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_J Immunol_ 2010; 184:6309-6319; Prepublished online 3 May 2010;
doi: 10.4049/jimmunol.0903891
http://www.jimmunol.org/content/184/11/6309
Superior Antitumor Response Induced by Large Stress Protein Chaperoned Protein Antigen Compared with Peptide Antigen

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Our previous studies have demonstrated that the natural chaperone complexes of full-length tumor protein Ags (e.g., gp100) and large stress proteins (e.g., hsp110 and grp170) with exceptional Ag-holding capabilities augment potent tumor protective immunity. In this study, we assess the peptide-interacting property of these large chaperones and, for the first time, compare the immunogenicity of the recombinant chaperone vaccines targeting two forms of Ags (protein versus peptide). Both hsp110 and grp170 readily formed complexes with antigenic peptides under physiologic conditions, and the peptide association could be further stimulated by heat shock. The large chaperones displayed similar but distinct peptide-binding features compared with hsp70 and grp94/gp96. Immunization with hsp110- or grp170-tyrosinase-related protein 2 (TRP2)175–192 peptide complexes effectively primed CD8⁺ T cells reactive with TRP2-derived, MHC class I-restricted epitope. However, the tumor protective effect elicited by the TRP2175–192 peptide vaccine was much weaker than that achieved by full-length TRP2 protein Ag chaperoned by grp170. Furthermore, immunization with combined chaperone vaccines directed against two melanoma protein Ags (i.e., gp100 and TRP2) significantly improved overall antitumor efficacy when compared with either of the single Ag vaccine. Lastly, treatment of tumor-bearing mice with these dual Ag-targeted chaperone complexes resulted in an immune activation involving epitope spreading, which was associated with a strong tumor growth inhibition of the established tumors. Our results suggest that high m.w. chaperones are superior to conventional chaperones as a vaccine platform to deliver large protein Ags, and provide a rationale for translating this recombinant chaperoning-based vaccine to future clinical investigation. The Journal of Immunology, 2010, 184: 6309–6319.

It is well known that stress/heat shock proteins (HSPs) act as molecular chaperones that are actively involved in almost every aspect of protein homeostasis (e.g., protein synthesis, folding/unfolding, oligomeric assembly, transportation, and degradation) (1). Stress proteins can be grouped into different families of protein based on their molecular size, which include hsp25/27, hsp70/grp78, hsp90/grp94, and hsp110/grp170 (2, 3). These molecules are constitutively expressed in mammalian cells, but can be strongly induced under stress conditions. As the most abundant and ubiquitous intracellular proteins, stress proteins play essential roles in the survival of cells because of their diverse housekeeping functions (4).

The immunologic feature of stress protein that has been the greatest focus of attention thus far is the ability to effectively direct associated Ags into the endogenous Ag presentation pathway of professional APCs (5). It has been well documented that stress protein–peptide complexes purified from tumors (e.g., hsp70, hsp90, grp94/gp96) are able to initiate an efficient tumor-specific CTL response and protective immunity (6, 7). Recent studies have implied that specific stress protein receptors on APCs are critical for the cross-presentation of stress protein-associated Ags (8–11).

High m.w. stress proteins, including hsp110 and grp170, belong to the hsp70 superfamily and represent large and highly diverged relatives of the hsp70s (3, 12). Chaperoning studies have shown that these large stress proteins are highly efficient peptide-chain binders compared with other classical chaperones (13, 14). Our earlier studies demonstrated that hsp110 and grp170 purified from tumors suppress the same tumor growth in mice and induce tumor-specific CTLs (15, 16). To overcome the technical difficulties associated with the conventional HSP vaccine approach (e.g., tumor tissue requirement, time-consuming preparation), we took advantage of the strong protein-holding capability of these large chaperones and developed a recombinant chaperoning vaccine approach by complexing a full-length protein Ag to hsp110 or grp170. The in vitro-generated complexes of the large stress protein and tumor protein Ags resemble the natural chaperone–client/partner protein complexes that are formed while the molecular chaperones execute their intracellular functions. We have demonstrated that the hsp110- and grp170–protein Ag complexes exhibit potent anti-tumor activities by stimulating Ag-specific immune responses in both prophylactic and therapeutic settings (17–19). Thus, these large chaperone molecules represent an

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Received for publication December 3, 2009. Accepted for publication March 27, 2010.

This work was supported by National Cancer Institute Grants CA129111, CA099362 and CA094045, American Cancer Society Grant RSG-08-187-01-LIB, the Harrison Endowed Scholarship, and the National Cancer Institute Cancer Center Support Grant to Massey Cancer Center.

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Abbreviations used in this paper: HSP, heat shock protein; i.d., intradermal(l)y; MHC I, MHC class I; TDLN, tumor-draining lymph node; TIL, tumor-infiltrating lymphocytes.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0903891
excellent choice for the development of synthetic and nontoxic vaccines for cancer immunotherapy.

