Chaperone Activity of Small Heat Shock Proteins Underlies Therapeutic Efficacy in Experimental Autoimmune Encephalomyelitis*

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Background: The small heat shock protein, HspB5, is therapeutic in experimental autoimmune encephalomyelitis.

Results: Eight other human sHsps, a mycobacterial sHsp, and a linear peptide from HspB5 were equally effective therapeutics.

Conclusion: All of the therapeutic proteins and peptides were also molecular chaperones.

Significance: Correlation between chaperone activity and therapeutic function supports data demonstrating sHsps bind inflammatory mediators in plasma.

To determine whether the therapeutic activity of αB crystallin, small heat shock protein B5 (HspB5), was shared with other human sHsps, a set of seven human family members, a mutant of HspB5 G120 known to exhibit reduced chaperone activity, and a mycobacterial sHsp were expressed and purified from bacteria. Each of the recombinant proteins was shown to be a functional chaperone, capable of inhibiting aggregation of denatured insulin with varying efficiency. When injected into mice at the peak of disease, they were all effective in reducing the paralysis in experimental autoimmune encephalomyelitis. Additional structure activity correlations between chaperone activity and therapeutic function were established when linear regions within HspB5 were examined. A single region, corresponding to residues 73–92 of HspB5, forms amyloid fibrils, exhibited chaperone activity, and was an effective therapeutic for encephalomyelitis. The linkage of the three activities was further established by demonstrating individual substitutions of critical hydrophobic amino acids in the peptide resulted in the loss of all of the functions.

Small heat shock proteins represent a large family of structurally diverse chaperones that form large dynamic oligomers, which bind partially unfolded regions of proteins and prevent their aggregation (1, 2). They are found in all biological kingdoms and appear to have evolved early in evolution. In contrast with large heat shock proteins, exemplified by the Hsp60 and Hsp90 families, sHsps do not bind ATP and do not refold their ligands (3). Their function is more fundamental. The sHsps are cytoprotective by limiting the concentration of partially unfolded proteins, which prevents the formation of amyloid or other protein aggregates known to be damaging to cells. In addition to this general function, several studies have established more specific interactions between human sHsps and cytosolic proteins such as p53 and Bax to inhibit apoptosis (4–7).

The crystal structures of sHsps from wheat, pea, tapeworm, Caenorhabditis elegans, and mycobacteria revealed that the family of proteins form a variety of dynamic oligomers with different stoichiometry, but in all cases the unit cell was a dimer with the monomer folding as a conserved β barrel with amino and carboxyl-terminal extensions (8–13). The extensions are integral in the formation and stabilization of dimers and aggregates of the dimers (14). The primary structures of the human sHsps are considered diverse compared with other Hsp families (1), ranging from 17 (HspB3 and B7) to 53% identity (HspB4 and B5), but when homologous residues are considered the similarity increases to a range of 53 to 84% homology (Table 1). Even greater similarity is observed when divergent amino and carboxyl termini are excluded and the strands of the characteristic β barrel are compared. In this region, the homology ranges from 66 to 92%. Conserved hydrophobic residues are seen in an alternating pattern characteristic of the barrel that is stabilized by hydrophobic contacts between the strands. The large variation in the termini is consistent with possible variations in the quaternary structures of the different sHsps. Nevertheless, many of the individual sHsps can form oligomers with other family members. For example, HspB5 can form aggregates with HspB4, HspB1, HspB2, and HspB8 (15).

Recent resolution of the Ig domain of human HspB5 revealed that the dimer is formed using different principles than observed in earlier structures (16, 17). The contact surface is between the edge strands and because the register of the strands...
is not symmetrical the interface forms a groove, which is postulated to be one of the principal hydrophobic binding sites. A conserved structural feature of the groove is the presence of salt bridges at both ends formed between a conserved arginine at residue 120 of one domain with a conserved aspartic acid at residue 110 in the adjacent subunit (16). A naturally occurring mutation of this arginine to a glycine compromises the structural integrity of the groove and the resultant chaperone activity of the protein (18). Most importantly, the mutation is physiologically relevant, and is linked to a human desmin myopathy (19, 20). The solution of the crystal structure of the mutant revealed a collapse of the groove between the subunits, but otherwise the crystallin domains were intact along with several pockets speculated to be ligand binding sites (21).

