Cancer Immunol Immunother (2006) 55: 339–346
DOI 10.1007/s00262-005-0020-y

SYMPOSIUM IN WRITING

**John G. Facciponte · Xiang-Yang Wang
Ian J. MacDonald · Jun-eui Park · Hilal Arnouk
Melissa J. Grimm · Ying Li · Hyung Kim
Masoud H. Manjili · Douglas P. Easton
John R. Subjeck**

Heat shock proteins HSP70 and GP96: structural insights

Received: 15 February 2005 / Accepted: 25 April 2005 / Published online: 20 July 2005

Abstract Several heat shock proteins (HSPs) act as potent adjuvants for eliciting anti-tumor immunity. HSP-based tumor vaccine strategies have been highly successful in animal models and are undergoing testing in clinical trials. It is generally accepted that HSPs, functioning as chaperones for tumor antigens, elicit tumor-specific adaptive immune responses. HSPs also appear to induce innate immune responses in an antigen-independent fashion. Innate responses generated by HSPs may contribute to anti-tumor immunity. Immunologically active chaperones with anti-tumor activity are referred to as "immunochaperones". Here, we review the studies that address the role of structural domains or regions of the immunochaperones HSP70 and GP96 that may be involved in the induction of adaptive or innate immune responses.

Keywords Heat shock proteins · Antigen presenting cells · Anti-tumor immunity · Adaptive immunity · Innate immunity

Abbreviations APCs: Antigen presenting cells · CTD: C-terminal domain · CTL: Cytotoxic T lymphocyte · DCs: Dendritic cells · HSPs: Heat shock proteins · IFN: Interferon · LPS: Lipopolysaccharide · LOX-1: Lectin-like oxidized low-density lipoprotein receptor · MHC: Major histocompatibility complex · NK: Natural killer · NO: Nitric oxide · NTD: N-terminal domain · PBD: Peptide-binding domain · PRR: Pattern recognition receptor · RANTES: Regulated on activation, normal T expressed and secreted · SR-A: Scavenger receptor-A · TLR: Toll-like receptor · TNF: Tumor necrosis factor

This article forms part of the Symposium in Writing "Thermal stress-related modulation of tumor cell physiology and immune responses", edited by Elfriede Noessner.

J. G. Facciponte (✉) · Y. Li
Department of Immunology, Roswell Park Cancer Institute,
Buffalo, NY 14263, USA
E-mail: john.facciponte@roswellpark.org
Tel.: +1-716-8454433
Fax: +1-716-8458899

X.-Y. Wang · I. J. MacDonald · M. J. Grimm · J. R. Subjeck
Department of Cellular Stress Biology,
Roswell Park Cancer Institute,
Buffalo, NY 14263, USA

H. Arnouk
Department of Pathology,
Roswell Park Cancer Institute, Buffalo, NY 14263, USA

H. Kim
Department of Surgery, Roswell Park Cancer Institute,
Buffalo, NY 14263, USA

X.-Y. Wang · H. Kim
Department of Urology, Roswell Park Cancer Institute,
Buffalo, NY 14263, USA

J. Park
Department of Medicine and Swim Across America Laboratory,
Memorial Sloan-Kettering Cancer Center,
1275 York Ave., New York, NY 10021, USA

M. H. Manjili
Department of Microbiology and Immunology,
Virginia Commonwealth University,
Richmond, VA 23284, USA

D. P. Easton
Department of Biology, State University of New York,
College at Buffalo, Buffalo, NY 14222, USA

Introduction

More effective therapies for treating many human cancers are needed. Several heat shock proteins (HSPs) elicit anti-tumor activity as demonstrated in pre-clinical studies. The HSPs that are effective as adjuvants include HSP70, HSP90, HSP110, GRP94 (GP96), GRP170 and calreticulin [1–4]. Autologous tumor-derived GP96 has been evaluated against several cancers in human clinical trials [5–8]. Positive human immunological responses have also been generated against tumors in vitro with HSP70 [9]. These HSPs are characterized as potent immunoadjuvants, helping the immune system recognize specific tumor antigens. Due to this property, HSPs are extremely important, since few conventional adjuvants to date are

safe for clinical testing. Since the primary role of HSPs is to act as molecular chaperones, these immunologically active chaperones that also possess anti-tumor activity are referred to as "immunochaperones" in this review [10].

HSPs in general are categorized as heat shock proteins or glucose regulated proteins (GRPs) based on the environmental stressors that induce their expression [11]. Both of these groups are collectively referred to here as HSPs or stress proteins. Within cells, HSPs are found in many organelles and in the cytoplasm, while the majority of GRPs are localized in the eukaryotic endoplasmic reticulum (ER).

The initial immunological interactions that lead to HSP-mediated anti-tumor immunity are not well defined. Studies are ongoing to attempt to characterize the mechanisms by which HSPs elicit immune responses to tumors. There appear to be two main functions of HSPs that are critical for their adjuvant properties. The first is their ability to chaperone a myriad of peptides and proteins. This property is believed to confer specificity to HSPs against individual tumors.

The second property is the ability of HSPs to bind to surface receptors on antigen-presenting cells (APCs). This interaction can result in receptor-mediated endocytosis of the associated peptides/proteins, facilitating cross-presentation via major histocompatibility class I (MHC class I). Exogenous antigen uptake usually results in classical MHC class II presentation that can generate CD4+ T cell activation. Cross-presentation is the process by which exogenous antigens are targeted for presentation by MHC class I for activation of CD8+ T cells, which in turn develops into cytotoxic T lymphocyte (CTL) responses. HSPs enable efficient cross-presentation in vitro with subsequent activation of antigen-specific CTL responses which can directly contribute to potent anti-tumor immunity [12, 13]. Thus, this second property of HSPs can result in antigen-specific, adaptive immune consequences.

