control}}\)) was
determined to be 0.096 ± 0.023 under the applied experimental conditions. Every FF value measured for interacting CFP/YFP-sHSP fusion proteins must be significantly higher than this value. For positive control, a CFP-YFP fusion protein was expressed (27). The measured FF value of 0.384 ± 0.037 is significantly different from FF_C (p < 0.0001) and indicates the range in which FF values for interacting proteins can be expected. For both, the interacting pairs of sHSPs and the controls, the CFP signal intensities before and after photobleaching of 10 randomly selected cells were measured. This allowed determining the significance of the differences of the FF values to the FF_C by the Student’s t test.

RESULTS

Phylogenetic Relationship of HSP22 among the Bilateria sHSPs—In the light of the divergent roles proposed for HSP22 (see Introduction and “Discussion”), we sought to gain additional information by a phylogenetic analysis. HSP22, like the other human sHSPs, consists of the conserved α-crystallin domain, a less conserved N-terminal domain, a variable central region, and variable N- and C-terminal tails (Fig. 1A). Based on the multiple alignment of all 10 human sHSPs, a hidden Markov model (sHSP-HMM) was built that was used to search the entire non-redundant protein data base (24). This sHSP-HMM identified 167 Bilateria sHSP-like proteins, including all known mammalian sHSPs as proteins with significant sequence similarity. The sHSP-HMM was used to align these 167 proteins, and the alignment was used to build a Bayesian inference phylogeny. An abbreviated version of this phylogeny illustrating the overall topology of Bilateria sHSPs while detailing the relationship of the HSP22 and HSP27 clades, is shown in Fig. 1B. The mammalian HSP22 form a monophyletic group which is sister to the HSP25/27 clade of Euteleostomi including mammals, birds and fish. Thus, HSP22 is most closely related to HSP27 among all proteins contained in the databases, while protein kinases or proteins related to the Herpes simplex protein kinase ICP10 were not identified.

Based on this analysis, HSP22 clearly is classified as a member of the superfamily of sHSPs. Therefore, we analyzed HSP22 for the property, which is characteristic for this superfamily: the ability to interact with itself and other sHSPs, which results in the formation of complexes.

Size Distribution of HSP22 Complexes in Monkey Heart—In order to determine the possible involvement of HSP22 in sHSP complex formation in tissues, a protein extract of a piece of Rhesus monkey heart was analyzed by gel filtration HPLC followed by SDS-PAGE and Western blotting. The proteins were extracted under moderate stringency conditions in order to leave sHSP complexes intact. HSP22 partitioned into all fractions corresponding to molecular masses between 25 kDa and 670 kDa with most of the proteins being found in the high molecular mass fractions (Fig. 2). For comparison, the size distribution of two further sHSPs, HSP27 and MKBP, was also analyzed. HSP27 showed a molecular mass partition, which is very similar to that of HSP22 and also to previous analyses (28), while MKBP partitioned into fractions with smaller molecular masses approximately between 25 and 200 kDa. These data suggest that (i) different sHSPs may have different size distributions, and (ii) HSP22 may interact with itself and/or other sHSPs, which results in the formation of complexes.

HSP22-HSP22 Interaction—Earlier biochemical data suggested that HSP22 forms homo-dimers (1). Here we examined the HSP22-HSP22 interaction in greater detail. Yeast TH as-
sHSP Interactions Involving HSP22

Fig. 2. Analysis by gel filtration HPLC of the apparent molecular masses of HSP22, HSP27, and MKBP complexes in an extract of a Rhesus monkey heart. A, elution profile as recorded at 280 nm. The position of the molecular mass marker proteins is indicated. B, distribution of HSP22, MKBP and HSP27 in the collected fractions as detected by SDS-PAGE/Western blotting.

says using the constructs 1 and 2 (Table I) were performed to determine the interaction of HSP22 with itself (Fig. 3A). Both reporter genes (his+, gal+) clearly indicated interaction of HSP22 with itself. This interaction was verified biochemically by co-IP using Myc- and FLAG-tagged HSP22 (constructs 7, 8) expressed in COS-7 cells (Fig. 3B). As expected, after transfection with HSP22-myc the anti-FLAG antibody did not precipitate HSP22-myc, while after transfection with Flag-HSP22 the anti-FLAG antibody did precipitate Flag-HSP22 (controls). After co-transfection with both HSP22-myc and Flag-HSP22, the anti-FLAG antibody pulled down both Flag-HSP22 and HSP22-myc indicating that HSP22 interacts with itself. Under the same conditions, actin remained in the supernatants of the precipitation steps suggesting that the HSP22-myc precipitation is due to the interaction with Flag-HSP22 and not due to a nonspecific protein precipitation or trapping of proteins in the immunoprecipitate (Fig. 3B).