Although the large stress proteins display highly efficient protein substrate/Ag chaperoning capability, the interaction of these molecules and peptide Ags have not been investigated. In this study, we have characterized the ability of large chaperones to interact with antigenic peptides in vitro under various conditions. Competition studies revealed that peptide binding to the large chaperones was specific. The reconstituted grp170-peptide complexes remain highly stable under high salt conditions, but are sensitive to low pH and high concentrations of reducing or oxidizing agents. We have compared the vaccine potency of large chaperones complexed with peptide Ags versus full length protein Ags. Interestingly, although the reconstituted peptide Ag chaperone complex was able to effectively elicit MHC class I (MHC I)-restricted, Ag-specific CD8+ T cell activation, the resultant anti-tumor response was significantly weaker than that generated by the full length protein Ag chaperone complex. Moreover, we have demonstrated that these large stress proteins are superior to conventional chaperones in holding tumor protein Ags. The use of full-length protein Ags in chaperone complex with these large stress proteins can be exploited as a platform for formulating recombinant chaperone vaccines that target one or more protein Ags.

Materials and Methods

Mice and cell lines

C57BL/6 and BALB/c mice purchased from the National Institutes of Health (Bethesda, MD) animal facilities were housed under pathogen-free conditions. Melanoma cell line B16-gp100 was maintained in DMEM, supplemented with 10% heat-inactivated FBS (Life Technologies, Grand Island, NY), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. All experimental procedures were conducted according to the protocols approved by the Institutional Animal Care and Use Committee.

Peptides and proteins

Recombinant proteins including hsp110, grp170, hsp70, gp100, and TRP2 were expressed in a BacPAK baculovirus expression system (BD Biosciences Clontech, Palo Alto, CA) and prepared using a nickel nitriloacetic acid (Ni-NTA)-agarose (Qiagen, Valencia, CA) column as previously described (14, 17). Tumor-derived grp170 was purified from culture media of B16 tumor cells that were engineered to secrete grp170 (20). Endotoxin levels in recombinant proteins (<3 EU/mg protein) were measured using a Limulus Amebocyte lysate kit (BioWhittaker, Walkersville, MD). The following peptides (underlined sequences represent the precise MHC I-binding epitope) were synthesized and purified by HPLC to >90% homogeneity: AH1423–431 (9 mer, SVYDFFVWL), and TRP2 175–192 (QIANC). Although both chaperones displayed the greatest peptide binding at 50˚C, a strong peptide interaction was readily detected under physiological condition (i.e., 37˚C). Similar

peratures in a thermostated cuvette. In some cases, excess protein substrate was added to the reaction solution. Light scattering by protein aggregation was determined by measuring the increase of OD at 320 nm with a spectrophotometer.

Immunization, ELISPOT and CTL assays

For preparation of protein Ag chaperone complexes, recombinant gp100 or TRP2 protein and stress proteins (1:1 molar ratio) were under heat shock conditions for 30 min, followed by incubation at 37˚C for 30 min (17). For preparation of peptide Ag chaperone complexes, peptides and stress proteins (50:1 molar ratio) were incubated at 50˚C for 30 min, followed by incubation at 37˚C for 30 min. Mice were immunized intradermally twice at 2-wk intervals with 30 µg of large stress proteins complexed with protein or peptide Ags. Two weeks after immunizations, splenocytes (5 × 10^6 per well) were stimulated with protein Ag (20 µg/ml), peptide Ag (1 µg/ml), or irradiated tumor cells (2.5 × 10^5 per well; splenocyte/tumor cell ratio = 20:1) at 37˚C for 24 h. IFN-γ production was assessed by an ELI-SPOT assay (17). For in vitro CTL assays, cells from spleen- or tumor-draining lymph nodes were stimulated with TRP2 or gp100 or mitomycin C-treated tumor cells and then cocultured with ^[3]Cr-labeled B16 cells at varying E/T ratios as described previously (17).

Tumor studies

For tumor challenge study, mice were inoculated intradermally with 1 × 10^5 B16-gp100 cells on the contralateral site after immunizations. Tumor growth was monitored every 2 d by measuring perpendicular tumor diameters using an electronic digital caliper. The tumor volume is calculated using the formula: volume = (shortest diameter^2 × longest diameter)/2. For tumor treatment, mice were established with tumors by injection of 2 × 10^5 B16 cells on day 0, followed by four treatments with protein Ag chaperone complexes or tumor-derived grp170 on days 3, 5, 7, and 9.

Isolation of tumor-infiltrating immune cell and intracellular IFN-γ staining

Freshly excised tumor tissues were gently minced into small pieces using a razor blade, and incubated in the digestion solution (400 U/ml collagenase D, 25 µg/ml hyaluronidase, 10 µg/ml DNase I) for 30 min at 37˚C. Single-cell suspensions were prepared using a mesh filter in combination with Lymphocyte-M centrifugation procedure (Cedarlane Laboratories, Burlington, NC). Cells were stimulated with CTL peptide epitopes from gp100/18 peptide overnight in the presence of IL-2. Cells were then treated with BD GolgiPlug protein transport inhibitor containing brefeldin A and Cytofix/CytoPerm solution (BD Biosciences, San Jose, CA) before intracellular staining with Abs for CD8 and IFN-γ was performed and analyzed on BD FACScalibur.

Statistical analysis

Comparisons between two groups were performed using Student t test. Comparisons between multiple groups were performed using ANOVA test. A value of p < 0.05 is statistically significant.

