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AN INTEGRATED VIEW OF THE ROLES AND MECHANISMS OF HEAT SHOCK PROTEIN GP96-PEPTIDE COMPLEX IN ELICITING IMMUNE RESPONSE

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1. ABSTRACT

Heat shock protein (HSP) gp96, or grp94 is an endoplasmic reticular (ER) paralog of the cytosolic HSP90. Being abundant and non-polymorphic, gp96 plays significant roles in maintaining protein homeostasis in the secretory pathway. This "house-keeping" role of gp96 has now been overshadowed by the intriguing findings that gp96 modulates both the innate and adaptive components of the immune system. It has been found that, (i) gp96 is one of the major peptide binding proteins in the ER, (ii) gp96 interacts specifically with receptors including CD91 and possibly toll-like receptors (TLRs), on the surface of professional antigen presenting cells (APCs), (iii) interaction with APCs leads to re-presentation of gp96-chaperoned peptides to the major histocompatibility complex (MHC) molecules of APCs, (iv) direct access of gp96 to APCs triggers functional activation of APCs. In this review, we will examine each of these immunological attributes of gp96 critically. As experimentalists, we will also propose specific experiments to examine the argument that gp96, perhaps along with other members of HSP family, is *the* antigenic carrier for mediating cross priming of antigen-specific T lymphocytes in vertebrates.

2. INTRODUCTION

2.1. Structurally unaltered gp96 and other HSPs are "tumor rejection antigens"

Fueled by the idea of immunosurveillance (1) and the dream that tumors can be dealt with by vaccinations, tumor immunologists have long been fascinated by the immunological differences between normal cells and their malignant counterparts. Successful tumor-specific vaccines are dependent on the proof and identification of tumor antigens, against which an immune response leads to tumor rejection. The existence of these so-called tumor rejection antigens (TRAs) was immunologically defined by a series of transplantation experiments in rodents using syngeneic, chemically-induced tumors performed as early as in the 1940s (2-6). Inactivated tumor cells were shown to immunize syngeneic animals against subsequent challenge with live tumors of the same origin. Soon it was found that this phenomenon was not restricted to tumor types or hosts. In addition, tumor immunity generated by immunization from whole tumor cells was shown to be exquisitely specific. Thus, tumor A can only immunize animals against the challenge of tumor A but not against that of tumor B, and vice versa, even if tumor A and B are derived from the same histological types, induced by the same carcinogen, or even developed in the same host. This suggests that the antigenic determinants vary among different tumors, and predicts that an effective cancer vaccine has to be derived from autologous tumors.

Since whole tumor cell lysate could also immunize and confer protection against tumors, a tedious biochemical approach was undertaken to identify the molecular basis for tumor rejection. Tumor cell lysates were fractionated biochemically. Each fraction was then injected to animals followed by live tumor challenge, to determine which fraction confers tumor protection. The active fraction was then further fractionated until a homogenous population of proteins was identified. This approach has led to the successful identification of several TRAs. Surprisingly, a majority of these TRAs turned out to be HSPs including HSP90 (7), gp96 (8-10), HSP70 (11), calreticulin (12), HSP110 and GRP170 (13), which collectively play critical roles in facilitating protein folding and unfolding in the cell (14).

Gp96 stands for glycoprotein of 96 kDa. In humans, only one true gene locus has been mapped and was named tra-1 (15). In the literature, gp96 is also referred to as GRP94, Erp99, endoplasmin, etc (16, 17). The first report to link gp96 with tumor immunity came from Srivastava and Das who showed that in a Wistar rat Zajdela ascitic hepatoma model, a homogenous preparation of a ~100 kDa protein (named ZAH-TATA) was able to immunize against challenge with the parental tumors (8). Although ZAH-TATA was not molecularly defined in the original report, its biochemical properties suggest that ZAH-TATA is the rat gp96 homolog.

Using methylcholanthrene (MCA)-induced fibrosarcoma (Meth A) as a model in BALB/c mice, a series of papers in the 1980s described the ability of gp96 to immunize naïve mice against challenge with the tumor from which gp96 was purified (9, 10, 18). Gp96 is expressed ubiquitously in both normal and tumor cells and turned out to be identical to Erp99, an abundant protein of the endoplasmic reticulum (19), although surface expression of gp96 was also demonstrated (9, 20). Subsequently, gp96 was found to be a *bona fide* protein chaperone and HSP because of heat or stress inducibility (20, 21), adenosine nucleotide binding and ATPase activity (22), and apparent unselective binding to unfolded proteins (16). Functionally, gp96 fulfills all the criteria for a TRA. Immunization of animals with tumor-derived gp96, but not gp96 purified from normal tissues or another tumor type, protects the animals against the tumor of gp96 origin. It was immediately speculated then that there are tumor specific alterations of gp96 itself (23). Yet, surprisingly, when the cDNA of gp96 was cloned and sequenced from both tumors and normal tissues, no such mutation of gp96 gene was discovered (24, 25).

2.2. Gp96 is not antigenic per se: the hypothesis and emergence of evidence for gp96 to chaperone antigenic peptides for adaptive immunity

The protective immunity elicited by gp96 vaccination is exquisitely specific. Various possibilities that might explain this specificity were considered (23). First, it was proposed that gp96 might be an extremely polymorphic molecule, much like MHC, T cell receptor and immunoglobulin. Second, gp96 was suggested to be especially prone for somatic mutations during tumorigenesis. Thus tumor A would have a mutation pattern of gp96 distinct from that of tumor B. These two possibilities were quickly rejected thanks to molecular cloning and sequencing. Third, extensive post-translational modification and the differences generated by it could have accounted for the differences in immunogenicity of gp96. This, too, was found to be incorrect. Gp96 was expressed abundantly in both normal and tumor cells. Gp96 from different sources was found to be identical in size and charge, and has overlapping behavior on various chromatographic columns. Fourth, gp96 itself is not the true antigen; the antigenicity of gp96 preparations is actually due to trace contamination of other proteins. This argument was thought to be unlikely due to the fact that gp96 preparations were found to be homogenous by all criteria tested, including: a single band on silver stained SDS-PAGE, single-peak UV absorption curve at the last step of chromatography of standard gp96 preparation, and undetectable materials with significant mass other than a 96 kDa protein by mass spectrometry. Finally, it was postulated that gp96 preparation harbor small molecular weight peptide moieties, given the fact that tumor-specific T cells recognize tumor-derived peptides in association with MHC molecules. This hypothesis was catalyzed by the landmark work from Townsend and others at the time, which showed peptides from all cellular proteins can be potentially charged to MHC class I molecules irrespective of their intended subcellular localization (26, 27).

This peptide-binding hypothesis, while offering an explanation for the specificity associated with gp96 vaccination, was limited by the fact that the mechanism

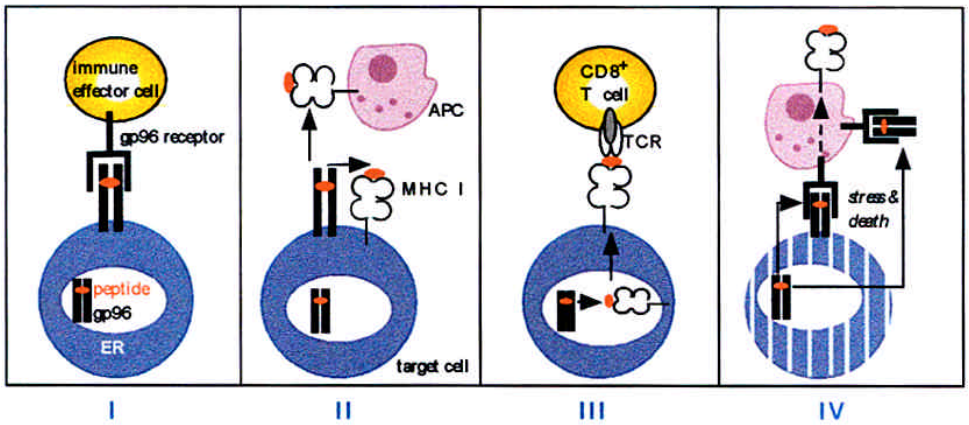

Figure 1. Models for the roles of gp96 in immune response. Gp96 is an abundant peptide binding protein in the ER. The observation that gp96-peptide complexes can immunize for peptide-specific immunity has led to speculations on the physiological roles of gp96 in immune responses. These ideas can be arbitrarily broken down into 4 distinct, albeit non-exclusive hypotheses: (I) gp96-peptide complex is the direct target of immune effector cells in a receptor dependent manner (II) surface gp96 mediates direct transfer of its associated peptides to MHC molecules on APCs and/or cells where gp96 originates (III) gp96 facilitates peptide transfer to MHC I in the ER (IV) extracellular gp96, due to cell surface expression and secretion, or release from dead cells, cross-present its associated peptides to MHC molecules on APC for cross-priming of T cells.

used by gp96-peptide complexes to activate an immune response was not clear. Various hypothetical mechanisms were proposed (23,25, 28-32), many of which have now been experimentally tested, and some of which have been validated (figure 1). These hypothesis and ideas have implications for the physiological roles of gp96 in immune response.

