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The messenger and the message: gp96 (GRP94)-peptide interactions in cellular immunity

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Abstract Vaccination of mice with tumor-derived stress proteins, such as Hsp70 and gp96 (GRP94), can elicit antitumor immune responses, yielding a marked suppression of tumor growth and metastasis. The molecular basis for this response is proposed to reflect a peptide-binding function for these proteins. In this view, stress proteins bind the antigenic peptide repertoire of their parent cell, and when provided to the immune system, tumor-derived stress protein–peptide complexes are processed by antigen-presenting cells (APCs) to yield the subsequent activation of tumor-directed cytotoxic T lymphocyte activity. This model predicts that stress proteins, whose primary intracellular function concerns the proper folding and assembly of nascent polypeptides, intersect with the cellular pathways responsible for the generation, processing, or assembly (or all) of peptide antigens onto nascent major histocompatability class I molecules. Recent insights into the pathways for peptide generation now allow this hypothesis to be critically examined, which is the subject of this review.

INTRODUCTION

The quest for clinically effective cancer vaccines has frequently been thwarted by the recurring observation that cancers tend to be weakly immunogenic or not immunogenic at all (Berd 1998; Marincola et al 2003; Mapara and Sykes 2004; Rosenberg 2004). In surmounting this obstacle, strategies to identify tumor rejection antigens (TRAs) are highly valuable (Boon et al 1995; Singh-Jasuja et al 2004), as are the now manifold means for promoting and enhancing CD8(+) T lymphocyte responses to tumor tissue (reviewed in Marincola et al 2003; Mapara and Sykes 2004). From this perspective, the likelihood of generating a clinically effective, antitumor immune response should be enhanced by targeting a diversity of tumor-specific antigens and by enhanced costimulatory and innate immune activation. Stress proteins, in particular gp96 (= GRP94), have been previously identified as tumor-specific antigens and, more recently, as regulators of antigen-presenting cell (APC) activation and so represent potentially ideal candidates for cancer immunotherapy (Srivastava et al 1998; Schild and Rammensee 2000; Srivastava and Amato 2001).

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Stress proteins as tumor-specific antigens

Investigations into the immunological identities of chemically induced sarcomas established the existence of TRAs, components of chemically induced sarcomas that provide a unique immunological identity to each tumor (Klein 1968). Although the subsequent search for the molecular basis of TRA activity proved largely futile, interest in TRAs was rekindled after the discovery, by Srivastava et al (1986), that gp96 could function as the long-sought tumor-specific antigen of chemically induced sarcomas. In this study, gp96 was purified from 2 immunogenic sarcomas, a MethA isolate and CMS5, and their ability to elicit protection against the 2 tumor cell types examined (Srivastava et al 1986). In a limited study size, no significant cross-protection was observed, a finding consistent with the proposed function of a TRA (Klein 1968; Srivastava et al 1986). These observations provided the foundation for the hypothesis that gp96 could serve as a tumor-specific (and thus patient-specific) vaccine (Srivastava 1994). Subsequent studies have identified tumor antigens that are shared by a diverse array of histologically distinct tumor types as well as tumor antigens that are shared among chemically induced sarcomas (Ikeda et al 1997; Klein 1997; Scanlan et al 2002). Nonetheless, the observation that tumor-derived gp96 can elicit tumor-di-

rected immune responses is fundamentally interesting and ultimately perplexing: how and why does a ubiquitous, highly abundant, highly conserved protein function to elicit tumor immunity? As noted above, the prevailing opinion is that gp96 function in tumor immunity reflects a robust peptide-binding activity, with gp96-peptide complexes serving as cross-priming antigens to initiate tumor-directed cytotoxic T cell (CTL) responses (Srivastava 1993; Srivastava et al 1998). If this hypothesis is correct, gp96 must functionally intersect with the pathways associated with peptide antigen generation, trafficking, or assembly (or all) onto major histocompatability (MHC) class I molecules. Recent advances in understanding, regarding the generation and fate of (antigenic) peptides in cells, now allow critical evaluation of this hypothesis.