To determine the interacting domains in this HSP22 homo-dimer, two fragments of HSP22, each covering the N- (N) or the C-terminal (C) part of the molecule (Fig. 1A), were cloned into the TH vectors (constructs 3–6) and used for interaction assays which also included full-length (F) HSP22 (construct 1). HSP22-N interacted with HSP22-F, itself, and also with HSP22-C (Fig. 3C), while HSP22-C interacted with HSP22-F and HSP22-N, but not with itself (Fig. 3D). Thus, HSP22 appears to have two interacting domains potentially involved in homo-dimer formation (N-N, N-C interactions).

Next we examined whether HSP22 can form homo-oligomers, similar to what has been shown for HSP27 (22). 293T cells were transfected (construct 8) to express FLAG-tagged HSP22 and treated with different concentrations of glutaraldehyde to cross-link cell proteins. HSP22 species were analyzed by SDS-PAGE and Western blotting by using anti-FLAG antibody (Fig. 3E). CL resulted in three major HSP22 bands with apparent molecular masses of ~30, 65, and 120 kDa, while at higher glutaraldehyde concentrations a background signal (smear) with a molecular mass above 250 kDa was obtained. Distinct bands indicate CL of HSP22 with a specific binding partner rather than with a nonspecific variety of cell proteins, which would result in a continuous smear. Since 293T cells do not contain any sHSP, it is very likely that the major bands at 65 and 120 kDa represent cross-linked homo-dimers and homo-tetramers. The determined molecular masses are somewhat higher than the calculated molecular masses (22.6 kDa for the monomer) of HSP22, however, a similar retardation of electrophoretic mobility has also been observed for other sHSPs. Using higher concentrations of glutaraldehyde resulted in high molecular mass background signals (a broad smear) probably indicating nonspecific CL involving several cell proteins. For control purposes, 293T cells were transfected, but not treated with glutaraldehyde (Fig. 3E), or not transfected, but treated with glutaraldehyde (not shown). None of these treatments resulted in the formation of high-molecular mass HSP22 signals. As a further control, the same blot was developed for actin. Actin was not cross-linked by any of the used glutaraldehyde concentrations which indicates that CL of HSP22 is specific and not due to general CL of cell proteins (Fig. 3E). Taken together, these data provide evidence that HSP22 forms homo-dimers and homo-oligomers.

HSP22-cvHSP Interaction—TH assays using the constructs 2 and 25 were performed to determine the interaction between full-length HSP22 and cvHSP. After double transformation, both reporter genes (his+, gal+) were activated indicating interaction between the two proteins (Fig. 4A). This interaction was verified biochemically by co-IP (Fig. 4B). For co-IP, COS-7 cells were transfected with vectors coding for full-length Myc-tagged HSP22 (construct 7) and FLAG-tagged cvHSP (construct 28). The expressed proteins were analyzed in a manner similar to the experiment described above. After expression of both proteins, the anti-FLAG antibody pulled down both the Flag-cvHSP and HSP22-myc indicating that HSP22 interacts with cvHSP. Actin was not precipitated indicating that the HSP22-myc precipitation is due to the interaction with Flag-cvHSP and not due to nonspecific protein precipitation.

As a further method to study the interaction of both proteins in vivo we used FRET microscopy. COS-7 cells were co-transfected with vectors that permit expression of HSP22-YFP and cvHSP-CFP (constructs 9, 29) fusion proteins (Fig. 4C). Fluorescence intensity of the CFP signal was determined before (panels a and c) and after (panels b and d) photobleaching of the YFP signal (panels c and d) signal in the region of interest. The fluorescence increase of the CFP signal was used to calculate the FRET Factor (FF) (0.24 ± 0.024, p = 0.0005), which is significantly different from the negative control (see “Experimental Procedures”) indicating interaction between the two proteins in vivo.