Receptor binding may also induce cell-signaling cascades, resulting in the induction of innate immune responses, including maturation of dendritic cells (DCs) and activation of macrophages. Other important non-antigen specific consequences by HSPs include secretion of pro-inflammatory cytokines by APCs and induction of natural killer (NK) cells that may modulate the immune response. We focus here on HSP70 and GP96 immunochaperones. In some cases, the described studies may be applicable to other stress proteins (e.g., calreticulin). However, in general one should be careful in extending these observations about HSP70/GP96 to other HSP/GRP stress proteins.

Immunochaperones: chaperoning ability and chaperokine function

Adaptive immune responses

Certain mouse sarcomas were initially found to be immunogenic in that immunization leads to rejection upon subsequent challenge with the same tumor. Intensive studies in search of tumor rejection antigens from these murine tumors led to the identification of HSPs (GP96) as the immunogenic component [14]. Since the identity of shared tumor antigens in the original sarcomas were lacking, GP96-derived peptides were consequently considered unique to each tumor [15, 16]. Therefore, the initial proposed mechanism for the generation of GP96-mediated immunity (and other immunochaperones) was based on the premise that these chaperones associate with immunologically relevant peptides from the tumor cells from which they were isolated [17]. HSP70 preparations purified from different murine sarcomas also elicits an immune response solely against the tumor from which the HSP70 was isolated [1]. Several early experiments support also this hypothesis for HSP70 as discussed below.

HSP70 can be viewed as having two structural domains, an N-terminal ATP-binding domain and a C-terminal peptide-binding domain (PBD). The PBD of HSP70 is responsible for binding unfolded protein substrates and is regulated by nucleotide binding in the ATP-binding domain [18]. The ADP-bound state favors polypeptide binding, while the ATP-bound state favors release [19]. Addition of ATP to tumor-derived HSP70 followed by removal of low molecular weight fractions depletes the associated antigenic peptides from HSP70 and abolishes the associated adjuvant function as measured by tumor rejection assays [20]. The ability of ATP to affect immune function of HSP70 was supported by results from another study that generated peptide-specific T cell responses after HSP70/influenza virus peptide immunization in mice. Addition of ATP rendered the HSP70 non-immunogenic as measured by T cell proliferative responses to the influenza peptide [21]. Therefore, HSP70 immunogenicity was attributed to physical association of HSP70 with peptides.

Other indirect data also suggests that peptides may be important for the specificity of HSPs. Immunization with HSP70 or GP96 preparations derived from normal tissues, *in general*, does not elicit immunity to tumors [1, 20]. This observation further strengthened the argument that peptide-specific immunity is generated by HSPs toward individual tumors. Recently, HSP70 and GP96 have been found to associate with tumor-derived peptides (e.g., the differentiation antigens Mart-1 and tyrosinase) from different human tumors and activate tumor antigen-specific CTL in vitro [9, 22]. Identification of immunogenic peptides found in complex with HSPs is a daunting task but may reveal new tumor-associated antigens [23, 24]. However, the biochemical basis for peptide interactions by chaperones, particularly GP96, is not entirely clear in vivo [25]. Additionally, GP96 exhibits very weak ATP-binding activity in vitro compared to HSP70 [26–28]. It is therefore difficult to conclude that GP96 uses ATP for peptide-binding reactions. Accordingly, observations made for HSP70 may not be directly applicable to GP96, since these

chaperones are distinct from one another in sequence, structure and function as discussed later.

HSP-peptide complexes have also been prepared in vitro as cancer vaccines. HSP70/GP96 complexes are as effective as tumor-derived HSPs for CD8+ T cell activation and anti-tumor immunity [29]. Picogram quantities of peptides complexed to HSP70 are sufficient for generation of antigen-specific CD8+ T cell responses [29, 30]. Complex formation between HSP70/GP96 and peptide appears to be required for generation of peptide-specific CTL responses [29, 31]. However, HSP-peptide complexing in vitro may not represent a natural chaperoning interaction. Reports have suggested that GP96-peptide complexing may induce tertiary conformational or multimeric changes in structure that may account for HSP-peptide complex immunogenicity [25, 32, 33].

Design of HSP fusion constructs, in the form of DNA or protein based vaccines, circumvent the need for non-covalent complex formation between HSP and peptide [34, 35]. However, HSP fusion proteins are non-native proteins and questions arise concerning proper protein folding, chaperoning activity and ability to bind to APC. As an example, the gene for E7 from Human Papilloma Virus 16 was genetically fused to HSP110 and expressed as a fusion protein. Upon immunization, this fusion protein was an inefficient anti-tumor vaccine, possibly due to one or more of the factors mentioned above (Li et al. unpublished observations).

The ability of HSP-peptide complexes to generate CTL also appears to correlate with the peptide-binding affinity by the HSP. The fusion of an immunogenic peptide with a high affinity linker protein, which resulted in an increased affinity for HSP70, was shown to enhance CTL responses [36].

These HSP-bound peptides are internalized by APCs with subsequent generation of adaptive responses, recapitulating what is believed to occur with tumor-derived HSPs [37]. Cross-presentation is only mediated by receptor-mediated endocytosis of the HSP-antigen complex, emphasizing the importance of endocytic receptors in the generation of adaptive immunity [38, 39].

Lastly, it must be noted that although antigen-specific CD8+ T cells can be activated by HSP immunization, it is difficult to conclude if T cell responses directly account for the measured partial responses in clinical trials [8]. NK cells and CD4+ T cells may also play a role in HSP-mediated immunity and is discussed in the next section and the structural studies section, respectively.