There are 10 members of the sHsp family in man, sHspB1–10, which differ in their tissue expression and their capacity to be induced by environmental stress (22). As a group they are expressed in relatively long-lived tissue, such as muscle (HspB1, -B2, -B3, -B5, -B6, -B7, and -B8), neurons (HspB1, -B5, -B6, -B7, and -B8), lenticular tissue (HspB4 and -B5), and the testes...
Briefly, the full-length clones of human sHspB1–8 were expression, and purification were described previously (30, 31). Gene expression profiling and mass spectrometry of tissue isolated by laser capture microdissection established HspB5 as a prominent protein at these sites.

A protective role for the protein was established when mice unable to express HspB5 were shown to exhibit greater paralytic symptoms of EAE than wild-type controls (25). Even though sHsps are intracellular, cytosolic proteins, which can be transported to the nucleus upon phosphorylation, exogenous though sHsps are intracellular, cytosolic proteins, which can be transported to the nucleus upon phosphorylation, exogenous intravenous injection of 10 μg every other day resulted in significant reduction of the symptoms of EAE. This was true in both HspB5 knock-out and wild-type mice. Subsequent studies have demonstrated that HspB5 is effective in reducing the lesion size in a murine model of stroke (26), reducing inflammatory and improving heart function in a model of myocardial infarction (27) and increased oligodendroglial survival in the optic nerve in a model of retinal ischemia (28). Further analyses in these animal models concluded that the protein was immunosuppressive. The anti-inflammatory property was not due to influencing the adaptive immune response directly, but rather there was binding and resultant modulation of the proinflammatory mediators in plasma (29). These observations are at the basis of the therapeutic effects seen in both autoimmune and ischemic models of disease, including EAE, stroke, myocardial infarction, and retinal ischemia (25–28).

The structural diversity in the family of human sHsps provides the opportunity to examine the relationship between variations in sequence and the resultant anti-inflammatory activities in the EAE model. The strategy in this study was to explore the therapeutic activity of HspB5 and related family members by testing whether any of the other human sHsps were active in modulating paralysis in EAE, and if so whether there are any differences in their potency. Eight of the 10 human sHsps were expressed in bacteria, purified, and their chaperone activity was compared. A naturally occurring point mutation of HspB5 that is known to affect the chaperone activity of the protein was analyzed along with a mycobacterial sHsp, whose crystal structure is known. In addition to the full-length proteins, a set of peptides corresponding to the HspB5 sequence, including linear regions of HspB1, -B4, and -B5, which have been shown to be chaperones, were analyzed for therapeutic activity in EAE as well. We demonstrate how an evolutionary diverse set of structures, derived from mycobacteria to humans, with shared structural motifs, exhibits common anti-inflammatory properties.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of T7-human HspB1-8, HspB5 G120, and acr-1 from M. tuberculosis—Cloning, expression, and purification were described previously (30, 31). Briefly, the full-length clones of human sHspB1–8 were obtained from Open Biosystems. An EcoRI, an ATG site, a HindIII, and stop site were introduced into each of the genes encoding a sHsp using PCR. The resulting sHsp PCR fragments were ligated into the EcoRI-HindIII restriction site of pET21b(+) (Novagen, Madison WI) in-frame with the amino-terminal T7-tag corresponding to the first 11 residues of the bacteriophage T7 gene 10 encoding the capsid protein, MASMTGGQQMG (30). The mutation of glycine for arginine at residue 120 of HspB5 was performed using the site mutation kit from Stratagene (Cedar Creek, TX) in which a primer encoding the mutation was synthesized, a clone with the mutation was generated by reverse PCR, the template plasmid was digested, and the plasmid encoding the mutated HspB5 was isolated. One-shot TOP10 cells (Invitrogen) were transformed with the resulting plasmids. For each sHsp the resulting colonies were selected, expanded, and the insertion was verified by restriction digest with EcoRI and HindIII, and sequencing. The proteins were produced in small scale by transforming BL21 Condon Plus cells (Stratagene) for protein expression. Larger scale production and purification of the T7-Hsps was accomplished by growing selected colonies in 250–1000 ml of LB broth with carbenicillin, induced with isopropyl-1-thio-β-D-galactopyranoside, and isolating the bacteria 4–12 h later. The cells were lysed with a bacterial protein extraction buffer (Thermo, Waltham, MA) with sonication while being cooled on ice, and the supernatant was collected after centrifugation; saturated ammonium sulfate was added to 20% (v/v), and the mixture centrifuged. Sufficient saturated ammonium sulfate was added to the supernatant to increase the concentration of the solution to 45% (v/v). After centrifugation, the pellet containing the sHsp was resuspended in 50 mM NaCl and 50 mM Tris, pH 8.0. Additional sHsp was recovered by extracting the initial pellet in the cellular lysis with 6 M guanidine hydrochloride 100 mM Tris, pH 8.0, and dialysis against 50 mM NaCl and 50 mM Tris, pH 8.0. The dialyzed mixture was spun and the supernatant combined with the resuspended pellet from the 50% ammonium sulfate precipitation and applied to DEAE fast flow column to remove the majority of the DNA and negatively charged glycosaminoglycans. The flow through was concentrated and applied to a Sephacryl S-300 column. The fractions corresponding to the large molecular mass sHsp (approximately 400 kDa) were pooled and concentrated, and finally applied to an anti-T7 column, and the T7-sHsps were eluted with glycine buffer, pH 3.0. The eluate was neutralized with 1 N Tris, pH 8.0, and concentrated. The affinity-purified material was dialyzed against PBS, and rerun through a DEAE fast flow column to remove as much LPS as possible. The purity of the protein was established using Coomassie-stained SDS-PAGE gels, and the structure was confirmed by mass spectrometry (data not shown). The quaternary structure was established using gel filtration on Sephacryl S-300 and by dynamic light scattering.

