The therapeutic benefit of the small heat shock protein \(\alpha\B-crystallin\) (HspB5) in animal models of multiple sclerosis and ischemia is proposed to arise from its increased capacity to bind proinflammatory proteins at the elevated temperatures within inflammatory foci. By mass spectral analysis, a common set of 70 ligands was precipitated by HspB5 from plasma from patients with multiple sclerosis, rheumatoid arthritis, and amyloidosis and mice with experimental allergic encephalomyelitis. These proteins were distinguished from other precipitated molecules because they were enriched in the precipitate as compared with their plasma concentrations, and they exhibited temperature-dependent binding. More than half of these ligands were acute phase proteins or members of the complement or coagulation cascades. Consistent with this proposal, plasma levels of HspB5 were increased in patients with multiple sclerosis as compared with normal individuals. The combination of the 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 and modulate inflammation.

\(\alpha\B-crystallin\) (heat shock protein B5, HspB5) is a member of the small heat shock protein (sHsp) family (1–3), whose members are temperature-sensitive chaperones known to inhibit the aggregation of partially denatured proteins (4, 5). Unlike the large heat shock family proteins, Hsp60 and Hsp90, the sHsps do not use ATP to refold proteins. They bind partially unfolded, hydrophobic structures and prevent their aggregation. In addition to lenticular tissue in which HspB5 was first identified (6), the protein is expressed in a variety of long-lived cells including neurons and muscle, where its expression is induced by a variety of cellular insults such as free radical oxidation, temperature increase, or hypoxia. The protein lacks a leader peptide and is found in both the cytosol and the nucleus, where it forms dynamic high molecular weight aggregates capable of stabilizing protein structures and limiting apoptosis (7) by binding and modulating the activity of several proteins including Bax and p53 (8–10). Recently, the protein has been shown to be secreted from cells via exosomes (11), which suggests that the protein might have additional physiological roles outside of cells.

The solution of the crystal structures of the dimers of \(\alpha\B-crystallin\) and HspB6 greatly aided the molecular understanding of the temperature-dependent, dynamic action of this class of molecules (12, 13). The \(\beta\) barrels of both Hsp molecules formed an extended \(\beta\) pleated sheet whose register varied between the two family members. Several empty hydrophobic pockets were noted in the structure that could bind ligands. The most prominent feature was a hydrophobic groove between the two monomers apparently occupied by residues from one of the amino-terminal extensions that could dissociate at elevated temperatures, creating two new binding sites for the chaperone and explaining the increase in chaperone function at temperatures greater than 37 °C.

Our group became interested in HspB5 when the gene encoding the protein was shown to be the most highly induced when tissue from human multiple sclerosis plaques was compared with normal brain tissue (14). A subsequent study, using mass spectrometry, established that the protein expression also

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**Significance:** The capacity of the heat shock protein to bind a spectrum of ligands represents a unique therapeutic reagent.

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**Background:** \(\alpha\B-crystallin\) is therapeutic in animal models of multiple sclerosis and ischemia.

**Results:** Crystallin binds ~70 plasma proteins; over half are members of the acute phase, coagulation, and complement pathways.

**Conclusion:** The heat shock protein can bind with apparent selectivity and modulate inflammation.

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\*2 To whom correspondence should be addressed: Dept. Neurology and Neurological Sciences, Beckman B002, 279 Campus Dr., Stanford, CA 94305-5316. Fax: 650-725-0627; E-mail: Steinman@stanford.edu.

\*3 The abbreviations used are: HspB5, heat shock protein \(\alpha\B-crystallin\); sHsp, small heat shock protein; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; RA, rheumatoid arthritis; SA, secondary amyloidosis; SAP, serum amyloid P;
was among the highest measured in the plaque (15). The importance of HspB5 in an animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), was established when HspB5 knock-out animals exhibited significantly greater degrees of clinical paralysis than genetically similar wild type animals (16). In addition to more severe paralysis, the mice with the HspB5 gene deleted had more widespread inflammation as compared with wild type animals, with increased infiltration of activated CD4+ lymphocytes and macrophages and significantly greater levels of glial apoptosis.

Surprisingly, when the putative intracellular protein was administered intravenously to mice exhibiting maximal symptoms of EAE including paralysis, the same inflammatory symptoms seen to increase in knock-out animals were reduced with administration of the exogenous αβ-crystallin (16). Exogenous administration of HspB5 has also been therapeutically beneficial in animal models of stroke (17), and ischemia-reperfusion injury in the eye (18) and the heart (19), demonstrating that the effects are not limited to autoimmune models and can be effective in acute ischemic conditions, where there is pathological evidence of inflammation. Collectively, the observed therapeutic benefit in animal models argues that the sHsps, and HspB5 in particular, are general anti-inflammatory agents, capable of acting systemically. Moreover, their temperature-dependent chaperone function might also make the sHsps particularly effective at sites of inflammation, where temperature is known to be elevated as compared with non-inflamed tissue.

Data in this study provide support for the hypothesis that HspB5 exerts its anti-inflammatory effects by temperature-dependent binding of a spectrum of proinflammatory proteins in plasma, whose activities influence both the innate and the adaptive immune responses. Reducing the concentration of these proteins at the sites of inflammation would be expected to indirectly modulate a variety of immune functions including lymphocyte and monocyte activation and cytokine production. The model for explaining how sHsps may influence inflammation was tested further by demonstrating that HspB5 modulates IL-6 levels in plasma after lipopolysaccharide (LPS) injection in mice, although there is no evidence that HspB5 directly binds the cytokine.