Hypothesis one: Gp96-peptide complexes are involved in presenting peptides directly to the adaptive immune system. This would mean that gp96 is a direct antigen-presenting molecule on the cell surface, something analogous to MHC itself. This would also mean that a gp96 receptor or ligand has to be expressed on the surface of cells of the immune system. This hypothesis is supported by suggestions that surface HSPs may be receptors for NK cells (33-35) and non-conventional T cells, such as Tgamma-delta (36, 37) and $CD4^{-}CD8^{-}$ double negative T cells (38, 39), as well as data showing gp96 on the surface of a number of cell types (20, 40, 41)

Hypothesis two: Gp96 can directly transfer peptides to MHC class I on the cell surface. There are two distinct scenarios. First, for antigen positive target cells such as tumor cells or virally infected cells, transfer of surface gp96-chaperoned peptides to MHC I can facilitate direct priming of antigen-specific T cells or potentiate the recognition of target cells by effector T lymphocytes. To date, this possibility still exists and has not been experimentally challenged. Second, transfer of peptides chaperoned by cell surface gp96 on target cells to MHC I of professional antigen presenting cells might result in more efficient cross priming of antigen-specific T cells by APCs. However, this possibility is unlikely to be true, due to the later finding that only receptor-mediated endocytosis of gp96-peptide complex leads to cross-presentation of peptides to MHC class I (42).

Hypothesis three: Peptides bound to gp96 in the ER eventually are released and associated with MHC class I in the target cells. The association of gp96-bound peptides with class I molecules is, in this case, proposed to be due to exogenous or tumor-derived gp96 mimicking the normal cellular role of gp96 within the APC after receptor-mediated endocytosis. Although there is no direct evidence for an involvement of gp96 in antigen presentation to class I molecules, the molecular properties of gp96, including peptide and ATP binding (22, 43-45), subcellular location (19, 46), aminopeptidase activity (47) and association with MHC class I (Z Li and PK Srivastava, unpublished observation), all suggest that gp96 may have a role in conventional antigen presentation which is being exploited during immunization.

Hypothesis four: The presence of gp96-peptide complexes in the ER plays insignificant roles in antigen presentation in a steady-state situation (32). Even in stress and death conditions, such as viral infections and transformation, the presence of enough chaperones in the ER and the requirement for as few as 100 MHC-peptide complexes to activate T cells would suggest that the role of gp96-peptide complexes is not to sensitize target cells for T cell recognition. Instead, the major function of gp96-peptide complexes is to be released or to be accessible to the immune system during stress and death, followed by specific interaction with APCs (30, 32). Such an interaction leads to the activation of APCs and cross-presentation of gp96-associated peptides from target cells to the MHC I of APCs. This highly efficient and regulated process has been shown to be dependent on gp96 receptors such as CD91 (48, 49), CD36 (50) and Toll-like receptors (TLRs) (H Schild, personal communications). This hypothesis has recently received a considerable amount of support. We shall now summarize and examine the supporting data in the following sections.

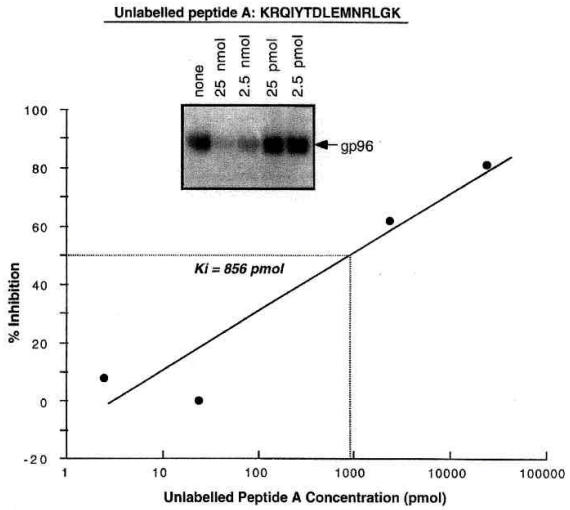

Figure 2. Gp96 binds to exogenous peptides *in vitro.*10 pmol of gp96 was incubated with 25 pmol I^{125}-labeled peptide A (KRQIYTDLEMNRLGK, single amino acid designation) in the absence or presence of increasing concentration of nonradioactive peptide A at 60°C for 10 minutes followed by incubation at 37°C for 30 min. Stable gp96 peptide complex is then resolved on 10% SDS-PAGE and visualized after autoradiograph (inset). The percentage of cold peptide inhibition was plotted as a function of the concentration of unlabelled peptide A. The dissociation constant (*Ki*) in this experiment was calculated to be 856 pmol.

3. SEARCHING FOR BIOCHEMICAL CLUES FOR THE ROLES OF GP96 IN ADAPTIVE IMMUNITY

3.1. Mechanistic and structural aspects of gp96-peptide interaction

Molecular chaperones are known to associate with polypeptide backbones to facilitate protein folding and unfolding. One such chaperon, HSP70, was studied extensively for its ability to associate with peptides. Using a peptide affinity column, Rothman and colleagues demonstrated direct association of peptides with GRP78, an ER lumenal HSP70 (51). This peptide binding ability was further characterized using a set of random peptides synthesized *in vitro* and testing the abilities of these substrates to stimulate the ATPase activity of GRP78. It was found that the peptide-binding site of GRP78 selects for aliphatic residues and accommodates them in an environment energetically equivalent to the interior of a folded protein (52). Using affinity panning of bacteriophage libraries that displayed random octapeptide or dodecapeptide sequences at the N-terminus of the adsorption protein (pIII), the peptide binding property of GRP78 was further investigated by Gething, Sambrook and their colleagues (53) It was found that GRP78 preferentially binds peptides containing a subset of aromatic and hydrophobic amino acids in alternating positions, suggesting that peptides bind in an extended conformation, with the side chains of alternating residues pointing into a cleft on the GRP78 molecule. This conclusion was supported by the finding that synthetic peptides with sequences corresponding to those displayed by GRP78-binding bacteriophage, bind to GRP78 and stimulate its ATPase activity. The peptide-binding fragment of a highly homologous prokaryotic HSP70 DnaK was structurally resolved (54).

The peptide binding property of gp96 was suggested primarily by the finding that structurally unaltered gp96, purified from tumor cells but not from normal tissues, could immunize for T cell immunity. This suggestion led immediately to the prediction that gp96 should bind to viral antigens, or other model antigens in cells. If so, immunization with gp96 purified from virally infected cells should be expected to induce T cell immunity against viral products. This prediction turns out to be true. Immunization with gp96 isolated from cells infected with SV-40 virus or influenza virus A induces CTLs against cells infected with those viruses (55). It was found that gp96 preparations from vesicular stomatitis virus (VSV)-infected cells elicited a specific CTL response against VSV viral proteins (56). Furthermore, the antigenic peptide (a H-2-K^b restricted 8-mer from VSV nucleoprotein) associated with gp96 was chromatographically demonstrated. Similarly, gp96 isolated from cells expressing beta-galactosidase (beta-gal) and minor H antigens induced CTLs specific for beta-gal as well as minor antigens (57). Up to the present, gp96 has been found to associate naturally with peptides derived from tumor antigens (44), viral antigens (56, 58), bacterial antigens (59), minor histocompatibility antigens (57), and various other model antigens (60) (table 1). Using a microsome based system and cross-linkable peptide substrates, it was found that gp96 is one of the major peptide acceptors in the lumen of the ER, in addition to protein disulfide isomerase (PDI) and GRP170 (61, 62).

In an attempt to define the parameters related to gp96-peptide interaction, it was found, accidentally, that the gp96-peptide complex is thermally stable (63). Incubation of gp96 with a radioactive peptide at 4°C results in no association of radioactive peptides with gp96. Raising the temperature up to 60°C led to complex formation, as measured by acquisition of radioactive material by gp96. Surprisingly, the complex is stable and not sensitive to SDS, since the radioactive complex can be resolved on SDS-PAGE and visualized by autoradiography (figure 2). This simple heat-dependent folding assay has allowed us to complex exogenous peptides to gp96 with ease. Gp96-peptide complexes reconstituted *in vitro* elicited antigen specific CD8+ cytotoxic T lymphocytes, in a manner that is indistinguishable from native gp96-peptide complexes (63). Using this assay, we have performed cold peptide competition experiments to calculate the association/dissociation constant (Ki) of gp96 in interaction with a 15-mer peptide (KRQIYTDLEMNRLGK) to be 856 pmol (figure 2). By shortening this peptide from either N or C-terminus, one residue at a time to the minimal 5-mer peptides, we generated 20 non-overlapping peptides. Each of these peptides was found to inhibit the binding of gp96 to the 15-mer peptides, albeit at different efficiency (table 2). This data has to be interpreted with caution since the sequence, solubility of the peptides, in addition to the peptide length, might influence the binding to gp96. It does seem that gp96 binds preferentially to 11-mer peptides.