**Peptide Synthesis—**Peptides were synthesized using solid phase techniques and commercially available Fmoc amino acids, resins, and reagents (PE Biosystems, Foster City, CA, and Bache, Torrance, CA) on an Applied Biosystems 433A peptide synthesizer as previously described (32). Fastmoc cycles were used with O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluro- nium hexfluorophosphate substituted for O-benzotriazole-
N,N,N′,N′-tetramethyluronium hexafluorophosphate/N-hydroxybenzotriazole as the coupling reagent. All Fmoc amino acids were commercially available (Bachem, San Diego, CA). The peptides were cleaved from the resin using 96% trifluoroacetic acid, 2% triisopropyl silane, and 2% phenol for between 1 and 12 h. The peptides subsequently were filtered from the resin, precipitated using diethyl ether, purified using HPLC reverse phase columns (Alltech Altima). The purity of the peptides was shown to be greater than 95% using a PE Biosystems 700E HPLC and a reverse phase column (Alltech Altima).

Chaperone Assays—The capacity of the proteins and peptides to inhibit DTT induced aggregation of the β chain of insulin was assayed using procedures described previously by several authors (33, 34). Briefly, 150 μg of bovine insulin (Sigma) was dissolved in 100 mM NaCl, 20 mM Tris, pH 7.4, with or without varying concentrations of the shSps in a total volume of 380 μl and incubated at 42 °C. DTT, 20 μl of a 100 mM stock solution, was added at time 0, and the aggregation was measured by the increase in absorption at 360 nm as a function of time over 20 min. In the case for peptides, 100 μg of insulin was used because of the limited solubility of several sequences.

Induction of EAE in Mice by Immunization with MOG and Adjuvant and Treatment with Hsb5—EAE was induced by procedures previously described (35). Briefly, EAE was induced in female C57BL/6J mice (Jackson Laboratories, Sacramento, CA) at 9 weeks of age by subcutaneous immunization in the flank with an emulsion containing 200 μg of myelin oligodendrocyte glycoprotein (35–55) (MOG35–55; MEVGYWRSPFS-RVVFHLRYNGK) in saline and an equal volume of complete Freund’s adjuvant containing 4 μg/ml of mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, MI). All mice were administered 400 ng of pertussis toxin (List Biological, Campbell, CA) intraperitoneally at 0 and 48 h post-immunization. Mice were given food and water ad libitum and were monitored daily for clinical symptoms. The neurological impairment was scored as follows: 0, no clinical disease; 1, tail weakness; 2, hindlimb weakness; 3, complete hindlimb paralysis; 4, hindlimb paralysis and some forelimb weakness; 5, moribund or dead. When animals exhibited level 2 symptoms they were injected in the peritoneum with 10 μg of Hsb1–8, 1 μg of peptide, or PBS daily. All animal protocols were approved by institutional IACUC.