Using the TH method, the interacting domains in the HSP22-cvHSP hetero-dimer were determined. The N- and the C-terminal halves of HSP22 and cvHSP (Fig. 1A) cloned into TH vectors (constructs 5, 6, 26, 27) were used, together with the full-length proteins (constructs 2, 25), for the interaction assays. HSP22-F interacted with cvHSP-C but not with cvHSP-N (Fig. 4D). HSP22-N did not interact with cvHSP-F, cvHSP-N, or cvHSP-C (Fig. 4E), while HSP22-C interacted with cvHSP-F.
TABLE I
Designation of the constructs, origin of the sHSP sequences, and cloning methods

| Construct no. | Construct designation | Source of sHSP cDNA/method of cloning | Used restriction sites | Primers |
|---------------|----------------------|--------------------------------------|-----------------------|---------|
| 1             | pAS-HSP22-F          | subcloning of pcDNA-HSP22-Fb         | EcoRI, XhoI/SalI      |         |
| 2             | pACT-HSP22-F         | PCR of pcDNA-HSP22-F/TopoTA          | EcoRI, XhoI           | 1, 2    |
| 3             | pAS-HSP22-N          | PCR of pcDNA-HSP22-F/TopoTA          | EcoRI, XhoI           | 3, 4    |
| 4             | pAS-HSP22-C          | PCR of pcDNA-HSP22-F/TopoTA          | Neol, Xhol            | 5, 6    |
| 5             | pACT-HSP22-C         | PCR of pcDNA-HSP22-F/TopoTA          | EcoRI, XhoI           | 7, 8    |
| 6             | pFlag-CMV2-HSP22-F   | PCR of pcDNA-HSP22-F/TopoTA          | KpnI, Xhol            | 9, 10   |
| 7             | pcDNA-HSP22-F-myc    | PCR of pcDNA-HSP22-F/TopoTA          | EcoRI, XhoI           | 11, 12  |
| 8             | pEYFPN1-HSP22-F      | PCR of pcDNA-HSP22-F/TopoTA          | EcoRI, KmI            | 13, 14  |
| 9             | pAS-HSP22-F          | PCR of pcDNA-HSP22-F/TopoTA          | EcoRI, KmI            | 15, 16  |
| 10            | pAS-**HSP27-F        | PCR of pcDNA-HSP27-F                 | BspHI/NcoI, XhoI/SalI | 17, 18  |
| 11            | pAS-**HSP27-N        | PCR of pcDNA-HSP27-F                 | BspHI/NcoI, XhoI/SalI | 17, 18  |
| 12            | pAS-**HSP27-C        | PCR of pcDNA-HSP27-F                 | EcoRI, XhoI           | 19, 20  |
| 13            | pAS-**HSP27-N        | PCR of pcDNA-HSP27-F                 | EcoRI, XhoI           | 19, 20  |
| 14            | pAS-**HSP27-C        | PCR of pcDNA-HSP27-F                 | Neol, Xmal            | 21, 22  |
| 15            | pACT-**HSP27-F       | PCR of pcDNA-HSP27-F                 | Neol, Xmal            | 21, 22  |
| 16            | pACT-**HSP27-C       | PCR of pcDNA-HSP27-F                 | Neol, Xmal            | 23, 24  |
| 17            | pECFPN1-**HSP27-F    | PCR of pcDNA-HSP27-F                 | EcoRI, BamHI          | 25, 26  |
| 18            | pECFPN1-**HSP27-C    | PCR of pcDNA-HSP27-F                 | EcoRI, BamHI          | 25, 26  |
| 19            | pECFPN1-**HSP27-N    | PCR of pcDNA-HSP27-F                 | EcoRI, BamHI          | 25, 26  |
| 20            | pACT-HSP22-F         | PCR of pcDNA-HSP22-F                 | EcoRI, XhoI           | 27, 28  |
| 21            | pACT-HSP22-N         | PCR of pcDNA-HSP22-F                 | EcoRI, XhoI           | 27, 28  |
| 22            | pACT-HSP22-C         | PCR of pcDNA-HSP22-F                 | EcoRI, XhoI           | 27, 28  |
| 23            | pAS-MKBP-F           | PCR of pcDNA-MKBP-F                  | EcoRI, XhoI           | 28, 29  |
| 24            | pAS-MKBP-N           | PCR of pcDNA-MKBP-F                  | EcoRI, XhoI           | 28, 29  |
| 25            | pAS-MKBP-C           | PCR of pcDNA-MKBP-F                  | EcoRI, XhoI           | 28, 29  |
| 26            | pAS-cvSP-H           | PCR of pcDNA-cvSP-F                  | EcoRI, XhoI           | 28, 29  |
| 27            | pAS-cvSP-N           | PCR of pcDNA-cvSP-F                  | EcoRI, XhoI           | 28, 29  |
| 28            | pAS-cvSP-C           | PCR of pcDNA-cvSP-F                  | EcoRI, XhoI           | 28, 29  |
| 29            | pFlag-CMV2-cvHSP-F   | PCR of pcDNA-cvHSP-F                 | KpnI, Xhol            | 41, 42  |
| 30            | pECFPN1-cvHSP-F      | PCR of pcDNA-cvHSP-F                 | EcoRI, BamHI          | 43, 44  |

