ISO: a critical evaluation of the role of peptides in heat shock/chaperone protein-mediated tumor rejection
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Heat shock/chaperone protein (HSCP)–peptide complexes can access the cross-priming pathways of antigen presenting cells (APCs), and the gp96-associated peptides can be re-presented on APC MHC class I molecules to elicit CD8+ T cell activation. This immunological circuit is thought to provide the functional basis for HSCP function in tumor immunity. Recent findings identifying a ‘natural adjuvant’ function for HSCPs suggest complementary, or perhaps alternative, mechanisms of HSCP-mediated tumor rejection.

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Abbreviations
APC antigen presenting cell
DC dendritic cell
HSCP heat shock/chaperone protein
MHC major histocompatibility complex
NK natural killer
TLR Toll-like receptor
TSTA tumor-specific transplantation antigen

Introduction
The remarkable discovery that immunization of mice with tumor-derived gp96 (also called GRP94) elicits anti-tumor immune responses has fostered the development of an emerging field of study, the immunobiology of heat shock/chaperone proteins (HSCPs), and has also provided promising new immunotherapeutic agents for the treatment of cancer and infectious diseases [1**]. In the basic science community, these observations have prompted the exploration of HSCP function where no function had been previously envisioned, including in the extracellular space, where such proteins reside following pathological cell death [2,3], the cell surface of antigen presenting cells (APCs), which bear receptors functioning both in receptor-mediated endocytosis of HSCPs [4–6] and HSCP-dependent activation of cellular signal transduction pathways [2,7**], and lastly, the endocytic compartments of APCs, which are accessed by these proteins following clearance from the extracellular space [8*]. Given these broad functions, HSCPs are uniquely poised to modify immune cell function and guide immunological responses to foreign pathogens or tumor cells. This review summarizes the current knowledge regarding the role of HSCP-bound peptides in the elicitation of cellular immune responses, provides a critical evaluation of the biochemical evidence for peptide binding as an in vivo function of HSCPs, and discusses recent literature on HSCP interaction with the innate immunity arm of the cellular immune response.

Establishing a molecular mechanism for heat shock/chaperone protein function in tumor rejection
With the passage of time, it has become apparent that HSCPs display a variety of interactions with cells of the immune system, most notably with APCs [1**]. With each new discovery, however, come new questions regarding the mechanism of HSCP function in tumor rejection. The founding experiments in the field, in which gp96 was observed to function as a tumor-specific transplantation antigen (TSTA), were highly influential in guiding the development of models for gp96-mediated tumor rejection [9]. In identifying a TSTA activity for gp96, Srivastava and colleagues [10] proposed that gp96, in a manner similar to MHC molecules, displayed tissue-restricted immunological identity. Though the molecular basis for this phenomenon was not immediately apparent, the knowledge that MHC molecules function to display peptides, coupled with the demonstration that the Hsp70 family members Hsc70, a cytosolic Hsp70, and BiP, the resident endoplasmic reticulum Hsp70, display in vitro peptide binding activity, providing a logical basis for gp96 immunogenicity: gp96 functions as a peptide-binding protein [1**,11]. Therefore, gp96 TSTA activity reflects the capacity of gp96 to be purified from tumor tissue in association with tumor-specific antigenic peptides [10].

A large array of immunological evidence has been marshaled in support of a peptide-binding function for gp96 in tumor rejection. Nonetheless, and in contrast to better characterized peptide-binding proteins, such as MHC class I/class II molecules, direct chemical evidence that gp96 is associated with a diverse array of bound peptides has proven very difficult to obtain (JJ Wassenberg, CV Nicchitta, AL Burlingame, unpublished observations; [1**]). In addition, the relatively limited characterization of gp96 peptide-binding activity has revealed several properties that distinguish gp96 from other peptide-binding proteins. Firstly, in vitro kinetic analyses of
gp96 peptide-binding activity have indicated maximal peptide-binding stoichiometries of 0.01–0.04 moles peptide:1 mole gp96 [12,13]. These data are in sharp contrast to data for proteins such as MHC class I, BiP and Hsc70, which display peptide-binding stoichiometries approaching 1 mole peptide:1 mole protein [11,14]. Secondly, in all published studies to date, gp96 peptide-binding activity is irreversible; by contrast, Hsc70/BiP–peptide interactions are reversible and regulated by cycles of ATP binding and hydrolysis [11–13]. Thirdly, the structural basis for peptide binding by MHC class I/class II and Hsp70 proteins has been confirmed in crystallographic studies and, for the Hsp70s, the peptide sequence determinants that yield optimal binding have been identified by combinatorial selection against synthetic peptide libraries and phage display [15,16]. For gp96, the lack of significant in vitro peptide-binding activity has precluded both crystallographic characterization of the putative peptide binding pocket and combinatorial analyses of peptide-binding determinants (see, however, [17]). In this context, it should be noted that influential experiments demonstrating that gp96-associated peptides are re-presented on APC class I molecules utilized gp96 preparations bearing synthetic peptides loaded in vitro by co-incubation at 50°C, conditions in which gp96 undergoes an irreversible tertiary conformational change and the observed peptide-binding stoichiometries remain exceedingly low [12,13,18].