Immune Cell Activation and Cytokine Analysis—Splenocytes and lymph node cells isolated from mice 9 days following induction of EAE using MOG (35–55) were incubated with MOG (35–55) (5, 10, and 20 μg/ml). The supernatants were collected at 48 h for IL-2 and IL-6, 72 h for TNFα and IFNγ, and 96 h for IL-17 measurement. Cytokine levels were quantified using anti-mouse OPTIA ELISA kits from BD Pharmingen (IFNγ, IL-2, and IL-6) and R&D Systems (TNFα and IL-17). For all activation assays, cells were pooled from three mice per group and triplicate wells were plated.

Thioflavin T Binding—The peptides corresponding to residues 73–92 of Hsb1, -B4, and -B5 and those with lysine substitutions were dissolved at 100 μg/ml, incubated at 37 °C overnight. The relative amount of amyloid present in each solution was measured by combining 100 μl of the peptide solution with 80 μl of PBS, pH 7.2, and 20 μl of thioflavin T in wells of a black 96-well microtiter plate. The emission fluorescence at 485 nm for each sample after excitation at 440 nm was measured using a SpectraMax 190 fluorescent microtiter plate reader.

Atomic Force Microscopy—The samples were prepared by drop casting 4 μl of 0.01 g/liter of amyloid solution on freshly cut silicon wafers, previously stored in a sealed box. The droplets were allowed to evaporate under house vacuum on a humid chamber for slower evaporation. Some wafers were treated with ozone plasma to increase their polarity. The imaging was performed on a Smena AFM from NT-MDT with a separate 50-μm bottom XY scanner. Piezo elements for all three axes have been equipped with capacitance sensors. Imaging was done in tapping (intermittent contact) mode at speeds between 0.6 and 1 Hz with commercial silicon tips from MicroMasch (R < 10 nm, k = 7.5 N/m). Minimal tip damping was employed with the set point typically within 20% of the maximum value to minimize the amyloid fiber distortion. No shifting of fibers has been observed after any of the experiments.

RESULTS

Quantification of the Chaperone Activity of Hsb1–8, Hsb5 G120, and Mycobacterium tuberculosis acr-1—Eight of the 10 known human shSps, Hsb1–8, a small heat shock protein from mycobacterial tuberculosis, acr-1, and the naturally occurring mutation of Hsb5 in which an arginine at residue 120 is substituted with a glycine, Hsb5 G120, were cloned into the pET 21b T7 plasmid, expressed in E. coli, and subsequently affinity purified to produce each protein with the 10-amino acid T7 tag expressed on the amino terminus of the protein. The capability of the modified human shSps to bind immunoglobulins in both solution and solid phase assays previously have been established (31). To extend their characterization and to confirm and compare their activity as chaperones, varying concentrations of each protein was incubated with bovine insulin under reducing conditions with DTT and the time-dependent light scattering produced by the association of the reduced β chain of insulin was monitored at 360 nm (33, 34). All of the proteins passed assay inhibited the aggregation of the β chain of insulin in a dose-dependent manner, including the Hsb5 mutant and the mycobacteria protein, acr-1 (Fig. 1, A–E). There were relatively small differences in their effectiveness as chaperones as can be seen when their half-maximal ED50 values were plotted in a common graph (Fig. 1F). Hsb1, -B2, -B7, and -B8 were the most potent, with Hsb5 and -B4 exhibiting equivalent activity. Hsb5 was approximately three times more potent than the G120 mutant, which was qualitatively similar to previous studies. acr-1 was similar to Hsb2, -B4, and -B5 in this assay. An important caveat is that only Hsb5 does not contain a cysteine, consequently some of the observed differences could be due to effects DTT has on the quaternary, or tertiary structure of the other proteins. Nevertheless, each of the proteins tested exhibited titratable chaperone activity.