Innate immune responses

Reports throughout the HSP literature have suggested mechanisms by which HSPs generate anti-tumor activity in addition to adaptive immune responses. HSPs derived from normal liver *can* have protective effects in a prophylactic setting. A delay in tumor onset and a small increase in survival were observed for mice immunized with liver-derived HSP70 compared to controls in the methylcholanthrene-induced sarcoma MC57X model [40]. In a seminal study, normal liver-derived GP96 had partial curative effects in a pre-established metastatic Lewis lung (D122) carcinoma model, suggesting a peptide-independent immunostimulatory activity by GP96 [16]. HSP70 derived from mouse B16 melanoma can immunize against the allogeneic tumor CT26 [41]. The anti-tumor immunity observed in this experiment may be generated from peptides derived from a viral gp70 protein that is shared between B16 and CT26 tumors. However, HSP70 generated minimal CTL activity against CT26 in vitro, suggesting an alternate mechanism of immunity. In another cross-presentation study in vitro, HSP70 purified from human melanoma was pulsed onto APCs, with subsequent activation of anti-melanoma CTL as measured by IFNγ secretion [42]. Curiously, IFNγ secretion by anti-influenza CTL was also observed when APCs were pulsed with high doses of melanoma-purified HSP70. This study suggested that CTL activation could be mediated by the natural adjuvant function of HSP70, although direct effector function such as CTL killing was not assayed in this study.

HSP70 or GP96 can also induce a number of innate immune responses in vitro [37, 43]. Both can upregulate surface expression of MHC class II and CD86 as well as stimulate secretion of pro-inflammatory cytokines IL-1 β, IL-6, TNF-α, and IL-12 [44, 45]. These HSPs can activate DCs via the Toll-like receptor 2/4 (TLR) pathway [46, 47]. Hsp70 can mediate pro-inflammatory signaling in a CD14- and MyD88-dependent fashion, described as the chaperokine activity of HSP70 [48–50]. However, these results and others have been called into question due to the immunostimulatory effects of endotoxin that may be present in HSP preparations [51, 52]. In addition to effects on APCs, HSP70 may also activate NK cells, part of the innate immune system. Tumor membrane-bound HSP70 may activate certain NK populations that lyse targets in an MHC class I-independent manner [53–55]. Synthetically generated peptide (14-mer), derived from the HSP70 amino acid sequence, was also found to stimulate NK cell activity in an endotoxin-free system [56]. HSPs may therefore have direct immunological effects on NK cells in the absence of endotoxin. In addition, human NK cells seem to express low levels of TLRs, suggesting that any endotoxin present in HSP preparations may exert minimal effects on NK cells [57].

HSPs were therefore proposed to generate anti-tumor immunity that did not require bound peptides [23, 58]. In addition to the aforementioned anecdotal reports, more recent evidence support this hypothesis also. Antigen-independent effects of GP96 have been observed in vivo in the *Xenopus* (frog) model. Larvae tadpoles are immunocompetent but they do not express MHC class I until metamorphosis into the mature frog. GP96 derived from tumor as well as normal tissue can equally generate anti-tumor responses in an antigen-independent manner in tadpoles [59]. Similarly, in adult

frogs immunized either with tumor or normal tissue derived-GP96, CD8+ T cell-depleted splenocytes display consistent potent in vitro cytotoxicity against MHC class I negative tumor but not against MHC class I+ lymphoblast targets [60]. An anti-NK antibody partially blocks killing against MHC class I negative targets by effectors that do not express the CD8 marker. Thus, in *Xenopus*, GP96 can generate NK-like activity that does not depend on the source of GP96, in addition to a tumor-specific CD8+ T cell response.

Proper activation of innate immunity may also be necessary for an effective adaptive response [61, 62]. A recently proposed mechanism for HSP-mediated anti-tumor activity bridges innate and adaptive responses in vivo. GP96 is believed to initially activate APCs, which then stimulates NK and CTL expansion. EG7 is an ovalbumin-transfected EL4 thymoma cell line that forms lethal tumors in syngeneic C57BL/6 mice. Immunization with EG7 cells engineered to secrete GP96-immunoglobulinG (IgG) fusion protein generates specific anti-tumor immunity that was abrogated with anti-CD8 antibodies [13]. A subsequent study demonstrated that NK1.1+ cell numbers increased 24 h after immunization while numbers of CD11c+ APCs also modestly expanded [63]. The authors suggested that EG7-GP96-Ig recruits CD11c+ APCs with subsequent expansion of NK cells, essential for subsequent CD8+ T cell expansion [64]. Cancer patients immunized with autologous tumor derived-GP96 appear to generate an inherently non-specific NK cell response in addition to a tumor-specific CTL response [5, 22]. Expansion of CTLs and generation of effective anti-tumor immunity by HSPs may therefore depend on innate immune stimulation. Experiments that investigate the effects of GP96 directly on NK cells may further support these findings. Both immune compartments should therefore be monitored in order to better understand the immune mechanisms that are elicited by various immunochaperones.

Structural studies: ATP-binding domains or peptide-binding domains?

To exploit immunochaperones for adjuvant use in humans, basic knowledge about the minimal regions required to generate adaptive as well as innate functions are desirable. Truncated fragments of HSPs that retain full immunological functions, compared to full-length protein, may reduce the likelihood of autoreactivity to self-HSPs [65, 66]. Different families of HSPs have different structures that are suited to different functions. The proposed dumbbell-shaped HSP70 may act as a molecular clamp, binding to newly synthesized polypeptides in the cytoplasm, promoting correct protein folding and preventing aggregation [10, 67, 68]. GP96 is proposed to exist as a homodimer, which may bind and help in the folding of immunoglobulin heavy chain in the ER [69–71]. In an attempt to define functional epitopes within HSP molecules, several groups have begun to investigate which regions and/or functional domains of HSP70 and GP96 stimulate adaptive or innate immune responses. The following sections review the literature on immune effects generated by the N- and C-terminal regions of HSP70 and GP96.