Table 1. Antigenic peptides chaperoned by gp96

Source of Antigenic peptides	Assay	Reference
Viral Antigens		
SV40	CTL	Blachere *et al* (58)
Influenza	CTL	Blachere *et al* (58)
Vesicular stomatitis virus	CTL	Suto & Srivastava (87)
	Chromato-graphy/CTL	Nieland *et al* (56)
Bovine herpesvirus 1	CTL/Ab	Navaratnam *et al* (116)
Hepatitis B	Chromato-graphy	Meng *et al* (148)
Tumor Antigens		
Meth A fibrosarcoma	Prophylaxis	Srivastava *et al* (9), Udono and Srivastava (149)
	Prophylaxis/therapy	Tamura *et al* (150)
CMS5	Prophylaxis	Srivastava *et al* (9)
CMS13	Cytotoxcity/ Prophylaxis	Palladino *et al* (10)
Lewis lung carcinoma	Prophylaxis/Therapy	Tamura *et al* (150)
B16.F10 melanoma	Prophylaxis/Therapy	Tamura *et al* (150)
CT26 colon carconoma	Therapy	Tamura *et al* (150)
UV6138	CTL/ Prophylaxis	Janetzki *et al* (151)
UV6139SJ	CTL/ Prophylaxis	Janetzki *et al* (151)
Dunning G Prostate cancer	Prophylaxis/Therapy	Yedavelli *et al* (152)
A20 B cell lymphoma	Prophylaxis	Graner *et al* (153)
15/0 lymphoma	Prophylaxis	Robert *et al* (154)
Bacterial Antigens		
Listeria monocyto-genes	CTL/ Prophylaxis	Zugel *et al* (59)
Mycobacte-rium tuber-culosis	Prophylaxis	Zugel *et al* (59)
Model Antigens		
Ovalbumin	CTL	Breloer *et al* (60)
Beta-galactosidase	CTL	Arnold *et al* (57)
Normal Cellular Antigens		
Minor H	CTL	Arnold *et al* (57)

Table 2. Peptide-binding specificity of gp96

Peptide/Length	Sequence	M. W.	Ki (pmol)
15 mer	KRQIYTDLEMNRLGK	2115	856
14 mer	RQIYTDLEMNRLGK	1987	3162
13 mer	QIYTDLEMNRLGK	1831	3383
12 mer	IYTDLEMNRLGK	1703	56
11 mer	YTDLEMNRLGK	1590	7
10 mer	TDLEMNRLGK	1427	1
9 mer	DLEMNRLGK	1326	464
8 mer	LEMNRLGK	1211	10000
7 mer	EMNRLGK	1098	11052
6 mer	MNRLGK	969	87332
5 mer	NRLGK	838	8733
14 mer	KRQIYTDLEMNRLG	1987	26
13 mer	KRQIYTDLEMNRL	1930	825
12 mer	KRQIYTDLEMNR	1802	1584
11 mer	KRQIYTDLEMN	1646	351
10 mer	KRQIYTDLEM	1532	4365
9 mer	KRQIYTDLE	1401	10000
8 mer	KRQIYTDL	1272	2015
7 mer	KRQIYTD	1159	3433
6 mer	KRQIYT	1044	3030
5 mer	KRQIY	943	3727

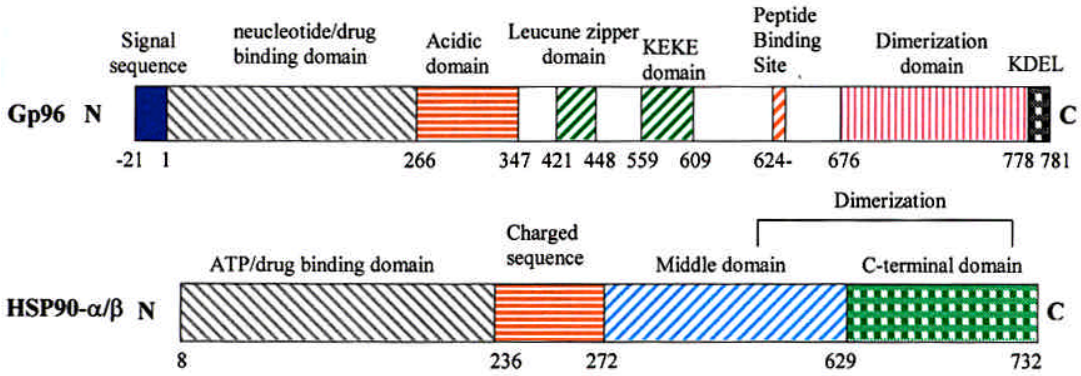

Figure 3. Gp96 is structurally similar to HSP90. Distinct from HSP90, gp96 possesses N-terminal signal peptide sequence and C-terminal KDEL motif responsible for ER retention of gp96 (160). Both proteins contain highly conserved N-terminal nucleotide/geldanamycin binding site, followed by acidic domain that regulates drug binding. This domain in gp96 is then followed by leucine zipper and KEKE domain, which are involved in protein-protein interaction (161). The single peptide binding site of gp96 is mapped at 624-630 (43). The C-terminal portion is crucial for dimerization (64). In addition to ATP/geldanamycin binding site in N-terminal domain of HSP90 (64), there is also a charged sequence which is relatively divergent, middle domain and C-terminal dimerization domain (68, 69). Both gp96 and HSP90 form homodimers constitutively (162, 163). HSP90 family includes HSP90-alpha and HSP90-beta, which are highly homologous to each other, with a slight difference in C-terminal domain. HSP90-beta dimerization is less stable than that of HSP90 (163, 164).

Wearsch *et al*. have initiated a series of studies on the structure/function relationships of gp96 in binding to its ligand. By analyzing a structure of native gp96, they showed that gp96 is an obligatory dimer with an extended, rod-like structure (64). Furthermore, they have identified that a discrete domain, corresponding to amino acid residues 676-719 regulates dimeric assembly and displays autonomous dimerization activity. The saturable, specific and temperature-sensitive peptide binding activity of gp96 was also demonstrated (65). In later studies, Wearsch *et al*. used environment-sensitive fluorescent probes to identify that the hydrophobic pocket in gp96 is a site of peptide binding (65). These results have been incorporated into a structural model of gp96 and provide a framework for future studies on its chaperone activity (figure 3).

Recently, Sastry and colleagues (43) as well as Wearsch *et al* (65) suggested that peptides bind to an open conformation of gp96 in a hydrophobic pocket and there may be a conformational change in gp96 for the loading of peptide. The peptide was found to bind to a hydrophobic region of gp96 in low salt conditions, and to a more hydrophilic region of the protein in high-salt conditions. In a related study, the peptide-binding site was mapped to amino acid residues 624-630 in a highly conserved region and the peptide-binding pocket abuts the dimerization domain of gp96 (figure 3). Pursuing a similar study, Linderoth *et al*. constructed a mutant form of gp96 protein lacking the dimerization domain, and then analyzed the structure of a wild type and a mutant gp96 and their peptide complexes. This result has shown that the bound antigenic peptide was located in a hydrophobic pocket and depletion of the dimerization domain affected the peptide-binding microenvironment (66). By using scanning transmission electron microscopy, it was found that gp96-peptide-complexes exist in higher order multimeric complexes, and some specific aromatic amino acid residues in the gp96 peptide-binding.

Many questions remain regarding the structural basis of gp96-peptide interaction. Studies into the regulation of its interaction both *in vitro* and *in vivo* would be insightful. Extensive mutagenesis, coupled with functional studies in a gp96 null cell line, as well as crystallographic studies, would provide a final answer to these questions.

3.2. ATP binding and ATPase activity of gp96: where is the functional link?

One of the functional hallmarks of many HSPs is their ability to associate with and hydrolyze ATP. In some cases ATP seems to regulate substrate binding, and in other cases it seems to act as a catalyst for rapid protein folding. The ATP binding and ATPase activity of the HSP90 family were controversial until two pivotal papers in 1997 (67,68), which demonstrated, by crystallography, the association of the N-terminal highly conserved 25 kD domain of HSP90 with ATP, as well as the association of the same domain with geldanamycin (GA). GA is one of the ansamycin drugs, which specifically target HSP90 (69) and it inhibits the HSP90 ATPase with nanomolar affinity. The importance of ATPase activity of HSP90 was unequivocally demonstrated by mutagenesis studies in yeast. Despite a slow ATPase activity *in vitro*, a single point mutation to abolish HSP90 activity is unable to rescue the lethal phenotype in yeast (70).

Gp96 is structurally similar to HSP90 (figure 3). Several studies have addressed whether gp96 is also an ATP binding protein and ATPase. Using photoaffinity labeling of purified gp96 to measure ATP binding by gp96, Li and Srivastava found that gp96 interacts with ATP directly *in vivo* (22). Highly purified gp96 was then measured *in vitro* for ATPase activity. Indeed, a Mg^{2+}-dependent ATPase activity was consistently observed. Moreover, this activity was stimulated by misfolded proteins, such as casein, *in vitro*, but not by peptides,

suggesting that the ATPase activity of gp96 is closely related to the chaperone function of gp96. Csermely *et al*. also have shown that gp96 has two ATP-binding sites and possesses an intrinsic autophosphorylation activity (71). Nichitta and colleagues contributed extensively to this question using an *in vitro* assay exclusively. They reported earlier that gp96 was able to bind to ATPs, but the low ATPase activity may not be the intrinsic property of gp96 (72). More recently it was shown that an adenosine derivative N-ethylcarboxamidoadenosine (NECA) binds to gp96 with a stoichiometry of 1 mol of NECA per 1 mol of gp96 dimer (73). In addition, NECA binding to gp96 can be wholly competed by geldanamycin, radicical, and ATP, suggesting that NECA binds to the N-terminal adenosine nucleotide-binding domain. These data are consistent with the notion that the N-terminal ATP-binding domain of gp96 is functional equivalent to HSP90, and that gp96 binds to ATP.

