ABSTRACT

Several studies have shown that when purified from a tumor, certain heat shock proteins (HSPs) can function as effective vaccines against the same tumor by virtue of their ability to bind tumor-specific peptides. However, only a small fraction of the associated peptides would be expected to be immunogenic, in addition to which, the clinical application of this vaccine requires the availability of a surgical specimen of sufficient quantity for purification of the HSP. The present study describes a new approach for the development of natural HSP vaccines that do not have these limitations. This approach uses a recombinant HSP that is noncovalently bound to a recombinant tumor protein antigen by heat shock. HSP110 has been selected for this purpose, because it has been shown to be a highly efficient molecular chaperone in binding to large protein substrates. We show that a "natural chaperone complex" between HSP110 and the intracellular domain (ICD) of human epidermal growth factor receptor 2 protein (HER-2/neu) is formed by heat shock. This HSP110-ICD vaccine elicited both CD8 + and CD4 + T-cell responses against ICD as determined by an antigen-specific IFN-γ production in an enzyme-linked immunospot assay (ELISPOT). In vitro depletion studies revealed that the CD8 + T-cell response was independent of CD4 + T-cell help. The HSP110-ICD complex also significantly enhanced ICD-specific antibody responses relative to that seen with ICD alone. No CD8 + T cell or antibody response was detected against HSP110. The use of recombinant HSP110 to form natural chaperone complexes with large protein antigens represents a new and powerful approach for the design of protein-targeted cancer vaccines.

INTRODUCTION

Certain HSPs1 have been shown to act as effective vaccines against a cancer when they are purified from the same cancer (1–4). This approach takes advantage of the peptide-binding properties of these stress proteins, which is also responsible for their functions as molecular chaperones in numerous processes (5–6). The peptides bound by these stress proteins would conceivably copurify a peptide “fingerprint” of the cancer cell of origin. This fingerprint would be expected to include a small subset of antigenic, tumor-specific epitopes against which an immune response would be mounted. Importantly, HSPs also possess other immunological properties, including their ability to bind to receptors on APCs as well as their ability to activate/mature DCs. These HSP-activated DCs secrete proinflammatory cytokines and up-regulate MHC class II molecules (7–9). By virtue of all of these properties, HSP preparations purified from tumor can be used as effective vaccines in the absence of an adjuvant. The success of HSP vaccines in preclinical animal studies has led to clinical phase II/II trials of tumor-derived HSP preparations, specifically using GRP944 (also called gp96) as autologous tumor vaccines (11, 12).

Only a few stress proteins have been shown to exhibit vaccine activity in animals when prepared in this way (the most frequently studied being GRP94/gp96 and constitutive HSP70). In addition, recent studies have shown that another HSP, known as HSP110, also exhibits this vaccine activity when purified from a tumor and applied as a preventative therapy (13). HSP110 has been shown to be representative of a recently discovered HSP family, which is expressed in (apparently) all of the eukaryotic cells. A hallmark of HSP110 is its ability to efficiently bind to and stabilize large protein substrates (14, 15).

As just described, the ability of HSPs to bind short peptides has formed the basis for their use as cancer vaccines. However, HSPs can also bind to and stabilize large proteins. Indeed, the activity of forming natural chaperone complexes with large proteins is an essential component of the HSP function in numerous cellular processes (14, 16, 17). The present study describes a new approach in the preparation of HSP vaccines. This takes advantage of this property of certain HSPs to naturally bind large proteins (14). Specifically, HSP110 is used for this purpose based on its strong ability to efficiently bind to and chaperone large protein substrates.

In this study, recombinant HSP110 is noncovalently complexed during heat shock with a recombinant tumor protein antigen in vitro. This natural chaperone complex is then evaluated as an effective vaccine. This vaccine would not be patient specific, as is the case with tumor-derived HSPs/GRPs, but could be applied to any patient with a tumor expressing that protein antigen and would not require a surgical specimen to prepare. Unlike tumor-derived HSPs, where only a small fraction of chaperoned peptide would be expected to be immunogenic, this approach will present a peptide "fingerprint" of the cancer cell of origin. This fingerprint would be expected to include a small subset of antigenic, tumor-specific epitopes against which an immune response would be mounted. Importantly, HSPs also possess other immunological properties, including their ability to bind to receptors on APCs as well as their ability to activate/mature DCs. These HSP-activated DCs secrete proinflammatory cytokines and up-regulate MHC class II molecules.