**EXPERIMENTAL PROCEDURES**

**Induction of EAE in Mice by Immunization with MOG and Adjuvant and Treatment with HspB5—**EAE was induced by procedures previously described (20). Briefly, EAE was induced in female C57BL/6J mice (The Jackson Laboratory, Sacramento, CA) at 9 weeks of age by subcutaneous immunization in the flank with an emulsion containing 200 μg of pertussis toxin, CA) at 9 weeks of age by subcutaneous immunization in the flank with an emulsion containing 200 μg of pertussis toxin, 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 two symptoms, they were injected in the peritoneum with either 50 μg of HspB5 or PBS daily. All animal protocols were approved by Institutional Animal Care and Use Committee (IACUC). Normal murine plasma was taken from age-matched healthy C57BL/6J mice.

**Disease Induction by Adoptive Transfer of Th17 Lymphocytes—**Female donor C57/BL/6 mice were immunized as previously described with MOG35−55 in Freund’s adjuvant followed by pertussis toxoid. 10 days after immunization, the spleen and draining lymph nodes were isolated and restimulated with 20 μg/ml MOG35−55 in the presence of 10 ng of IL-23 for 3 days. Lymphocytes (30 × 10⁶ cells) were injected intraperitoneally into female recipient C57/BL/6 mice. Mice were examined daily for clinical signs of EAE and were scored on a five-point scale as described previously. When animals exhibited level two symptoms, they were injected in the peritoneum with either 50 μg of HspB5 or PBS daily (21).

**Isolation of Mouse CD4 T Cells—**Spleen and lymph nodes were harvested from 8–12-week-old BALB/C (The Jackson Laboratory) or MOG T cell receptor transgenic C57BL/6 (2D2 clone) mice and homogenized through a strainer. Red blood cells were lysed, and lymphocytes were isolated by density centrifugation using Lympholyte-M (Cedarlane Laboratories). CD4+ T cells were sorted via negative isolation by AutoMACS (Miltenyi Biotech).

**Stimulation and T Lymphocyte Proliferation Assay—**For polyclonal T cell receptor stimulation, 96-well flat bottom culture plates were first coated with secondary anti-hamster IgG antibody (Vector Laboratories) in 50 mM sodium bicarbonate buffer. Plates were washed and then coated with the indicated concentration of hamster anti-mouse CD3 antibody (145-2C11, eBioscience) in PBS. Plates were rinsed prior to the addition of 0.05 × 10⁶ CD4 T cells with the indicated amount of soluble anti-CD28 (37.51, eBiosciences) in the absence or presence of recombinant HspB5. For MOG stimulation, the non-lymphocyte AutoMACS fraction was mitomycin-C-treated and used as antigen presenting cells. 3 × 10⁶ antigen presenting cells were mixed with 1 × 10⁶ CD4 T cells, along with the indicated amount of MOG35–55 peptide, in the absence or presence of recombinant HspB5. Cell cultures were pulsed with 1 mCi of [methyl-³H]thymidine (Amersham Biosciences) for 6 h during the last 72 h of stimulation, harvested onto filters (Wallac), and read on a 1205 Betaplate liquid scintillation counter (Wallac).

**B Cell Activation—**A20 mouse B cell lymphomas (ATCC), cultured in RPMI-C, were stimulated with LPS (0.5 μg/ml) in the presence or absence of 50 μg/ml HspB5 for 24 h. Cells were stained with activation marker antibodies specific for CD80, CD83, and CD86 and analyzed by flow cytometry (Stanford FACs Facility).

**Cloning, Expression, and Purification of T7-Human HspB5—**Cloning, expression, and purification were described previously (22, 23). Briefly, the proteins were expressed in Escherichia coli and purified using ammonium sulfate precipitation, anion ion exchange, gel filtration, and affinity purification using commercially available anti-T7 resin (EMD Chemicals, Gibbstown, NJ).
sHsp Binding of Inflammatory Proteins in Plasma

The purity of the protein was assayed by SDS-PAGE, its identity was assayed by mass spectrometry, and its quaternary structure was confirmed by gel filtration and dynamic light scattering. The behaviors of the T7-tagged protein in gel filtration and dynamic light scattering were indistinguishable from the unmodified protein. In addition, the T7-tagged protein exhibited equivalent therapeutic effects in the EAE animal model and was shown to be therapeutically beneficial in the murine model of stroke (17).

**Blood Samples**—Under the Institutional Review Board protocol used in this study, the human blood samples were provided without further information other than that they were collected from patients at the Stanford Multiple Sclerosis, Amyloidosis, and Rheumatology Clinics from patients older than 18 years of age with each of the respective clinically defined indications. The control group was composed of healthy, unrelated adult subjects. Murine blood was taken from 9-week-old C57BL/6 mice (The Jackson Laboratory) exhibiting maximal symptoms of experimental allergic encephalomyelitis.

HspB5-Ligand Precipitation from Human and Murine Plasma, Reduction/Alkylation, and Trypsinization—5 μg of T7 HspB5 were added to 300-μl aliquots of fresh human or murine plasma and incubated at 23, 37, or 42 °C for 2 h, after which 50 μl of anti-T7-Sepharose beads (Clontech) were added, and the mixture was incubated an additional 2 h at the relevant temperature. The resin was separated from the plasma by centrifugation, and the plasma was saved for later analysis, whereas the resin was washed with PBS, pH 7.4. The HspB5 and its ligands were eluted with 100 μl of 100 mM glycine, pH 1.8. 50 ml of trichloroacetic acid (100% w/v) (Sigma) was added, the samples were cooled to 4 °C for 1 h and centrifuged, the supernatants were discarded, and the pellets were washed twice with cold acetone and dried. The protein pellets were reconstituted in 15 μl of 8 M urea, pH 7.8, and 20 μl of 0.2% Protease Max (Promega, Madison, WI). The samples were solubilized and reduced with 5 mM DTT at 55 °C for 30 min, after which they were alkylated using acrylamide. Trypsin (0.3–0.5 μg, Promega) was added, and the samples were digested overnight at 37 °C. The samples were acidified with formic acid and purified using STAGE tips (Thermo Scientific). The amount of sample injected was controlled to avoid detector saturation, which is critical for quantitative analyses.