Results

Large stress proteins are capable of forming complexes with antigenic peptides in vitro

We first investigated the ability of grp170 and hsp110 to bind peptides by incubating purified recombinant proteins with a molar excess of labeled peptides (protein/peptide molar ratio = 1:50). The presence of biotinylated AH1 peptide associated with grp170 or hsp110 proteins was traced using gel electrophoresis and immunoblotting. All experimental procedures were conducted according to the protocols approved by the Institutional Animal Care and Use Committee.

Peptide chaperone complex formation and characterization

Grp170 or hsp110 (2 µg) was incubated with biotinylated peptides at a 1:50 molar ratio in PBS under different conditions as indicated for 30 min, followed by incubation at room temperature for an additional 60 min. The complexes were purified using a Centricon-10 filter to remove unbound peptides and resolved on nonreducing 10% SDS-PAGE, followed by immuno blotting analysis using streptavidin-HRP. The complexes were visualized using chemiluminescence on radiographic film. For ATP/ADP binding study, the formed complexes were applied to an ADP-agarose column (Sigma-Aldrich, St. Louis, MO), washed, and eluted with buffer (20 mM Tris acetate, 20 mM NaCl, 3 mM MgCl2, pH 7.5) containing either 5 mM ADP or ATP.

Protein thermal aggregation assay

One hundred fifty nanomolars protein Ag alone or in the presence of a 1:1 molar ratio of hsp70, hsp110, or grp170, were equilibrated to room temperature in 25 mM HEPES, pH 7.4, 5 mM magnesium acetate, 50 mM KCl, 5 mM β-mercaptoethanol followed by incubation at the indicated tem
observations have been made when large stress proteins were incubated with several other antigenic peptides, including HPV E7,49-57 and melanoma Ag gp10025-32 (data not shown).

Next, we assessed the effect of peptide concentrations on the complex formation at 50°C, a heat shock condition that stimulated the highest peptide binding. Grp170 (Fig. 1B, top) or hsp110 (Fig. 1B, bottom) was incubated with different concentrations of biotinylated AH1 peptides. Increased peptide concentrations resulted in a dose-dependent increase in the complex formation. It was estimated that the binding reached the highest levels when grp170 or hsp110 was incubated with AH1 peptide at a molar ratio of ∼25:1.

We also examined the grp170–AH1 peptide complex formation in the presence of unlabeled AH1 peptides. Addition of excess, unlabeled AH1 peptide effectively competed off the labeled AH1 peptides bound to grp170 in a dose-dependent manner (Fig. 1C, top). Furthermore, the presence of unrelated TRP2175-192 peptides, which contains an immunodominant CTL epitope (SVYDFFVWL) derived from melanoma Ag tyrosinase-related protein 2, also markedly inhibited AH1 peptide binding (Fig. 1C, bottom). In addition, grp170 was seen to bind to AH1 (18 mer) peptides and AH1 (9 mer) peptides in a comparable manner (Fig. 1D, top). The addition of unlabeled short AH1 (9 mer) peptides interfered with the complex formation between grp170 and the long AH1 (18 mer) peptides (Fig. 1D, bottom), suggesting that the size of peptides does not appear to significantly affect the peptide binding affinity of grp170. These studies suggest that grp170 is able to specifically bind a diverse array of peptides and displays no recognition preference for the peptide sequences under the conditions used here.

We next examined the competition effect of proteins on the grp170 interactions with AH1 peptides. As expected, the non-chaperone molecule BSA showed only marginal peptide binding, whereas chaperones grp170 and grp78/BiP bound labeled AH1 peptides efficiently. The presence of excess BSA had no effect on the interaction between grp170 and AH1 peptides. Intriguingly, the addition of grp78/BiP to the solution of grp170 and AH1 peptides enhanced peptide bound to not only grp170, but also grp78/BiP (Fig. 1E).

Large stress protein grp170 is more efficient in binding peptides than other chaperones

We next characterized the non-covalent interaction between grp170 and antigenic peptides in vitro under redox conditions. Grp170–peptide complex formation remained intact under reducing conditions (i.e., 1 mM DTT). However, a 5 mM concentration of DTT started to diminish the peptide complexing (Fig. 2A, top). In addition, the grp170 binding of peptides became weak in the presence of increased concentrations of hydrogen peroxide (i.e., H2O2); 5 mM H2O2 completely dissociated the grp170–AH1 complex (Fig. 2A, middle). Surprisingly, high salt concentrations up to 2 M had a negligible effect on the grp170 interaction with AH1 peptides (Fig. 2A, bottom). The peptide-binding activity of grp170 was also evaluated under different pH conditions. It is evident that the complex of grp170 and AH1 peptides was highly susceptible to acidic conditions; a pH value of 6 resulted in reduction in peptide association with grp170 (Fig. 2B). Another peptide TRP2 was also tested and showed an identical binding pattern (data not shown).
lanes 1–3. Grp170– and hsp70–AH1 peptide complexes were generated by incubation of chaperones with biotinylated AH1 (18 mer) peptide at 37˚C. The AH1 peptide was eluted from column with PBS containing 5 mM ADP (lanes 1–3) or ATP-agarose (lanes 2 and 3) column, followed by washing with 500 mM NaCl containing PBS. Grp170 or hsp70 was eluted from column with PBS containing 5 mM ADP (lanes 1 and 3) or ATP (lanes 2 and 4). Eluted fraction was examined by immunoblot using streptavidin-HRP.