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3 The abbreviations used are: HSP, heat shock protein; DC, dendritic cell; APC, antigen presenting cell; ICD, intracellular domain; GRP, glucose regulated protein; CFA, Complete Freund’s Adjuvant; ELISPOT, enzyme-linked immunospot assay; HRP, horseradish peroxidase; IFA, Indirect Fluorescent Antibody; mAb, monoclonal antibody; NBT, nitroblue tetrazolium; Ab, antibody; BCIP, 5-bromo-4-chloro-3-indolyl phosphatase.
4 GRPs are endoplasmic reticulum resident members of HSP families (10).
MATERIALS AND METHODS

**Mice.** Studies were performed in A2/Kb transgenic animals purchased from Harlan Sprague Dawley (La Jolla, CA). This model was used for comparison of data obtained in the present study with peptide immunization approach using the HSP110-peptide complex (HLA-A2 epitopes from HER-2/neu) in a separate investigation. In addition, studies were reproduced using C57/Bl6 mice (obtained from the Department of Laboratory Animal Resources at Roswell Park Cancer Institute) in a confirmatory experiment. Data obtained using A2/Kb mice are presented. All of the animals used in this study were 6–8-week-old females.

**Recombinant Proteins.** Recombinant mouse HSP110 is being routinely prepared in our laboratory using bacPAKHis vector (Clontech Laboratories Inc., Palo Alto, CA). This vector carrying the HSP110 gene was cotransfected with bacPAK6 viral DNA into Sf21 insect cells using a bacPAK Baculovirus Expression System kit (Clontech Laboratories Inc.) followed by amplification of the recombinant virus and purification of HSP110 protein using Ni-NTA-Agarose (Qiagen, Venetica, CA). Concentration of the recombinant HSP110 was determined using Bio-Rad protein assay kit. Highly purified recombinant human ICD was provided by Corixa Corp. This protein was produced in Escheria coli and purified from solubilized inclusion bodies via High Q anion exchange followed by Nickel resin affinity chromatography. A control recombinant protein was also made in E. coli and purified in a similar way as the ICD.

**In Vitro HSP110-Antigen Binding.** The HSP110-ICD complex (3–6 μg each in 1 ml PBS) was generated by incubation of the mixture in a 1:1 molar ratio at 43°C for 30 min and then at 37°C for 1 h. The binding was evaluated by immunoprecipitation as described previously (14) with some modifications. Briefly, the HSP110-ICD complex was incubated with either rabbit antimouse HSP110 antisera (1:200) or rabbit antimouse GRP71 antisera (1:100), as a specificity control, at room temperature for 1–2 h. The immune complexes were then precipitated by incubation with protein A-Sepharose CL-4B (20 μl/ml; Amersham Pharmacia Biotech, AB, Uppsala, Sweden) and rocking for 1 h at room temperature. All of the proteins were spun for 15 min at 4°C to precipitate any aggregation before use. Samples were then washed eight times with washing buffer [1 M Tris-Cl (pH 7.4), 5 M NaCl, 0.5 M EDTA (pH 8.0), and 0.13% Triton X-100] at 4°C to remove any nonspecific binding of the recombinant proteins to protein A-Sepharose. The beads were then added with 2 × SDS sample buffer, boiled for 5 min, and subjected to SDS-PAGE (10%) followed by either Gel-blue staining or probing with mouse antihuman ICD antisera (1:10000; provided by Corixa Corp.) in a Western blotting analysis using HRP-conjugated sheep antiserum IgG (1:5000; Amersham Pharmacia Biotech, Piscataway, NJ) and 1 min incubation of the nitrocellulose membrane with chemiluminescence reagent followed by exposure to Kodak autoradiography film for 20 s.