a Described in 1.
b Construct 1, 2.
c MKBP (pGAD424) and HSPB3 (pGBT9) cDNAs were obtained from A. Suzuki (Yokohama, Japan).
d Used restriction sites in the PCR product and in the vector are compatible.
and cvHSP-C, but not with cvHSP-N (Fig. 4F). These data suggest that HSP22 and cvHSP interact through their C-terminal domains (C-C interaction).

**HSP22-MKBP Interaction**—TH assays using the full-length HSP22 and MKBP (constructs 2, 20) activated both reporter genes (his+, gal+) indicating interaction between the two proteins (Fig. 5A). This interaction was also verified biochemically by co-IP using full-length FLAG-tagged HSP22 and Myctagged MKBP (constructs 8, 29)(Fig. 5B). Similar to the experiments described above, after co-transfection with both Flag-HSP22 and MKBP-myc the anti-Myc antibody pulled down both proteins indicating that HSP22 interacts with MKBP. Again, actin was not precipitated suggesting that the Flag-HSP22 precipitation is due to a specific interaction with MKBP-myc.

For FRET measurements, COS-7 cells were co-transfected with vectors which permit expression of HSP22-YFP and MKBP-CFP (constructs 9, 24) fusion proteins (Fig. 5C). From the fluorescence intensity of the CFP signal (panels a and b) before (panels a and c) and after (panels b and d) photobleaching of the YFP (panels e and d) signal the FF (0.21 ± 0.0024, p = 0.0036) was calculated. This value is significantly different from the negative control (see “Experimental Procedures”). Thus both proteins interact in vivo.

We also sought to determine the interacting domains in the HSP22-MKBP hetero-dimer using the N- and the C-terminal halves of both proteins (constructs 5, 6, 21, 22; Fig. 1A), together with the full-length proteins (constructs 2, 20), in TH assays. HSP22-F did not interact with any fragment of MKBP (Fig. 5D). HSP22-N interacted with MKBP-F, but not with MKBP-N or MKBP-C (Fig. 5E). Finally, HSP22-C did not interact with MKBP-F, MKBP-N, or MKBP-C (Fig. 5F). These data suggest that HSP22-MKBP interaction involves the N-terminal half of HSP22, but appears to require full-length MKBP (N-F interaction).