Peptides and proteins as sources of immunogenic activity
A frequently overlooked issue in the study of HSCP immunogenicity is that of protein purity. Protein purification, by nature, is a process that approaches, but very rarely achieves, the goal of absolute purity. Deciding on a standard of purity requires consideration of what the protein will be used for: protein sufficiently pure for one use may contain co-purifying molecules making it unsuitable for another. Arthur Kornberg [19] observed “No enzyme is purified to the point of absolute homogeneity. Even when other proteins constitute less than 1% of the purified protein and escape detection by our best methods, there are likely to be many millions of foreign molecules in a reaction mixture. Generally, such contaminants do not matter unless they are preponderantly of one kind and are highly active on one of the components being studied.”

The standard of protein purity is particularly relevant when considering the immunological properties of HSCPs such as gp96. This HSCP displays natural adjuvant activity [2,20,21] and so, by definition, will enhance the processing and re-presentation of any ‘bystander’ proteins. Recent work from our laboratory indicates that enzymologically significant levels of ‘bystander’ proteins are present in typical gp96 preparations [22]. For example, we found that electrophoretically pure gp96 displayed several enzymatic activities, including an experimentally introduced ‘tracer’ enzyme, β-galactosidase, a gp96-directed kinase, and a gp96-directed aminopeptidase. In each case, the contaminants were present at levels only discernable by enzymological assay and, in each case, they could be separated from gp96 by further purification.

Could such ‘bystander’ proteins themselves act as antigens and thereby contribute to the apparent tissue specificity of gp96-elicited immune responses? It has been estimated that 30 × 10⁶ antigenic peptides are delivered to and presented by dendritic cells (DCs) per microgram gp96 [1**]. We have calculated, with respect to contaminating aminopeptidase, that electrophoretically pure gp96 preparations contain at least 26 × 10⁶ molecules of aminopeptidase per microgram of gp96 [22]. In addition, such preparations of gp96 also contain at least 125 × 10⁶ molecules of casein kinase II-like activity per microgram of gp96 [22]. It is likely that numerous other protein contaminants are present at similarly low but discernible levels. Though these appear to be biochemically minute quantities, it should be noted that 200 pg of ovalbumin (∼500 × 10⁶ molecules), provided in the absence of accompanying adjuvant, is sufficient to elicit a CD8⁺ response in mice [23]. These calculations suggest, but by no means prove, that gp96 preparations contain quantities of ‘bystander’ proteins sufficient to contribute to adaptive immune responses.

Given these observations, it is of value to consider an alternative view: the physiological function of gp96 in tumor rejection is independent of bound peptides (but see [1**.24]). For this discussion, we focus not on peptide binding to gp96 per se, but rather on the broader question of whether peptide binding is a physiologically relevant function of HSCPs in vivo. Though it has been well documented that chaperone proteins, such as Hsc70 and BiP, can bind peptides in vitro, it is not known whether such activity occurs in vivo. Consider the following: peptide binding to Hsp70 family members is a low-affinity process, occurring with micromolar to millimolar equilibrium binding constants [11]. Given such low binding affinities, even modest HSCP–peptide occupancy states would require steady-state intracellular peptide concentrations of tens to hundreds of micromolar. However, current experimental data do not support the existence of significant levels of free intracellular peptides [25].

