Chaperoning Function of Stress Protein grp170, a Member of the hsp70 Superfamily, Is Responsible for its Immunoadjuvant Activity

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Abstract

When used as vaccines, tumor-derived stress proteins can elicit antitumor immune responses. For members of the hsp70 superfamily, like grp170, this seems to be due to (a) the chaperoning of antigenic peptide by the stress protein and (b) the binding of the stress protein to receptor(s) on antigen-presenting cells (APC) and subsequent antigen presentation. This suggests that domains exist on the stress protein for each function. In this study, we determine the ability of grp170 and its structural domains to (a) bind to and present melanoma-associated antigen gp100 to the immune system and (b) to bind to receptors on APCs. A direct correlation between chaperone function, binding to APCs in a receptor-like manner, and antitumor immunity was observed. Two mutants that share no common sequence, yet are both effective in their antitumor activities, compete with one another for APC binding. Studies of other members of the hsp70 superfamily, hsp110 and hsp70, or their domain deletion mutants, further confirmed that APC binding segregates with chaperone function and not sequence. Therefore, these studies suggest that molecular chaperoning is involved in stress protein interactions with APCs, antigen binding, and in eliciting antitumor immunity, thus bridging this ancient function of stress proteins in prokaryotes to their ability to elicit immunity in higher organisms. (Cancer Res 2006; 66(2): 1161-8)

Introduction

Several studies have examined stress proteins/heat shock proteins (HSP) as tumor rejection antigens, most notably, hsc70 and grp94/grp96 (1–3). In some instances, exogenous stress proteins seem to act as vehicles for the delivery of antigens to professional antigen-presenting cells (APC) resulting in cross-priming. It has been shown that hsp70 and hsp90 family members can interact with various receptors on APCs leading to HSP-peptide uptake and antigen cross-priming (4–6), secretion of pro-inflammatory cytokines (7, 8), and maturation of dendritic cells (9). Thus, the adjuvant activity of some stress proteins seems to be several-fold in that they induce both innate and adaptive immunity. The fact that stress proteins of entirely different sequence each possess similar immune functions suggests that a fundamental underlying property of the HSP is involved.

Stress proteins are molecular chaperones, and during stress (e.g., heat shock), act to inhibit the aggregation of other damaged proteins and, in concert with other chaperones, can often refold and reactivate damaged proteins. Molecular chaperones also participate in numerous normal cellular processes such as protein folding, transport, and peptide processing and trafficking (10, 11). The cellular functions of chaperones are essential to all living organisms from prokaryotes to man (12, 13). Grp170 is a major stress protein/molecular chaperone resident in the endoplasmic reticulum (ER; refs. 14–17) that is induced by stress conditions such as hypoxia, ischemia, and interference in calcium homeostasis (18). Studies have shown that grp170 is associated with the folding/processing of secretory proteins such as thyroglobulin and immunoglobulin chains (14, 19), suggesting that it may be involved in protein/peptide import into the ER (20–23).

We have recently described a novel approach to HSP vaccine formulation that uses the potent chaperoning property of hsp110 to form natural chaperone complexes with denatured protein antigens by heat shock (24, 25). Although heat is used as the denaturant of the mature protein in these studies, such chaperone complexes reflect folding and transport intermediates with nascent proteins characteristic of the natural functions of some molecular chaperones (26). In a recent study, a chaperone complex of hsp110 and the melanoma differentiation antigen gp100 was shown to activate antigen-specific T cell responses, leading to growth inhibition of B16 melanoma tumors (25). Importantly, these studies showed that it is the heat-induced chaperone complex itself that is required for immunologic activity and that other preparations containing hsp110 and gp100 (e.g., mixing without heat) show no antitumor activity. In this chaperone complex with gp100, mouse hsp110 (in mouse) was more effective as an adjuvant than was complete Freund’s adjuvant (CFA), suggesting potentially significant clinical applications.