**Liquid Chromatography and Mass Spectrometry**—Liquid chromatography was done using an Eksigent nano-2D LC (Dublin, CA) coupled to an in-house packed C18 analytical column. A Bruker Advance Source (Auburn, CA) was used for nano-electrospray mass ionization at a flow of 750 nl/min. A 1-h linear gradient from 2% mobile phase B (acetonitrile 0.1% formic acid) to 40% B was used. Data were acquired using an LTQ Orbitrap Volos mass spectrometer (Thermo Fisher), in which data acquisition was performed in a data-dependent fashion (MSMS on top of the eight most intense precursor ions). The RAW files were converted to mzXML format and searched on a Sorcerer (Sagan, Milpitas, CA) processing system using SyQuest against the appropriate database (ipi Human or ipi Mouse). The search results were then uploaded into a Scaffold (Proteome Software, Portland, OR) work station for visualization, data filtering, and statistical analysis. Finally, the data were exported to Microsoft Excel.

**Mass Spectrometry Data Analysis**—A global proteomic approach was used, identifying the tryptic peptides (thereby protein assignment) as well as quantitatively measuring each of the samples using a label-free spectral counting approach (24). The data were filtered to a false discovery rate at the peptide level of 5% and at the protein level of 0.1%. The higher fidelity for protein assignment was the result of the constraint in which a minimum of two peptides was required to assign an individual protein. Statistical analysis of protein expression profiles of different sample sets was done using a Fisher’s exact test, and all p values were appropriately reported (25). In addition to identifying each of the proteins precipitated, the software places a quantitative value (spectral count) on each peptide, and thereby protein is assigned. This step allowed the HspB5 to be used as an internal standard, which enabled the direct comparison of the relative amounts of each of the client proteins between different samples. After normalizing the samples based on HspB5 content, the putative ligands could be placed in a rank hierarchy and compared with their concentration in plasma and with their concentration relative to different precipitates. Reproducibility was remarkably good, both between triplicate injections of the same sample (technical triplicates) and between plasma of different patients (biological replicates). The protein composition was consistent with variation seen principally in the constituents composing the lowest percentage composition (low spectral counts), whereas the quantitative values varied by less than 2% with only a few outliers (shown in Fig. 2 and supplemental Fig. 1 as error bars). The highest reproducibility corresponded to the proteins whose relative concentration in the precipitate increased commensurate with a rise in the incubation temperature for the assay.

**Inhibition of Plasma Levels of IL-6 in Vivo by HspB5 after LPS Challenge**—LPS from purified lyophilized extract of E. coli endotoxin (011:B4, Sigma) was dissolved in sterile saline and sonicated for 5 min before use and injected at a concentration of 5 mg/kg intraperitoneally into C57BL/6J mice at 9 weeks of age. Mice were treated with an intraperitoneal injection of HspB5 at concentrations of 2.5, 5, and 10 mg/kg at time of LPS injection followed by a second injection 3 h later.

**Determination of Protein Concentrations in Plasma**—ELISA tests were used to determine the plasma concentration of HspB5, serum amyloid P, complement C4b, C1s, C1r, C5 (Cedarlane, Burlington, NC), serum amyloid A (Antigenix America, Huntington Station, NY), C-reactive protein (eBioscience, San Diego, CA), apolipoprotein E (Mabtech, Cincinnati, America, Huntington Station, NY), C-reactive protein (eBioscience, San Diego, CA), apolipoprotein E (Mabtech, Cincinnati, OH) and complement factor H (Kamiah, Seattle, WA) and mouse αB-crystallin (Cedarlane), serum amyloid P (Alpo, Salem, NH), serum amyloid A (Trident Diagnostics, Boonton Township, NJ), and IL-6 (BD Biosciences) as described by the manufacturer.

**RESULTS**

HspB5 Reduces Paralytic Symptoms in EAE, but Not When Disease Is Induced by Adoptive Transfer of Myelin-specific Th17 Lymphocytes—As shown previously (16), daily intraperitoneal injection of 50 μg of HspB5 rapidly and effectively reduces the
symptoms of the disease (Fig. 1A). However, cessation of the protein therapy resulted in return of the paralytic symptoms soon after discontinuation of the administration of the protein. This indicates that the protein acts as a biological inhibitor, with the therapeutic effects dependent on maintaining a serological level above a defined concentration, rather than inducing a long-acting immunological state of reduced inflammation or tolerance.

Further information on the mode of action was provided by the inability of the identical injection regime of HspB5 to modulate the symptoms of EAE when the disease was induced by direct transfer of myelin-specific Th17 lymphocytes (Fig. 1B).

The discrepancy of the effect of HspB5 in the two models indicates that the protein might not directly affect T lymphocytes, but rather limits the innate inflammation induced by the Toll-like receptor ligands used in the induction of EAE with complete Freund’s adjuvant. Complete Freund’s adjuvant is known to induce Toll-like receptor (TLR) and innate immunity (26).

**HspB5 Does Not Directly Affect T or B Lymphocyte Proliferation**—The hypothesis that the protein did not affect T lymphocytes was supported by the demonstration that incubation of varying amounts of HspB5 did not inhibit either antigen-nonspecific proliferation induced by anti-CD3 or anti-CD28 (Fig. 1C) or antigen-specific stimulation of MOG-specific T
cells (Fig. 1D). In addition, incubation of 50 μg/ml HspB5 had no effect on the expression of the activation markers CD80, CD83, and CD86 characteristic of B cell proliferation induced by LPS (Fig. 1E). The effect of the protein on monocytes cannot easily be assayed because of the trace amounts of LPS in the purified protein, which is known to activate the cells. The inability to affect cells composing the adaptive immune response focused our attention on the possible role of HspB5 on the innate immune response and as a chaperone, so we focused on the interaction of HspB5 with plasma proteins.