Stress proteins as peptide-binding proteins

Extending from the premise that the antigenic diversity common to chemically induced sarcomas (Klein 1968) is a ubiquitous and predictive phenotype of all tumors, Srivastava proposed that gp96 functions as a peptide-binding protein, with the population of bound peptides serving to provide the unique immunological identity of its parent tissue (Srivastava et al 1986; Blachere et al 1993; Srivastava 1993). Embodied within this provocative hypothesis are a number of predictions: (1) gp96 is capable of binding a diverse array of peptides suitable for assembly, either directly or after further processing, with nascent MHC class I molecules (Blachere et al 1993; Srivastava 1993; Srivastava et al 1994; Przepiorka and Srivastava 1998); (2) gp96-peptide interactions are stable to biochemical purification; (3) gp96-peptide interactions are intrinsically reversible, with peptide release accompanying uptake and processing by APC (Srivastava 1993; Srivastava et al 1994); and (4) after internalization by APC, gp96-bound peptides are ''relayed'' to the endoplasmic reticulum (ER) through a stress protein–based shuttle system (Srivastava 1993; Srivastava et al 1994). This latter prediction, in particular, suggests a fundamental and dynamic role for stress proteins in the trafficking of peptide antigens between the cytosol and ER compartments of the cell.

The immunological evidence consistent with this hypothesis is extensive but, because of the immunological readout in the absence of known tumor antigens, indirect (Srivastava et al 1998). The reader is referred to a very comprehensive review of available immunological data supporting a role for stress proteins as peptide chaperones in immune responses (Srivastava et al 1998). Importantly, numerous studies have concluded that tumor-derived stress proteins can elicit antitumor immune responses in either prophylactic or, to a limited degree, therapeutic experimental settings (Tamura et al 1997; Asea et al 2000; Baker-LePain et al 2002; Manjili et al 2002; Rivoltini et al 2003; Wang et al 2003). Based on these data, gp96 is currently in phase III clinical trials as an immunotherapeutic for renal cell carcinoma and malignant melanoma (see Castelli et al 2004).

In vitro studies with different stress proteins have, in many instances, directly demonstrated peptide-binding activity (Flynn et al 1989, 1991; Blond-Elguindi et al 1993; Suto and Srivastava 1995; Wearsch and Nicchitta 1997; Vogen et al 2002). The state of knowledge regarding stress protein–peptide interactions is most developed in the case of the heat shock protein 70 family member, BiP. Three important studies on BiP-peptide interactions provide a biochemical framework for the analysis of stress protein–peptide interactions. In the groundbreaking study of Flynn et al (1989), it was demonstrated that BiP could function as a peptide-binding protein and that BiP-peptide interactions were governed by cycles of adenosine triphosphate (ATP) binding and hydrolysis. Significantly, this study demonstrated that peptides could be used as mimetics of (poly)peptides and provided an avenue for analysis of the structural basis for (poly)peptide substrate selection. This important question was subsequently answered in 2 studies: in 1 approach, preferred peptide substrates were affinity selected from combinatorial peptide libraries and subsequently identified by direct sequencing (Flynn et al 1991). In an alternative approach, phage display was used to identify preferred peptide substrates by affinity panning (Blond-Elguindi et al 1993). Both approaches yielded similar answers—BiP displayed a distinct preference for short peptides enriched in aromatic and hydrophobic amino acids (Flynn et al 1991; Blond-Elguindi et al 1993). The phage display data identified a preferred sequence emphasizing aromatic and hydrophobic amino acids in alternating positions (Blond-Elguindi et al 1993). Biochemical analyses of BiP-peptide interactions demonstrated a low affinity for peptides, with equilibrium dissociation constants of 50–500 μM being commonly observed (Flynn et al 1989; Blond-Elguindi et al 1993). As will be later discussed, such relatively low equilibrium affinities confer specific kinetic restrictions on stress protein–peptide interactions in the cell.