When purified from tumors, grp170 has been identified as a stress protein that can elicit antitumor immune responses. Immunization with tumor-derived grp170 can elicit tumor-specific CD8+ T cell responses and also significantly reduce pulmonary metastatic disease (27, 28). In order to gain insight into the mechanisms of grp170 immunogenicity, we now characterize complexes of gp100 with full-length grp170 and with mutants of grp170 that lack one or more of its structural domains (29). We continue to use melanoma-associated antigen gp100 as the substrate protein for chaperoning, paralleling our previous studies with hsp110, due to the potential clinical significance of this antigen (30, 31).

We show here that the full-length grp170-gp100 chaperone complex generates an effective antigen-specific antitumor immunity in vivo. In addition, domain deletion mutants of grp170 in a complex with gp100 also elicit a potent antitumor immune
response comparable to the full-length grp170-gp100 complex, as long as the deletion mutants are functional as chaperones. In parallel, we examine the ability of grp170 and its mutants to bind to macrophages. It is shown that only those mutants with chaperoning ability bind to macrophages in a receptor-like manner. Two of these grp170 mutants, exhibiting equal chaperoning and immunologic capability, share no common domains or sequence. Therefore, grp170 has two distinct sequence domains that are equally efficient in eliciting immunologic responses when in chaperone complex with melanoma-associated antigen gp100. Two other stress proteins, hsp110 and hsp70, are also examined with regard to APC binding. Again, binding of these proteins or selected domain mutants to macrophages was found to strictly segregate with the ability of the protein to function as a molecular chaperone. The results indicate that it is not an element of sequence or specific domain that is integral to a stress protein's ability to bind to macrophages and elicit an immune response, but is simply its ability to function as a molecular chaperone. Because chaperoning is a promiscuous function, these studies suggest a unifying principle that may explain how entirely different stress proteins of different sequence are able to bind to a variety of receptors on APCs.

Materials and Methods

Mice and cell lines. Eight- to 12-week-old female C57BL/6 mice were purchased from the Department of Laboratory Animal Resources at Roswell Park Cancer Institute or Jackson's Laboratory (Bar Harbor, ME) and maintained in the Department of Laboratory Animal Resources animal facility at Roswell Park Cancer Institute. Human gp100-transduced B16 cells (B16-gp100) were kindly provided by Dr. Alexander Rakhmilevich from University of Wisconsin-Madison (32). The cells were maintained in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Grand Island, NY); 2 mmol/L of l-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The RAW264.7 mouse macrophage cell line from American Type Culture Collection (Manassas, VA) was maintained in DMEM with 4 mmol/L glucose, 4.5 mg/ml sodium bicarbonate, 10% FBS at 37°C and 5% CO2 and 95% air.

Construction and expression of mouse grp170, hsp110, hsp70, and deletion mutants. Grp170 domains were determined by molecular modeling and comparison with the crystal structure of the homologous hsp70 as described previously (29). Grp170 cDNA (mouse) was cloned into a baculovirus transfer vector and a polyhistidine tag sequence was added to grp170, BSA, or ovalbumin in 0.1 mol/L sodium bicarbonate/carbonate buffer. The mixture was incubated for 50°C for 30 minutes. The excess free FITC was removed with a Sephadex G-25 column (Pharmacia, Piscataway, NJ). The F/P (fluorescence/protein) ratio was calculated according to the manufacturer's instructions using the absorbance at 495 nm (FITC absorbance) and 280 nm (protein absorbance). Proteins were then subjected to SDS-PAGE for analysis of the FITC conjugation.

Protein binding to cell surface. FITC-conjugated proteins were incubated with 1 × 10^5/mL of APCs in 100 μL PBS containing 1% BSA on ice for 20 minutes. Unbound protein was removed with three washes with 1% BSA in PBS. The cells were fixed with 1% paraformaldehyde (Fisher, Fair Lawn, NJ) and analyzed by flow cytometry. FITC-conjugated BSA or ovalbumin were used as negative binding controls. For the competition studies, 1, 2, 5, or 10 times mass excess of unlabeled gp170, hsp110, or ovalbumin was added to RAW264.7 cells in the presence of FITC-conjugated grp170 and incubated for 20 minutes.