**Increased Plasma Levels of HspB5 in MS and EAE**—The recent experiments demonstrating that HspB5 can be excreted via exosomes (11) suggest that the protein might have a previously undefined extracellular role of binding plasma proteins, which could help explain the therapeutic effects of the exogenously administered protein. If true, the protein should be detectable in plasma, and its concentration would be expected to be higher in patients than in normal individuals. To test this idea, the level of HspB5 in plasma from patients with multiple sclerosis was measured using an enzyme-linked immunosassay and compared with plasma from healthy individuals (Fig. 1F). The amount of HspB5 was ~5-fold higher in MS patients (12.4 ± 3.7 ng/ml) as compared with healthy controls (2.7 ± 2.2 ng/ml), which was a statistically significant difference based on a Mann–Whitney U test. Similarly, HspB5 concentration was almost 3-fold higher in mice exhibiting maximal symptoms of EAE (16.3 ± 0.99 ng/ml) as compared with healthy littermates (6.0 ± 1.6 ng/ml).

Interestingly, equivalent levels of HspB5 were found in the plasma of mice in which EAE was induced with adoptive transfer of Th17 cells. These data support the concept that HspB5 could act systemically via its presence in the plasma. This would represent an activity distinct from its established intracellular role where it provides protection from stress. Finding HspB5 in MS plasma is concordant with recent measurements of HspB5 in the plasma from stroke patients (17). However, the endogenous levels found in mice with EAE were insufficient to reverse the symptoms of EAE. We believe that this might be similar to other potentially protective molecules such as corticosteroids. As in the case of the steroids, augmentation of endogenous plasma levels of HspB5 can provide therapeutic benefit.

**Definition of Specific HspB5 Ligands in Plasma**—The set of proteins precipitated by HspB5 differs from the composition of plasma. To test whether the mode of action of HspB5 is based on its ability to bind proteins in blood, T7 HspB5 (5 μg) was added to plasma from six patients with multiple sclerosis (300 μl). The concentration of the protein in the plasma was chosen to approximate the therapeutic doses of the protein used in animal models of multiple sclerosis, stroke, and ischemia/reperfusion injury (16–19). Separate plasma samples from the patients were incubated at 23, 37, and 42 °C based on the hypothesis that relevant interactions between the sHsp and possible client proteins should exhibit a temperature-dependent increase in binding, whereas the majority of nonspecific interactions would be temperature-independent.

To determine whether HspB5 exhibited any specificity in binding, the composition of the HspB5 precipitate when incubated at 42 °C from the first patient was compared with the total protein precipitated from the plasma by trichloroacetic acid (TCA). The TCA precipitate reflected the published composition of plasma (27, 28), with albumin and the different immunoglobulins composing greater than half of the precipitate. In contrast, HspB5 precipitated only ~0.1% of the total protein found in the TCA precipitate, with albumin and the immunoglobulins represented, but not the predominant molecules in the mixture. When the compositions of the two samples were normalized based on total measured units of protein, even greater differences in their composition were seen (Fig. 2, A and C, supplemental Fig. 1, and Table 1). Of the 350 proteins identified in the TCA precipitate, 125 were observed in all six MS samples. The Spearman’s correlation using the rank hierarchy of the concentration of the 125 proteins in each of the samples demonstrates that protein composition of the HspB5 precipitates is similar among the six MS samples (mean of 15 correlations, r² = 0.761), but most importantly, statistically different from the composition of the TCA precipitate (mean correlation, r² = 0.411), p value = 5.29 × 10⁻⁴. Several abundant cytoplasmic proteins such as hemoglobin, actin, talin, filamin, gelsolin, and myosin exhibited the greatest variations in both content and amount in repeated experiments, consistent with their variable leakage during the generation of the plasma and nonspecific interactions with HspB5. Reducing the sample size to the 100 most prominent proteins in the precipitates, by removing the set of cytoplasmic proteins, revealed that the small heat shock protein bound only a small fraction of the most prominent proteins in plasma such as albumin, IgG1, IgG2, IgG3, transferrin, Ig κ and λ light chains, haptoglobin, apolipoprotein A-I, apolipoprotein A-II, IgA, IgM, serpin A1, hemopexin, α-1B-glycoprotein, and prothrombin (partially shown in Fig. 2, A and B, with the full set of data shown in supplemental Fig. 1). Removal of the cytosolic proteins did not significantly affect statistical correlations, with the mean between the MS samples being r² = 0.764, whereas the correlation with the TCA precipitate exhibited r² = 0.382, p value = 5.16 × 10⁻⁴.

In contrast, when the relative amounts of the 100 most prominent proteins in the precipitate from the plasma of the first MS patient were compared with the TCA precipitate, 70 proteins were present at higher concentration in the HspB5 precipitate than in the TCA pellet (Fig. 2, A and C, Table 1, and supplemental Fig. 1). Correlation analysis of this set of proteins among the MS precipitates demonstrated an increased correlation (r² = 0.828) with a lower correlation with the composition in the TCA precipitates (r² = 0.401), arguing that HspB5 specifically binds this set of proteins in MS plasma.

Compared with the original 350 proteins identified in the TCA precipitate, the 125 proteins enhanced in the HspB5 pellet (Table 1) had a statistically significant enrichment in members of the acute phase response (43/125; 34% as compared with 49/350; 14% p value = 1.59 × 10⁻⁴), complement (27/125; 22% as compared with 32/350; 9% p value = 3.28 × 10⁻³), and coagulation pathways (14/125; 11% as compared with 16/350; 5% p value = 2.04 × 10⁻²). Taken together, members of the three pathways represented 65/350 proteins in the TCA precipitate (19%), which increased to 55/125 (44%, p value = 5.57 × 10⁻⁵). Percentage of the proteins that were members of the three
pathways increased to 54% of the most common 100 proteins and to 59% of the 70 proteins whose concentration was enhanced in the HspB5 precipitate.