HSP70

In addition to the N-terminal ATP-binding domain (A) and β-sheet containing the PBD (B), HSP70 family members have an α-helical domain present at the C-terminus (H) [72]. The HSP70 H domain acts as a "lid" which decreases on/off rates for bound substrate [73]. The ATP-binding domain of HSP70 has ATPase activity and influences the affinity of the PBD for unfolded proteins [18]. ATP nucleotide hydrolysis leads to conformational changes in the PBD, indicating communication between these two domains [74]. Interdomain communication results in modulation of peptide affinity and ATPase activity [75–77]. The mechanism of HSP70 chaperoning function has been modeled after the mechanism of interactions of hexokinase and actin with substrate. The tertiary structure of the HSP70 ATP-binding domain is similar to the ATP-binding domains of hexokinase and actin that also have open and closed conformations and are substrate dependent [78].

The N-terminal region of HSP70 was shown to generate CTL activity upon immunization in two early studies. These initial studies utilized HSP70 constructs fused to peptide and expressed as a fusion protein. Ovalbumin peptide was fused to four segments of mycobacterial HSP70; the ATP-binding domain (N-terminal and C-terminal halves), and the PBD and H domains. The C-terminal half of the ATP-binding domain of mycobacterial HSP70 (amino acids 160–370) was found to be essential for generation of CTL against the ovalbumin peptide [79].

Another group cleaved 100 amino acid long fragments starting from the N-terminus of murine HSC70, the constitutively expressed homologue of HSP70, and fused the resulting mutants to a peptide derived from a *Plasmodium* protein at the C-terminus of the deletion mutants [80]. The critical region of HSC70 required for generation of CTL responses was mapped to residues 280–385 in the ATP-binding domain, although the PBD also induced CTL responses. The authors of these studies suggest that the ATP-binding domain may activate APCs, up-regulating costimulatory molecules and inducing secretion of pro-inflammatory cytokines [79, 80]. The latter group suggests that APC binding may occur via pattern-recognition receptors such as TLRs that have been identified as receptors for HSPs [47, 49]. The proximal region of the ATP-binding domain and the adjacent PBD and C-terminal regions of HSP70 may form a structure that permits binding to APCs and subsequent endocytic uptake of HSP70 fusion proteins and activation of CTL [80]. However, the possible lack of proper tertiary folding of these deletion mutants in

these studies may complicate the observed responses as discussed earlier in this review.

One recent study dissected the peptide-binding activity of HSP70 and its ability to generate CTL from inherent innate activity. Human DCs pulsed with mycobacterial HSP70-peptide complexes efficiently generated peptide-specific CTL as measured by CTL lysis [30]. The HSP70-peptide complex was found to be more potent than high concentrations of peptide alone or LPS-stimulated DC pulsed with peptide, indicating that CTL generation is not simply due to activation of DC. The wild-type peptide-binding region of HSP70 without the ATP-binding domain, (i.e., HSP70 PBD mutant), with high affinity for peptide, was sufficient for eliciting a CTL response in vitro. Other HSP70 PBD domain deletion mutants, with amino acid substitutions that significantly reduce peptide affinity, were unable to generate an antigen-specific CTL response. However, these HSP70 PBD mutants were able to induce release of chemokines and cytokines by DCs, separating the innate and adaptive functions of HSP70 [30]. Furthermore, the innate compartment was shown to augment antigen-specific CTL activation. Addition of excess peptide-free HSP70 to DCs could increase CTL killing when a sub-optimal concentration of wild-type HSP70/peptide was tested as a stimulus. However, peptide-bound HSP70 is necessary for efficient CTL generation; peptide-free HSP70 or excess free peptide led to minimal CTL lysis [30, 81]. The authors proposed that the PBD of HSP70 could induce innate responses that can augment CTL responses produced by the antigen-complexed PBD of HSP70.

Several groups have begun to investigate which regions and/or functional domains of HSPs bind to specific receptors on immune system cells and result in stimulation of adaptive or innate responses. All three domains of HSP70 (ATP-binding domain, peptide-binding domain and C-terminal helices) are required for binding to a macrophage cell line as visualized by immunofluorescence [82]. In addition to synthetic peptides derived from HSP70, the C-terminal domain (CTD) of HSP70 stimulates the cytolytic activity of naive NK cells against HSP70-positive tumor target cells. Binding of recombinant HSP70 CTD to NK cell line (YT) was also demonstrated by immunofluorescence studies [55].

In addition to binding to the NK cells, the CTD of HSP70 was shown to bind to the CD40 receptor on APCs. Mycobacterial HSP70 CTD containing the PBD stimulated mononuclear cells to release the chemokine RANTES [83]. Secretion of the chemoattractant was not inhibited by polymyxin B, which is believed to inhibit the effects of endotoxin. These observed innate immune effects by mycobacterial HSP70 was dependent on the cell surface expression of CD40. A follow-up study demonstrated that the CTD of mycobacterial HSP70 is also responsible for secretion of T_H1-polarizing cytokine IL-12, as well as TNF-α and NO by human monocytes [65]. Different epitopes within the peptide-binding domain of mycobacterial HSP70 were found to induce or suppress cytokine production by monocytes and DCs, as well as maturation of DCs [84]. Thus, in addition to chaperoning antigens that generate adaptive immune responses, the HSP70 PBD may also induce receptor-mediated innate responses, similar to that observed by MacAry et al. [30].

The underlying theme in most of the studies described here is the role of surface receptors on APCs for initiation of adaptive and innate responses [39]. Endocytic receptors for HSPs include scavenger receptors CD91, LOX-1, SR-A, and CD36 [85–88], while activating receptors include TLR2/4 and CD40 [46, 47, 83]. Identification of HSP receptors and their consequences on immune cells is in its infancy and may play a pivotal role in our understanding of the generation of adaptive and innate immunity. Data suggests the CTD of HSP70 can interact with specific receptors of immune cells such as CD40; other identified receptors await future study. Studies that address the structural domains of GP96 that interact with specific receptors should also be informative.