**Comparison of peptide binding ability of different stress proteins.** Grp170, gp96/grp94, or hsp70 was incubated with biotinylated AH1 peptides in PBS at 37˚C or 50˚C for 30 min, followed by incubation at room temperature for additional 1h. Representative data from three independent experiments are shown.

**FIGURE 2.** Large stress protein grp170 is more efficient in binding peptides than other chaperones. A. Effect of reducing, oxidative agents, and salt concentrations on the complex formation. Grp170 was incubated with biotinylated AH1 peptides in the presence of DTT, H2O2, or NaCl at different concentrations as indicated. B, pH effect on the complex formation. Grp170 was incubated with biotinylated AH1 peptides in PBS with a different pH, and analyzed by immunoblot. Membranes were stripped and reprobed with Abs against grp170. C. Effect of ATP/ADP on grp170–AH1 peptide complex. Grp170– and hsp70–AH1 peptide complexes were generated by incubation of chaperones with biotinylated AH1 (18 mer) peptide at 37˚C. The AH1 complexes were loaded onto ADP-agarose (lanes 1 and 3) or ATP-agarose (lanes 2 and 4) column, followed by washing with 500 mM NaCl containing PBS. Grp170 or hsp70 was eluted from column with PBS containing 5 mM ADP (lanes 1 and 3) or ATP (lanes 2 and 4). Eluted fraction was examined by immunoblot using streptavidin-HRP. D. Comparison of peptide binding ability of different stress proteins. Grp170, gp96/grp94, or hsp70 was incubated with biotinylated AH1 peptide in PBS at 37˚C or 50˚C for 30 min, followed by incubation at room temperature for additional 1h.

It has been demonstrated that a conformational change associated with ATP binding stimulates release of bound peptides from hsp70 (23). Therefore, we examined the effect of ATP/ADP on peptides bound to grp170. Stress protein–AH1 peptide complexes were incubated with ADP- or ATP-agarose beads and eluted with ADP or ATP, respectively. Analyses of eluted samples showed that grp170 bound ATP and ADP efficiently, and excessive ATP resulted in partial dissociation of peptides from grp170 (Fig. 2C). In contrast, the presence of ATP completely removed the AH1 peptides from hsp70, which agrees with the earlier report of the presence of peptides bound to Hsp70 after ADP purification and their absence after ATP purification (24). Given the highly efficient protein holding property of large chaperones, we compared the peptide-binding abilities of grp170 and two other conventional stress proteins (i.e., grp94/gp96 and hsp70). Whereas grp94/gp96 was a weak peptide-binder at 37˚C, the grp170–peptide complexes readily formed at the physiologic condition (Fig. 2D). Similar results were obtained when different peptides were tested (data not shown). Although heat shock at 50˚C modestly stimulated peptide association with grp170 and hsp70, it markedly increased the amount of peptide bound to grp94/gp96, which is consistent with the previous finding that an elevated temperature elicited an irreversible tertiary conformational change that is required for its enhanced peptide-binding activity (25, 26). Quantification of the peptides associated with grp170 by liquid chromatography-mass spectrometry indicates that grp170–AH1 complexes contained ∼8.2 ng peptides per 1 µg of grp170 (data not shown), which is a 5-fold increase of the reported peptides bound to grp94/gp96 (27).

**Grp170 enhances the immunogenicity of associated antigenic peptides in vivo**

Given that large stress protein–peptide complexes can be reconstituted in vitro, we next examined the ability of grp170–AH1 peptide complexes to elicit an Ag-specific immune response in vivo. Neither AH1 peptide alone nor grp170 alone were able to generate a measurable immune response against AH1. However, immunization of mice with the grp170–AH1 peptide complex promoted a potent AH1-specific T cell activation, as indicated by an increase in the frequency of IFN-γ producing cells compared with other groups. Mice receiving grp170 mixed with AH1 also developed an AH1-specific immune response, probably caused by the peptide-binding at room temperature (Fig. 3A). We also immunized mice with the grp170–AH1 peptide complexes via different injection routes. Whereas i.m. vaccination produced the weakest AH1-specific response, s.c. and i.p. injections both generated a modest AH1-specific T cell activation. Intradermal (i.d.) delivery of the grp170–AH1 peptide complex appeared to be the most effective for induction of T cell response against associated AH1 peptides (Fig. 3B).

In a similar manner, melanoma Ag TRP2-derived precursor peptide TRP2175–192, which contains a K b restricted epitope SVYDFFVWL(180–188), was evaluated for its interaction with grp170. The complexes between grp170 and TRP2175–192 were readily formed, and increasing temperatures stimulated the amount of peptide bound to the grp170 (Fig. 3C). The same binding pattern was also seen with hsp110 (data not shown). The hsp110– or grp170–TRP2175–192 complexes generated in vitro were used to immunize C57BL/6 mice. ELISPOT assays showed that both TRP2 chaperone peptide complexes induced a more effective TRP2180–188-specific CTL response than did the TRP2175–192 peptide alone (Fig. 3D). Mice were also immunized with grp170 complexed with precisely processed MHC I-restricted peptide TRP2180–188. These mice developed only a slightly higher T cell response against the CTL epitope than did those immunized with grp170 complexed with TRP2175–192 peptide complex.
Protein Ag chaperone complex generates a more potent anti-tumor response than peptide Ag chaperone complexes in vivo