**Relevant Ligands of HspB5 Exhibit Temperature-dependent Binding**—Increasing the temperature from 37 to 42 °C modifies the conformation of HspB5, resulting in exposure of additional hydrophobic sites and an increase in chaperone function (reviewed in Refs. 5 and 7). Consequently, specific ligand binding by HspB5 might be distinguished further from nonspecific interactions by analyzing the changes in the amount of protein bound at 23, 37, and 42 °C. Of the 100 most prominent proteins in the T7 HspB5 precipitate, 84 exhibited a pattern of binding that was temperature-sensitive (partially shown in Fig. 2, B and D, and completely listed in supplemental Fig. 1). The correlation within this set of proteins in the MS samples was close to that observed for the enhanced set ($r^2 = 0.815$), also with low statistical correlation with the TCA precipitate ($r^2 = 0.339$).

Of the 70 proteins whose concentration was enhanced in the HspB5 precipitate, 67 were temperature-sensitive (Table 1 and Fig. 2). The $\alpha$ and $\beta$ chains of fibrinogen and CD5, a protein known to associate with IgM in plasma, were the only exceptions. This set of 67 proteins, based on their enrichment in the HspB5 precipitate and the increase in their binding with temperature, encompass the most attractive ligands of the sHsp within the MS samples and 0.373 correlation with the TCA samples ($p$ value $= 2.46 \times 10^{-4}$). Collectively, the proteins precipitated among the six samples were highly similar and supported the initial observation that acute phase, complement, and coagulation proteins were highly represented in the HspB5 precipitates ($39/67, 58\%$).

By using two independent criteria, enrichment in the HspB5 precipitate and an increase in binding with temperature, to define specificity, a collection of ligands has been identified in the plasma of six multiple sclerosis patients. These two characteristics also distinguish specific from nonspecific binding of both highly expressed proteins and proteins found at trace levels.

**FIGURE 2.** Comparison of relative concentrations of proteins precipitated by either HspB5 or TCA from plasma from two multiple sclerosis patients and effect of incubation temperature on binding. A and C, the relative concentrations of the proteins precipitated by either HspB5 or TCA are compared with those whose relative concentration was enhanced (black bars) in the HspB5 precipitate as distinguished from those that were not (gray histograms). B and D, incubation with HspB5 at increasing temperatures results in the greater amount of some, but not all, proteins in the precipitate. The proteins that did not exhibit temperature dependence (*) correspond to highly expressed plasma proteins, such as albumin, serotransferrin, fibrinogen, and the immunoglobulins. Error bars correspond to S.D. between triplicate measurements of two different plasma samples (six separate measurements). *Insulin-like growth factor bind prot, insulin-like growth factor-binding protein.*
TABLE 1
Compilation of the 100 most prominent plasma proteins precipitated by HspB5 segregated by their enrichment relative to their plasma concentration defined by precipitation with trichloroacetic acid

The fold increase for each protein is shown whether it was enhanced (upper) or not (lower). The fold increase was calculated by dividing the concentration of the protein in the HSPB5 precipitate at 42 °C by its normalized concentration in the TCA precipitate. The 84 proteins that exhibited temperature dependent binding are listed in normal font, while 16 that did not are shown in bold and italics. Members of the complement (†), coagulation (‡), and acute phase (*) pathways are noted. AMBP, α-1-microglobulin/bikunin precursor; IGHM, immunoglobulin heavy constant.

| -Fold increase enhanced | -Fold increase not enhanced |
|-------------------------|----------------------------|
| α-Crystallin B chain    | Haptoglobin-related protein |
| 34.43                   | 0.91                        |
| Coagulation factor V     | Fibrinogen γ chain*         |
| 147.79                  | 0.91                        |
| Complement C1qA*         | α-2-HS-glycoprotein*        |
| 90.09                   | 0.91                        |
| Complement C5*          | IgG2                        |
| 64.64                   | 0.89                        |
| Thrombospondin-1        | IgHM                        |
| 57.25                   | 0.89                        |
| Ficolin-3*              | α-2-HS-glycoprotein*        |
| 53.28                   | 0.87                        |
| Complement C2*          | β-2-Glycoprotein 1*         |
| 50.60                   | 0.87                        |
| Carboxypeptidase B2*    | Complement factor H*        |
| 47.93                   | 0.84                        |
| Phosphatidylinositol-glycan-specific phospholipase D | Platelet factor 4 |
| 43.93                   | 2.18                        |
| Vitamin K-dependent protein S | Complement C3* |
| 41.28                   | 32.93                       |
| Mannose-oligosaccharide 1,2-α-mannosidase IA | C-reactive protein* |
| 41.28                   | 23.59                       |
| Collagen acidic protein 1 | Biotinidase               |
| 39.94                   | 21.31                       |
| Mannan-binding lectin serine protease 1 | Platelet factor 4 |
| 35.95                   | 18.70                       |
| SerpinA10 protein Z-dependent protease inhibitor | Complement C8 α chain |
| 35.40                   | 18.07                       |
| Insulin-like growth factor-binding protein | Complement C5 |
| 33.69                   | 15.09                       |
| Heat shock protein β-1 | Complement C1qC* |
| 33.28                   | 13.71                       |
| Mannan-binding lectin serine protease 2* | C-reactive protein* |
| 26.63                   | 9.36                        |
| Precocyte oxidase 1     | α-1-antichymotrypsin*       |
| 25.30                   | 7.72                        |
| Coagulation factor X     | IgG1                        |
| 23.99                   | 6.55                        |
| Biotinidase             | SerpinA3                  |
| 21.31                   | 6.52                        |
| Platelet factor 4        | α-1-antitrypsin*            |
| 18.70                   | 6.03                        |
| Carboxypeptidase N subunit 2 | IgG3 |
| 18.18                   | 5.95                        |
| Complement C7*          | Fibrinogen α chain*         |
| 16.60                   | 5.26                        |
| Complement C8 α chain † | C-reactive protein*         |
| 15.29                   | 4.95                        |

Fold increase indicates -fold increase in HspB5 precipitate.
amounts. That the acute phase, complement, and coagulation proteins were highly represented in this set supports the hypothesis that the collection of HspB5 ligands is not formed by random associations, but rather specific interactions that could predominate in the elevated temperatures of sites of inflammation.