Assessment of Human and Mycobacterial shSps as Therapeutic Agents in EAE—Previous studies have established that intravenous or intraperitoneal administration of Hsb5 reduces the
paralytic symptoms in EAE and was immunosuppressive (25). The similarity in structure and chaperone function of the other sHsp family members predicts that each family member might be effective in treating EAE. To determine whether any of the other sHsps were therapeutic and if so, whether any were significantly more potent than HspB5, the 10 sHsps were separately tested using this animal model. Treating groups of 10 mice with EAE at the peak of disease with daily injections of 10 μg of HspB1, -B4, and -B5 resulted in reduction of paralytic symptoms compared with mice injected with PBS (Fig. 2A).

The magnitude of the reduction of symptoms was not significantly different between the three proteins. Therapeutic effects statistically different from animals treated with PBS were observed when HspB2, -B3, and -B6–8 were administered (Fig. 2, B and C). However, no statistically different therapeutic effects could be assigned to the different protein therapeutics primarily because of the inherent variation in the induction of the disease between sets of animals. Nevertheless, all of the human sHsps tested were therapeutic. Treatment of diseased mice with mycobacterial sHsp, acr-1, also modulated symptoms of EAE when 10 μg was administered daily (Fig. 2D). An important aspect of this experiment was that cessation of treatment resulted in complete return of the paralytic symptoms (Figs. 2D and 3, A and B). This result was surprising based on the knowledge that chaperone activity of the mutant was less than the parent sequence (19, 20) and confirmed in the insulin chaperone assay (Fig. 1E). To determine this result was not due to the administration of an excessive amount of protein, mice were treated with HspB1, which was the most potent protein inhibiting the insulin aggregation, or HspB5 G120. Groups of 10 animals were treated with daily injections of 0.1, 1, or 10 μg of each protein (Fig. 3). In each case the reduction of symptoms was dose dependent, but a statistically significant different therapeutic effect between the two proteins, HspB1 and HspB5 G120, was not observed. All of the sHsps analyzed exhibited chaperone function and were equally effective in this animal model.

Treatment with each protein modulated the inflammatory cytokines, IL-2, IL-6, and IFNγ, but not IL-17 and TNFα from splenic lymphocytes (Fig. 4A). Treatment with HspB5 G120 modulated the inflammatory cytokines similar to HspB5, reducing IL-2, IL-6, and TNFα from splenic lymphocytes (Fig. 4B).

**Analysis of sHsp Peptides as Chaperones**—Peptides by definition do not exhibit a single well defined conformation and consequently are rarely efficient surrogates for protein exhibiting equivalent activity. However, in the case of the chaperone activity of HspB4 and -B5, Sharma and colleagues (33, 36–38) have established that a peptide sequence corresponding to residues 73–92 in HspB5 exhibits equivalent activity as the intact protein. To confirm this result and explore whether there are other active peptides within HspB5, a set of overlapping 15-mers were synthesized. Those that were freely soluble in aqueous
buffers, a total of seven, were analyzed for their ability to act as a chaperone (Fig. 5A). Only residues 71–85 were effective, as were residues 73–92 from HspB5, which corresponds to the region defined by Sharma and colleagues (33, 36–38) (Fig. 5B). The homologous regions of B1 and B4 were equally active. As was the case of the different sHsps, the ED50 values for chaperone activity of the three peptides were very similar.

**Therapeutic Efficacy of the Peptide Chaperones in EAE**—The fact that only residues 71–85 exhibited chaperone activity allowed the hypothesis that this activity was essential for therapy, a situation that was unable to be tested with the set of intact proteins. Sets of mice with hind-limb paralysis were injected daily with 1 μg of residues 11–25, 151–165, or 71–85 in the peritoneum and their symptoms were monitored over 2 weeks (Fig. 6A). Only in the animals treated with residues 71–85 were...
the symptoms significantly reduced. The symptoms in the animals treated with 11–25 or 151–165 were indistinguishable from animals treated with PBS (Fig. 6A).