Current understanding regarding the biochemical basis for peptide interactions with gp96 is far less developed. The history of gp96-peptide interactions begins with the landmark study by Suto and Srivastava, where it was reported that in vitro assembled complexes of gp96 and synthetic peptides can be re-presented by APC to yield T cell activation (Suto and Srivastava 1995). These experiments provided proof of principle that gp96 can direct peptides into the class I antigen processing pathway of APCs. From the perspective of the global hypothesis regarding gp96 function in tumor immunity, chemical characterization of the bound peptide pool(s) is of critical im-

portance—it is the proposed immunological ''message'' necessary for the elicitation of tumor-directed CD8(+) T lymphocyte activity. In principle, insight into the identity of the gp96-bound peptide pool should be readily achievable—steal a page from immunological history and as was done with MHC class I, purify gp96, elute bound peptides, and characterize the structural features of the pool by mass spectrometric analysis. Progress on this front has been reported, but the field is more noteworthy for the paucity, rather than the abundance, of identified peptide sequences (Nieland et al 1996; Ishii et al 1999; Meng et al 2001; Liu et al 2004). This is an issue of particular importance to the validation of the gp96-peptide hypothesis; in the absence of such data, a peptide basis for gp96 function in tumor immunity remains a matter of speculation.

Studies on gp96-peptide interactions have emphasized a single peptide substrate, VSV8, the K^b epitope of VSV G protein. First used by Suto and Srivastva for in vitro studies, VSV8 was identified in a bound peptide fraction obtained from gp96 derived from VSV-infected, but not control, EL4 cells (Nieland et al 1996). This finding, too, is an essential ''proof of principle'' observation in support of the peptide basis for gp96-elicited tumor immunity. Surprisingly, however, Nieland et al were able to identify VSV8 in the gp96 fraction derived from H-$2K^b$–negative cells (Nieland et al 1996). This observation distinguishes VSV8, as available data on the pathways for peptide antigen generation indicate that peptides lacking a suitable MHC class I binding partner are highly unstable (Falk et al 1990; Yewdell et al 1999).

Two groups have published identifications of the gp96-peptide (VSV8)–binding site (Linderoth et al 2000; Vogen et al 2002). Whereas Vogen et al identified the N-terminal nucleotide-radicol–binding domain of gp96 as the peptide-binding site, Linderoth et al concluded that a region adjacent to the C-terminal gp96 dimerization domain serves as the peptide-binding site (Linderoth et al 2000; Vogen et al 2002). Such differing conclusions likely reflect the distinct methodologies used by the 2 laboratories to identify the peptide-binding domain(s). In the study of Linderoth et al (2000), VSV8 bearing an azido-moiety was complexed with gp96 at 50°C, photolyzed, and the prominent cross-linked product identified by mass spectrometric analysis of the proteolyzed complex. In the study of Vogen et al (2002), radiolabeled peptide binding to recombinant domains of gp96 was examined where it was observed that VSV8 bound to the N-terminal domain (NTD) of gp96 but much less so to a recombinant construct bearing the proposed near C-terminal peptide-binding domain. It is not immediately apparent why the 2 experimental approaches should yield such disparate results because both experimental methodologies have previously been used successfully to identify ligand-binding domains.