GP96

GP96 is a homologue of the cytosolic HSP90 and resides in the ER [89, 90]. GP96 exists as an obligate homodimer, and adopts a tail-to-tail orientation [71, 91]. Overall, the relationship between GP96 structure and function, such as peptide binding, is poorly understood compared to HSP70 [25]. A peptide-binding site for an extended version of VSV8 was mapped to a highly conserved region of GP96, next to the dimerization domain at the C-terminus [92]. VSV8 is an immunogenic peptide epitope of Vesicular Stomatitis Virus N protein. The peptide-binding site of GP96 was found by an in vitro peptide cross-linking technique followed by peptide sequencing and confirmed by mass spectroscopy [92].

Initial studies suggest that innate immunity may originate at the N-terminus of GP96 and may be independent of the HSP-bound peptides. Two GP96-secreting cell lines (4T1 and NIH 3T3) were tested as adjuvants against 4T1 mammary tumor challenge. Interestingly, the allogeneic 3T3 fibroblast cell line secreting GP96 was effective against 4T1 tumor, in addition to the autologous 4T1 tumor cell secreting-GP96, for suppression of tumor growth [93]. The possibility of shared antigens bound to GP96 post secretion by 3T3 cells could not be entirely excluded. Therefore, cell lines were designed that secrete only the N-terminal domain containing the ATP-binding domain of GP96 (NTD). Surprisingly, immunization with 4T1 secreting-GP96 NTD suppressed 4T1 tumor growth. Control of tumor growth was found to be independent of anti-4T1 CTL generation, and immunization with GP96 NTD had only a modest effect on NK activity. GP96 NTD was shown to bind various APCs in vitro as measured by flow cytometry. Supernatants from either GP96 or GP96

NTD-secreting cells activated DCs as measured by CD86, CD40 and MHC class II up-regulation. These observations prompted the investigators to hypothesize alternate mechanisms generated by the secreted GP96 NTD.

In a follow-up study, immunization of BALB/c mice with syngeneic KBALB fibroblasts secreting GP96 or GP96 NTD stimulated TNF-α and IFNγ production by CD11b/c(+) cells in the spleen and draining lymph nodes [93]. After depleting mice of APCs with carageenan, immunization with KBALB fibroblasts secreting GP96 NTD resulted in a decrease in cytokine secretion by CD4+ T cells [94]. The authors proposed that GP96 NTD appears to delay tumor growth by stimulating APCs that in turn stimulate cytokine secretion by CD4+ T helper cells. These observations suggest effector mechanisms for GP96 other than CTL that may result in anti-tumor responses. Although HSPs may also be involved in the generation of CD4+ T cell responses through MHC class II presentation, the role of class II presentation and CD4+ T cell effectors in HSP-mediated responses is only beginning to be explored [21, 95].

In addition to localization of a PBD at the CTD for GP96, other studies have identified a peptide-binding site for VSV8 at the N-terminus [96, 97]. This site is present within the GP96 NTD in the above described secretion models [93]. Peptide-binding activity within the NTD cannot therefore be entirely ruled out. Lastly, characteristics of receptor binding include specificity, saturation and competition. Demonstration of these attributes for GP96 NTD binding to APCs can indicate a ligand-receptor interaction. A recent study demonstrates the NTD of GP96 can also lead to the generation of CTL (Hepatitis B virus peptide-specific) as measured by ELISPOT assay [98]. This GP96 NTD construct also contains the putative peptide-binding site and may account for CTL activation in this report.

Taken together, it is unclear where bona-fide peptide-binding region(s) for GP96 are located. In addition to the different methodologies used for identification of the peptide-binding site for GP96, the proposed N-terminal region has such a low affinity for VSV8 peptide that it is difficult to ascertain if this site is physiologically relevant [25, 96, 97]. It is possible that GP96 possesses two or more peptide-binding sites, similar to HSP90 [99]. Unless the GP96-peptide interaction is spatially defined, separating the peptide-binding function from the natural innate function of GP96 will be a complex undertaking.

In contrast to the ATP-binding domain of the HSP70 family, the ATP-binding region of the HSP90 family (GP96) forms part of a family called the GHKL proteins that shares structural similarity with diverse proteins such as DNA gyrase and DNA topoisomerase II [100]. Mechanisms of chaperone activity for HSP90 have been modeled after DNA gyrase [101]. As previously discussed, the mechanism regulating the interaction between GP96 and peptide/polypeptide substrates remains largely unknown as compared to HSP90. In addition to weak ATP-binding activity, GP96 has little ATP hydrolysis activity in vitro [26–28]. Crystal structure data of the N-terminus of GP96 suggests that a nucleotide-dependent conformational switch induces dimerization at the N-terminal region that may facilitate client protein binding [91]. HSP70 and GP96 N-terminal regions are therefore structurally and functionally different and may account for the observed differences in the immunological activity of these immunochaperones.

Concluding remarks

The long-standing paradigm that chaperoned peptides of GP96 or HSP70 confer peptide-specific adaptive immunity *that predominantly contributes to anti-tumor immunity* is one that requires revision given evidence summarized here. While chaperoned peptides by HSPs appear to be critical for adaptive immune consequences, the observation that the source of these HSPs for anti-tumor effects is not as crucial as once believed and therefore lack of specificity suggests inherent functions of HSPs, most likely sufficient for suppressing tumor growth.

The natural innate function of HSPs has been underappreciated in the generation of effective immune responses against tumor. Recent evidence suggests DCs and NK cells can cross-talk during infection or anti-tumor immune responses and may contribute to the CD8+ T cell response [102–104]. GP96-mediated immunity may also elicit this interaction between DCs and NK and may augment adaptive immune responses [13, 64].