Statistical analysis. Student's t test was done for statistical analysis and P < 0.05 was considered significant.

Results

Grp170 structure and construction of its deletion mutants. Our previous studies have shown that a tumor protein antigen chaperoned by a large HSP (i.e., hsp110) can be efficiently

Unpublished data.
cross-presented, leading to antigen-specific tumor immunity (25). A detailed relationship between structure and molecular chaperoning function has been recently defined for grp170, another major stress protein related to both hsp110 and hsp70 (29). In order to identify functional domains of grp170 and determine whether immunoadjuvant activity of grp170 is related to chaperoning function, we engineered several domain deletion mutants.

The predicted secondary structure of grp170 was determined using a cassette of programs and by homology modeling with hsp110 and hsp70 as described previously (17, 29). Based on these comparisons, grp170 has an NH₂-terminal ATP binding domain (A), followed by a β-sheet domain (B). These two domains are homologous to the ATP- and peptide-binding domains of hsp70. This is followed by a predicted acidic loop domain (L) that is not seen in hsp70, but is similar to a region seen in hsp110. The loop domain is then followed by α-helical domain (H) that is somewhat similar to the COOH-terminal regions of both hsp70 and hsp110. Based on this secondary structure, we constructed structural deletion mutants as follows (Fig. 1A): no ATP-binding domain (i.e., BLH), no ATP-binding domain and no β-sheet domain (LH), the H domain only (H), a protein lacking only the H domain (ABL), and a protein with ATP-binding domain and β-sheet domain (AB).

Our previous molecular chaperoning studies showed that all of these mutants, except AB, bound to and stabilized heat-denatured luciferase (17). Importantly, these studies indicate that grp170 has two discrete domains, each independently capable of binding and chaperoning “denatured” protein substrate. In the following studies, we use this information to examine chaperoning abilities of grp170 and its mutants employing the melanoma associate antigen gp100. Observations are then extended to other members of the hsp70 superfamily, hsp70 itself and hsp110.

**Grp170 and certain domain deletion mutants prevent aggregation of heat-denatured gp100.** First we determined the ability of full-length grp170 (ABLH in Fig. 1) to inhibit heat-induced aggregation of melanoma-associated antigen gp100. For this purpose, 50°C was selected as the heat shock temperature because our previous studies showed that this is the approximate temperature threshold at which gp100 begins to denature and aggregate (25). Grp170 itself is a relatively heat resistant protein, i.e., it aggregates at 70°C (data not shown). Recombinant gp100 was incubated alone, or in the presence of ovalbumin, grp170 or hsp110 at 50°C. Light scattering was used to measure gp100 aggregation (Fig. 1B). Although ovalbumin was not able to prevent aggregation of gp100, gp100 aggregation is largely inhibited by the addition of full-length grp170 (i.e., ABLH). When grp170 was replaced by BLH, LH, H, or ABL mutant, a similar level of protection of gp100 aggregation was observed. All studies used a 1:1 molar ratio of grp170 or mutant with gp100. The AB mutant did not inhibit gp100 aggregation, despite the fact that it contains the putative “peptide-binding domain” (B) based on homology modeling with hsp70. These studies were confirmed by direct analysis of gp100 protein levels in the supernatant (soluble) and pellet (insoluble) fractions following centrifugation. In the case of grp170, direct binding to gp100 was shown by immunoprecipitation using an anti-grp170 antibody and Western analysis using a gp100 antibody (data not shown). Thus, grp170 (ABLH) and mutants BLH, LH, H, and ABL form efficient “chaperone complexes” with gp100 following heat shock, and protect it from heat-induced aggregation.