Indeed, there may be a strong biological rationale for maintaining intracellular peptide concentrations at very low levels. Foremost, peptides efficiently compete with client proteins for binding to HSCPs and thus act as competitive inhibitors of chaperone activity [26–28]. In addition, if the concept of peptide binding is extended to protein–protein interactions in general, it can be appreciated that many, if not all, proteins that engage in
protein–protein interactions, can function as peptide-binding proteins. This conclusion extends from the observation that the recognition process for various protein–protein interactions can be recapitulated in vitro with isolated structural domains and small peptide motifs. For example, crystal structures of the amino-terminal domain of the tetratricopeptide (TPR) binding protein Hop, in association with a synthetic TPR peptide (GPTIEEVD; one letter amino acid code) representing the Hsp70 carboxy-terminal TPR domain, detail the structural determinants for Hop–Hsp70 interactions [29]. Valuable as such studies are, it is not generally concluded that Hop functions in vivo as a peptide-binding protein. The use of peptides to mimic or disrupt protein–protein interactions has also been demonstrated in vivo. In one example (of many), peptide sequences of interest have been attached to a “Trojan peptide”, the penetratin-1 fragment of the Drosophila antennapedia homeodomain transcription factor, which enables peptide entry into the cytosol [30,31]. Using this approach, Steitz and colleagues [30] were able to disrupt specific protein–protein recognition processes displayed by the HuR RNA-binding protein. The experimental power of this technique is based on the empirical observation that when presented to cells at micromolar concentrations the Trojan peptide conjugates specifically disrupt the targeted protein–protein interaction.

When considering chaperone–peptide interactions in vivo, one can thus envision a thorny problem of numbers. Because HSCPs display low affinities for their peptide substrates, high intracellular peptide concentrations would be needed to achieve broad representation of the cell’s antigenic repertoire on HSCPs. However, at such concentrations, pleiotropic disruptions of protein–protein and chaperone–client protein interactions would be expected. These issues, although conjectural, highlight the need for further investigation into the physiological basis for HSCP–peptide interactions.

**Heat shock/chaperone proteins: activators of innate immunity**

An additional immunological role for HSCPs has been defined in recent years; HSCPs from both mammalian and microbial sources act as natural adjuvants and thereby elicit cytokine secretion, upregulation of co-stimulatory molecule expression and upregulation of MHC class II expression in APCs [2,20,32,33]. These effects are thought to reflect HSCP-dependent activation of the NF-κB signalling pathways [2,32]. As a member of the HSCP family, gp96 is known to activate IL-12, TNF-α and IL-1β secretion in DCs and macrophages [2,20] and to induce DC maturation [2,20,34]. Importantly, the activation of innate immunity by HSCPs is thought to be independent of their ability to function as peptide-binding proteins, as these responses occur with HSCPs derived from a variety of tissue sources and from antigen-negative HSCP preparations [2,20,35]. Thus, HSCPs signal the activation of cells of the innate immune system in a manner similar to either microbial byproducts, such as lipopolysaccharide and peptidoglycan, or immunostimulatory mammalian cytokines, such as TNF-α. The involvement of HSCPs in the invocation of innate immunity suggests a role for these proteins as endogenous immunological ‘danger’ signals [36]. These proteins are released from cells upon pathological cell death [2,3,37] and thus their presence in the extracellular space serves as a physiological signal of pathological cell stress or damage. The receptors proposed to function in the recognition of extracellular HSCPs include scavenger receptors such as CD91 ([5], but see [38]), CD36 [39] and the Toll-like receptors (TLRs) 2 and 4 [7,32,33,40,41]. HSCP clearance by scavenger receptors is consistent with a role for these proteins as ‘danger’ signals and activators of innate immunity, as many microbial ‘danger’ signals are also cleared by these receptors. HSCP signaling through the TLRs is also indicative of their important role in innate immunity, as these receptors are uniquely poised both for the stimulation of innate immune responses and the direction of subsequent adaptive immune responses [42]. Thus, the encounter of HSCPs with cells of the mammalian immune system sets into motion an immunological program involving both the innate and adaptive elements of the host immune response. As illustrated in Figure 1, the interaction of gp96 with the adaptive element of the cellular immune system is thought to involve the processing of gp96-bound peptides, which are in turn routed to the cross-priming pathways of APCs to elicit CD8+ T cell activation. In addition, gp96 interacts with innate elements of the host immune system, where it elicits cytokine/chemokine expression, DC maturation, and upregulation of co-stimulatory molecules.