The controversy surrounding the ATPase activity of gp96 may stem from the fact that the activity *in vitro* is weak, and it is therefore sensitive to varying conditions associated with assays used by different investigators (16). The ultimate solution would be to precisely mutate the potential residues involved in ATP binding and hydrolysis and study if mutant molecules are associated with functional loss of gp96. Precisely this was done by Randow and Seed recently (74). By subjecting a pre-B cell line to random frame shift mutation and selecting a variant for loss of endotoxin responsiveness, a cell line with both gp96 alleles mutated was serendipitously discovered. This gp96 null mutant is unable to export TLR2 and TLR4 to the cell surface. The phenotype can be rescued by transfecting wild type gp96 cDNA back to the cell, but not by transfecting gp96 mutants with point mutations to disrupt ATP binding and ATPase activity of gp96. It was further shown that gp96 interacts with TLR directly, thus establishing that gp96 is a chaperone for TLRs, and the interaction with ATP is vital for this process.

The other functional link between ATP and gp96 comes from the study of peptide transport into an *in vitro* microsome system (75). By using DIDS (4,4'-diisothiocyanatostilbene 2,2'-disulfonic acid), an inhibitor of the ER-resident ATP transporter and a photo-crosslinkable peptide, it was found that the transport of peptides both into and out of the lumen of microsomes (75) and binding to gp96 and PDI are strictly dependent on ATP (Pieter Spee, personal communication).

3.3. Requirement for further trimming of antigenic peptides in the ER and the potential role for gp96 in the process

MHC class I molecules present peptide antigens to $CD8^+$ T lymphocytes. The assembly of MHC class I – peptide complexes in the ER is a dynamic and highly coordinated process. There is now strong evidence for the idea that a substantial proportion of peptides generated in the cytosol due to proteosomal action are extended on the amino terminus with respect to the MHC I-binding epitopes (76). These peptides need to be further trimmed in the ER, although redundant cytosolic trimming mechanisms have also been demonstrated (77). A number of studies suggest that MHC I guides the trimming in the ER, perhaps by protecting the octamer and allowing the 'overhang' to be acted upon by an aminopeptidase (78), while other studies suggest a lack of MHC I involvement in the process (79, 80). Menoret *et al*. have shown recently that gp96 is an aminopeptidase *in vitro* (47). Gp96 can trim amino terminus extended 19mer precursors of an octamer epitope derived from the vescicular stomatitis virus to the octamer.

The peptidase activity of gp96 needs to be further characterized with regards to the Km and maximum velocity. Furthermore, the regulation of this activity by other molecules such as ATP, other ER chaperones, or MHC itself needs to be considered. The most relevant question to understand is whether the activity observed *in vitro* is operative *in vivo* (see section 5.1). We are actively studying this process by using gp96 null cell lines.

4. IMMUNOREGULATORY ACTIVITIES OF EXTRACELLULAR GP96: UNVEILING THE "DIRTY LITTLE SECRET" OF IMMUNOLOGY?

The challenge for adaptive immunity is to tolerate self, but at the same time to reject non-self, such as virus-infected cells, or altered self, such as malignancy. This specificity is achieved to a large extent by central and peripheral tolerance mechanism by which autoreactive T cells are negatively selected in the thymus (81), and rendered anergic/apoptotic in the periphery (82). Since neither of these two selection processes is perfect, and autoreactive T cells are readily detectable in the periphery, other mechanisms must exist to ensure the appropriateness of immune responses. Recent developments indicate that the context in which antigens are delivered to the immune system plays important roles in the initiation of immune responses. It is proposed that productive immune responses occur only if there is a "dangerous" environment, which harbors natural or endogenous adjuvants (the need for an adjuvant was called "the dirty little secret of immunology" by Charles Janeway, Jr (83) that are normally not present in the microenvironment of healthy tissues (84). Since dendritic cells play central roles in the initiation of adaptive immunity, such endogenous adjuvants are expected to activate immunogenic DCs (85).

4.1. Extracellular gp96 interacts with APCs

The necessity of APCs for gp96 to induce strong T cell immunity came from studies showing that depletion of the phagocytic function of APCs during the immunization phase abolished the effect of gp96 (86). It was soon discovered that macrophage-like cells in peritoneal exudates were able to re-present or cross-present peptides chaperoned by gp96-peptide complexes to their own MHC class I molecules (87). The unexpected potent efficiency of gp96-peptide complex vaccination and the dependence on APCs led Srivastava *et al*. to suggest the presence of receptor(s) for gp96 on APCs (28). By immunofluorescence and electron microscopy, multiple groups have indeed provided convincing evidence for receptor-dependent interaction of gp96 with macrophages, dendritic cells and B cells (88-91). Furthermore, by using

Table 3. HSPs modulate the function of antigen presenting cells *in vitro*

HSPs	APC	Cytokine Release	Surface Expres-sion	Reference
Soluble gp96	Mouse macro-phage	TNF-alpha, IL-12, Il-1 beta		Basu *et al* (98)
	Mouse DC		MHC-II, CD86	Basu *et al* (98)
	Human DC	IL-12, TNF-alpha	CD 86, CD83	Singh-Jasuja *et al* (89)
Membrane gp96	Mouse DC	IL-12, IL-1beta, MCP-1	MHC-I, MHC-II, CD80, CD86, CD 40	Zheng *et al* (100)
Soluble HSP 70	Mouse Macro-phage	IL-12 TNF-alpha IL-1beta		Basu *et al* (98)
	Mouse DC		MHC-II, CD80, CD86	Basu *et al* (98)
	Human Mono-cyte	IL-6, IL-1beta, TNF-alpha		Asea *et al* (155)
	Human DC		CD40, CD86, CD86	Kuppner *et al* (156)
	Mouse DC	Il-1beta, IL-6, IL-12, TNF-alpha		Moroi *et al* (157)
Soluble HSP 90	Mouse macro-phage	TNF-alpha, IL-1beta, IL-12		Basu *et al* (98)
Soluble HSP 60	Mouse macro-phage	IL-1, IL-6, IL-8, TNA-alpha		Breloer *et al* (158)
	Mouse DC	IL-1, IL-6, IL-8, TNF-alpha	MHC-II, CD86	Breloer *et al* (158)
	Human Macro-phage	IL-6		Kol *et al* (159)
	Human PBMC	IL-6		Kol *et al* (159)

affinity purification with gp96-conjugated column, Binder *et al*. purified the cell surface ligand (receptor) for gp96 from a macrophage cell line, RAW 264.7 to homogeneity. A polyclonal antibody raised against this protein can block the re-presentation of gp96 chaperoned peptide to MHC class I (91). Microsequencing of this molecule by mass spectrometry confirmed it to be CD91, a protein known as alpha 2-macroglobulin receptor or the low-density lipoprotein-related protein. CD91 as a receptor for gp96 was further supported by the evidence that alpha 2-macroglobulin, a previously known CD91 ligand, inhibited re-presentation of gp96-chaperoned antigenic peptides by macrophages, as did antibodies against CD91 (49, 91). It is possible that CD91 is not the only receptor that gp96 can bind, given the fact that the interaction of gp96 with APC triggers a cascade of events that are important for productive immunity.

4.2. Peptide-independent activation and maturation of dendritic cells by gp96

Agents that activate and mature DCs can be broadly classified into two groups: exogenous agents typified by bacterial product LPS (92) and unmethylated CpG molecules (93, 94); endogenous agents such as inflammatory cytokines IL-1 beta and TNF-alpha (95). In the last two years, gp96 and other members of HSP family have emerged as the likely universal DC activators.

The presence of universal (expressed in every cell) endogenous DC activators has long been postulated. Such molecules are expected to be present only in a situation when an immune response is needed. Gallucci *et al* showed that necrotic cells, but not healthy or apoptotic cells can mature bone marrow derived murine DCs (96). Later, Sauter *et al*. showed that human DCs could also be activated by necrotic cells or their supernatant (97). These observations are in agreement with the notion that abnormal death (necrosis) is proinflammatory due to the activation of DCs by soluble molecules liberated. Recent studies suggested that HSPs are the molecular definition of these proinflammatory molecules. Basu *et al* showed that necrotic cells, but not apoptotic cells, release HSPs including gp96, HSP70, HSP90 and calreticulin (98). They also found that soluble gp96 purified from normal tissues, can stimulate bone barrow derived DCs to express MHC-II and the co-stimulatory molecule B7.2, whereas non-HSPs were not able to do so at the same concentration (49, 89, 98). The activation of DCs by soluble HSPs has been independently confirmed by many groups in human and murine system, supporting the hypothesis that HSPs are the sensors for non-physiological death because they are the endogenous activators of DCs (table 3).