**HSP22-HSP27 Interaction**—In a previous publication we reported that HSP22 (construct 2) interacts with a mimic of phosphorylated HSP27 (\(^{33P}\)HSP27; construct 11), but not with wild-type HSP27 (\(^{33P}\)HSP27; construct 10) (1). In the present study we confirmed these data (Fig. 6A). However, when the vectors and sHSP inserts were exchanged in the TH assays (constructs 1, 15, 16), there was also an activation of both reporter genes when \(^{33P}\)HSP27 cDNA was used indicating interaction between HSP22 and HSP27, regardless of the phosphorylation state of HSP27 (Fig. 6B). In order to resolve these conflicting TH data, a number of co-IP, CL and FRET experiments were performed. While the co-IP and CL experiments were unable to demonstrate interaction of HSP22 with any form of HSP27 (not shown), the FRET measurements did detect interaction. For FRET analysis, COS-7 cells were co-transfected with vectors that permit expression of HSP22-YFP (construct 9) and either \(^{33P}\)HSP27-CFP, \(^{33P}\)HSP27-CFP or \(^{33P}\)HSP27-CFP (constructs 17–19) fusion proteins (Fig. 6, C–E, respectively). The \(^{33P}\)HSP27 construct permits the expression of a non-phosphorylatable HSP27 fusion protein in which all three MAPKAPK-2 phosphorylation sites in HSP27 were replaced by alanine residues. The fluorescence intensity of the CFP signal (panels a and b) was determined before (panels a and c) and after (panels b and d) photobleaching of the YFP (panels c and d) signals. The calculated FF for \(^{33P}\)HSP27 (0.44 ± 0.045, p < 0.0001), \(^{33P}\)HSP27 (0.32 ± 0.047, p = 0.0006), and \(^{33P}\)HSP27 (0.34 ± 0.042, p < 0.0001) are all significantly different from the control (see “Experimental Procedures”). Thus, by FRET assay, HSP22 interacted with all forms of HSP27 regardless of the phosphorylation status of HSP27.

We also sought to identify, by TH assays, the interacting domains of HSP22 and HSP27. In these experiments, we tested whether HSP22-F, HSP22-N, or HSP22-C (constructs 2, 5, 6) can interact with the various forms of HSP27 including \(^{33P}\)HSP27-F, \(^{33P}\)HSP27-F, \(^{33P}\)HSP27-N, \(^{33P}\)HSP27-N, and \(^{33P}\)HSP27-C (constructs 10–14). The results show that HSP22-F interacted with HSP27-C, but not with \(^{33P}\)HSP27-N or \(^{33P}\)HSP27-N (Fig. 6F). When the fragments of HSP22 were assayed for interaction with HSP27, none of them, in combination with any form of HSP27, activated both reporter genes (Fig. 5, G and H). Taken together, these data suggest that 1) HSP22 interacts with HSP27 regardless of the phosphorylation status of HSP27, and 2) HSP27 has one binding domain (C

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**Fig. 4. HSP22-cvHSP interaction.** A, TH assay shows interaction of HSP22-F with cvHSP-F (constructs 2, 25). B, Co-IP assay shows interaction of Flag-cvHSP with HSP22-myc. Immunoprecipitation (IP) of Flag-cvHSP pulls down HSP22-myc only after double transfection of COS-7 cells with constructs 28 and 7, as shown by SDS-PAGE/Western blotting (WB). Under the same conditions, actin remained in solution (S, supernatant versus P, precipitate). C, FRET assay using doubly transfected COS-7 cells shows interaction of HSP22-YFP with cvHSP-CFP (constructs 9, 29). The CFP signals (a, b) were determined before (a, c) and after (b, d) the bleaching of YFP (c, d) in the selected area. The FF of 0.24 indicates interaction between both proteins. D, TH assay shows interaction of HSP22-F (construct 2) with cvHSP-C (construct 27), but not with cvHSP-N (construct 26). E, TH assay shows that there is no interaction between HSP22-N (construct 5) and cvHSP-F, cvHSP-N, or cvHSP-C (constructs 25–27). F, TH assay shows interaction of HSP22-C (construct 6) with cvHSP-F and cvHSP-C (constructs 25, 27), but not with cvHSP-N (construct 26). TH controls: C1–C6 as in Fig. 3.
terminus), which can interact with HSP22, but full-length HSP22 appears to be required for this interaction (F-C interaction).

**DISCUSSION**

The biological role of HSP22 has been controversially discussed. While some groups considered HSP22 to be an sHSP, others classified it as a protein kinase homologous to the large subunit of Herpes simplex virus type 2 ribonucleotide reductase (ICP10) which also has a protein kinase activity (4–6).

Aligning human HSP22 (accession AAF65562) and ICP10 (accession 1813262A), we found only weak similarity (11% identical, 13% similar; or 14% identical, 17% similar using the C-terminal part of ICP10; not shown) and could not reproduce the value of 30% identity reported previously (4). In order to learn about the phylogenetic position of HSP22, we performed an analysis using a sHSP-HMM and Bayesian inference as described (24). All 167 proteins identified by the sHSP-HMM were previously described as sHSPs or sHSP-like proteins of Bilateria (Metazoa: Animalia: Eumetazoa: Bilateria). In this
phylogeny, the mammalian HSP22 proteins form a well-supported, monophyletic group which is sister to the clade of HSP25/27 of Euteleostomi (Bilateria: Chordata: Craniata: Vertebrata: Euteleostomi) including mammals, birds and fish. Based on this analysis, we conclude that human HSP22 belongs to the superfamily of mammalian sHSPs and that it is most closely related to HSP27 among all proteins covered by the databases.