Do the interactions of gp96 with the innate immune system contribute to its ability to trigger anti-tumor responses? Evidence for peptide antigen-independent, gp96-mediated tumor rejection has been obtained in experiments demonstrating that preparations of gp96 from normal tissue elicit tumor suppression (see Figure 1 in [43], Figure 1 in [44], and [45]). Other agents that function to initiate innate immune responses can also elicit tumor rejection; activation of innate immunity with bacillus Calmette–Gurin (BCG), for example, is used in the immunotherapy of bladder cancer [46]. In this regimen, BCG-dependent activation of natural killer (NK) cell activity is known to be essential to the anti-tumor response [47]. Interestingly, NK [48,49] and NKT [50] cells are key mediators of tumor rejection in a variety of experimental systems, and these cells are in turn activated by their interaction with DCs and immunostimulatory cytokines [51–53]. Similarly, the function of HSCPs in microbially-induced inflammatory and autoimmune disease, at one time thought to reflect only immune cross-reactivity between microbial and endogenous...
Mechanisms of gp96-mediated tumor rejection: two emerging views. The mechanisms underlying the TSTA activity of gp96 are thought to consist of two major components. (a) gp96 elicits specific adaptive immune responses against its repertoire of bound peptides. In this response, gp96–peptide complexes are internalized by APCs by means of specific cell-surface receptors. In the endocytic compartments of the APC, peptides are transferred onto MHC molecules for re-presentation on the cell surface, where they are recognized by T lymphocytes bearing T cell receptors specific to the antigen–MHC complex. (b) gp96 binds to cell-surface receptors of APC and activates signal transduction pathways associated with innate immune responses. Signal transduction mediated by the TLRs, including TLR2 and TLR4 (not shown), and yielding activation of NF-κB, are thought to play a role in these processes. The result of this activation is the stimulation of cytokine and nitric oxide production and the maturation of DCs, which in turn may influence APC interactions with effector cells such as NK and NKT cells. These functions support a role for gp96 as a natural adjuvant of the mammalian immune system.

Conclusions
The current paradigm for HSCP function in tumor rejection is intuitively appealing and consistent with considerable immunological data. Yet, important questions remain regarding the physiological basis for peptide binding, the identification of an appropriately diverse HSCP-bound peptide pool, and the contribution of bystander proteins to HSCP-elicted immune responses. Recent insights into the interactions of HSCPs with the innate immune system, and the role(s) of innate immunity in tumor rejection, provide promising new avenues of investigation into the mysteries surrounding the diverse cellular functions of these ancient proteins.

Update
In a recent publication [55**], the binding of radiolabeled peptides to the amino-terminal domain of GRP94 is demonstrated and differences between GRP94 and BiP in preferred peptide substrates are reported. A detailed analyses of peptide VSV8 binding indicated a very high apparent Kd (0.8 millimolar) and a remarkably slow off rate, with saturation requiring a 36 hour incubation at room temperature. Consistent with past studies, peptide binding was irreversible under native conditions.
References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


A very comprehensive and personal account of the identification and analysis of gp96 function as a tumor-specific translocation antigen. Contains a thorough summary of the data supporting a peptide-basis for gp96 immunogenicity.


In extending insights into the role of gp96 in innate immunity, this report provides evidence indicating that gp96 binding to TRLS 2/4 elicits DC activation.


The first report to identify a potential subcellular trafficking pathway for representation of gp96-bound peptides. The authors provide evidence that gp96-bound peptides can be transferred onto structurally mature MHC class I molecules.


The authors report that gp96 and Hsp70 activate nitric oxide production in APCs. These data provide further evidence of a potentially important role for gp96 and Hsp70 in innate immunity.


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38. Berwin B, Hart JP, Pizzo SV, Nicchitta CV: Cutting edge: CD91- independent cross-presentation of GRP94(gp96)-associated peptides. J Immunol 2002, 168:4282-4286. This article provides data demonstrating that gp96 uptake into APCs can occur via a CD91-independent pathway and that this pathway for receptor-mediated uptake of gp96 can yield peptide re-presentation.


This report identifies an innate immunity pathway in the immunosurveillance of methylcholanthrene-induced sarcomas, the tumor model used in the original identification of gp96 tumor-specific transplantation antigen activity.


55. Vogen S, Gidalevitz T, Biliwas C, Simen BB, Stein E, Gulmen F, Argon Y: Radicicol-sensitive peptide binding to the N-terminal portion of GRP94. J Biol Chem 2002, 277:40742-40750. The binding of radiolabeled peptides to the amino-terminal domain of GRP94 is demonstrated, and differences between GRP94 and BiP in preferred peptide substrates are reported.