Cellular trafficking of gp96 has also been shown to have an impact on immunity. In non-stressful conditions, gp96 resides in the lumen of the ER. Upon stress, both surface expression and secretion of gp96 have been reported (20, 46) Although the cell biological basis of gp96 “ectopic” transport is unknown, its effect on an immune response has been studied. By deleting carboxyl terminal KDEL ER retention signal and fusing with the Fc fragment of immunologlobulin, Podack and his colleagues have engineered a gp96 secretory molecule. Tumor cells secreting gp96-Ig were found to be much more immunogenic (99). Similarly, by fusing a transmembrane domain with the C-terminus of gp96, Zheng *et al* have found that gp96 surface expressing tumor cells induced robust maturation and activation of DCs, as evidenced by up-regulation of MHC-II, CD80, CD86, CD40, and secretion of pro-inflammatory cytokines such as IL-1 alpha, IL-12 and chemokine MCP-1 (100). The activation of DCs by HSPs such as gp96 mirrors the effect by lipopolysaccharide (LPS). It was shown that gp96 activates DCs at least partially through NF-kappa B, although the kinetics is clearly different from that of LPS (98).

4.3. Gp96 induces the migration of dendritic cells to lymphoid organs

Migration of DCs to secondary lymphoid organs is an essential step in priming an adaptive immune response. Accumulating evidence demonstrates that the capacity of DCs to migrate to draining lymph nodes is mediated by chemokines interacting with chemokine receptors (101-103). Inflammatory chemokines, such as MIP-3 alpha (104), MIP-1 alpha and MCP-1 (105) are responsible for recruiting cells to inflammatory sites, while constitutive chemokines, such as MIP-3 beta (105), are responsible for the trafficking of immune cells to lymphoid organs. Immature DCs express high levels of receptors for inflammatory chemokines, such as CCR1, CCR2, CCR5 and CCR6, which guide them to the inflammatory site, where they pick up antigens. Upon maturation, receptors for constitutive chemokines, such as CCR7, are up-regulated, which leads to the migration of DCs to secondary lymphoid organs, where DCs prime naïve T and B cells (104, 106).

Soluble gp96 can induce migration of DCs to draining lymph nodes *in vivo* (107). Immunization of mice with as little as 1 mug of gp96 induced a 5-7 fold enlargement of the draining lymph nodes within hours after injection. This enlargement is the result of accumulation of CD11c+ cells. These presumed Langerhan's cells obtained a mature phenotype and were able to stimulate naïve T cells. DCs activated by cell surface-bound gp96 also have enhanced migratory ability to chemokine MIP-3beta (H Zheng and Z Li, unpublished observation), indicating that chemokine-chemokine receptor interaction is involved in gp96 induced migration of APCs.

4.4. Chaperoning antigenic peptides by gp96 to MHC I of antigen presenting cells: fulfilling a role in cross-presentation?

In general, endogenous antigens are presented to MHC I by non-professional APCs for recognition by CD8+ T cells, whereas exogenous antigens can be picked up, processed and presented to the MHC class II pathway by APCs (108). Since non-APCs do not prime $CD8^+$ T cells due to lack of expression of co-stimulatory molecules, antigens from these cells have to be cross-presented to the MHC I of APCs (109). This cross-presentation pathway is necessary for priming of $CD8^+$ T cells against tumor antigens (110), viral antigens (109) and a variety of model antigens (111). When soluble HSPs, including gp96, were found to immunize effectively for tumor-specific CTLs without the use of an exogenous adjuvant, it was immediately suggested that gp96 plays an important role in cross-presentation (28). Indeed, it was found that exogenous peptides chaperoned by gp96 were presented to MHC I by Pristane-induced peritoneal macrophages (87). Furthermore, gp96 isolated from VSV infected cells of H-2^d background could induce H-2^b restricted VSV-specific CTLs, demonstrating that antigens chaperoned by gp96 can be cross-presented to MHC I of APCs (87). Similar findings were obtained using other model antigens such as minor histocompatibility antigens and beta-galacatosidase (112). Impressively, compared with soluble peptides, the amount of gp96-peptide complexes required to prime T cell response is several orders of magnitude less (63). As discussed previously, this unusual high efficiency led to the suggestion and eventual confirmation that cross-presentation of gp96 chaperoned peptide is receptor mediated.

4.5. Characterization of the gp96 receptors

As described in Section 4.1, interaction of gp96 with its receptor(s) on APCs is expected to induce profound molecular events, leading to the maturation, activation, migration of DCs, as well as cross-presentation of gp96-chaperoned antigens. Not surprisingly, energy was focused on cross-presentation and the identification of key molecules in this process. Using affinity chromatography, the receptor responsible for cross-presentation of gp96 was identified as CD91 (48). Antibodies to CD91 completely inhibited the re-presentation of peptides bound to gp96, supporting the previous evidence that, without receptor-mediated endocytosis, gp96-peptide complexes cannot initiate cross-presentation (42). Further studies by Basu, *et al*. showed that CD91 is a receptor not only for gp96, but also for other peptide chaperones known to induce immune responses via cross-presentation of antigens, namely, calreticulin, HSP70 and HSP90 (49).

It is doubtful that CD91 is also a functional receptor for mediating the effects of gp96 in maturing and activating DCs, since CD91 itself has limited signaling capacity. Interestingly, using a mutant cell line that is defective of signaling by LPS, it was found that gp96 binds and chaperones TLR-2 and TLR-4 selectively, presumably in the lumen of ER (74). This finding raises the possibility that extracellular gp96 interacts with cell surface TLRs for signaling. Indeed, by using various TLR knockout mice and *in vitro* transfection of TLR genes into HEK293 cells, it was shown that gp96 utilizes both TLR2 and TLR4 for signaling (H Schild, personal communication). This data could correlate nicely with the observation that gp96 stimulation of DCs and macrophages leads to translocation of NF kappa B to the nucleus (98). This transcription factor could be extremely important in the downstream effects of gp96 stimulation described above. The toll-like receptors are attractive candidate receptors for gp96 because, like gp96, their function is primitive, and their evolutionary conservation is great. The importance of toll-like receptors in innate immunity supports the hypothesis that gp96 was an important molecule in host defense long before the adaptive immune system developed.

In addition to TLRs and CD91, there is evidence that the scavenger receptor CD36, present on macrophages and other phagocytic cells, can also interact with gp96. One of the clues is that the gp96 receptor(s) as well as CD36 were down-regulated on the surface of mature dendritic cells (89). Evidence for CD36 as a receptor for gp96 includes: CD36 null macrophages bind 52% less gp96 than their $CD36^+$ counterparts, and cells transfected to express human CD36 bound more gp96 than untransfected, $CD36^-$ cells (50) (Srivastava PK, personal communications). CD36 also has the potential to be a

Table 4. Cellular requirements for immunization with soluble gp96, tumor cells secreting gp96 (gp96-IG) or tumor cells expressing gp96 on cell surface (gp96-TM)

CELL TYPES	PRIMING PHASE			EFFECTOR PHASE		
	Soluble gp96 (1)	gp-96-Ig (2)	gp-96-TM(3)	Soluble gp96 (1)	gp-96-Ig (2)	gp-96-TM(3)
$CD4^+$T cell	--	--	+	+	--	+
$CD8^+$ T cell	+	+	+	+	+	+
Phagocytes	+	--	--	+	--	NP

(1) Udono, *et al*. (86)
(2) Ymazaki, *et al* (99)
(3) J Dai and Z Li, unpublished

\+ required
-- not required
NP not performed

signaling receptor based on the presence of one tyrosine kinase domain sequence within its cytoplasmic tail (50).

The details of gp96 effects due to receptor-mediated endocytosis and receptor signaling will most definitely become clearer very soon. Such knowledge undoubtedly will help us understand the basis and regulation of the complex interactions of gp96 with APCs. It may also open doors for generating novel pharmacological reagents for manipulating immune responses for therapeutic purpose.

4.6. Immunological circuit as a result of gp96 vaccinations

An insight into the mechanisms through which gp96 interacts with the immune system to induce antigen-specific cellular immunity was first made by Udono *et al* (86). Mice immunized with irradiated intact Meth A cells required $CD4^+$ T cells during the priming phase to generate an anti-tumor response; in contrast, mice immunized with soluble gp96 derived from Meth A cells required $CD8^+$ T cells but not $CD4^+$ T cells for priming (table 4). However, in the effector phase of tumor rejection, both $CD4^+$ and $CD8^+$ T cells were necessary. In both the priming and effector phases, phagocytic cells were necessary for gp96, but not for whole cell vaccinations. This is consistent with the requirement of APCs to cross-present gp96-bound peptides.