The dose of the peptide chosen in the previous set of experiments is close to equimolar with the doses of proteins administered in Fig. 2 (i.e. $1\mu g$ of the peptide $M_r=2,000$ compared with $10\mu g$ of HspB5 $M_r=22,000$). To confirm the peptide exhibited equivalent potency as the intact protein, a set of animals with EAE were treated with $10\mu g$ of HspB5 and compared in the same experiment with another set treated with $1\mu g$ of residues 73–92 of HspB5. The effects of the two treatments were equivalent (Fig. 6B). The molar equivalence of the therapeutic potency of the peptides and proteins also was demonstrated using HspB1 and HspB4 and their corresponding peptides (Fig. 6C), and in all cases, splenocytes for the peptide-treated animals exhibited reduction in their production of the inflammatory cytokines, IL-2, IL-6, and IL-17, but not IFNγ. These results are consistent with those observed in splenocytes isolated from animals treated with the intact proteins (Fig. 4).

The effect of the HspB5 and HspB5(73–92) treatment on inflammation in the CNS was assessed by quantifying the number of inflammatory foci in the meninges and parenchyma (Table 2). Although there was no statistically significant difference between the treated and untreated mice, there was a ~44% decrease in the number of inflammatory foci in mice treated with HspB5 and a 39% decrease in mice treated with HspB5(73–92).

**Identification of Residues within 73–92 Critical for Amyloid Formation and Therapeutic Function**—Tanaka and colleagues (38) have previously shown that the chaperone activity of residues 73–92 of HspB4 arises from its ability to form amyloid fibrils, which can explain how a relatively short peptide can exhibit equivalent biologic function as a fully folded protein. Solutions of residues 73–92 from HspB1, -B4, and -B5 all exhibited increased fluorescence at 485 nm when incubated with thioflavin T and excited at 440 nm, characteristic of amyloid formation (Fig. 7A). The relative amount of fluorescence was significantly higher for HspB4 than -B5 or -B1, but the signals observed for the latter two solutions were significantly greater than background. Consistent with this measurement, amyloid fibrils were found in each of the solutions when analyzed by atomic force microscopy (data not shown).

Crystallographic solutions of amyloid fibrils have established that amyloid fibrils are composed of two self-complementary $\beta$-pleated sheets, whose strands are as short as six amino acids (39–41). In addition, several groups have written algorithms to predict amyloid forming regions based on $\beta$ sheet propensity (42–44) and the capacity to form complementary segments...
capable of close packing characteristic of the zipper-like configuration observed in the crystal structures (45). In the 73–92 region, the algorithm developed by Goldschmidt and Eisenberg (45) predicted two regions within the peptide, residues 76–82 and 89–95, and would exhibit the greatest propensity to aggregate and form an amyloid. Both regions correspond to a β-pleated sheet in the crystal structure of the intact protein with alternating hydrophilic and hydrophobic amino acids. Residues 89–95 extended outside of 71–85 and 73–92 and consequently residues 76–82 appeared to be central to the activity.

To test whether the pattern of hydrophobic residues between 76–82 were central to the biological activity of the peptide, each of three hydrophobic amino acids, 77, 79, and 81, were separately replaced with lysine (Table 3). The three peptides with the individual lysine substitutions were tested for thioflavin T binding, chaperone function, and whether they could inhibit the paralytic symptoms of EAE (Fig. 7, A–C). In each assay removal of any of the three hydrophobic amino acids resulted in failure to bind thioflavin T, loss of chaperone function, and loss of therapeutic activity (Fig. 7, A–C).

### DISCUSSION

The structural basis of the therapeutic mode of action of HspB5 in reversing paralysis in EAE was explored by determining whether any of the other human sHsps were also effective. Compared with the 65- and 90-kDa Hsps, the set of human

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**TABLE 2**

Quantification of inflammatory foci in brain and spinal cord samples of mice with EAE treated with HspB5 protein and HspB5(73–92) peptide

|             | Meninges | Parenchyma | Total | p value |
|-------------|----------|------------|-------|---------|
| No treatment| 116.4 ± 28.8 | 127.8 ± 23.1 | 244.2 ± 46.4 | 0.0941 |
| HspB5       | 64.6 ± 6.7  | 72.4 ± 13.8 | 136.4 ± 20.4 | 0.1869 |
| HspB5(73–92)| 71.4 ± 15.7 | 76.8 ± 22.5 | 148 ± 37.3  |         |