TRP2 is highly expressed in human primary and metastatic melanomas, and it has also been defined as a potential tumor-rejection Ag in murine B16 melanoma (28). In light of the efficient priming of Ag-specific T cells by both protein and peptide chaperone complexes, we sought to compare the anti-tumor activities generated by these two forms of Ag chaperone complexes. We first prepared recombinant human TRP2 protein using a BacPAK Baculovirus expression system, which not only offers a high yield of recombinant protein with proper tertiary structure and posttranslational modifications, but also greatly minimizes potential endotoxin contamination in protein preparation. Protein purity was assessed by SDS-PAGE stained with Coomassie blue and immunoblotting using anti-TRP2 Abs and anti–His-tag Abs (Fig. 4A). Because the partially denatured peptide chain of a protein is recognized by chaperone molecules and stimulates their chaperoning-binding function, we determined the thermal sensitivity of TRP2 protein Ag using an in vitro aggregation assay (17). This study indicated that the melting point of TRP2 protein is ~45°C (data not shown), which was then used for subsequent complexing experiments. Protein aggregation assay showed that the large chaperone hsp110, but not OVA, effectively blocked heat-induced TRP2 protein aggregation (Fig. 4B), which is consistent with our previous observations with another melanoma Ag gp100 (17). The ability of the TRP2 protein chaperone complex to generate Ag-specific CTL response was examined using an ELISPOT assay. After vaccination, splenocytes from animals immunized with the hsp110– or grp170–TRP2 protein complex vaccines displayed high levels of IFN-γ production compared with those from untreated or OVA–TRP2 protein immunized mice (Fig. 4C). However, mice immunized with the grp170–TRP2 175–192 peptide complex developed a stronger T cell activity recognizing the CTL epitope TRP2 180–188 than did the TRP2 protein complex (Fig. 4C).

Because the TRP2 180–188 has been previously characterized as the most immunogenic epitope in the TRP2 aa sequence, and this conserved nonmutated epitope can be recognized by anti-B16 melanoma CTLs as well as human HLA-A2.1 restricted anti-melanoma CTLs (28, 29), we performed tumor prevention studies to compare the immunogenicity of TRP2 175–192 peptide and full-length TRP2 protein when complexed to grp170. Although grp170–TRP2 175–192 peptide vaccine induced a considerably higher T cell response against CTL epitope TRP2 180–188 than did the TRP2 protein complex (Fig. 4C), this vaccine did not provide a modest tumor inhibitory effect. In contrast, the grp170–TRP2 protein chaperone complex augmented a robust anti-tumor response (Fig. 4D). We also showed that vaccination with grp170 protein alone, heated TRP2 protein, or grp170 mixed with irrelevant Ag OVA failed to induce tumor-protective effects, indicating that chaperone complex formation is required for the observed anti-tumor activity. This result is
consistent with our previously published data on gp100-targeted chaperone vaccines (17, 19).

We next performed the chromium release assays to measure the cytolytic activity of T effector cells from immunized mice. It was shown that CD8+ T cells from the grp170–TRP2 protein complex-immunized mice exhibited significantly higher cytotoxic activity compared with those from the grp170–TRP2175–192 peptide complex-treated mice (Fig. 4E), suggesting that protein Ag-targeted chaperone vaccines efficiently promote functional activation of CTLs.

Large stress proteins exhibit a superior Ag-holding capability

We have shown previously that hsp110 and grp170 are highly efficient at preventing aggregation of reporter protein luciferase and tumor-associated protein Ags such as gp100 upon heat shock at a 1:1 molar ratio, compared with hsp70 and grp94/gp96 in vitro (17, 20, 30). We next compared the Ag-holding capabilities of hsp110 and hsp70 by performing the similar protein aggregation assays with the addition of excess substrate (i.e., Ag) under heat shock conditions. While the chaperoning activity of hsp70 was negligible at <4-fold excess of protein substrate, hsp110 was able to protect gp100 from heat-induced protein aggregation, even with >10-fold excess gp100 (Fig. 5A). The same observations were made with TRP2 protein Ag, although hsp110 prevented aggregation of TRP-2 less efficiently than did gp100 (Fig. 5B). In addition, we showed that grp170 has more capacity for preventing heat shock-induced aggregation of excess model Ag luciferase compared with hsp70 and hsp110 in vitro (Fig. 5C). These results indicate that large stress proteins chaperone or bind to Ag substrate in a quantitatively greater degree than do conventional hsp70.