The structural domains and regions of immunochaperones required for generating effective anti-tumor immunity is an area of current interest. The peptide-binding activity of HSP70 correlates with generation of CTL responses. Surprisingly, the CTD of HSP70 may also have a dual role of eliciting chaperokine effects as well as adaptive responses.

The NTD of GP96 has modest anti-tumor activity and seems to induce innate immunity via activation of APCs and generation of cytokines by CD4+ T cells [93]. CD4+ T helper cells can play a role as effector cells in anti-tumor immunity and may play a role in GP96 NTD-mediated immune responses as well [105–108]. The identification of a chaperoning site within the NTD of GP96 prevents any definitive conclusions to be made at this point regarding a possible innate function of the NTD of GP96. Design of HSP70 NTD-secreting tumor models would be informative in addition to full-length hsp70-secreting models [109]. Comparable regions of these immunochaperones may have qualitatively different immunological effects as anti-cancer vaccines.

Adaptive and innate compartments of immunity contribute significantly to the overall immune response to infection and likely for anti-tumor immunity as well. An effective HSP70 or GP96-mediated anti-tumor response may similarly result from interplay between both compartments of immunity. In order to generate a robust immune response, the innate effects of HSPs and a

source of complexed antigen for targeting tumors may require the entire immunochaperone for a clinical therapeutic effect.

The next several years should be exciting due to a better understanding of HSP-mediated mechanisms of immunity and should bring us closer to developing more effective HSP-based vaccines. Elucidation of the interrelatedness of adaptive and innate immunity by immunobiologists will also, undoubtedly, shed light on HSP-mediated mechanisms as well.

Acknowledgements This work was supported by NIH grants RO-1 CA099326-13, PO-1 CA94045-01A2SUB (JRS) and by shared resources of the Roswell Park Cancer Center Support Grant P30 CA16056, as well as a Department of Defense Pre-Doctoral Grant (JGF). Views and opinions of, and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense.

References

1. Udono H, Srivastava PK (1994) J Immunol 152(11):5398–5403
2. Manjili MH, Wang XY, Chen X, Martin T, Repasky EA, Henderson R, Subjeck JR (2003) J Immunol 171(8):4054–4061
3. Wang XY, Chen X, Manjili MH, Repasky E, Henderson R, Subjeck JR (2003) Cancer Res 63(10):2553–2560
4. Basu S, Srivastava PK (1999) J Exp Med 189(5):797–802
5. Janetzki S, Palla D, Rosenhauer V, Lochs H, Lewis JJ, Srivastava PK (2000) Int J Cancer 88(2):232–238
6. Belli F, Testori A, Rivoltini L, Maio M, Andreola G, Sertoli MR, Gallino G, Piris A, Cattelan A, Lazzari I, Carrabba M, Scita G, Santantonio C, Pilla L, Tragni G, Lombardo C, Arienti F, Marchiano A, Queirolo P, Bertolini F, Cova A, Lamaj E, Ascani L, Camerini R, Corsi M, Cascinelli N, Lewis JJ, Srivastava P, Parmiani G (2002) J Clin Oncol 20(20):4169–4180
7. Castelli C, Rivoltini L, Rini F, Belli F, Testori A, Maio M, Mazzaferro V, Coppa J, Srivastava PK, Parmiani G (2004) Cancer Immunol Immunother 53(3):227–233
8. Lewis JJ (2004) Proc Natl Acad Sci USA 101(Suppl2):14653–14656
9. Noessner E, Gastpar R, Milani V, Brandl A, Hutzler PJ, Kuppner MC, Roos M, Kremmer E, Asea A, Calderwood SK, Issels RD (2002) J Immunol 169(10):5424–5432
10. Craig EA, Gambill BD, Nelson RJ (1993) Microbiol Rev 57(2):402–414
11. Subjeck JR, Shyy TT (1986) Am J Physiol 250(1Pt1):C1–C17
12. Udono H, Levey DL, Srivastava PK (1994) Proc Natl Acad Sci USA 91(8):3077–3081
13. Yamazaki K, Nguyen T, Podack ER (1999) J Immunol 163(10):5178–5182
14. Srivastava PK, DeLeo AB, Old LJ (1986) Proc Natl Acad Sci USA 83(10):3407–3411
15. Srivastava PK (1996) Semin Immunol 8(5):295–302