**Gp100 elicits specific tumor immunity when chaperoned by grp170 or its functional mutants.** Next we determined whether vaccination of mice with the full-length grp170-gp100 chaperone complex could generate a specific immune response using an ELISPOT assay. Splenocytes derived from animals immunized with the gp100-grp170 complex showed significant IFN-γ production upon gp100 stimulation in vitro, whereas those obtained from naïve mice or mice vaccinated with gp100 or grp170 alone did not (Fig. 2A). Additionally, gp100 mixed with grp170 without heat shock treatment (i.e., no chaperone complex formed) only resulted in a marginal increase in IFN-γ production. Interestingly, a mixture of CFA and gp100 generated a response similar to the gp100-grp170 complex. To exclude the possibility that the IFN-γ-positive spots were due to lipopolysaccharide contamination of the recombinant proteins, irradiated B16 cells transfected with human gp100 (B16-gp100) were used to stimulate splenocytes in vitro. It was found that only splenocytes derived from mice immunized with the gp100-grp170 complex responded to stimulation with the B16-gp100 tumor cells, as determined by IFN-γ secretion. Interestingly, in this study splenocytes from CFA/gp100 immunized mice did not respond to stimulation with the B16-gp100 tumor cells (Fig. 2B). Moreover, sera from mice were also examined to evaluate antigen-specific antibody response. Mice immunized with the gp100-grp170 complex developed significantly high levels of gp100-specific IgG following immunization, compared with other vaccine regimen–immunized animals (Fig. 2C).

Because full-length grp170 in chaperone complex with gp100 elicits a specific immune response, we next examined the structural deletion mutants of grp170.
involvement of its structural domains in this immune response. For this purpose, we examined the grp170 mutants described above, heat-shocked in the presence of gp100, and again determined antigen-specific immune responses. Splenocytes were obtained and assessed for IFN-γ production upon gp100 stimulation using an ELISPOT assay. It was seen that gp100-BLH, gp100-LH, gp100-H, and gp100-ABL complexes all elicited a robust gp100-specific immune response approximately equivalent to that obtained with the chaperone complex of full-length grp170 (ABLH) (Fig. 2D). The AB mutant of grp170 that lacks chaperoning ability was also examined. Splenocytes from AB-gp100–immunized mice also produced IFN-γ, but significantly lower than that induced by grp170 or its functional mutants.

In view of the antigen-specific immune response elicited by gp100 complexed with grp170 and its functional mutants, we next addressed whether these chaperone complexes could also protect mice against subsequent tumor challenge. We used human gp100 cDNA-transduced B16 melanoma cells (B16-gp100) as a tumor model (25, 32). C57BL/6 mice were immunized twice with different vaccine formulations or left untreated. Two weeks after the second immunization, mice were challenged intradermally with B16-gp100 cells (Fig. 2E). Although tumor developed aggressively in naïve mice and mice receiving gp100 alone, a significant tumor growth inhibition was observed in mice vaccinated with the gp100-grp170 (ABLH) complex (versus gp100, P = 0.000043 at day 21), gp100-BLH (P = 0.000033 at day 21), gp100-LH complex (P = 0.00024 at day 21), gp100-ABL complex (P = 0.00277 at day 21), and gp100-H (P = 0.00312 at day 21). Although the mutant AB consisting of the ATP-binding domain and the predicted peptide-binding domain do not exhibit chaperoning functions, immunization with a heated mixture of gp100 and mutant AB did generate a marginal antitumor response. However, it was significantly weaker than full-length grp170 and other functional mutants. From this data and that presented above, once grp170 or its functional mutants bind to gp100 in a chaperone-like manner, the complex can present antigen and generate antigen-specific tumor immunity. Interestingly, the putative “peptide-binding domain” of grp170 (B) is not required for binding to gp100 or for the immunologic functions of the chaperone complex.