Large stress protein-based chaperone vaccines targeting dual protein Ags enhance Ag-specific tumor immunity

Given their unique and highly efficient Ag-holding capabilities, these large chaperones can be used as a platform to deliver multiple Ags that could enhance immunotherapeutic efficacy against tumor. We examined whether the vaccine efficacy might be further improved by simultaneous immunization with hsp110-based chaperone vaccines directed against two melanoma Ags instead of one. Hsp110–gp100 protein complex and hsp110–TRP2 protein complex were prepared separately under heat shock conditions. Mice were immunized with either single protein Ag complexed with hsp110 or the combination of gp100 and TRP2 chaperone vaccines. The frequency of gp100 protein-specific IFN-γ–producing spleenocytes was significantly higher in mice immunized with the gp100 chaperone vaccine and the polyvalent vaccines than in mice untreated or immunized with TRP2 chaperone vaccine. Similarly, a TRP2-specific immune response was also specifically provoked in hsp110–TRP2 or the combined hsp110–gp100/TRP2 complex...
vaccine immunized mice, not in hsp110–gp100 immunized animals (Fig. 6A). These results suggest that immunization with individual melanoma Ags complexed with hsp110 does not augment immune reactivity against each other, and the combined vaccinations do not appear to interfere with the activation of Ag-specific immune responses induced by the individual chaperone vaccine.

To investigate the feasibility and anti-tumor efficacy of the polyvalent protein chaperone vaccines targeting both gp100 and TRP2, mice were immunized with different vaccine regimens, followed by B16 tumor challenge. The polyvalent vaccines resulted in an effective protection against subsequent tumor challenge, which was more potent than that achieved with either of the single Ag-chaperone complexes alone (Fig. 6B), suggesting that this polyvalent vaccine simultaneously targeting dual Ags promotes the overall immune response against B16 tumors.

Because the B16 tumor protective immunity was induced by administration of chaperone-based protein vaccines, we next sought to determine whether the combined vaccines elicited gp100 and TRP2-specific CD8+ T cell responses. Splenocytes from immunized or naive animals were stimulated with MHC I-restricted CTL epitopes, gp100 25–33 or TRP2 180–188 peptides, and assayed for IFN-γ production. In contrast to naive or mice immunized with TRP2 protein chaperone vaccine alone, those immunized with the gp100 protein vaccine or combined gp100/TRP2 protein chaperone vaccines displayed greater activation of gp10025–33-specific T cells. Similarly, TRP2 180–188-specific T cells were also primed in mice immunized with vaccine formulations containing TRP2 protein Ag. In addition, irradiated B16 tumor cells were used to stimulate splenocytes harvested from treated mice. Compared with individual protein Ag chaperone vaccines (i.e., gp100 or TRP2), administration of the dual chaperone vaccines markedly enhanced the B16 tumor-specific IFN-γ production, suggesting that the combined vaccinations resulted in an improved immune recognition of the B16 tumor cell target (Fig. 6C).

Combined grp170 chaperone vaccines targeting gp100 and TRP2 protein Ags result in a greater anti-tumor immunity compared with tumor-derived grp170

We compared the anti-tumor activities of recombinant grp170–gp100/GRP2 protein Ag complex and tumor-derived/purified
grp170 in a therapeutic setting. Tumor-bearing mice were treated with grp170–TRP2 complex, grp170–gp100/TRP2 complexes, or grp170-derived/purified from B16 tumor cells. It was observed that TRP2 protein Ag carried by grp170 exhibited comparable anti-tumor activities to tumor-derived grp170. However, treatment with combined protein chaperone vaccines targeting both gp100 and TRP2 led to a significantly more effective inhibition of tumor growth than did single Ag chaperone vaccine or tumor-derived grp170. We next examined whether the enhanced anti-tumor effect by recombinant grp170–gp100/TRP2 complexes resulted from increased molar ratio of tumor-associated Ags in the chaperone vaccine formulation. To this end, we removed endogenous Ags associated with B16 tumor-derived grp170 using ATP-agarose column as previously described by Peng et al. (24), and subsequently complexed recombinant gp100/TRP2 proteins to the stripped grp170. Tumor-bearing mice were treated with B16-grp170, the stripped grp170, or gp100/TRP2-reloaded grp170. As expected, the stripped grp170 lost its immunogenicity against B16 tumors. However, the enhanced therapeutic effect was observed in

![Graph A](http://www.jimmunol.org/)

**FIGURE 7.** Chaperone vaccines targeting dual Ags induce potent therapeutic anti-tumor immunity. A, Superior therapeutic efficacy generated by the combined chaperone vaccines. Mice (n = 5) were established with B16 tumors 3 d prior to vaccine treatment. Grp170–TRP2 complex, tumor-derived grp170 or grp170–gp100/TRP2 complexes were administrated every 3 d for a total four treatments. B, Priming of T cells reactive with gp100 25–33 following TRP2-chaperone vaccine treatment. Splenocytes from treated mice were stimulated with gp100 25–33 or TRP2 180–188 and subjected to an ELISPOT assay. Data shown are from two independent experiments. C, Potent anti-tumor immunity augmented by endogenous grp170 complexed with gp100/TRP2 proteins. B16 tumor-bearing mice were treated with tumor-derived grp170, Ag stripped grp170, or stripped grp170 complexed with gp100/TRP2 protein Ags. Tumor growth was followed as described in Figure 7A. D, High frequency of gp100/TRP2-specific TILs elicited by the chaperone vaccines. TILs were isolated from pooled tumor tissues after treatment and stimulated with gp100 25–33/TRP2 180–188 peptides, followed by intracellular staining for IFN-γ. Cells were analyzed using FACS by gating on CD8+ cells. E, Robust tumor-killing activity of TDLN cells from chaperone vaccine-treated mice. Single-cell suspensions were prepared using TDLNs pooled from treated mice. Cells were stimulated with mitomycin and IFN-γ-treated B16 cells for 5 d. T cells were used as effector cells in cytotoxic assays against B16 cells as targets. *p < 0.005. Data shown are from two independent experiments.
mice treated with endogenous grp170-loaded with gp100/TRP2 compared with those treated with B16-grp170 (Fig. 7C).