16. Tamura Y, Peng P, Liu K, Daou M, Srivastava PK (1997) Science 278(5335):117–120
17. Srivastava PK, Maki RG (1991) Curr Top Microbiol Immunol 167:109–123
18. Liberek K, Skowyra D, Zylicz M, Johnson C, Georgopoulos C (1991) J Biol Chem 266(22):14491–14496
19. Schmid D, Baici A, Gehring H, Christen P (1994) Science 263(5149):971–973
20. Udono H, Srivastava PK (1993) J Exp Med 178(4):1391–1396
21. Roman E, Moreno C (1996) Immunology 88(4):487–492
22. Rivoltini L, Castelli C, Carrabba M, Mazzaferro V, Pilla L, Huber V, Coppa J, Gallino G, Scheibenbogen C, Squarcina P, Cova A, Camerini R, Lewis JJ, Srivastava PK, Parmiani G (2003) J Immunol 171(7):3467–3474
23. Baker-LePain JC, Reed RC, Nicchitta CV (2003) Curr Opin Immunol 15(1):89–94
24. Demine R, Walden P (2005) J Biol Chem
25. Nicchitta CV, Carrick DM, Baker-LePain JC (2004) Cell Stress Chaperones 9(4):325–331
26. Wearsch PA, Nicchitta CV (1997) J Biol Chem 272(8):5152–5156
27. Soldano KL, Jivan A, Nicchitta CV, Gewirth DT (2003) J Biol Chem 278(48):48330–48338
28. Rosser MF, Trotta BM, Marshall MR, Berwin B, Nicchitta CV (2004) Biochemistry 43(27):8835–8845
29. Blachere NE, Li Z, Chandawarkar RY, Suto R, Jaikaria NS, Basu S, Udono H, Srivastava PK (1997) J Exp Med 186(8):1315–1322
30. MacAry PA, Javid B, Floto RA, Smith KG, Oehlmann W, Singh M, Lehner PJ (2004) Immunity 20(1):95–106
31. Srivastava PK, Menoret A, Basu S, Binder RJ, McQuade KL (1998) Immunity 8(6):657–665
32. Linderoth NA, Simon MN, Hainfeld JF, Sastry S (2001) J Biol Chem 276(14):11049–11054
33. Linderoth NA, Simon MN, Rodionova NA, Cadene M, Laws WR, Chait BT, Sastry S (2001) Biochemistry 40(5):1483–1495
34. Suzue K, Zhou X, Eisen HN, Young RA (1997) Proc Natl Acad Sci USA 94(24):13146–13151
35. Chen CH, Wang TL, Hung CF, Yang Y, Young RA, Pardoll DM, Wu TC (2000) Cancer Res 60(4):1035–1042
36. Moroi Y, Mayhew M, Trcka J, Hoe MH, Takechi Y, Hartl FU, Rothman JE, Houghton AN (2000) Proc Natl Acad Sci USA 97(7):3485–3490
37. Srivastava P (2002) Nat Rev Immunol 2(3):185–194
38. Singh-Jasuja H, Hilf N, Scherer HU, Arnold-Schild D, Rammensee HG, Toes RE, Schild H (2000) Cell Stress Chaperones 5(5):462–470
39. Singh-Jasuja H, Hilf N, Arnold-Schild D, Schild H (2001) Biol Chem 382(4):629–636
40. Ciupitu AM, Petersson M, Kono K, Charo J, Kiessling R (2002) Cancer Immunol Immunother 51(3):163–170
41. Casey DG, Lysaght J, James T, Bateman A, Melcher AA, Todryk SM (2003) Immunology 110(1):105–111
42. Castelli C, Ciupitu AM, Rini F, Rivoltini L, Mazzocchi A, Kiessling R, Parmiani G (2001) Cancer Res 61(1):222–227
43. Hilf N, Singh-Jasuja H, Schild H (2002) Int J Hyperthermia 18(6):521–533
44. Singh-Jasuja H, Scherer HU, Hilf N, Arnold-Schild D, Rammensee HG, Toes RE, Schild H (2000) Eur J Immunol 30(8):2211–2215
45. Zheng H, Dai J, Stoilova D, Li Z (2001) J Immunol 167(12):6731–6735
46. Vabulas RM, Braedel S, Hilf N, Singh-Jasuja H, Herter S, Ahmad-Nejad P, Kirschning CJ, Da Costa C, Rammensee HG, Wagner H, Schild H (2002) J Biol Chem 277(23):20847–20853
47. Vabulas RM, Ahmad-Nejad P, Ghose S, Kirschning CJ, Issels RD, Wagner H (2002) J Biol Chem 277(17):15107–15112
48. Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, Calderwood SK (2000) Nat Med 6(4):435–442
49. Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, Stevenson MA, Calderwood SK (2002) J Biol Chem 277(17):15028–15034
50. Asea A (2003) Exerc Immunol Rev 9:25–33
51. Reed RC, Berwin B, Baker JP, Nicchitta CV (2003) J Biol Chem 278(34):31853–31860
52. Manjili MH, Wang XY, MacDonald IJ, Arnouk H, Yang GY, Pritchard MT, Subjeck JR (2004) Expert Opin Biol Ther 4(3):363–373
53. Multhoff G, Botzler C, Wiesnet M, Eissner G, Issels R (1995) Blood 86(4):1374–1382
54. Multhoff G, Botzler C, Jennen L, Schmidt J, Ellwart J, Issels R (1997) J Immunol 158(9):4341–4350
55. Gross C, Hansch D, Gastpar R, Multhoff G (2003) Biol Chem 384(2):267–279