Several independent groups further documented that gp96-peptide complexes are capable of inducing antigen-specific $CD8^+$ and $CD4^+$ T cells. In these *in vivo* and *in vitro* experiments, a broad range of models has been employed, covering mouse and human tumor models, as well as native and reconstituted gp96-peptide complexes (table 1). Using an MHC-II restricted $CD4^+$ T cell clone against a mutated peptide from the ribosomal protein L11 (113), it was found that gp96 can also associate with this peptide or its precursor(s) (Srivastava PK, personal communications). Pulsing of APCs with gp96-peptide complexes isolated from Meth A containing the mutant protein effectively stimulated the proliferation and cytokine production of IL-5 by an L11-specific clone. The effect could be inhibited by anti-CD91 antibody, suggesting that the presentation of MHC II epitope associated with gp96 utilizes the same upstream molecules.

Tumor cells engineered to secrete gp96 are more immunogeneic in multiple tumor models (99). It was demonstrated that anti-tumor immunity induced by tumors secreting a gp96-Ig fusion protein was dependent on $CD8^+$ T cells without the need for $CD4^+$ T lymphocytes.

Similarly, by attaching a transmembrane domain to gp96 and transfection, Zheng *et al*. have been able to target gp96 expression to cell surface. A single immunization with gp96 surface-expressing tumor cells stimulates IFN-gamma-producing lymphocytes with high-frequency, leading to tumor regression (100). Interestingly, this immunity is dependent on both $CD4^+$ and $CD8^+$ T cells. Phagocytic cells, however, are not required (J Dai and Z Li, unpublished observation). These studies suggest that surface gp96 might prime tumor-specific immunity in a distinct mechanism, perhaps as a result of high avidity interaction of surface gp96 with gp96 receptors due to cross-linking, or perhaps due to direct exchange of peptides chaperoned by gp96 to MHC I and MHC II. Alternatively, the differential requirement for phagocytes, in addition to $CD4^+$ or/and $CD8^+$ T cells, might be related to different tumor models or/and different immunization protocols (table 4). Recent studies suggest that cross-presentation of cell-based antigens is more efficient than that of soluble antigens (114). The doses of gp96 and routes of immunization also have a profound impact on HSP induced immunity (section 5.3) (115). Higher than optimal doses of gp96 can even down regulate anti-tumor immunity. Therefore, careful comparison among different vaccination strategies of gp96 is necessary to understand the underlying mechanisms, as well as for future clinical applications.

Whether or not gp96 can interact with B cells remains unclear. The existence of a phagocyte-independent antigen presentation pathway, the gp96 receptor on B cells (42), together with the findings that peptides associated with gp96 can be re-presented via both MHC-I (87) and MHC-II pathways (T Matsutake and PK Srivastava, personal communications) leads to speculation that B cells could be directly or indirectly involved in this process. Binding of gp96 to B cells has been observed (42) by using fluochrome-labeled gp96. Navaratnam *et al*. reported that by immunizing with gp96 isolated from cells expressing glycoprotein D of bovine herpesvirus 1(BC-gD), mice were able to generate specific CTLs and antibodies against BC-gD as well (116). Interestingly, natural autoantibodies (IgD) for gp96 exist at a low but sustained level, which couldn't be further boosted by immunization with gp96 from the livers of syngeneic mouse (117). Meanwhile,

antibodies to HSP90, a cytosolic paralog of gp96, play dual roles in down regulating antigen-specific T cell responses and activation of peripheral blood mononuclear cells. These observations highly suggested that autoantibodies are involved in maintaining balance between activation and inactivation of HSP-induced immune responses and that the production of antibodies is finely regulated by as yet unknown mechanisms.

It has been reported recently that platelets also expresses high level of CD91, a common HSP receptor (H Schild, personal communication). Platelets are small, non-nucleated cell fragments derived from myeloid progenitors that are important for hemostasis. The association of the actin-rich cytoskeleton with rapidly phosphorylated HSP27 has been shown when platelets were activated (118). A phosphorylated complex of HSC70, HSP90, and the catalytic and myosin-targeting subunits of protein phosphatase 1 has been demonstrated to undergo rapid disassociation and dephosphorylation after adhesion of platelets to collagen (119). These results indicate that HSPs may be involved in actin polymerization or serving as signaling scaffolds. It was found that gp96 is able to bind to human platelets specifically through CD91. Binding of gp96 to platelets does not interfere with the intrinsic function of platelets, such as activation and aggregation induced by ADP or collagen (H Schild, personal communications). However, the presence of platelets inhibits gp96-induced DC maturation *in vitro*, suggesting that the sequestration of gp96 by platelets *in vivo* may provide a negative regulation of immune responses. Such an anti-inflammatory response might be helpful for wound healing, therefore the physiological function of the interaction between gp96 and platelets needs further investigation.

5. AN INTEGRATED VIEW OF GP96 IN IMMUNE RESPONSE

5.1. Exploring the role of gp96 in antigen presentation

It has long been known that MHC class I molecules contain peptides, however, the details of their source and the path that they follow before binding the MHC in the endoplasmic reticulum has proven to be quite complicated. We now know that most peptides degraded in the cytosol come from newly synthesized proteins (120-122). The proteosome and immunoproteosome components that participate in this degradation pathway have also been well characterized, although their specificity in generating class I peptides remain somewhat mysterious. After proteosome processing, these defective ribosomal products (DRiPs) become the source of peptides that get loaded onto MHC class I molecules in the ER. Despite the rapid and continuous production of these peptide products in the cytosol, very few peptides can be isolated from the cytosol of a normal cell. To explain this, it has been postulated that abundant chaperones in the cytosol, including HSP90, participate as a relay and sink for newly generated peptides (28). It is known that the TAP heterodimer on the ER membrane is responsible for shuttling peptides from the cytosol into the ER lumen. To date, there is no reported association of HSP90 with TAP. The evidence for the association of HSP70 with TAP is weak, so the exact details of which peptides get delivered from the cytosol and how are still unclear (123). As discussed previously, there is also evidence for resident cytosolic and/or ER aminopeptidases, given that the proteasome creates peptides with carboxy termini exhibiting high affinity for the MHC class I peptide binding site, but amino termini that are not ideal for recognition by class I. Indeed, two new cytosolic, proteosome-independent aminopeptidases were recently identified (77).

A role for gp96 in antigen presentation was proposed as early as in 1991. Supporting evidence continues to emerge, although no definitive evidence has materialized. There are several factors which implicate gp96 as a candidate in the presentation of peptide antigens onto class I molecules (29, 32). First, gp96 is extremely abundant in the endoplasmic reticulum. It makes up approximately 3% of all ER proteins, making the likelihood of interaction with class I molecules very high. In fact, in cells where there is a disproportionately abundant amount of empty MHC class I, the association of MHC I with gp96 can be demonstrated (Z Li, unpublished observation). Second, gp96 is known to avidly bind peptides (section 3.1), suggesting that gp96 could be a carrier for peptides being delivered by TAP, holding onto them until the class I molecules have reached the correct conformation to allow their binding. Third, although the peptide-binding specificity of gp96 is not nearly as selective as that of class I, gp96 has been implicated as an aminopeptidase *in vitro* (47). As stated above, the proteosome does not always generate peptides of the correct length and sequence to bind class I molecules, especially at the amino terminus. Therefore, there is a requirement for an aminopeptidase either in the ER or the cytoplasm. Although the aminopeptidase activity of gp96 *in vitro* was relatively low, it is reasonable to assume the kinetics would be enhanced in the lumen of the ER, given that the ER lumen has carefully regulated osmotic conditions, pH, and accessory molecules that an *in vitro* system cannot replicate. Therefore, gp96 could be cleaving either MHC I-bound peptides, or peptides it binds before handing them over to class I molecules. Fourth, gp96, like other molecules in the class I antigen presentation pathway, is up-regulated in response to IFN-gamma stimulation (124). This fact indicates that gp96, along with MHC I heavy chain, TAP and immunoproteasome components, is coordinately regulated during viral infection. Fifth, gp96 harbors ATPase activity (22), which has recently been implicated in its functional abilities (74), and which supports an active role within the ER. Given that the assembly of MHC I-peptide complex is ATP-dependent (125), ATP binding and ATPase activity of gp96 therefore also fulfills a functional requirement in assisting antigen presentation. Sixth, gp96 has the ability to cross-prime, as described above, indicating that it can somehow deliver peptides to class I MHC molecules when delivered exogenously. Although it is still unclear whether this exchange of peptides from exogenous gp96 to MHC class I molecules happens directly, either in an endosomal compartment or in the ER via retrograde transport of endocytosed gp96-peptide complexes, or indirectly, via release of gp96-bound

peptides into the cytosol to be transported into the ER, the mere fact that endocytosed gp96-bound peptides can be represented on class I molecules lends strong support for a role for gp96 in presentation of normal endogenous antigens.