Any discussion of the methodologies used to identify the (a) gp96-peptide–binding site(s) should mention the unusual (and rarely discussed) characteristics of the in vitro interaction of gp96 with peptides (Suto and Srivastava 1995; Wearsch and Nicchitta 1997; Wearsch et al 1998; Linderoth et al 2000; Vogen et al 2002; Gidalevitz et al 2004). The study of Suto and Srivastava (1995) introduced a somewhat unorthodox methodology for assembling gp96-peptide complexes. In this assay, synthetic peptides are incubated with gp96 at 50°C for 10 minutes, cooled to room temperature, and free peptide is removed by ultrafiltration. This peptide-binding assay system is very reproducible—multiple laboratories have used this, or closely related assays, to examine the biochemical basis for peptide binding to gp96 (Blachere et al 1993; Wearsch and Nicchitta 1997; Wearsch et al 1998; Linderoth et al 2000; Vogen et al 2002; Gidalevitz et al 2004). The initial reports did not make clear, however, why incubation at such high temperatures was necessary to observe peptide binding. Subsequent investigations into the molecular basis for this phenomenon have found that incubation of gp96 at 37°C for very extended time periods (≥24 hours), or at elevated temperatures (50°C) for brief periods, elicited an irreversible tertiary conformational change that was associated with exposure of a hydrophobic domain(s) and enhanced peptide-binding activity (Wearsch and Nicchitta 1997; Wearsch et al 1998). Importantly, efforts to determine if such a gp96 conformational state exists in vivo have so far proven negative (Rosser and Nicchitta, unpublished data). The described conformational change is also associated with a marked propensity for homo-oligomerization, likely the consequence of the time- or temperature-induced exposure of a hydrophobic domain(s) and is displayed by recombinant gp96 N-terminal geldanamycin or adenosine nucleotide-binding domain as well (Rosser et al 2004). Significantly, when incubations are performed in the presence of ligands for the gp96 NTD (ATP or adenosine diphosphate, radicicol or geldanamycin), the conformational conversion is dramatically suppressed (Rosser et al 2004). These latter findings are of relevance to the peptide-binding assay used by Vogen et al (2002) and Gidalevitz et al (2004), where maximal VSV8 binding to the gp96 NTD required extended (36 hours) incubation at 37°C, was prevented by prior addition of radicicol, and displayed a remarkably low affinity for peptide, with half-maximal peptide binding being observed at ca 0.5 mM peptide and maximal peptide binding at 0.8 mM peptide. The question of whether peptides are ever present at such concentrations in cells will be discussed in the next section. As noted previously, such data suggest that peptide binding to the gp96 NTD can only occur to that gp96 fraction that has

undergone the time- or temperature-dependent conformational conversion to a peptide-receptive conformation (Wearsch and Nicchitta 1997; Wearsch et al 1998; Vogen et al 2002). In critically analyzing the body of literature on gp96-peptide interactions, the difficult question must, therefore, be posed: is the low affinity, poorly reversible peptide association phenomenon that has been studied by many laboratories (including the author's) of cell biological relevance? Again, peptide binding is limited to that fraction of gp96 that has undergone a tertiary structural conversion that, to date, lacks an in vivo correlate. As will be discussed later, recent investigations into the cell biology of peptide production or consumption indicate that peptides have an exceedingly brief lifetime in cells and do not accumulate to significant chemical concentrations. Analyses of peptide-binding specificity raise an additional concern. Where this has been most thoroughly investigated (Vogen et al 2002), semi-log plots of fractional peptide binding vs competitor peptide concentration were linear, which suggest that peptide association with gp96 occurs through an adsorptive, rather than bioselective, mechanism. Further analyses of the kinetics of peptide binding and, ideally, atomic-level structures of multiple gp96 NTD-peptide complexes will be helpful in distinguishing between adsorptive and bioselective peptide binding mechanisms.

A BRIEF HISTORY OF THE LIFE OF A PEPTIDE

The above discussion is intended to promote investigation into whether the in vitro gp96-peptide–binding reaction is of physiological relevance and thus whether it recapitulates the peptide-binding process proposed to occur in vivo (Srivastava 1993; Srivastava 1994; Srivastava et al 1994). In posing this question, it should again be reiterated that in vitro assembled complexes of gp96 and synthetic peptides are efficiently internalized through APCs and their bound peptides re-presented on MHC class I molecules to yield peptide-specific T cell activation (Blachere et al 1993; Srivastava et al 1994; Navaratnam et al 2001; Berwin et al 2002; Hilf et al 2002; Staib et al 2004). These findings are of clear immunotherapeutic significance, particularly in light of recent findings demonstrating the validity of this approach in human cell-based assays (Staib et al 2004).