Figure 2. The gp100-grp170 chaperone complex elicits gp100-specific tumor immunity. A, C57BL/6 mice (five per group) were immunized i.p. with 30 μg of the gp100 alone, grp170 alone, gp100 emulsified in CFA, gp100-grp170 complex, gp100 mixed with grp170, or left untreated. The vaccinations were repeated 2 weeks later. Two weeks after the second immunization, splenocytes (5 × 10^6 cells/well) were stimulated in vitro with gp100 (20 μg/mL) or concanavalin A (5 μg/mL) for 24 hours, and IFN-γ production was examined using an ELISPOT assay. B, splenocytes were isolated from immunized animals and stimulated with irradiated B16-gp100 cells for 24 hours. ELISPOT assay was done to determine IFN-γ production. C, serum was collected from immunized mice and gp100-specific IgG levels were measured by an ELISA assay (gp100-grp170 group versus gp100 group; P < 0.05). D, mice were immunized with chaperone complexes gp100-grp170 (ABLH), gp100-BLH, gp100-LH, gp100-H, gp100-ABL, or heat shocked gp100/AB mixture. Two weeks after the second immunization, antigen-specific IFN-γ production by splenocytes was measured. E, following immunization with gp170 or its mutant-gp100 complexes, mice (five per group) were challenged with 1 × 10^6 B16-gp100 cells. Similar results were obtained from three independent experiments.
Grp170 and its functional deletion mutants bind to APCs in a saturable and competitive manner. Interaction between HSP and APCs plays a critical role in cross-presentation of HSP-chaperoned antigen and initiation of tumor immunity. It has been suggested that immunologic functions of HSPs are at least partially due to their capability to deliver associated antigens to professional APCs through a receptor-mediated process (34). To extend our studies to the cellular level, we determined the ability of grp170 and its mutants to bind to RAW264.7 macrophages, which can take up exogenous HSP-peptide complexes and represent them on their MHC I molecules in the same manner as primary APCs (5). Cell surface binding of grp170 was measured by mean fluorescent intensity with flow cytometry using FITC-conjugated grp170. FITC-conjugated grp170 binds to RAW264.7 macrophage cells at 4°C in a concentration-dependent manner, and the binding is saturable at ~50 μg/mL (500 nM). FITC-conjugated BSA does not bind to the surface of RAW264.7 cells, indicating the specificity of the binding event (Fig. 3A). Similar results were observed when mouse bone marrow–derived dendritic cells, freshly isolated mouse macrophages, or the JAWS II mouse dendritic cell line were used (data not shown). A competition assay showed that unlabeled grp170 competes effectively with FITC-grp170 for APC surface binding, but unlabeled albumin does not (Fig. 3B). A 10-fold molar excess of unlabeled grp170 reduces the amount of surface bound FITC-conjugated grp170 by ~60%. Therefore, grp170 binds to RAW264.7 macrophages with the characteristics of receptor-ligand interaction. Hsp110, another member of the hsp70 superfamily, is also seen to inhibit grp170 APC binding (Fig. 3B), suggesting that grp170 and hsp110 are likely to bind to the same receptor(s). It is interesting that, at a low concentration of hsp110, some cooperative interaction (i.e., increasing APC binding) is evident.

To investigate which structural domain of grp170 is important for cell surface receptor binding, all grp170 deletion mutants were covalently labeled with FITC and their interaction with APC examined. In contrast to an earlier report showing that hsp70 deletion mutants are unable to bind to the surface of APCs (35), our binding studies showed that all of the grp170 domain deletion mutants (BLH, LH, H, and ABL), except mutant AB, bind to the RAW264.7 cells in a saturable manner, although each seems to have differing affinities (Fig. 3B). In addition, binding of grp170 mutants can be inhibited with unlabeled full-length grp170 (ABLH; Fig. 3C).

The studies shown in Fig. 1B indicate that BLH, BL, H, and ABL deletion mutants are equally efficient at chaperoning heat-denatured gp100 whereas AB is not. Thus, the results shown in Fig. 3A show a direct correlation between the ability of the mutants to bind to APCs and their ability to chaperone gp100. The studies shown in Figs. 2B and C show a correlation between chaperoning and the capacity to elicit an antigen-specific immune response and inhibit B16 tumor growth. In addition, two grp170 deletion mutants studied, i.e., ABL and H, differ totally in sequence. Yet each exhibits a similar ability to chaperone gp100, bind to APCs, and elicit a specific immune response. Thus, the ancient property of molecular chaperoning seems to be the common denominator underlying these observations.