We subsequently investigated Ag-specific tumor-infiltrating lymphocytes (TILs) following the therapeutic vaccinations. Intracellular IFN-γ staining of Ag-stimulated TILs showed that recombiant chaperone vaccine resulted in a significant increase in the frequency of gp100/TRP2 reactive CD8+ T cells in the tumor site compared with control or B16-grp170 (Fig. 7D). This finding is not surprising, because the Ags in the chaperone vaccine formulation are highly concentrated, whereas only a small percentage of Ags carried by B16-grp170 is likely to be tumor-specific. In addition, cytotoxic T cell assays were performed to assess the effector function of T cells from tumor-draining lymph nodes (TDLNs). TDLN-derived CD8+ T cells from the grp170–gp100/TRP2 chaperone vaccine treated mice displayed a higher cytolytic activity than their counterparts from B16-grp170 treated mice (Fig. 7E). Collectively, these results demonstrate the superior capability of the recombinant chaperone vaccines to augment robust anti-tumor immunity targeting carried protein Ags.

Discussion
Several lines of evidence have shown that stress proteins, including hsp70, hsp90, and grp94/grp96, are peptide-binding chaperones, and stress protein-peptide complexes released into the extracellular milieu as a consequence of pathologic cell death might play an important role in Ag cross-presentation. Given the strong Ags potency exhibited by high m.w. chaperones, we have investigated the peptide-binding ability of large stress proteins and the immunogenicity of hsp110- and grp170-peptide complexes reconstructed in vitro. We have demonstrated for the first time that full-length protein Ag chaperone complex is superior to peptide chaperone complex in augmenting an anti-tumor immune response in both prophylactic and therapeutic settings, strongly supporting the idea of using large chaperone-protein Ag complexes as a new clinical approach to the treatment of cancers or other diseases.

Our results show that hsp110 and grp170 readily form complexes in vitro with a variety of antigenic peptides derived from viral or tumor Ags. Competition studies reveal that the peptide binding can be efficiently blocked in the presence of excess unlabeled peptides or peptides of different sequences or length, suggesting that these large chaperones bind peptides in a promiscuous manner. The complexes formed in vitro are highly stable as indicated by their resistance to high salt concentrations. However, grp170 association with antigenic peptides is susceptible to acidic pH, which can have implications for grp170-mediated peptide loading/release process in vivo, because MHC I molecules were also found to be able to exchange peptides under acidic conditions (31). Indeed, earlier studies have shown that grp170 interacts with transporter associated with Ag processing translocated peptides and is involved in peptide trafficking in the Ag presentation pathway (32, 33).

Hsp70 interactions with peptides in vitro have been shown to be promoted by oxidative conditions (34). However, no significant stimulation of peptide association with grp170 or hsp110 (data not shown) is observed under oxidative or reducing conditions, although they represent highly diverged and distant family members of hsp70, suggesting that different chaperones bind to peptides through distinct mechanisms. Compared with the hsp70 as shown here and grp94/grp94 with a reported adenine nucleotide independent peptide binding activity (25, 26), ATP only partially dissociates antigenic peptides from grp170, suggesting that ATP plays a regulatory role in the grp170-involved peptide binding and release processes in vivo. One interesting finding was that the presence of grp78/BiP significantly stimulates peptide association with both grp170 and grp78, indicating the existence of the functional interactions between these two chaperones (35). In fact, Lhs1p, the grp170 homolog in yeast, can serve as an alternative nucleotide exchange factor, and it is believed that the coordination of both grp78/BiP (Kar2p) and grp170 (Lhs1p) promotes productive folding of unfolded polypeptide substrate (36). In a similar scenario, either sequential or simultaneous association with the substrate has been proposed for the hsp110–hsp70 complex (37). However, how exactly grp78/BiP works together with grp170 to promote peptide association under conditions used in this study remains unclear.

High temperature appears to stimulate grp170 association with peptides, but not as dramatically as it does to grp94/grp96 (4-fold increase) (26). It has been reported that the transient heat shock results in the formation of higher order oligomers of stress protein; however, it is not responsible for the increase in peptide-binding activity of grp94. Indeed, the activation of peptide interaction directly correlates with the exposure of a hydrophobic pocket (26). It should be noted that the binding of peptides to large stress proteins is probably not optimum under our experimental conditions, likely because of the nonphysiologic parameters of the experiment, and the lack of co-chaperones. Nevertheless, the strong grp170 association with peptides at 37˚C implies that grp170 might be more efficient in interacting with and actively participate in shaping the antigenic peptide repertoire in vivo compared with other chaperones, which might contribute to the highly potent vaccine activity of this molecule (15). In contrast to the reported weak CTL responses elicited by hsp70-peptide complex (38), the grp170-peptide complex effectively induces T cell activation against associated antigenic peptides, which are in line with several reports suggesting that the peptide-binding affinity is important for the generation of a CTL response by chaperone-peptide complexes in vivo (39–41).