**Immunizations.** Preliminary studies showed that s.c. and i.p. routes of injection of the HSP110-ICD complex stimulated comparable levels of cell-mediated immune responses, but i.p. injection was better in s.c. injection in eliciting Ab responses (data not shown). Thus, all of the groups were injected i.p. except for mice immunized s.c. with ICD together with CFA and boosted together with IFA. Mice (five per group) were injected with 25 μg of the HSP110-ICD complex in 200 μl PBS on days 0 and 14. Control groups were injected with 25 μg of the HSP110, ICD, ICD together with CFA/IFA, or left unvaccinated. The splenocytes were removed 14 days after the booster and subjected to ELISPOT assay to evaluate CTL responses. Sera were also collected on days 0, 14, and 28 to measure isotype-specific antibodies (IgG1 and IgG2a) against the ICD or HSP110 using ELISA technique. Groups of animals (five per group) were also depleted from CD4+ T cells (g 2% 15% T cells) and CD8 T cells and then injected once a week. The splenocytes were then subjected to ELISPOT assay. **In Vivo Ab Depletion.** In vivo Ab depletions were carried out as described previously (20). The GK1.5, anti-CD4 and 2.43, anti-CD8 hybridomas were kindly provided by Dr. Drew Pardoll (John Hopkins University, Baltimore, MD), and the ascites were generated in SCID mice. The depletions were started 4 days before vaccination. Each animal was injected i.p. with 250 μg of the mAbs on 3 subsequent days before and twice a week after immunization. Animals were depleted from CD4+, CD8+, or CD4+/CD8+ T cells. Depletion of the lymphocyte subsets was assessed on the day of vaccination and weekly thereafter by flow cytometric analysis of spleen cells stained with mAbs GK1.5 or 2.43 followed by FITC-labeled rat antimouse IgG (PharMingen, San Diego, CA). For each time point analysis, >98% of the appropriate subset was achieved. Percentage of CD4+ T cells did not change after CD8+ T-cell depletion and percentage of CD8+ T cells also did not change after CD4+ T-cell depletion. The representative data are shown in Table 1.

**ELISPOT Assay.** Generation of T-cell responses by the immunized animals were evaluated using ELISPOT assay as described by others (21). Briefly, the 96-well filtration plates (Millipore, Bedford, MA) were coated with 10 μg/ml of rat antimouse IFN-γ Ab (clone R4–6A2; PharMingen) in 50 μl PBS. After overnight incubation at 4°C, the wells were washed and blocked with RPMI 1640 containing 10% fetal bovine serum (RF10). Red cells were lysed by incubation of the splenocytes with Tri-NH4Cl for 5 min at room temperature followed by washing twice in RF10. Cells (50 μl; 107 cells/ml) were added into the triplicate wells and incubated with 50 μl of the ICD (20 μg/ml) or HSP110 (20 μg/ml) at 37°C in a atmosphere of 5% CO2 for 20 h. Positive control wells were added with Con-A (5 μg/ml), and background wells were added with RF10. A control recombinant protein made in E. coli was also used (20 μg/ml) in a confirmatory experiment using the HSP110-ICD or ICD-immunized animals (data not shown). The plates were then washed extensively (10 times) and incubated with 5 μg/ml biotinylated IFN-γ Ab (clone XMG1.2; PharMingen) in 50 μl PBS at 4°C overnight. After washing six times, 0.2 units/ml alkaline phosphatase avidin D (Vector Laboratories, Burlingame, CA) in 50 μl PBS, was added and incubated for 2 h at room temperature and washed on the following day (the last wash was carried out with PBS without Tween 20). IFN-γ spots were developed by adding 50 μl BCIP/NBT solution (Boehringer Mannheim, Indianapolis, IN) and incubating at room temperature for 20–40 min. The spots were counted using a dissecting microscope.