This analysis did not identify any eukaryotic, prokaryotic or viral protein kinase, nor any protein related to ICP10. Although sHSPs are known to have conserved a few protein kinase catalytic domains, which may be the basis for the observed autophosphorylation activities (4, 29), they are not known to be biologically significant protein kinases. At present there is no convincing biochemical evidence (e.g. K_m-value, specific activity, stoichiometry of the enzymatic reaction, substrate requirements) available which would support a role of HSP22, aB-Cry, or any other sHSP as a protein kinase with biological significance.

With HSP22 being a member of the sHSP superfamily, we reasoned that in addition to interacting with HSP27 it may also interact with other sHSPs abundant in muscles. The HPLC analysis of the monkey heart protein extract shows that HSP22 forms high molecular mass complexes which may well result from such interactions. To determine the interaction of HSP22 with other sHSPs, we used several approaches including genetic (TH), immunological (co-IP), chemical (CL), and cell biological (in vivo FRET) methods. The use of several methods helps to minimize both false-positive and false-negative results, which are known pitfalls of each of these methods. We showed using different methods that HSP22 interacts with itself forming homo-dimers, which further dimerize to form tetramers. Thus, HSP22 shares the complex-forming characteristics, which are typical for the other studied sHSPs. Similarly, we showed by independent methods that HSP22 forms hetero-dimers with cvHSP and MKBP.

For HSP22 interaction with HSP27, the obtained TH data were conflicting. Originally, only 3DHSP27 (mimicking phosphorylation) but not wHSP27 was found to interact with HSP22 using a certain TH environment (1). In the present study we confirmed this finding, which clearly suggests that the insertion of negative charges at the HSP27 phosphorylation sites causes conformational changes, which support interaction with HSP22. However, as shown in this study, when the sHSP inserts and TH vectors were exchanged, there was a comparable interaction of both 3DHSP27 and wHSP27 with HSP22. Thus, the conformational change caused by substitution of serine by aspartate residues is not critical for interaction under these conditions. We conclude from these results that in such fusion proteins the Gal4 (+)-transcription activation and -DNA binding domains have an impact on the steric accessibility of the proteins to be tested in these binding assays. Indeed, similar asymmetric results in TH experiments have been observed by others (30).

We conducted a series of biochemical and cell biological experiments to further address the issue of whether HSP22 and HSP27 interact in a phosphorylation-dependent way. Using different approaches (including expression of wHSP27, 3DHSP27, and HSP22 in COS-7 cells, followed by co-IP, native PAGE, native IEF, or CL), we did not observe evidence of interaction between these two sHSPs (not shown). However, we could demonstrate in vivo interaction between HSP22 and HSP27 by FRET using CFP/YFP-fusion proteins, regardless of whether the wHSP27, 3DHSP27, or 3DHSP27 constructs were used. Thus, the available data suggest that phosphorylation of HSP27 does not contribute to its interaction with HSP22, though it causes a conformational change in HSP27, which can be detected in TH experiments under certain conditions.