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**FIGURE 6.** Peptide fragments of HspB1, -B4, and -B5 corresponding to the region of chaperone activity reduced the symptoms of EAE. A, 1 μg of HspB5 residues 11–25, 71–85, and 151–165 was injected intraperitoneally daily at the peak of disease (n = 6–7). PBS was injected as control (n = 16). Bar represents duration of treatment. Values in the graph represent mean ± S.E. *, p < 0.05 by Mann Whitney U test for residue 71–85. Full protein (10 μg) or residue 73–92 (1 μg) for HspB5 (panel B) and B1 and B4 (panel C) were injected intraperitoneally daily at the peak of disease (n = 7–14). PBS was injected in control littermates (n = 23). Bar represents the duration of the treatment. Values in the graph represent mean ± S.E. *, p < 0.05 by Mann Whitney U test. D, splenocytes from mice treated with HspB1, -B4, and -B5 residue 73–92 were stimulated with 0, 5, 10, and 20 μg/ml of MOG(35–55) and the levels of cytokines were measured (n = 3). *, p < 0.001 and **, p < 0.01 by two-way analysis of variance for all peptides. #, p < 0.01 for B5 peptide and ##, p < 0.01 for B1 peptide by two-way analysis of variance.
hSps are more structurally diverse, and were expected to exhibit a range of potency, which could be correlated with structural similarities and differences. To provide an even more diverse range of structural variation, a mycobacterial sHsp was included in the analysis. We hypothesized that the characteristic responsible for the therapeutic activity in EAE would correlate with the known chaperone function of the sHsp. We therefore also tested a naturally occurring mutant of HspB5 with arginine 120 substituted with glycine. We further hypothesized that this mutant protein with its reduced chaperone activity (18, 20) would have limited therapeutic activity.

The 10 recombinant proteins were shown to be active chaperones, inhibiting the aggregation of the β chain of insulin under reducing conditions, and their ED_{50} values reflected a relatively tight range of variation. When tested as therapeutics to reduce the degree of paralysis in EAE all eight of the known 10 human sHsps were effective. These experiments did not provide any structural insight due to inherent variation in disease induction in the model and the practical limit of the size of the groups of the animals. Consequently statistically significant variation in their therapeutic effects was not observed. The HspB5 mutant, G120, also was therapeutically effective and when compared with HspB1 in the same set of animals, was equally effective at doses over 2 orders of magnitude. Even the mycobacterial sHsp, acr-1, reduced the paralytic symptoms. The latter result not only emphasizes that a more structurally diverse member of the sHsp family that exhibits different qualities. Because even though the loss of a central hydrogen bond apparently central to the formation of a central groove, the rest of crystallin domain of each monomer is indistinguishable from the wild-type protein and consequently any ligand binding pockets would be conserved (21).

To determine whether the crystallin domain was responsible for therapeutic activity, peptides corresponding to linear regions of the protein were analyzed for chaperone function, based on earlier reports by Sharma et al. (33, 36, 37, 46) that residues 72–93 in HspB5 and the corresponding sequence in HspB4 bound both the hydrophobic dye, 1,1'-bis-(4-anilino)-naphthalene-5,5'-disulfonic acid, and was a chaperone. Our data confirmed that the peptide was a chaperone, established that this region was a unique region in HspB5, and when used as a therapeutic for EAE, was equally potent in its effects as the intact protein on a molar basis. The homologous regions of HspB1 and HspB4 were equally effective therapeutics. In addition to reducing the paralytic symptoms of EAE, treatment with the proteins or the chaperone peptides reduced the capacity of splenocytes to produce inflammatory cytokines as was previously shown for HspB5 (25) demonstrating their mode of action was immunosuppressive. The serological half-life for HspB5 was of the order of 6 h, and that for the peptides are expected to be equally short. Cessation of the injection of the proteins or peptides resulted in return of the symptoms, establishing that they act as biological inhibitors, and did not stimulate a long acting immunological cascade. Most importantly, only the peptide exhibiting chaperone activity was therapeutic, establishing a correlation between the two activities. No peptide tested that was not a chaperone modulated the symptoms of EAE.

Tanaka and colleagues (38) previously has shown that the chaperone activity of residues 73–92 of HspB4 arises from its ability to form amyloid fibrils, which can explain how a relatively short peptide can exhibit equivalent biologic function as a fully folded protein. In addition to establishing 73–92 as a chaperone, Sharma and colleagues (36) also demonstrated that the peptide was able to arrest fibril formation of Aβ peptides and...