Given the abovementioned concerns regarding the physiological validity of current in vitro assays of gp96-peptide interactions and in an effort to precisely define the mechanism of gp96-elicited tumor immunity, it is also of value to critically examine the null hypothesis that stress protein function in the induction of antitumor immunity can be independent of bound peptides (Baker-LePain et al 2002). Where this has been examined, experiments have demonstrated that gp96 derived from nontumor cells can elicit antitumor immune responses, though the mechanistic basis for this response also remains uncertain (Tamura et al 1997; Baker-LePain et al 2002). In addressing the null hypothesis, it is necessary to define if, when, and how stress proteins, and in particular gp96, intersect with the processes of peptide production, peptide trafficking, or peptide loading (or all) onto MHC class I molecules in the cell.

The past few years have been a rich period of discovery into the mysteries of peptide antigen generation and capture by MHC class I molecules. Of particular relevance to this discussion, Schubert et al (2000) found that a surprisingly high fraction of newly synthesized proteins represent ''defective ribosomal products,'' or DRiPs, which undergo rapid proteasome-mediated degradation (Schubert et al 2000; Yewdell et al 2001). Furthermore, quantitative analysis of the rates of DRiP processing and MHC class I capture of an assumedly abundant cellular supply of peptides identified a very inefficient capture process—on average approximately 2000 protein molecules were degraded to yield a single MHC class I–peptide complex (Princiotta et al 2003). At face value, such findings should weigh in favor of the formation of stress protein–peptide complexes; with stress proteins being among the most abundant proteins in the cell, it would be expected that these proteins would serve as an abundant ''sink'' for the highly active process of peptide generation. However, detailed investigations into the intracellular fate of peptides demonstrated that peptides have lifetimes noteworthy for their brevity, with model peptides displaying half-lives of seconds (Reits et al 2003, 2004). The remarkably transient existence of intracellular peptides is a consequence of intracellular aminopepidases, in particular the peptidases TPPII and thimet oligopeptidase, which rapidly degrade proteasome products (York et al 2003; Reits et al 2004). The cytosol is not the only subcellular compartment with a healthy appetite for peptides. After transporter associated with antigen processing (TAP)-mediated transfer across the membrane of the ER, the ER-resident aminopeptidase ERAAP acts to trim peptides to the ideal length (8–9) amino acids necessary for assembly onto nascent MHC class I molecules (Saric et al 2002; Serwold et al 2002; York et al 2002).

The studies referenced above identify a surprisingly active process of peptide generation and degradation, with peptide assembly onto MHC class I molecules being a quite rare event. In addition, quantification of the diffusion rates of small, fluor-labeled peptides in cells yielded diffusion rates significantly higher than that of a peptide-(stress)protein complex (Reits et al 2003). Thus, and though quite distinct experimental approaches have been applied to the question, there is as yet no evidence to implicate cytosolic or ER stress proteins, such as Hsp70 or gp96, as physiologically relevant antigenic peptide-

binding partners. The question of whether cytosolic stress proteins influence the metabolic fate of peptides was also addressed in a study from the Shastri laboratory (Kunisawa and Shastri 2003). Using a detailed analytical–immunological protocol to track the processing of proteolytic intermediates in the class I antigen processing pathway, Kunisawa and Shastri (2003) discovered that proteolytic intermediates could be identified in high molecular weight cellular complexes. Cell fractionation, stress protein immunoprecipitation, and RNA interference (RNAi) experiments conclusively identified TriC, a chaperonin thought to function primarily in the assembly of actin and tubulin, as a recipient for early proteolytic ovalbumin intermediates (Kunisawa and Shastri 2003). Whether other cytosolic chaperones, in particular those that might be assembled onto DRiPs, participate in the processing pathways for (poly)peptide precursors, promises to be an interesting area of study.