HspB5 Capable of Binding Similar Set of Proteins from Normal Plasma—When HspB5 was used to precipitate proteins from normal plasma, many of the same proteins were precipitated (73/125 common proteins), but at significantly lower levels. Consequently, when the rank hierarchies of the proteins precipitated from normal plasma were compared between the six MS samples, a weak correlation was observed, \( r^2 = 0.411 \) \( p \text{ value} = 1.40 \times 10^{-3} \). As was seen earlier in the plasma of patients, albumin and the nine Ig family members were the principal members of the set of proteins that did not exhibit an increase in binding with temperature. These data reveal that HspB5 binds a specific set of proteins in the plasma of patients and can bind similar proteins in normal plasma. However, because the compositions of normal plasma and the plasma of patients do not contain equivalent amounts of inflammatory proteins, the resultant precipitates are similar, but not identical. In addition, there is significantly less HspB5 in normal plasma (Fig. 1F), and the frequency of inflammatory loci with their inherent increased temperatures secondary to the inflammatory response would also be relatively rare in healthy individuals. Consequently, these two factors would be expected to limit the role of HspB5 in healthy individuals.

Anti-T7 Resin Precipitates the Most Concentrated Proteins in Plasma—The anti-T7 resin precipitated between 45 and 55 proteins of the 125 proteins shared by the six MS precipitates and the proteins precipitated by TCA, with only between 5 and 15 exhibiting temperature-dependent binding. In addition to albumin, the lgs, and serotransferrin, the other proteins, apolipoprotein A-I, transferrin, coagulation factor II, \( \alpha-1 \)-glycoprotein, complement factor H, transthyretin, \( \alpha-2 \)-HS-glycoprotein, vitronectin, and fibrinogen \( \alpha, \beta, \) and \( \gamma \) chains are all highly expressed in plasma. In contrast to precipitation with T7 HspB5, the resin appears to bind a spectrum of proteins representative of the most concentrated proteins in plasma, characteristic of nonspecific adhesion to the resin. The six MS samples and the resin samples exhibited a poor correlation, mean \( r^2 = 0.220 \), emphasizing the difference between the population precipitated by HspB5 and this control.

Depletion of Proteins from Plasma—As unexpected and interesting as the apparent preferential binding of complement
and acute phase proteins by small heat shock proteins is, these results support, but certainly do not prove, that this interaction explains the therapeutic mode of action. Only if the plasma concentrations of the relevant proinflammatory proteins are significantly reduced by the shsp could the interaction be viewed as a possible therapeutic mechanism. The concentration of HspB5 (5 μg in 300 μl, 0.83 μM) used in the precipitation reactions was significantly less than the concentration of many of the 65 common proteins found in the precipitates from MS, RA, SA, and EAE plasma. Consequently, HspB5 might not affect the serological concentration of many of the proteins listed in Table 1.

The serological levels of the acute phase proteins, C-reactive protein, serum amyloid A, serum amyloid P, and apolipoprotein E were measured by ELISA in each of the plasma samples from MS and RA patients after the precipitation with HspB5. There was a correlation between an increase in the amount of protein precipitated as a function of the incubation temperature with a corresponding reduction in the amount remaining in the supernatant for each of these proteins (Fig. 3, A–P). When a set of other proteins expressed at higher relative concentration in MS and RA plasma such as apolipoprotein A-I, transthyretin, complement 1r, complement 1s, and α-2-macroglobulin, was analyzed, a different pattern was observed. In these cases, there was a temperature-dependent increase in binding, but not a measurable reduction that could be titrated in their concentrations in the supernatant of the plasma.

Interestingly, the plasma concentrations of C-reactive protein, serum amyloid A, and apolipoprotein E were all in low μg/ml or high ng/ml levels, concentrations similar to that of HspB5, and consequently, the amounts in the plasma could be modulated. In contrast, the concentrations of complement 1r, complement 1s, complement 5, and factor H were all above 20 μg/ml, with some being in the hundreds of micrograms, concentrations too high for HspB5 to reduce. An exception was SAP, whose plasma concentration was between 49 and 86 μg/ml, which should be too large for HspB5 to modulate. Nevertheless, there was a temperature-dependent reduction in the plasma levels after incubation with HspB5 (Fig. 3, M and N). This difference could be rationalized by the known propensity of SAP to aggregate, which could result in a small amount of shsp precipitating a larger molar amount of SAP.

A very similar pattern was observed when plasma depleted from mice with EAE was analyzed (Fig. 4). The concentrations of C-reactive protein, serum amyloid A, serum amyloid P, and apolipoprotein E were all reduced in plasma depleted by HspB5 in a temperature-dependent fashion. An example of the role of the concentration of the ligand as a determinant of whether it could be modulated was evident in the case of complement factor H. Precipitation of factor H by low micromolar amounts of HspB5 in MS and RA plasma (~600 and 500 μM, respectively) was not seen to affect the plasma levels. However, in EAE plasma, there was a titratable reduction of ~15% because the initial levels were 10 times lower in murine plasma.

As was the case in human plasma, a set of proteins expressed at relatively high concentration, such as apolipoprotein A-I, transthyretin, complement 1r, complement 1s, and α-2-macroglobulin, was analyzed. There was a temperature-dependent increase in binding, but not a measurable, titratable reduction in their concentration in the HspB5 depleted plasma (data not shown).