Based on multiple alignment, structural domains of sHSPs have been identified: the conserved α-crystallin domain, the less conserved N-terminal domain, the variable central region, and the variable region of the C-terminal tails (Ref. 24; Fig. 1A). A number of previous reports suggested the involvement of at least two binding sites, one in the α-crystallin domain and one in the N-terminal domain, in sHSP complex formation. In a TH study it was shown that the conserved C-terminal domain is essential for the interaction between αA-Cry, αB-Cry, and HSP27 subunits (31). For HSP27-HSP27 and HSP27-αB-Cry interaction, the major interacting site in the rat HSP27 sequence was defined as being amino acid residues 141–176 (23). Also yeast HSP42p interacts with itself via a conserved C-terminal site (32). The isolated C-terminal domain of mammalian αA-Cry forms dimers or tetramers, while the isolated N-terminal domain was still able to form a high molecular mass complex (33). Also for Caenorhabditis elegans HSP16–2, the N-terminal domain was shown to be essential for oligomerization into high molecular mass complexes (34). Two binding sites were found to be involved in HSP27-HSP27 interaction: one in the α-crystallin domain (which was insensitive to phosphorylation), the other in the far N terminus (which was sensitive to phosphorylation of Ser90) (22). It was proposed that HSP27 forms stable dimers through the α-crystallin domain, and these dimers then further multimerize through the phosphorylation-sensitive N-terminal domain. Based on these data we reasoned that HSP22 also may have two binding sites which are involved in interaction with other sHSPs. For that reason, we separated the cDNA of HSP22 and three of its binding partners, MKBP, cvHSP, and HSP27 (including phosphorylation site mutants) into N- and C-terminal parts and tested these fragments in TH assays. The data suggest that HSP22 has at least two binding sites, which have specificity in interaction with other sHSPs. HSP22-N interacts both with itself and with HSP22-C, while HSP22-C does not interact with itself. This N-N and N-C interaction would permit the formation of homo-dimers and homo-oligomers, as has been detected in transfected cells. In contrast to this interaction, HSP22 and HSP42 interact exclusively through their C-terminal (C-C interaction). The interaction between HSP22 and HSP27 involves the C terminus of HSP27, but apparently requires full-length HSP22. Similarly, the interaction of HSP22 and MKBP involves the N terminus of HSP22, but appears to require full-length MKBP. At this time, it is not clear whether the lattermost interactions indeed require one full-length binding partner, or whether this results from false-negative TH data.

Taken together, the present study shows, by in vivo and in vitro approaches, that HSP22 has the ability to bind to itself and three other sHSPs (cvHSP, MKBP, HSP27) which are abundant in muscles. These results also indicate that HSP22, like the other studied sHSPs, has at least two binding domains, which bind specifically to defined sites of other sHSPs. In a forthcoming study, the interactions between HSP22 and the remaining muscle sHSPs (αB-Cry, HSP20, HSPB3) will be described. It is hoped that this detailed analysis of the sHSP interactions will contribute to a better understanding of the biological role of the sHSP complexes in muscles and other tissues.

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REFERENCES

1. Benndorf, R., Sun, X., Gilmont, R. R., Biederman, K. J., Molloy, M. P., Goodmurphy, C. W., Cheng, H., Andrews, P. C., and Welsh, M. J. (2001) J. Biol. Chem. 276, 26751–26761

2. Charpentier, A. H., Bednarek, A. K., Daniel, R. L., Hawkins, K. A., Laflin, J. J., Gaddis, S., MacLeod, M. C., and Aldaz, C. M. (2000) Cancer Res. 60, 5977–5983

3. Kappe, A., Depre, C., Hase, M., Gaussin, V., Zajac, A., Wang, L., Hittinger, L., Ghaleh, B., Yuan, X., Kudej, R. K., Wagner, T., Sadoshima, J., and Vatner, S. F. (2002) J. Biol. Chem. 277, 5983–5989

4. Smith, C. C., Yu, Y. X., Kulka, M., and Aurelian, L. (2000) J. Biol. Chem. 275, 25690–25699

5. Aurelian, L, Smith, C. C., Winchurch, R., Kulka, M., Gyotoku, T., Zaccaro, L., Kappe, A., Boelens, W. C., and De Jong, W. W. (2001) J. Biol. Chem. 276, 36640–36644

6. Sugiyama, Y., Suzuki, A., Kishikawa, M., Akutsu, R., Hirose, T., Waye, M. M., Tsui, S. K. W., Yoshida, S., and Ohno, S. (2000) J. Biol. Chem. 275, 3652–3657

7. Krief, S., Faivre, J. F., Robert, P., Le Douarin, B., Brument-Larignon, N., Lefrere, I., Bouzyk, M. M., Anderson, K. M., Greller, L. D., Tobin, F. L., Souchet, M., and Bril, A. (1999) J. Biol. Chem. 274, 36592–36600

8. Sikorska, M., Gilmont, R. R., Benndorf, R. (2000) Nat. Genet. 25, 1095–1104

9. Vicart, P., Caron, A., Guichene, P., Li, Z., Prevost, M. C., Faivre, A., Chateau, D., Chapon, P., Tome, F., Dupret, J. M., Paulin, D., and Fardeau, M. (1998) Nat. Genet. 20, 92–95