To pursue this last point concerning ABL and H, we examined whether these two entirely different mutant proteins can cross-compete with each other for APC surface binding. As shown in Fig. 4A, unlabeled ABL or unlabeled H, but not ovalbumin, effectively competed for APC binding of FITC-conjugated ABL (Fig. 4A). Similarly, unlabeled ABL or H competed for APC binding of FITC-conjugated H (Fig. 4B). Therefore, these two sequence-independent components of grp170, each with comparable chaperone and vaccine abilities, bind to the same receptor(s).

Binding of hsp110 and hsp70 and their H domains to macrophages. Grp170 is a member of the hsp70 superfamily (17) and exhibits a structural organization similar to hsp110 and hsp70 as described above (Fig. 1). As in the case of grp170, hsp110, and...

**Figure 3.** Grp170 and its functional mutants interact with APCs in a receptor-mediated fashion. A, grp170 and its functional mutants bind to RAW264.7 macrophage cells in a specific, saturable manner: FITC-conjugated grp170 or its domain deletion mutants (BLH, LH, H, ABL, or AB) were incubated with 10^6/mL RAW264.7 macrophages at 4°C for 20 minutes. Cells were washed thrice with 1% BSA in PBS, fixed with 1% paraformaldehyde, and subjected to flow cytometry analysis. B, grp170 binding was competed by unlabeled excess amount of grp170 and hsp110. FITC-conjugated grp170 (50 μg/mL) were incubated with macrophages at 4°C in the presence of unlabeled grp170, hsp110, or ovalbumin with different concentrations. The cells are subjected to flow cytometry after removing excess unbound protein. C, FITC-conjugated grp170 or its functional deletion mutants (BLH, LH, H, or ABL; 50 μg/mL) were incubated with macrophages at 4°C in the presence of unlabeled full-length grp170. Surface binding of FITC-labeled proteins was measured as described. Mean fluorescent intensity (MFI) of each domain deletion mutant is set to 100% binding. Data are representative of at least three separate experiments.
hsp70 also possess a COOH-terminal α-helical domain (H). Both hsp110 and hsp70 are molecular chaperones and can inhibit heat-induced protein aggregation (26). However, whereas grp170-H can bind to and protect heat-denatured protein (e.g., gp100), hsp110-H and hsp70-H cannot (i.e., they do not possess notable molecular chaperoning ability; refs. 29, 36). We therefore examined the ability of the hsp110 and hsp70 as well as their H domains to bind to macrophages in parallel to the above described grp170 and grp170-H studies. As shown in Fig. 5, full-length hsp110 and hsp70 bind to RAW264.7 cells. In contrast, their H domain mutants show little or no binding ability, again demonstrating a relationship between the ability to act as a chaperone and the ability to bind to macrophages.

Discussion
Grp170 is a major molecular chaperone/stress protein resident in the ER. It is distantly related in sequence to both hsp110 and hsp70 families, but represents a member of “its own” grp170 stress protein family (15–17). Studies have suggested that grp170 is an important element of the protein processing machinery of the ER (14, 19, 20) and the molecular chaperoning properties of grp170 have been recently described (29). These chaperoning studies show that, unlike hsp110 and hsp70, grp170 has two distinct chaperoning domains as determined using luciferase as a reporter protein. One seems to be the classic β-sheet peptide binding domain, characteristic of this family of stress proteins, with some COOH-terminal sequence. The second is its COOH-terminal α-helical domain. Utilizing recombinant grp170 and several of its domain deletion mutants, the present study characterizes (a) their ability to bind and chaperone melanoma-associated antigen gp100, (b) their interactions with APCs, and (c) their antigen-presentation capacities.