The data in Figs. 3 and 4 establish that with the exceptions of aggregated proteins, HspB5 at the therapeutic doses used in EAE, stroke, and animal models of cardiac and retinal ischemia can modulate only plasma proteins whose concentrations are low micromolar to high nanomolar. This reduces the possible therapeutic targets from ~80 (Table 1) to less than 50 proteins, but did not significantly reduce the percentage of acute phase, coagulation, and complement proteins that constitute ~60% of the set.

HspB5 Can Reduce Plasma IL-6 Levels after LPS Injection—To establish that the anti-inflammatory effects of small heat shock proteins arise from modulating the concentration of inflammatory proteins in plasma is a difficult task because of the manifold ligands and their diverse effects. To provide further support for the mode of action, the ability of HspB5 to reduce plasma levels of IL-6 after LPS injection in C57BL/6 mice was analyzed. IL-6 is a pleiotropic cytokine and mediator of inflammation whose synthesis and secretion are induced rapidly after LPS engagement with TLR4 (29). The cytokine is particularly relevant to mode of action of HspB5 because recent studies have demonstrated that IL-6 has a important role in regulating the balance between IL-17-producing Th17 cells and regulatory T cells (30), and inhibition of the production of the cytokine has been shown to be therapeutic in EAE (31). The peak concentration of the cytokine only reaches between 50 and 100 ng/ml in plasma, which appears to be too low to be bound directly by HspB5 because of the large molar excess of other plasma proteins. Consequently, reduction of IL-6 plasma levels by the administration of HspB5 would provide support for the indirect action of the chaperone, which would reduce

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**TABLE 3**

Correlation values ($r^2$) comparing the populations of proteins precipitated from two RA, SA, and EAE plasma with the MS populations. 

| Set of proteins observed in all MS and TCA samples, cytosolic samples | MS1 | RA1 | RA2 | SA1 | SA2 | EAE1 | EAE2 |
|---------------------------------------------------------------------|-----|-----|-----|-----|-----|------|------|
| **MS1**                                                             | 1.00| 1.00|     |     |     |      |      |
| **RA1**                                                             | 0.78| 1.00|     |     |     |      |      |
| **RA2**                                                             | 0.76| 0.75| 1.00|     |     |      |      |
| **SA1**                                                             | 0.45| 0.52| 0.61|1.00 |     |      |      |
| **SA2**                                                             | 0.51| 0.52| 0.58| 0.88|1.00 |      |      |
| **EAE1**                                                            | 0.53| 0.63| 0.58| 0.46| 0.52|1.00  |      |
| **EAE2**                                                            | 0.40| 0.53| 0.48| 0.31| 0.35| 0.82 | 1.00 |

| Set of proteins observed in all MS and TCA samples, cytosolic samples | MS1 | RA1 | RA2 | SA1 | SA2 | EAE1 | EAE2 |
|---------------------------------------------------------------------|-----|-----|-----|-----|-----|------|------|
| **MS1**                                                             | 1.00| 1.00|     |     |     |      |      |
| **RA1**                                                             | 0.78| 1.00|     |     |     |      |      |
| **RA2**                                                             | 0.76| 0.73| 1.00|     |     |      |      |
| **SA1**                                                             | 0.43| 0.46| 0.55| 1.00|     |      |      |
| **SA2**                                                             | 0.51| 0.50| 0.56| 0.85| 1.00|      |      |
| **EAE1**                                                            | 0.52| 0.58| 0.47| 0.36| 0.42| 1.00 |      |
| **EAE2**                                                            | 0.31| 0.40| 0.29| 0.21| 0.24| 0.82 | 1.00 |
other proteins that modulate IL-6 production, such as serum amyloid A, which in turn would reduce levels of the cytokine. When C57BL/6 mice were injected with increasing amounts of *E. coli* LPS, from 50 to 200 μg, and treated at the time of injection and 3 h later with 100 μg of HspB5, the IL-6 levels in plasma at 6 h were reduced as compared with untreated animals. The amount of reduction was inversely related to the amount of LPS injected (Fig. 5A). IL-6 was reduced by 80% at the lowest concentration of LPS, rising to 20% reduction when 200 μg of LPS were administered. In the reciprocal experiment where the amount of LPS injected was kept constant at 100 μg and the dose of two injections of HspB5 was increased from 50, 100, and 200, the amount of IL-6 was reduced by 10, 25, 45, and 60% (Fig. 5B). The titratable modulation of IL-6 in this well established model provides support for the anti-inflammatory activity of the molecule and the hypothesis that the mode of action of sHsps is the binding and reduction in concentration of plasma proteins, which in turn have direct and indirect effects on inflammation.

**DISCUSSION**

Exogenous administration of HspB5 is therapeutic in animal models of multiple sclerosis, stroke, and cardiac and retinal ischemia. *In vivo* administration of HspB5 reduced cellular immune responses and production of concomitant inflammatory cytokines. The beneficial effects occur relatively quickly, a day or two after injection, with cessation of treatment resulting in the rapid return of symptoms. The pattern is characteristic of a biological inhibitor, whose activity requires maintenance of a threshold concentration of the drug, and not a molecule that induces an immunosuppressive cascade, such as the generation of regulatory T cells.
The inability to modulate T or B cell proliferation in vitro combined with the lack of observable therapeutic effects in EAE when the disease was induced by adoptive transfer of Th17 lymphocytes was consistent with the protein inhibiting inflammation, and not directly modulating the adaptive immune response. Of the many possible roles the protein could function, we were guided by our recent study demonstrating that the therapeutic activity of sHsps and some of their peptides are correlated with their chaperone function. As a chaperone, the protein would be expected to bind partially unfolded species in plasma more likely than cell surface receptors, which logically led to our attempts to define the serological ligands of human HspB5 in human and murine plasma with the goal of mapping a specificity that could explain the therapeutic effects. No effort was taken to simplify the complexity of the plasma samples by removing the most prominent proteins because if the small heat shock protein is effective in vivo, then its effects should be apparent in the presence of all of the components of plasma.