**ELISA.** ELISA technique was carried out as described elsewhere (22). Briefly, 96-well ELISA plates were coated with ICD (20 μg/ml) or HSP110 (20 μg/ml), and then blocked with 1% bovine serum albumin (BSA) in phosphate buffer saline (PBS) after incubation at 4°C overnight. After washing with PBS-0.05% Tween 20, wells were added with 5-fold serial dilutions of the sera starting at 1:50, then incubated at room temperature for 1 h, washed three times, and added with horseradish peroxidase (HRP)-labeled goat antimouse IgG1 or IgG2a Ab (Caltag Laboratories, Burlingame, CA). The reactions were developed by adding 100 μl/well of the TMB Microwell peroxidase substrate (KPL, Gaithersburg, MD) and reading at 450 nm after stopping the reaction with 50 μl of 2 M H2SO4. Specificity of the binding was assessed by preincubation of the primary sera or staining of the ICD with the pooled immune sera (1:2000) collected from the HSP110-ICD-immunized animals in a Western blot. Data are presented as mean values for each Ab isotype.

**Statistical Analysis.** Unpaired two-tailed Student’s t test was used to analyze the results. Data are presented as the ± SE. P ≤ 0.05 was considered significant (23).

**RESULTS**

**Noncovalent Binding of the HSP110 to ICD at 43°C.** On the basis of our previous finding that HSP110 binds to Luciferase and Citrate Synthase at a 1:1 molar ratio at 43°C, we examined whether

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<th>Table 1 Flow cytometric analysis of the presence of T-cell subsets after in vivo antibody depletion</th>
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<td><strong>Animals</strong></td>
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The depletion of CD4+ and CD8+ T cells was accomplished by i.p. injection of GK1.5 or 2.43 antibodies (250 μg), respectively. The CD4+CD8+ T cells were also depleted by i.p. injection of both GK1.5 and 2.43 antibodies (250 μg each). The depletion was performed on 3 subsequent days before immunization and followed by twice a week injections. Spleen cells were stained for CD4+ or CD8+ T cells using FITC-labeled rat antimouse IgG and subjected to flow cytometry showing that ~98% of the lymphocyte subsets were depleted without any affect on other T-cell subsets.
Vaccination with the HSP110-ICD Complex Induces Antigen-specific IFN-γ Production. ELISpot assay is a sensitive functional assay used to measure IFN-γ production at the single-cell level, which can, thus, be applied to quantify antigen-specific CD8+ or CD4+ T cells. Depletion of T-cell subsets was also performed to determine the source of IFN-γ production. We first explored whether the HSP110-ICD complex, without any adjuvant, could elicit antigen-specific IFN-γ production. Fig. 2 demonstrates that the HSP110-ICD-immunized animals elicited significant IFN-γ production on stimulation with ICD in vitro. No IFN-γ spot was detected in the background wells (nonstimulated cells). The HSP110-ICD complex was as efficient as the CFA-ICD, i.e., there was no significant difference between the two vaccines in their ability to induce IFN-γ production. Splenocytes collected from all of the groups did not produce IFN-γ on in vitro stimulation with HSP110. Mice immunized with ICD only did not show IFN-γ production on stimulation with the antigen.

Vaccination with the HSP110-ICD Complex Induces Both CD8+ and CD4+ T-Cell-mediated Immune Responses. To identify which cell populations were involved in the antigen-specific IFN-γ production, in vivo lymphocyte subset depletion was performed with injections of the mAb 2.43 or GK1.5 to deplete CD8+ or CD4+ T cells, respectively. A group of animals were also depleted from both CD8+ and CD4+ T cells. Fig. 3 shows that all of the animals vaccinated with the HSP110-ICD complex and depleted from the CD8+ or CD4+ T cells showed IFN-γ production on in vitro stimulation with the antigen. Animals depleted from both CD8+ and CD4+ T cells did not show any IFN-γ production on either ICD or Con A stimulation in vitro. There was also no significant difference between the CD8+-depleted cells and CD4+-depleted cells to produce antigen-specific IFN-γ in vitro (P = 0.95).

To additionally explore whether activation of CD4+ T cells may promote activation of CD8+ T cells, we carried out CD4+ T-cell depletion in the HSP110-ICD-immunized animals 1 week after the booster. Although frequency of IFN-γ-producing cells was slightly higher in these animals than that in animals depleted from CD4+ T cells before vaccination, this difference was not statistically significant (P ≥ 0.16).