56. Multhoff G, Pfister K, Gehrmann M, Hantschel M, Gross C, Hafner M, Hiddemann W (2001) Cell Stress Chaperones 6(4):337–344
57. Muzio M, Bosisio D, Polentarutti N, D'Amico G, Stoppacciaro A, Mancinelli R, van't Veer C, Penton-Rol G, Ruco LP, Allavena P, Mantovani A (2000) J Immunol 164(11):5998–6004
58. Nicchitta CV (2003) Nat Rev Immunol 3(5):427–432
59. Robert J, Gantress J, Rau L, Bell A, Cohen N (2002) J Immunol 168(4):1697–1703
60. Goyos A, Cohen N, Gantress J, Robert J (2004) Eur J Immunol 34(9):2449–2458
61. Biron CA (1999) Curr Opin Microbiol 2(4):374–381
62. Degli-Esposti MA, Smyth MJ (2005) Nat Rev Immunol 5(2):112–124
63. Strbo N, Yamazaki K, Lee K, Rukavina D, Podack ER (2002) Am J Reprod Immunol 48(4):220–225
64. Strbo N, Oizumi S, Sotosek-Tokmadzic V, Podack ER (2003) Immunity 18(3):381–390
65. Wang Y, Kelly CG, Singh M, McGowan EG, Carrara AS, Bergmeier LA, Lehner T (2002) J Immunol 169(5):2422–2429
66. Hong SH, Misek DE, Wang H, Puravs E, Giordano TJ, Greenson TJ, Brenner DE, Simeone DM, Logsdon CD, Hanash SM (2004) Cancer Res 64(15):5504–5510
67. Shi L, Kataoka M, Fink AL (1996) Biochemistry 35(10):3297–3308
68. Kiang JG, Tsokos GC (1998) Pharmacol Ther 80(2):183–201
69. Melnick J, Aviel S, Argon Y (1992) J Biol Chem 267(30):21303–21306
70. Wearsch PA, Nicchitta CV (1996) Biochemistry 35(51):16760–16769
71. Sastry S, Linderoth N (1999) J Biol Chem 274(17):12023–12035
72. Erbse A, Mayer MP, Bukau B (2004) Biochem Soc Trans 32(Pt4):617–621
73. Zhu X, Zhao X, Burkholder WF, Gragerov A, Ogata CM, Gottesman ME, Hendrickson WA (2004) Science 272(5268):1606–1614
74. Buchberger A, Theyssen H, Schroder H, McCarty H, Virgallita G, Milkereit P, Reinstein J, Bukau B (1995) J Biol Chem 270(28):16903–16910
75. McCarty JS, Buchberger A, Reinstein J, Bukau B (1995) J Mol Biol 249(1):126–137
76. Mayer MP, Schroder H, Rudiger S, Paal K, Laufen T, Bukau B (2000) Nat Struct Biol 7(7):586–593
77. Moro F, Fernandez V, Muga A (2003) FEBS Lett 533(1–3):119–123
78. Bork P, Sander C, Valencia A (1992) Proc Natl Acad Sci USA 89(16):7290–7294
79. Huang Q, Richmond JF, Suzue K, Eisen HN, Young RA (2000) J Exp Med 191(2):403–408
80. Udono H, Yamano T, Kawabata Y, Ueda M, Yui K (2001) Int Immunol 13(10):1233–1242
81. Javid B, MacAry PA, Oehlmann W, Singh M, Lehner PJ (2004) Biochem Soc Trans 32(Pt4):622–625
82. Zimmer C, Henics T (2002) Cell Stress Chaperones 7(3):243–249
83. Wang Y, Kelly CG, Karttunen JT, Whittall T, Lehner PJ, Duncan L, MacAry P, Younson JS, Singh M, Oehlmann W, Cheng G, Bergmeier L, Lehner T (2001) Immunity 15(6):971–983
84. Wang Y, Whittall T, McGowan E, Younson J, Kelly C, Bergmeier LA, Singh M, Lehner T (2005) J Immunol 174(6):3306–3316
85. Binder RJ, Harris ML, Menoret A, Srivastava PK (2000) J Immunol 165(5):2582–2587
86. Delneste Y, Magistrelli G, Gauchat J, Haeuw J, Aubry J, Nakamura K, Kawakami-Honda N, Goetsch L, Sawamura T, Bonnefoy J, Jeannin P (2002) Immunity 17(3):353–362
87. Berwin B, Hart JP, Rice S, Gass C, Pizzo SV, Post SR, Nicchitta CV (2003) Embo J 22(22):6127–6136
88. Panjwani N, Popova L, Srivastava PK (2000) Cell Stress Chaperones 5:392
89. Mazzarella RA, Green M (1987) J Biol Chem 262(18):8875–8883
90. Argon Y, Simen BB (1999) Semin Cell Dev Biol 10(5):495–505
91. Immormino RM, Dollins DE, Shaffer PL, Soldano KL, Walker MA, Gewirth DT (2004) J Biol Chem 279(44):46162–46171
92. Linderoth NA, Popowicz A, Sastry S (2000) J Biol Chem 275(8):5472–5477
93. Baker-LePain JC, Sarzotti M, Fields TA, Li CY, Nicchitta CV (2002) J Exp Med 196(11):1447–1459
94. Baker-LePain JC, Sarzotti M, Nicchitta CV (2004) J Immunol 172(7):4195–4203
95. Doody AD, Kovalchin JT, Mihalyo MA, Hagymasi AT, Drake CG, Adler AJ (2004) J Immunol 172(10):6087–6092
96. Vogen S, Gidalevitz T, Biswas C, Simen BB, Stein E, Gulmen F, Argon Y (2002) J Biol Chem 277(43):40742–40750
97. Gidalevitz T, Biswas C, Ding H, Schneidman-Duhovny D, Wolfson HJ, Stevens F, Radford S, Argon Y (2004) J Biol Chem 279(16):16543–16552
98. Li H, Zhou M, Han J, Zhu X, Dong T, Gao GF, Tien P (2005) J Immunol 174(1):195–204
99. Scheibel T, Weikl T, Buchner J (1998) Proc Natl Acad Sci USA 95(4):1495–1499
100. Dutta R, Inouye M (2000) Trends Biochem Sci 25(1):24–28
101. Wigley DB, Davies GJ, Dodson EJ, Maxwell A, Dodson G (1991) Nature 351(6328):624–629
102. Andrews DM, Scalzo AA, Yokoyama WM, Smyth MJ, Degli-Esposti MA (2003) Nat Immunol 4(2):175–181
103. Andrews DM, Andoniou CE, Scalzo AA, van Dommelen SL, Wallace ME, Smyth MJ, Degli-Esposti MA (2005) Mol Immunol 42(4):547–555
104. Fernandez NC, Lozier A, Flament C, Ricciardi-Castagnoli P, Bellet D, Suter M, Perricaudet M, Tursz T, Maraskovsky E, Zitvogel L (1999) Nat Med 5(4):405–411
105. Matsutake T, Srivastava PK (2001) Proc Natl Acad Sci USA 98(7):3992–3997
106. Egilmez NK, Hess SD, Chen FA, Takita H, Conway TF, Bankert RB (2002) Cancer Res 62(9):2611–2617
107. Hess SD, Egilmez NK, Bailey N, Anderson TM, Mathiowitz E, Bernstein SH, Bankert RB (2003) J Immunol 170(1):400–412
108. Corthay A, Skovseth DK, Lundin KU, Rosjo E, Omholt H, Hofgaard PO, Haraldsen G, Bogen B (2005) Immunity 22(3):371–383
109. Massa C, Guiducci C, Arioli I, Parenza M, Colombo MP, Melani C (2004) Cancer Res 64(4):1502–1508