There are, however, some data that argue against a role for gp96 in antigen presentation. First, cells expressing high levels of antisense cDNA of gp96 constitutively expressed drastically reduced level of gp96 protein. These cells failed to exhibit decreased MHC class I surface expression or inability to be lysed by antigen-specific T cells (126). This experiment was limited by the inability to convincingly eliminate 100% of the gp96 protein and by the fact that only one antigenic epitope was tested, that for VSV nucleocaspid protein. Second, a recently identified mutant pre-B cell line that has two truncated gp96 alleles failed to exhibit a noticeable deficit in class I surface expression, however, the structural conformation and peptide binding ability of the detected class I molecules was not described (74). While there is also no evidence that gp96 associates with any of the molecules that have been identified so far in the MHC complex (TAP, Tapasin, calnexin, calreticulin, or Erp57), the often used immunoprecipitation in the presence of detergent might not be the optimal assay for studying protein-protein interaction. It could also be argued that the abundance and phylogenetic conservation of gp96, all the way back to cells and organisms that have no adaptive immunity or antigen presentation systems, indicate its main role is probably more fundamental than that of an ER protein required for antigen presentation. However, it is not beyond the realm of possibility that as adaptive immunity and antigen presentation evolved, such systems made use of this protein, given that it is so abundant, and is a peptide-binding protein.

Our discussion so far is limited to the role of gp96 in *de novo* presentation of endogenous antigens to MHC I pathway. Cross-presentation of gp96-chaperoned peptides to MHC I of APC requires receptor-mediated endocytosis, and translocation of either gp96 or gp96 ligand across the plasma membrane to cytosol, since it has been shown that cross-presentation is dependent on TAP and the proteasome (49). The cell biology basis is unclear. An experiment to allow visualization of the peptides bound to gp96 as they enter the cell would be the ideal way to discern the details of the endocytic pathway, however, this is difficult to do with current technology. Electron microscopy has shown that endocytosed gp96 localizes to non-acidic endocytic compartments and colocalizes with class I and class II molecules (88). Whether this colocalization is enough to allow peptide exchange is unknown. Retrograde transport is another possible mechanism by which gp96 could be mediating cross-priming. There are examples of molecules that find their way to the trans-golgi network via endocytosis mediated by special receptors and then are taken back to the ER by retrieval proteins which recognize their KDEL retention sequences. The *E. coli* toxin VT-1 and the Shiga toxin, ricin, are two such proteins. Given that gp96 has a KDEL signal, retrograde transport of endocytosed gp96-peptide complexes by KDEL receptor is an attractive mechanism for cross-presentation.

5.2. Gp96 (and other HSPs) are the molecules in cross-priming the adaptive immunity

Intracellular antigens in non-APCs, such as tumor antigens or viral antigens, have to be cross presented from these cells to the class I molecules of APCs for the priming of $CD8^+$ T cells. The requirement for cross-priming by APCs is dictated by two considerations: First, only APCs have the capacity to express essential co-stimulatory molecules for T cell activation; second, naïve T cells generally stay in secondary lymphoid organs, whereas APCs are localized in all tissues and organs for sampling antigens. Priming of CTLs across MHC I was operationally and functionally defined by Bevan using minor histocompatibility antigens (127, 128). This phenomenon has now been substantiated in T cell responses against tumor antigens, viral antigens, bacterial antigens and other model antigens (section 4.1.3). The molecular basis for it has now been studied. All data supports the notion that only bone marrow-derived APCs are able to cross-present exogenous antigens. The nature of antigens also dictates whether an antigen can be cross-presented or not. In general, cross-presentation of soluble antigens is extremely insufficient, unless there is a specific receptor expressed on the surface of APCs. For antigens captured by antibody, the Fc receptor on APCs might serve this purpose (129). For a vast majority of intracellular antigens, a generic system including carrier molecules for antigens and the shuttling of antigens through a common receptor for the carrier might suffice. The candidate carrier molecules responsible for such a mechanism are expected to have the following features: universal expression in all somatic cells, binding non-selectively to intracellular antigens, specific interaction with APCs, and capacity to escort exogenous antigens to MHC class I of APCs. As evident from Section 2, HSPs including gp96 possess all of these features and are thus the best candidate molecules so far for chaperoning peptides from non-APCs to MHC class I of APCs.

As discussed previously, the best evidence for the role of HSPs in cross-presentation is the presence of receptors for HSPs on the surface of APCs. CD91 binds directly to gp96, and is functionally involved in presenting peptides chaperoned by gp96 to MHC I molecules *in vitro*. In addition, HSPs including gp96 are able to stimulate DC maturation as indexed by increased expression of cell surface activation markers, such as MHC I, MHC-II, CD40, CD80 and CD86 (table 3).

Since HSPs are intracellular proteins, the presence of an HSP receptor on APCs and the proposal that HSPs are involved in cross-presentation must mean that HSP-peptide complexes have to be released extracellularly. There are several possibilities. First, release of HSPs occurs after cell lysis. It has been shown that HSPs are released after necrosis, but not after apoptosis (98). This is consistent with the notion that only non-physiological cell death is proinflammatory. Apoptosis is generally a physiological process that is not expected to trigger immune responses. Second, active extracellular expression

such as secretion and cell surface expression should also be expected to prime immune response as suggested by cellular engineering (99, 100). Both surface expression and secretion of gp96 by tumor cells have been shown to increase the immunogenicity of tumors. In stress conditions, it was found that surface expression and secretion of gp96 is an active process, and not the result of cell death. It is unclear which pathway (cell death or active cellular transport) plays predominant roles in cross presentation, the clear knowledge on the molecular basis of extracellular trafficking and the regulation of it would be instrumental to our understanding of the roles of HSPs in immune response *in vivo*.

Are HSPs essential for cross-priming *in vivo*? This question has proven to be difficult to address. The description of a gp96 null cell line may help to address this question, although it is virtually impossible to eliminate all HSPs from a cell line. Cell lines with defective TAP can still prime, indicate that ER HSPs such as gp96 are not essential (130). An alternative approach would be to perform the same tedious biochemical experiment that led to the discovery of HSPs as "tumor rejection antigens". If whole cell lysate can cross-prime, biochemical fractionation and characterization of each fraction for their abilities to cross prime T cells would lead to the discovery of the active components necessary for this process. Generation of transgenic animals that export HSPs might also lead to some clues as to whether the interaction of HSPs with their receptors might have immunological consequences.

5.3. Gp96 (and other HSPs) regulate the immune response

The roles of HSPs in the effector phase of an immune response have not been looked at closely. Moreover, studies so far are heavily biased towards the study of HSPs in productive cellular immunity. At least in one scenario, it was found that immunization with higher than optimal dose of gp96 might lead to immunosuppression (115). Therefore, it would be prudent to examine the possibility whether or not gp96 also performs immunoregulatory functions.

Recently, the suppressive or regulatory functions of $CD4^+CD25^+$ T cells have attracted a considerable amount of attention (131). These cells produce high levels of IL-10, IL-4 and TGF-beta upon stimulation, which suppress proliferation of other T cells in both a cytokine (132) and cell-cell contact-dependant manner (133). Another subset of regulatory cells has been cloned and described by Zhang and colleagues (134). These cells express a unique combination of cell surface markers (alpha beta-TCR^+ $CD4^-$ $CD8^-$ $CD25^+$ $CD28^-$ $CD30^+$ $CD44^-$) and secrete a distinct profile of cytokines (IFN-gamma, TGF-beta, TNF-alpha) from that of Th1, Th2, or $CD4^+$ $CD25^+$ regulatory T cells. Inhibition of $CD8^+$ T cells occurs by the Fas-FasL pathway, but only if the target cells have the same TCR specificity.

Another subset of regulatory cells is NKT cells which express alpha beta TCR as well as NK cell receptor (135). Their development is dependent on MHC-I like CD1 molecules (135). NKT cells are highly heterogenous population, at least three subsets ($CD4^+$, $CD8^+$, $CD4^-CD8^-$) of NKT cells have been identified (136, 137). Functionally, they can regulate immune response both positively and negatively. For example, it has been shown that NKT cells are essential in IL-12 mediated tumor rejection (138, 139), indicating their role in positively regulating immune response. On the other hand, Terabe *et al*. found $CD4^+$ NKT cells could suppress tumor immunosurveillance through the production of IL-13 (140). NKT cells can also prevent autoimmune diseases, such as IDDM, by producing IL-14 or IL-10 (141), supporting that they also play inhibitory roles in regulating immune responses.

There is also evidence suggesting that gamma delta T cells can function as regulatory cells (142). Gamma delta T cells can contribute to the resolution mechanism of pathogen induced inflammatory immune response by killing activated inflammatory macrophages . Also, a population of regulatory gamma delta T cells which have a similar cytokine profile to $CD4^+CD25^+$ regulatory T cells has been identified (143). These cells can be found in tumor infiltrating lymphocytes, which inhibit anti-tumor immunity (144).

Limited data indicate that gp96 may contribute to the regulation of these regulatory cells. Chandawarkar *et al*. have found that immunization with optimal doses of tumor-derived gp96 elicits tumor immunity (115). Immunization with a 5-10 fold higher dose of gp96 actually down regulates anti-tumor immune responses. This regulatory effect is antigen specific and is dependent on $CD4^+$ T cells as demonstrated by adoptive transfer. Further characterization of these cells has not been performed, leaving the question as to whether these T cells are $CD25^+$ regulatory cells or NKT cells. It is also unclear how these T cells are generated, whether gp96 interacts directly with these T cells, and what is the mechanism for the suppressive function. Studies also suggest that NKT cells express some level of CD91 (H.L. Aguila, personal communication), indicating that gp96 might interact with NKT cells.