Vaccination with the HSP110-ICD Complex Induces both IgG1 and IgG2a Ab Responses against the ICD. It has been reported that noncovalent binding of HSPs with a peptide could elicit potent T-cell
responses to the bound peptide, whereas the covalent binding complexes elicit the potent Ab responses (24, 25). Therefore, we decided to examine whether in vitro loading of HSP110 with a large tumor antigen, ICD, in a form of noncovalent complex may be able to elicit a strong Ab responses in addition to cell-mediated immunity. We collected blood from animals that were used to monitor cell-mediated immunity by ELISPOT assay. Sera were prepared and tested for antigen-specific Ab responses by ELISA. Using HRP-labeled antimouse isotype specific antibodies, IgG1 or IgG2a, we identified that both IgG1 and IgG2a Abs were elevated remarkably in the immunized animals (Fig. 4A). Both IgG1 and IgG2a Ab levels were significantly higher in the HSP110-ICD-immunized animals than those in the ICD-immunized animals 14 days after immunization ($P \leq 0.0001$). However, IgG2a Ab reached the same levels in the two groups on day 28. The IgG1 was the major Ab, which stayed significantly higher in the HSP110-ICD-immunized animals than in the ICD-immunized animals 28 days after immunization ($P \leq 0.0001$). Western blot analysis of the pooled immune sera collected from the HSP110-ICD immunized animals revealed specificity of the Ab for the ICD (Fig. 4B, Lane 1). Mouse antihuman ICD Ab (1:10000) was used as a control to stain the ICD (Fig. 4B, Lane 2). No anti-HSP110 Ab was detected before or after immunization.

**DISCUSSION**

HSPs purified from tumors have been shown to act as anticancer vaccines (1–4, 26, 27). The clinical application of this approach requires the availability of a sufficient surgical specimen from which the HSP can be purified. We describe here an alternative approach, which uses the natural chaperoning function of certain HSPs, specifically HSP110. We have found that HSP110 efficiently binds the $M_r$ 84,000 ICD of HER-2/neu at $\sim 1:1$ molar ratio after heat shock (Fig. 1). We have shown that immunization with this HSP110-ICD complex resulted in an antigen-specific immune response against the ICD that was as potent as adding CFA to the ICD. This is important in that the HSP110 used to immunize mice is obtained using the mouse sequence, in contrast to the numerous foreign and microbial constituents present in CFA. Indeed, mice immunized with HSP110 alone did not show any IFN-$\gamma$ production on in vitro stimulation with the mouse HSP110, indicating that as a self-protein, it did not elicit an autoimmune response. Additionally, animals immunized with ICD alone also failed to show IFN-$\gamma$ production on in vitro stimulation with the ICD. That this strong response against HSP110-ICD is tumoroidal is indicated by recent data using neu-transgenic mice that develop spontaneous breast tumors (28). In this system, the HSP110-ICD vaccine significantly delayed the development of spontaneous breast tumors when used at the time of puberty (data not shown). Moreover, this approach to vaccine formulation is not restricted to the protein antigen studied here (i.e., ICD). Recent studies using the melanoma antigen gp100 similarly complexed with HSP110 also demonstrated a strong anti-gp100 immune response and inhibition of B16 tumor growth.6

HSP110 is representative of a family of stress proteins conserved from *Sacharomyces cerevisiae* and *Schizosaccharomyces pombe* to

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man and is distantly related to the HSP70 family (10). Recent characterization of the molecular chaperoning properties of HSP110 demonstrates that it exhibits important functional differences when compared with HSP70 (10). One attribute of HSP110 is its high efficiency in binding to and stabilizing denatured proteins. HSP110 complexes with reporter proteins and totally inhibits their heat induced aggregation at a 1:1 molar ratio. Because interactions of HSPs and partially denatured protein substrates within cells is an important natural function of these molecular chaperones, complexes between HSP and denatured substrate protein as used in this study would reflect chaperone complexes, which are present naturally (13, 14, 16). HSPs have been proposed to be “danger signals,” which alarm the immune system of the presence of tumor or damaged tissues (29). This hypothesis envisions the release of HSPs, carrying peptides from necrotic or damaged cells and their interaction and uptake by APCs. The release of HSPs as a putative danger signal from damaged cells would be expected to also encompass the presentation of HSP-protein complexes, as studied here, in addition to HSP-peptide complexes.