Although the increasingly sophisticated understanding of the processes governing antigenic peptide production and presentation has yet to identify a significant role for the proposed peptide-binding function of gp96, the plethora of immunological data indicating that gp96 can function as a cross-priming antigen suggests otherwise (Srivastava et al 1994; Singh-Jasuja et al 2000; Li et al 2002). This possibility was addressed in 2 recent investigations. In 1 study, cells were engineered to produce the model antigen ovalbumin in different subcellular compartments, and the efficiency of cross-priming was examined in vivo (Shen and Rock 2004). In this experimental design, similar levels of antigenic peptide antigen (SIINFEKL) were produced, and K^b/SIINFEKL complexes assembled, regardless of the subcellular location of the antigen (Shen and Rock 2004). Nonetheless, the efficiency of cross-priming correlated most closely with the relative stability of the particular ovalbumin construct, suggesting that ovalbumin itself, or relatively large fragments thereof, served as the relevant cross-priming antigen (Shen and Rock 2004). Furthermore, cross-priming antigen activity could be readily separated from major cellular stress proteins, leading the authors to conclude that stress protein–peptide complexes are unlikely to serve a significant role in the phenomenon of cross-priming (Shen and Rock 2004). This particular conclusion was mirrored in a recent study into the molecular basis of cross-priming (Norbury et al 2004). This study demonstrates that proteasomal substrates, but not proteasomal products or stress protein–peptide complexes, function as cross-priming antigens (Norbury et al 2004). By comparing cross-priming activity in cells engineered to synthesize either ovalbumin or ovalbumin antigenic peptide, Norbury et al (2004) demonstrated that only those cells engaged in the synthesis of protein antigen precursor exhibited in vivo cross-priming activity. In addition, Norbury et al (2004) demonstrate that altering protein stability, through inhibition of proteasome activity, markedly enhances cross-priming activity. These workers, too, conclude that stress protein–peptide complexes are unlikely to contribute to the phenomenon of cross-priming, rather, larger (poly)peptide proteasome substrates serve as the physiological message (Norbury et al 2004). Consistent with this view, Fleischer et al (2004) reported that though complexes of gp96 and synthetic melanoma peptide antigens can be efficiently processed by APC to yield stimulation of melanoma reactive CTL clones, no significant melanoma reactive CTL activity was obtained from APC pulsed with melanoma-derived gp96.

CONCLUSIONS

It is well established that gp96 can elicit antitumor immune responses. Despite considerable skepticism from the scientific community, this founding observation has proved quite reproducible. The guiding hypothesis for the immunogenicity of gp96 identifies an antigenic peptide-binding function for gp96, with gp96-peptide complexes serving as surrogate cross-priming antigens in the elicitation of tumor-directed CTL. However, in in vivo experiments conducted with model antigens, no physiological role for putative gp96-peptide complexes in cross-priming has been identified. In addition, recent advancements in our understanding of the cell biological basis of antigenic peptide generation, processing, trafficking, and assembly onto MHC class I molecules do not identify a function for gp96-peptide interactions. From a cell biological perspective, because it is becoming increasingly apparent that peptides are highly unstable and very unlikely to accumulate to significant chemical concentrations, it is now necessary to consider that gp96, as well as other stress proteins, do not interact with small peptides but rather solely with (poly)peptides, as they fulfill their cellular roles as molecular chaperones (Nicchitta and Reed 2000).

The question, however, remains. What is the mechanism of gp96-elicited tumor immunity? We favor the hypothesis that the physiologically relevant aspect of gp96 function in tumor immunity reflects its capacity to activate the innate immunity arm of the cellular immune response (Baker-LePain et al 2002; Reed et al 2003; Baker-LePain et al 2004). In this view, we suggest that observed tumor antigen-specificity is derived from (poly)peptide contaminants present in biochemically purified preparations of gp96 (Reed et al 2002), a proposal consistent with recent opinions on the physiologically relevant structural form of cross-priming antigens (Norbury et al 2004; Shen and Rock 2004). This hypothesis, too, is currently undergoing critical evaluation.

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