10. Suzuki, A., Sugiyama, Y., Hasiwada, N., Nonoaka, I., Ishii, S., Arahata, K., and Ohno, S. (1999) J. Cell Biol. 144, 1113–1124

11. Lutsch, G., Vetter, R., Offhausu, U., Wieske, M., Grone, H. J., Klemenz, R., Schimke, I., Stahl, J., and Benndorf, R. (1997) Circulation 96, 3466–3476

12. Martin, J. L., Mestril, R., Hipol-Dundan, E., Brunton, L. L., and Dillmann, W. H. (1997) Circulation 96, 4343–4348

13. Yamboliev, I. A., Hedges, J. C., Mutnick, J. L., Adam, L. P., and Gerthoffer, W. T. (2000) Am. J. Physiol. Heart Circ. Physiol. 278, H11899–H11907

14. Beall, A., Bagwell, D., Woodrum, D., Stoming, T. A., Kato, K., Suzuki, A., Rasmussen, H., and Brophy, C. M. (1999) J. Biol. Chem. 274, 11344–11351

15. Kato, K., Hasegawa, K., Geto, S., and Inaguma, Y. (1994) J. Biol. Chem. 269, 11274–11278

16. Mehlen, P., Kretz-Heym, C., Briolay, J., Fostan, P., Mirault, M. R., and Arrigo, A. P. (1995) Biochem. J. 313, 367–375

17. Augustyn, R. C., Ghiggino, K. P., and Puttilina, T. (1995) Biochim. Biophys. Acta 1162, 61–71

18. Sun, T. X., and Liang, J. J. (1998) J. Biol. Chem. 273, 286–290

19. Bova, M. P., Mchaourab, H. S., Han, Y., and Fung, B. K. (2000) J. Biol. Chem. 275, 1035–1042

20. Ehrnsperger, M., Lilie, H., Gaestel, M., and Buchner, J. (1999) J. Biol. Chem. 274, 14867–14874

21. Rogalla, T., Ehrnsperger, M., Preville, X., Kotlyarov, A., Lutsch, G., Ducasse, C., Paul, C., Wieske, M., Arrigo, A. P., Buchner, J., and Gaestel, M. (1999) J. Biol. Chem. 274, 18947–18956

22. Lambert, H., Charette, S. J., Bernard, A. F., Guimond, A., and Landry, J. (1999) J. Biol. Chem. 274, 9378–9385

23. Liu, C., and Welsh, M. J. (1999) Biochem. Biophys. Res. Commun. 255, 256–261

24. Fontaine, J. M., Rest, J. S., Walsh, M. J., and Benndorf, R. (2003) Cell Stress Chaperones 8, 62–69

25. Siegel, R. M., Ka-Ming Chan, F., Zacharias, D. A., Swafford, R., Holmes, K. L., Tsien, R. Y., and Lenardo, M. J. (2000) Science’s Sites 38, 1–6

26. Zacharias, D. A., Violin, J. D., Newton, A. C., and Tsien, R. Y. (2002) Science 296, 913–916

27. Hoppe, A., Christensen, K., and Swanson, J. A. (2002) Biophys. J. 83, 3652–3664

28. Arrigo, A. P., Suhani, J. P., and Welsh, W. J. (1988) Mol. Cell. Biol. 8, 5059–5071

29. Kantorow, M., and Piatigorsky, J. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 3112–3116

30. Maslov, S., and Steppen, K. (2002) Science 296, 910–913

31. Boelens, W. C., Croes, Y., de Ruwe, M., de Reu, L., and de Jong, W. W. (1998) J. Biol. Chem. 273, 28085–28090

32. Wetton, D., Freeman, R., and Shure, D. (1998) J. Biol. Chem. 273, 2717–2723

33. Merck, R. B., De Haard-Hoeckman, W. A., Oude Essink, B. B., Bloemendal, H., and De Jong, W. W. (1992) Biochim. Biophys. Acta 1130, 267–276

34. Leroux, M. R., Melki, R., Gordon, B., Bateliers, G., and Candido, E. P. (1997) J. Biol. Chem. 272, 24646–24656