The ability of HSP110 to chaperone and present the ICD of HER-2/neu to the immune system and the resulting strong immune response indicates that HSP is processed via an intracellular pathway, which requires degradation of ICD in APCs into a repertoire of antigenic peptides. This would be expected to include the presentation of both CD8+ as well as CD4+ T-cell epitopes from ICD by APCs, because immunization with the HSP110-ICD complex was able to induce both CD8+ and CD4+ T cells to produce IFN-γ. Moreover, evaluation of the ICD-specific Ab responses in the immunized animals revealed that the HSP110-ICD complex could elicit both Tp1 and Tp2 cells as evaluated by production of IgG2a and IgG1 antibodies, respectively, additionally indicating that the vaccine complex could provide the immune system with CD4+ T-cell epitopes. These results are consistent with previous studies showing that HSPs are able to route exogenous antigens into an endogenous presentation pathway for presentation by MHC class I, as well as class II molecules (30). The mechanism by which HSP110 complexed protein antigens charge MHC class I molecules remains to be determined.

Depletion studies also demonstrated that the HSP110-ICD complex could stimulate CD8+ T cells in the absence of CD4+ T cells. This is consistent with previous studies showing that depletion of CD4+ T cells in the priming phase did not abrogate the immunity elicited by gp96 (7, 31). One explanation for this phenomenon is that HSPs may replace CD4+ T-cell help to convert APCs into the cells that are fully competent to prime CD8+ T cells (32, 33).

Whereas this HSP110 protein vaccine lacks some of the polyvalent benefits of the tumor-derived HSPs, which presumably carries a spectrum of unknown peptides, it also offers important benefits. First, because HSP110 is able to efficiently bind large proteins at approximately an equivalent molar ratio, a highly concentrated vaccine would be expected to carry immunogenic epitopes. The HSP110 vaccine would include numerous peptide epitopes (a single copy of each represented in each full-length protein) bound to each HSP110 molecule. Thus, such a preparation would not only be “partially polyvalent” but would provide both CD4 and CD8 antigenic epitopes. The vaccine would also circumvent HLA restriction, because a large reservoir of potential peptides would be available. Additionally, such a recombinant protein vaccine would not be an individual specific vaccine, as are the tumor-derived HSP vaccines (34), but could be applied to any patient with a tumor expressing that tumor antigen. Furthermore, if an antigenic protein is shared among several tumors, the HSP110 protein complex could well be applied to all of the cancers expressing that protein. For example, in the case of HER-2/neu, HSP110-HER-2 vaccines would be applicable to the treatment of numerous patients with breast cancer as well as some ovarian, prostate, lung, and colon cancers. Lastly, preparation of such protein vaccines would be much less labor intensive than purification of tumor-derived HSP from a surgical specimen. Indeed, a surgical specimen is not required to prepare such a vaccine. The vaccine would also be available in unlimited quantity, and a composite vaccine using more than a single protein antigen (e.g., gp100, MART1, and so on, for melanoma) could be easily prepared.

Aluminum adjuvant, together with calcium phosphate and a squelone formulation are the only adjuvants approved for human vaccine use. These approved adjuvants are not effective in stimulating cell-mediated immunity but rather stimulate a good Ab response (35). We have shown here that HSP110 is a safe mammalian adjuvant in molecular targeting of a well-known tumor antigen, ICD of HER-2/neu, being able to activate both arms of the immune system. In addition, no CD8+ T cell or Ab response was detected against HSP110. This property of HSP110 is particularly interesting in light of the paucity of adjuvants judged to be effective and safe for human use. Because of these points, natural chaperone complexes of HSP110 and protein antigens could be of significant benefit in the treatment of human cancers.

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Development of a Recombinant HSP110-HER-2/neu Vaccine Using the Chaperoning Properties of HSP110


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