Plasma is a remarkably complex mixture of proteins whose concentration varies over 12 logarithms in concentration (27, 28). To identify the relevant ligands responsible for the therapeutic effects is challenging, particularly for a putative nonspecific molecule such as a small heat shock protein, which binds unnaturally exposed hydrophobic regions of a protein, and not specific sequences (5). However, previous studies defining the association of HspB5 with desmin, desmin-related myopathy, titan, and several molecules involved in apoptosis argue that this small heat shock protein does exhibit some degree of specificity and that it can bind specific ligands (8–10, 32).

Distinguishing more relevant interactions from those arising from nonspecific adhesion was done by comparing the relative amounts of the plasma proteins in the HspB5 precipitates from that seen in a TCA precipitate of plasma. This analysis identified of the order of 70 proteins that were enriched in the HspB5 precipitate, with ~30 proteins being enhanced 10 times over their relative amounts in the corresponding plasma from two patients with multiple sclerosis. Interestingly, a highly statistically significant percentage of these proteins consisted of acute phase proteins or components of the complement or coagulation cascades (p value 3.68 × 10^-6). Confirmation that this set of proteins was associated with binding the sHsp came from analyzing the correlation of the relative amount of each protein in the HspB5 precipitate as a function of incubation temperature. As the temperature increased from 23–37 to 42 °C, the relative amount of protein in the precipitate increased by 76 and 200%, emphasizing the ability of the protein to increase its binding capacity as a function of temperature (7). We believe that this feature of the small heat shock protein is a critical aspect of its therapeutic function, allowing the protein to be more effective at sites of inflammation whose temperature is higher than the rest of the organism.

Identifying ligands whose binding exhibited temperature dependence distinguished ~80–100 proteins in the different samples, whose number did not vary significantly regardless of whether 100 or 300 proteins were in the precipitate. In most analyses, the highly abundant proteins, albumin and the immunoglobulins, did not exhibit temperature-dependent binding.

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most likely because of a relatively high nonspecific component. This was confirmed by their predominance in the precipitate obtained using the anti-T7 resin alone. Interestingly, 67 of the 70 proteins that were enriched in the HspB5 precipitate also exhibited temperature-dependent binding, arguing that they specifically were bound by the sHsp. A statistically significant percentage of this population also consisted of members of acute phase, complement, or coagulation pathways, with multiple sclerosis, but also rheumatoid arthritis and amyloidosis. Although there was variation in the relative amounts of proteins in the precipitates, there was a high percentage of similar proteins found for each indication, supporting the hypothesis that the mode of action of HspB5 is to bind a common set of proinflammatory proteins in plasma and not to bind a unique set of ligands for each indication. A similar pattern of ligands was also precipitated from plasma of mice with EAE, which was an important confirmation of the breadth of the specificity and supports the hypothesis that the binding of the ligands could be linked to the therapeutic effects of the protein.

Binding of the ligands is necessary, but not sufficient, to establish a mode of action. ELISA tests measuring serological levels of C-reactive protein, serum amyloid A, SAP, and APOE were used to establish that the concentration of these ligands in plasma were reduced by HspB5 in a temperature-dependent fashion. Similar analyses emphasized that the concentrations of several proteins, such as complement components C3, C5, C1r, and C1s, were too great in the plasma samples to be significantly reduced. The results establish that the addition of low microgram amounts of HspB5 can modulate plasma proteins found at low micromolar to high nanomolar levels, but not proteins whose concentration is above 10 μM. However, in vivo, this stoichiometry might not be limiting because HspB5 is known to be found at high local concentrations at inflammatory sites due to both release from apoptotic cells and exocytosis from cells in milieu, consistent with high concentrations of HspB5 found in plaques in multiple sclerosis patients (17). Inflammatory proteins should also be found at high concentrations at these sites. Taken together, these two events, combined with the elevated temperature at the inflammatory site, would lead to optimal conditions where the sHsp would bind a high percentage of partially unfolded or aggregated proteins, many of which were acute phase, complement, and coagulation proteins, modulating the inflammation within the plaques. A proteomic study on laser-captured microdissected lesions from MS brain showed a large accumulation of molecules from the coagulation cascade at the site of inflammation (17). The role of coagulation proteins in neuroinflammation is supported by the therapeutic effects of thrombin inhibitor hirudin and activated protein C.

Because the sHsps only bind, and do not refold their ligands, we envision the complexes being transported away from the site of inflammation with consequent dissociation. In this model, the sHsp functionally reduce the concentration of the partially unfolded, or aggregated, inflammatory proteins at the sites of inflammation, limiting a variety of deleterious signaling pathways in a wide spectrum of cells, particularly neutrophils, macrophages, and dendritic and endothelial cells. The systemic amounts of HspB5 found in plasma of MS (Fig. 1F) and stroke patients (17) are consistent with the model that HspB5 is secreted to regulate inflammation, and the exogenous administration of the protein appears to supplement the naturally occurring pathway. The ability of HspB5 to inhibit IL-6 in mice injected with LPS demonstrated that the protein could be effective in an acute indication, and most importantly, that it modulated a cytokine that was never present in any of the precipitations. In addition, because inhibition of IL-6 production has been shown to modulate the induction of myelin antigen-spe-
specific Th17 cells with the reduction of symptoms of EAE (31, 33), these results by themselves could rationalize the therapeutic effects of HspB5 in this animal model.

The observations that the concentration of HspB5 increases in MS plasma and mice with EAE as compared with plasma from normal individuals and that the protein is highly concentrated in MS plaques (14, 15) support the hypothesis that HspB5 is a naturally occurring modulator of inflammation, both when expressed in intracellular compartments and when administered exogenously. The capacity of the protein to bind a spectrum of ligands starkly distinguishes it from a monoclonal antibody and thus represents a unique therapeutic reagent exploiting the properties of these molecular chaperones.

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