HSPs such as gp96 can clearly modulate the immunological climate by inducing cytokine production by DCs. Efforts so far have been focused exclusively on the induction of APCs to produce pro-inflammatory cytokines such as IL-1, IFN-gamma, TNF-alpha and IL-12. In order to gain some clues as to how HSPs interact with the immune system, it would be insightful to analyze down stream signaling events, and profile all the potential cytokines or other target molecules induced or suppressed by HSPs on a large scale.

6. THE CHALLENGES AND PERSPECTIVES

The unfolding of the story of gp96 is exciting. The unraveling of the details has proven to be nothing but a challenging task both conceptually and experimentally. An open-minded approach is crucial as we put together all the pieces of the gp96 puzzle.

6.1. Conceptual challenges

The immunological roles of gp96 must be examined dynamically. Examination of the context in which gp96 positions itself in the immune response holds the key for resolving the confusions currently surrounding gp96. It is worthwhile to discuss a few examples of conditions in which gp96 may play complimentary or even distinctive roles in the generation of immune responses.

6.1.1. APCs vs. non-APCs

There are two obvious differences between APCs and non-APCs. APCs are the only cell types that can present exogenous antigens to MHC molecules, a process defined as cross-presentation. The second difference is the ability of APCs to express co-stimulatory molecules that are required to activate naïve T cells. Non-APCs do not cross-present antigens and rarely express co-stimulatory molecules. These functional differences between APCs and non-APCs would require distinct roles of gp96 in antigen presentation and re-presentation. For example, gp96-associated peptides can only be cross-presented by APCs. This apparent cell type selectivity cannot be attributed solely to the expression of gp96 receptor CD91, since CD91 is also expressed on non-APCs such as fibroblast, platelets and hepatocytes. It is possible that only in APCs there exists a specialized compartment where MHC I, MHC II and CD91 are in close enough physical proximity for peptide exchange to occur. It is equally possible that there is a retrograde transport mechanism of gp96-peptide complex from early endosome to the ER that is unique to APCs. Moreover, there could be more efficient direct transfer of gp96-chaperoned peptides to MHC I in professional APCs. Finally, *de novo* antigen presentation to MHC I has not been carefully compared between APCs and non-APCs. The possibility remains that molecular chaperones may play more important roles in antigen presentation in APCs than in non-APCs.

6.1.2. Extracellular vs. intracellular gp96

We have now begun to appreciate the differences between extra cellular gp96 and intracellular gp96 in the presentation of antigens. Intracellular gp96 is likely important for all cells, whereas extra cellular gp96 exerts its function simply through APCs. While the distinction is acknowledged, there seems to be a tendency in the field to make at least two assumptions. First, the generation of extra cellular gp96 is simply by a messy cell death, referred to as necrosis. A well-described phenomenon of secretion and surface expression of gp96, particularly under stress conditions, has not received adequate attention. Consequently, there has not been a careful study on the cell biological basis of "ectopic" transport of gp96 and the regulation of it in the context of immune response. Second, the trafficking of extra cellular gp96 and intracellular gp96 in APCs is entirely distinct. There has not been enough emphasis on possible convergence of these two pathways in APCs. Dissecting these two pathways carefully will be instrumental to our understanding of the roles of gp96 in antigen presentation.

6.1.3. Priming vs. effector phase

Much energy has been devoted towards the understanding of the roles of gp96 in priming, a process defined as activation and expansion of naïve antigen-specific T or B cells. However, little attention has been paid to the role of gp96 in the effector phase of an anti-tumor immune response. For example, the presence of anti-gp96 antibody has been described. In the case when gp96 is expressed on a cell surface, anti-gp96 antibody might contribute to the elimination of these cells. It was reported that a gp96 receptor exists on APCs and B cells, but not T cells. But it is unclear if the receptor expression is regulated during the activation of T cells. Future work needs to examine this question, and to test if gp96 receptor can be expressed by other cell types such as NK cells and gamma delta T cells.

6.1.4. Steady state vs. stressed situation

Induction of HSPs by heat has been shown to facilitate migration of DCs to the draining lymph nodes and potentiate the ability of DCs to prime T cells. Moreover, heat shocked cells have been shown to have increased immunogenicity. Therefore, both the expression level and peptide repertoire associated with HSPs such as gp96 are likely to be different between resting and stressed cells. In the acute stress conditions such as virus infection and rapid proliferation of malignant cells, the demand for efficiency of antigen presentation is high, the roles for accessory molecules might therefore become more significant. During non-stressed steady state conditions, the roles of HSPs in direct antigen presentation might be subtle. There is even a possibility for constitutively expressed HSPs in non-stress conditions to deliver tolerogenic signals to the immune system, and thus contribute to peripheral tolerance. Thus, the roles of gp96 have to be analyzed in the context of stressful, or pathological conditions.

6.1.5. Quantity vs. quality

As eloquently pointed out by Jonathan Yewdell, immunologists and cell biologists have profound tendency to focus on the qualitative, rather than the quantitative aspects of cell physiology (145). In the non-disturbed situation, approximately one-third of newly synthesized proteins (approximately $5x10^6$ proteins per minute) are degraded (termed DRiPs, for 'defective ribosome products') by proteasome within minutes of their synthesis. Since MHC I-peptide complexes are formed in the ER at a rate of roughly 150 per minute, 10,000 proteins, on average, are degraded for each class I-peptide complex generated. This suggests that the production of MHC I-associated peptides is an extremely inefficient process in the steady state, which raises a question as to how then a viral or tumor antigen can be rapidly presented in the stress conditions. It is safe to say that the dynamics of protein synthesis, turnover, and transport in these stress conditions would be very different from the resting state. The tremendous inductions of HSPs could surely contribute to the regulation of these dynamic processes. But to sort out the contributions of gp96 or other HSPs, it is clear that qualitative science is not enough. The kinetics of peptide generation, MHC I folding and transport, as well as the accumulation of all the intermediate products have to be carefully analyzed and quantified, preferentially by comparing cells that have normal and no induction of HSPs.

6.2. Experimental challenges

The difficulties surrounding gp96 also stem from numerous experimental challenges, as described below. These challenges must be overcome by ingenious experimental approaches so that a verdict on the roles of gp96 in immune response can be delivered.

6.2.1. Essential non-immunological functions:

Gp96 associates with a broad spectrum of substrates in the secretory pathway. This apparent "lack" of specificity is probably because of its general roles in facilitating protein folding. This is supported by the finding that disturbance of protein homeostasis in the ER leads to further induction of gp96 expression. Direct introduction of misfolded proteins to the ER strongly induced the synthesis of gp96. In addition to its broad substrate binding capacity, gp96 has also been suggested to have the following enzymatic activities: endo-beta-D-glucuronidase (heparanase) (146), ATPase (22), aminopeptidase (47), and foldase (to facilitate protein folding). Therefore, unlike tapsin or TAP (transporter associated with antigen presentation), gp96 most definitely has multiple non-immunological functions. It is experimentally difficult to separate the generalized functions of gp96 in the ER from its more specialized immunological functions.

6.2.2. Functional redundancy

The HSP family is composed of numerous members. In the lumen of the ER where gp96 normally resides, there are multiple HSPs that have similar biochemical properties to gp96, such as GRP78, calreticulin, calnexin and GRP178, protein disulfide isomerase. Although each of the players has been found to perform somewhat unique roles in antigen presentation, the roles of gp96 have not been finalized. Even if a gp96 negative cell line is used, it is not clear whether the loss of gp96 can be functionally compensated by other molecular chaperones. This clearly poses an experimental challenge.

6.2.3. Lack of genetic tools

Gp96 is one of the most abundant molecules in the lumen of the endoplasmic reticulum. Polymorphisms or natural mutants of gp96 have not been found. The ubiquitous and constitutive expression suggests that gp96 possibly plays an essential function in cells. This is probably the reason why the manipulation of the expression of gp96 remains difficult. Anti-sense cDNA constructs targeting to the coding sequence (126) or promoter region of gp96 (147) have met with some limited success in a transient system. Homologous recombination has been used to generate gp96 null mice. Unfortunately, gp96 null embryos die very early at embryonic day of 5.5, which has prohibited further study of gp96 function at the organismal level (Rinni de Crom, personal communications). By subjecting a pre-B cell line to a frameshift mutation *in vitro*, a cell line with both alleles of gp96 truncated at the c-terminus has been described recently (74). Conditional gp96 knockout mice using the cre-lox P system are being generated. This new development will undoubtedly facilitate our research in dissecting both immunological and non-immunological roles of gp96.

7. CONCLUSION

The immunological roles of gp96 were discovered serendipitously. The pieces of the puzzles of gp96 in interacting with peptides and dendritic cells have now been placed, and substantiated. Putting all the pieces together has proven to be challenging, however rewarding the process may be. But it is our belief that the extra cellular presence of heat shock proteins like gp96 plays important roles in alarming, mobilizing and activating the immune system in the face of cancers, infections and other hazardous environments. Studies of the functions of gp96 in these conditions by creating novel genetic tools and animal models should help to more completely uncover the physiological roles of gp96 in immune responses.

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