Coincident with the observations of highly efficient peptide-binding capability, the protein aggregation assays in the current study have demonstrated that large stress proteins display superior capability to stabilize and hold full-length client proteins/substrates compared with hsp70 and grp94/grp96. These results are consistent with our previous observations that both hsp110 and grp170 can efficiently stabilize thermally denatured luciferase in a folding-competent state (13, 14, 20). Studies of grp170 deletion mutants show that grp170 contains two essential substrate-binding regions (i.e., the β-sheet domain and the C-terminal helix domain) (14). Therefore, it is evident that these large stress proteins, grp170 in particular, represent a unique vaccine vehicle for shutting protein Ags. Strikingly, we found that the TRP2 protein Ag chaperone complex augments a more potent tumor protective response against B16 melanoma than the grp170–TRP2175–192 peptide complex, although T cell activity specific for a major CTL epitope (i.e., TRP2190–198) appears to be higher in mice immunized with the TRP2 peptide complex. We reason that full-length protein Ag contains CD4 T helper epitopes and other unknown CD8 epitopes that may have contributed to the observed robust anti-tumor effect. The result agrees with our earlier studies showing that CD4+ cells are involved in the protein chaperone complex-induced anti-tumor immunity (16).

Using an experimental murine model of melanoma, we have demonstrated that immunization with large chaperone vaccines targeting two different melanoma Ags (i.e., gp100 and TRP2) dramatically enhances overall anti-tumor efficacy compared with either single Ag-targeted vaccine. Furthermore, treatment of B16 tumor-bearing mice with the polyvalent grp170 chaperone complex vaccines result in a more potent therapeutic efficacy against established tumors when compared with tumor-derived grp170. This finding has two implications for the use of chaperoning-based
vaccine approach. First, these large stress proteins can be used as a platform or a building block method to form multivalent vaccines targeting multiple protein Ags. Second, although it is not clear whether the improved anti-tumor activity is due to additive or synergistic effects derived from the combined vaccines, such a multivalent vaccine design is expected to significantly reduce the possibility of tumor escape as a result of Ag loss during Ag-targeted immunotherapy. Interestingly, we also show that reloading of the stripped endogenous grp170 with gp100/TRP2 protein Ags recapitulated the enhanced therapeutic effect of the recombinant chaperone vaccines-targeting gp100/TRP2, suggesting that the high concentrations of tumor Ags contribute to the improved anti-tumor activities elicited by the chaperone vaccines. However, it should be noted that, given the nature of the Ag targets in these two different forms of vaccine regimens (protein versus peptide; well-defined Ags versus undefined and presumably polyvalent), it is possible that the immunostimulatory actions of these two vaccine approaches involve distinct mechanisms.

Of particular interest, we found that administration of a TRP2-targeted chaperone vaccine in the therapeutic setting augmented an immune activation against another melanoma Ag gp100. It is likely that the observed epide ice spreading was caused by tumor destruction and subsequent cross-presentation of tumor cell-associated Ags following the chaperone vaccine therapies. In addition, the TRP2-specific T cell activities induced by the combined chaperone vaccines are significantly higher than those generated by the grp170–TRP2 complex in this melanoma treatment model, suggesting that administration of dual-Ag chaperone vaccines are able to amplify the immune responses specific for targeted Ags after the initial tumor killing event. It is not clear whether T cells specific for melanoma Ags other than gp100 and TRP2 have been stimulated in this setting. B16 melanoma is a more relevant model for evaluating anti-tumor immunity, because it expresses multiple melanoma-associated Ags. In addition, the sequences of these murine melanoma Ags, including gp100, TRP2, and tyrosinase, are all highly homologous to their human counterparts (42, 43). Therefore, immunization by combining different tumor protein Ags using the same chaperoning formulations should be clinically beneficial relative to the use of tumor-derived stress proteins. A recent clinical trial using patient-specific autologous grp94/gp96 showed that overall survival in the treatment arm is statistically indistinguishable from that in the control arm (44). However, retrospective analyses show that patients in certain subtypes receiving a larger number of grp94/gp96 immunizations survived longer than those receiving fewer such treatments (44). Unfortunately, the yield of tumor-derived stress proteins was a limiting factor and many patients were unable to participate or complete the vaccine trial. Clearly, the synthetic production of the recombinant chaperone complexes will provide several advantages over the preparation of autologous or tumor-derived HSP vaccines, including no requirement for tumor specimen, unlimited quantities of vaccine with significant uniformity from batch to batch, broad applicability to all patients with Ag(s)-positive tumors, increased chance of stimulating polypeptide directed cellular and humoral responses, and posttreatment immunomonitoring by using well-defined whole protein Ags. Because a tumor specimen is not required for vaccine production, patients with no measurable disease or inaccessible tumor can still be treated using this approach. This also makes it an ideal approach for eventual adjuvant therapy in high-risk patients with completely resected disease. Collectively, the recombinant large chaperone-based vaccines targeting dual or multiple melanoma Ags presented in this study warrant further development and evaluations in the clinic for treatment of human melanoma. In addition, this synthetic building block approach of complexing Ag with immunostimulatory large stress proteins can serve as a model to evaluate other different Ags, either alone or in combination vaccines.

**Disclosures**

The authors have no financial conflicts of interest.

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