We show here that grp170 forms a molecular chaperone complex with melanoma-associated antigen gp100 during heat shock, protecting it from heat-induced aggregation (Fig. 1B). Next, we show that immunization of mice with gp100 chaperoned by grp170 elicits a strong immune response against gp100 as determined by antigen-specific IFN-γ production, antibody response, and antitumor immunity (Fig. 2). In our earlier work, we demonstrated the immune functions of T cells following vaccination with hsp110 (a cytosolic homologue of grp170)-gp100 complexes using cytotoxicity and in vivo T cell subset depletion assays (25). In combination, these studies argue strongly for the involvement of a gp100-specific T cell response in the antitumor immunity generated by the grp170-gp100 chaperone vaccine. We also observed that grp170, as an immunoadjuvant for vaccine formulation, is superior to CFA for priming antigen-specific immune responses (Fig. 2B). These results are in agreement with our recent studies using hsp110 in complex with heat-denatured gp100 (25). In those studies, it was shown that the hsp110-gp100 chaperone complex was highly effective in inhibiting in vivo tumor growth and that it was the chaperone complex that was essential for immune function (e.g., a mixture of hsp110 and gp100 was ineffective).
The present study also examines the immunologic and anti-tumor activities of domain deletion mutants of grp170. These mutants were constructed based on computer modeling as described previously and the predicted structures were confirmed, in some cases, by circular dichroism (29). We show that some of these mutants are able to bind to and stabilize heat-denatured gp100 with a chaperoning efficiency comparable to full-length grp170. When examined as vaccines, each of these grp170 mutant-gp100 complexes activated antigen-specific immune responses, and exhibited antitumor activity. Moreover, these mutants are found to be as efficient as the full-length grp170-gp100 vaccine, but only when the mutant used had chaperoning function (Fig. 2D and E). The AB mutant, which did not exhibit apparent chaperoning ability or APC binding, only induced a marginal IFN-γ production and antitumor immunity. It is possible that the AB mutant may still retain some weak protein-binding activity. Alternatively, AB may have the capacity to stimulate immune components that are not identified. Most recently, it was shown that the NH2-terminal domain deletion mutant of grp94/grp96, a region lacking peptide-binding ability, could still activate innate immunity and generate an antitumor response (37, 38). Interestingly, two of these grp170 mutants with both chaperone and vaccine activities share no common sequence, i.e., the ABL and H mutants. Thus, the generation of antigen-specific tumor immunity depends on the ability to act as a molecular chaperone and is independent of any sequence element or structural domain.

Earlier studies have shown that tumor-derived stress proteins including grp94/grp96, hsc70, calreticulin, hsp110, and grp170 can each exhibit antitumor immunity (2, 3, 27, 39). These chaperones have no common sequence elements and the means by which entirely different autologous chaperones mediate antigen presentation has remained an unanswered question. The studies presented here could explain how various stress proteins/molecular chaperones that derive from sequence-independent families can both bind to APCs and act in antigen presentation.