**sHsp Chaperones as Therapeutic Agents**

suppress their toxicity on rat pheochromocytoma cells (PC12). Intact HspB5 recently has been shown to also inhibit fibril formation of this peptide (47). That both a soluble amyloid and sHsps can bind unfolded or proteins with their interior β sheet edges exposed would be consistent with their shared therapeutic efficacy in EAE. This hypothesis also argues that misfolded proteins and/or amyloids are involved in the pathogenesis of EAE, a disease that previously was not considered to have amyloid involvement. That amyloid forming peptides are immunosuppressive and therapeutic is counterintuitive to the manifold publications emphasizing their correlation with inflammation. However, we have observed similar effects when β amyloid peptides are used as treatment for EAE (48) and currently are exploring the molecular basis for these effects.

In another manuscript (29) we have explored this hypothesis by defining the set of proteins HspB5 binds in plasma from patients with multiple sclerosis, rheumatoid arthritis, and amyloidosis. In each case, the sHsp precipitate was enriched relative to the normal plasma concentration with a set of ~65 proteins, over half of which are members of the acute phase, complement, and coagulation pathways. The ligands were bound in a temperature-dependent fashion, characteristic of known behavior of heat shock protein binding. The combination of thermal sensitivity of the HspB5 combined with the high local concentration of these ligands at the site of inflammation is proposed to explain the paradox of how a protein believed to exhibit nonspecific binding can bind with some relative apparent selectivity to proinflammatory proteins and thereby modulate inflammation. Consequently, we believe the chaperone activity of both intact sHsps and amyloid forming peptides is the basis for the observed therapeutic activity in EAE. However, the mechanistic details of how the peptides bind the set of ligands appears to be different from the sHsps. Hydrophobic binding pockets formed by the secondary structure of the protein, or by interfaces between subunits, have been postulated to be responsible for the chaperone activity of the sHsps (16, 17, 21). Residues 73–92 could contribute amino acids to one of these pockets when part of the tertiary structure of the protein. However, as an amyloid, an entirely different binding surface would be created, which could bind a similar set of ligands in plasma as the intact sHsps. Alternatively, the peptides could form heteroamyloid structures and modify the length or biological activity of endogenous amyloids (recently reviewed in Ref. 49).

Support for the differences in the details of the binding pockets is provided in the role of arginine 120. The solution of the crystal structures of several human sHsps revealed that arginine 120 forms a critical salt bridge, which when disrupted compromises the chaperone activity of the protein (16, 17). In contrast, the peptide containing this arginine, 111–125, did not exhibit chaperone activity, did not form an amyloid, and was not therapeutic. Consequently, the residue was unimportant in the activity of the peptides, distinguishing critical residues between the two species of chaperones. The current model is that both sHsps and amyloids can reduce unfolded proteins and/or amyloid formation at sites of inflammation. Future experiments are planned to determine whether they accomplish this function by similar or dissimilar mechanisms.

The similar therapeutic activities of the eight human sHsps elicits a number of questions about their common, and possible differential, functions. If they are all similarly protective, why is a HspB5/B2 knock-out mouse more susceptible than a wild-type animal to EAE and stroke? Why does the apparent redundant function of the other family members not protect the animals? Several authors have speculated that the corresponding set of their ligands of each sHsp would overlap, but would be distinct, which can be supported by data in studies of two different yeast sHsps (12). One explanation is that not all sHsp are expressed equally at sites of inflammation due to their varying abilities to be excreted. Serological levels of HspB5 have been shown to increase in stroke and MS patients (26, 29) compared with healthy controls, which is consistent with its ability to reach extracellular sites of inflammation. Because exogenous injection of the protein has a beneficial effect, loss of any expression of the protein would be expected to result in greater inflammation. A significant drop in concentration of HspB5 in plasma could explain the phenotype of the knock-out animals. Whether this is the case can be tested experimentally.

Regardless of the unexpected therapeutic equivalence of the diverse set of human and bacterial proteins and the peptides, each was shown to inhibit the aggregation of the β chain of insulin and consequently was a chaperone. Only those proteins or peptides that exhibited chaperone activity were therapeutic in EAE, supporting the idea that chaperone function and anti-inflammatory properties of the sHsps are highly correlated.

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