Previous studies by others, employing different end points and approaches, have examined the immunologic activity of mutants of hsp70. These studies seem to come to somewhat different conclusions with regard to whether it is the NH2- or COOH-terminal region of hsp70 that is necessary for its immunologic activity. Two of these studies suggest that “immune function” segregates with the COOH-terminal region of hsp70 that is necessary for its immunologic activity. Two of these studies suggest that “immune function” segregates with the COOH-terminal region that contains the hsp70 peptide-binding domain (40, 41). The other two studies, using fusion constructs, implicate the NH2-terminal part of the hsp70 protein in its immune functions (42, 43). A recent study by MacAry et al, shows that the ability to induce cytokine secretion and to present antigen are separable functions for microbial hsp70. Although we look at APC binding and not cytokine expression, we find that antigen presentation and chaperone functions coincide. It could be argued that the correlation of chaperoning and receptor binding observed here is coincidental. Although possible, this seems unlikely. To expand on this point, we also examined two other stress proteins, hsp110 and hsp70. Both exhibit domain structures similar to grp170 (29, 33). The H domain of hsp110 (hsp110-H) shows similarity in sequence and organization to grp170-H. However, unlike grp170-H, hsp110-H has no significant chaperoning ability (29). When examined for its ability to bind to macrophages, hsp110-H was not able to bind whereas full-length hsp110 itself exhibits binding similar to that of grp170 (Figs. 3A and 5A). Similar results were obtained using hsp70 and its nonfunctional hsp70-H mutant (Fig. 5B). These findings further emphasize the importance of chaperoning in receptor binding and extend this observation to two other major stress protein families. Perhaps, the more significant difference between MacAry et al. and this study is that MacAry and all of the other abovementioned hsp70 studies employ a microbial hsp70 protein which is itself a potent immunogen (44, 45). Wang et al. reported that microbial but not human hsp70 elicit cytokine release in a system using human monocytc cells (40). In our studies, we use autologous (mouse in mouse) grp170. However, we found that mouse hsp110 or grp170 could simulate cytokine release from mouse bone marrow-derived dendritic cells (46, 47). Another important difference between the present study and other studies is that we examine grp170-protein interactions similar to previously described interactions exhibited by grp170, hsp70 and other stress proteins in situ (14, 19, 48), and not HSP-peptides. Studies using tumor-derived autologous HSPs (3, 49) may also include proteins as well as peptide antigens, although peptides have been the key point of discussion. The binding of a diverse background of different proteins in HSPs/glucose-regulated proteins purified from tumor might not be readily detectable.

This and our previous study of grp170 define two sequence-independent chaperoning domains in B (-L) and H (29). Assignment of one of these regions to the B domain is based on structural analysis of grp170 and on earlier studies of other members of this stress protein family, i.e., hsp70 and hsp110 (33, 50). Grp170 is homologous to and possesses a domain structure similar to both hsp70 and hsp110, including a β-sheet domain which has been identified in hsp70 by crystallographic analysis as its peptide binding domain (50). Nonetheless, a slight degree of caution is still warranted in immediately accepting this interpretation for grp170-B and further studies of B (-L) are necessary. In addition, the specific basis for the chaperoning ability of grp170-H requires a more detailed analysis. Additional studies to precisely define the exact peptide binding motifs in each of these domains are under way.

The present study has shown that two domain deletion mutants (i.e., ABL and H) of grp170 exhibit equal chaperoning and immunologic capabilities whereas sharing no common sequence elements. Furthermore, we have shown that different stress proteins, hsp110 and grp170, compete with one another for APC binding and exhibit strong vaccine activity when chaperoning gp100 (present work; ref. 25). In all of these studies, only chaperone functional stress proteins or mutants are effective immune adjuvants. Thus, it seems to be function and not sequence that is the essential feature involved in efficient APC binding, antigen presentation, and anti-tumor activity.

It is clear from the studies described here that grp170 and its functional mutants are capable of binding to macrophages (Fig. 3). In addition, they do so irrespective of whether grp170 or the derived chaperone is binding a substrate protein or not, because we observed that a bound protein substrate does not inhibit binding to APCs (data not shown). This would argue for the existence of different domains for APC binding and chaperoning of substrate protein. However, the present study suggests that this seems to be incorrect. It is possible that the molecular chaperone itself may be able to bind to and chaperone more than a single substrate protein in the same chaperone binding region, thus accommodating both the protein antigen and the receptor simultaneously at the same site. Studies examining this point are under way.
The ability of stress proteins to act as molecular chaperones underlies their essential functions in all living organisms. The present study argues for the extension of this ancient property of prokaryotic stress proteins to APC receptor binding, antigen presentation and immune function at the mammalian level. Because stress proteins share a common function of molecular chaperoning, these studies may provide insight into how entirely different stress proteins express APC receptor-binding ability and different immunological activities.

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Chaperoning Function of Stress Protein grp170, a Member of the hsp70 Superfamily, Is Responsible for its Immunoadjuvant Activity

Jun-Eui Park, John Facciponte, Xing Chen, et al.