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Specific Immunogenicity of Heat Shock Protein gp96 Derives from Chaperoned Antigenic Peptides and Not from Contaminating Proteins\textsuperscript{1}

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The peptide-binding property of MHC is central to adaptive immunological functions. A similar property of heat shock proteins (HSPs) hsp70 and hsp90 has been implicated in Ag presentation by MHC and in cross-priming. The peptide-binding pocket of hsp70 has been characterized structurally and functionally and a peptide-binding site in gp96 (of hsp90 family) has been defined. Nonetheless, questions persist whether the specific immunogenicity of HSP preparations derives from the peptides chaperoned by the HSPs or by proteins contaminating the HSP preparations. Because absolute purity of a protein preparation is a metaphysical concept, other approaches are necessary to address the question. In this study, we demonstrate that the specific immunogenicity of gp96 preparations isolated from cells expressing β-galactosidase derives from the MHC I epitope precursors associated with the gp96 and not from contaminating β-galactosidase protein nor unassociated fragments derived from it. Although the observations here are limited to a single HSP and antigenic peptides chaperoned by it, they can be extended broadly.


A pparently homogeneous preparations of selected heat shock proteins (HSPs)\textsuperscript{3} gp96, hsp90, hsp70, calreticulin, hsp110, grp170 have been shown to elicit specific CD8\textsuperscript{+} T cell response to Ags expressed by the cells from which the HSPs have been purified (for review, see Ref. 1). Such immunity has been shown to be protective against the cancers, viruses, or intracellular bacteria that express the cognate Ags. The same HSPs purified from normal tissues or from tissues expressing irrelevant Ags show no immunogenicity. When the observations were originally made, it was suggested that the genes encoding HSPs were hypervariable (2) and, thus, lent themselves to be sources of a tremendous range of antigenic variation. However, the sequencing of cDNAs of HSPs from normal tissues and tumors failed to show any evidence of the proposed hypervariability of the hsp genes. Since 1) the HSP preparations appeared, by all of the methods available at the time, to be homogenous and devoid of detectable contaminating proteins, 2) elution of HSP bands from polyacrylamide gels led to isolation of immunologically active material (3), and 3) glycosylation did not appear to play a role in immunogenicity, it was suggested in 1991 (4, 5) that the immunogenicity of HSP preparations was derived from antigenic peptides associated with the HSPs. That idea has derived significant support since then. Thus, defined antigenic peptides derived from viral Ags (6–8), tumor Ags (9), mycobacterial Ags (10), model Ags (11), or minor histocompatibility Ags (12) have been isolated from gp96, hsp90, or hsp70. Furthermore, immunological evidence has been reported for association of these same HSPs with well-characterized epitopes of viral Ags (7, 8), tumor Ags (13–15), or model Ags (11, 16). Peptide-binding sites of hsp70 (17, 18) and gp96 (19, 20) have been demonstrated by crystallography and modeling studies. Nonetheless, the notion that specific immunogenicity of HSP preparations derives from peptides noncovalently and physiologically associated with the HSP molecule has been questioned recently (21–24). The questioning is based on the idea that HSP preparations are contaminated with trace amounts of other cellular proteins and that this trace contamination and not the HSP-chaperoned peptides is responsible for the specific immunogenicity of the HSP. This criticism has not been experimentally substantiated, but is a reasonable one and merits experimental scrutiny.

In this study, we provide such a scrutiny, using the HSP gp96 purified from murine P13.4 cells, which are P815 cells transfected with the gene encoding β-galactosidase (β-gal). The choice of the Ag was influenced by the fact that it is detectable structurally as well as enzymatically, such that logarithmically smaller quantities of β-gal can be detected than possible for other well-used model Ags. Furthermore, a range of immunologically relevant tools are available for this Ag. Using a range of sensitive, complementary, and redundant tools, our data clearly demonstrate that the specific immunogenicity of gp96 preparations isolated from β-gal-expressing cells derives from gp96-chaperoned peptides and not from contaminating β-gal.

Materials and Methods

Mice and reagents

Mice were purchased from The Jackson Laboratory and maintained in the Center for Laboratory Animal Care facilities at the University of Connecticut Health Center. Female C57BL/6 mice were used at 6–8 wk of age. gp96 was purified from P13.4, P815, or PIK23 cells in identical procedures as previously described (3). Peptides were stripped off gp96 exactly as previously published (9). Untransfected P815 and β-gal- transfected P13.4
cells were maintained in complete medium (RPMI 1640 supplemented with 5% FCS and 1% each of nonessential amino acids, glutamine, and pyruvate) in addition to geneticin (Invitrogen Life Technologies) for P13.4 cells. Cells transfected with pNEBR-R1 were supplemented with geneticin (400 μg/ml); transfectants with pNEBR-X1Hygro 3C5 also required the addition of 400 μg/ml hygromycin B (Invitrogen Life Technologies). Purified recombinant β-gal was purchased from Calbiochem and the polyclonal antiserum against it from Oncogene. LPS was removed from β-gal by incubation of solubilized protein with endotoxin-removal Sepharose beads (Pierce) for 12 h, followed by fresh beads for an extra hour. Beads were removed by centrifugation and the LPS content was subsequently determined to be lower than can be detected (0.01 endotoxin units) by the Limulus amebocyte lysate assay (BioWhittaker). Anti-β-gal Ab used for immunoblotting was purchased from StressGen. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) was used at a final concentration of 2.5 mM in the β-gal detection assay. Enzymatic digestion of X-GAL was monitored by measuring absorbance at 595 nm.

**Immunization and measurement of CTL activity**

Mice were immunized s.c. twice (1 wk apart) in the nape of the neck. One week later, spleens were removed and splenocytes were incubated in complete medium and relevant peptide as indicated for 1 wk. On day 5 after one restimulation, cytotoxicity was measured in a chromium release assay as described. Briefly, peptide-pulsed or unpulsed target cells were loaded with 3HCr and incubated with titrated numbers of T cells. Amount of 3HCr released was measured and percent specific release was calculated. Percent lysis of un-pulsed cells (<2% in all mice) was subtracted from percent lysis of peptide-pulsed cells.

**Preparative gel electrophoresis**

Proteins were applied to a preparative gel (Bio-Rad) under denaturing conditions. Proteins were resolved at 100 V and eluted into 3-ml fractions under conditions specified by the manufacturer.

**Anion exchange chromatography**

Proteins were applied to a HiPrep 16/10 DEAE FF column (Amersham Biosciences) attached to a System Gold HPLC (Beckman Coulter) and used according to the manufacturer’s recommendations. Proteins were eluted over increasing salt concentrations from 200 to 800 mM NaCl.

**Plasmids**

The plasmid pNEBR-R1 is available from New England Biolabs (NEB) as part of their Rheoswitch Mammalian Inducible Protein Expression System. The KOVAK plasmid was a gift from N. Shastri (25). The coding region for the KOVAK protein (beginning at the Pvu II site) was amplified via PCR using a forward primer 5′-CACCATGGCTGACATCAAGCCAAGAG-3′ and reverse primer 5′-AAAAGGGAAAAACCATCTGCAAAAG-3′ and inserted into the directional TOPO cloning vector pBAD200-DTOPO (Invitrogen Life Technologies). This construct was verified to produce the protein of interest, fused to the V5 epitope and 6xHis. His tag via sequencing and expression studies in Escherichia coli. The KOVAK protein was subcloned from this vector using the forward primer 5′-CTCGAGAATGTTGCAAGATCAAGCCAGAGCCTG-3′ and reverse primer 5′-GGATCCGGGCGGCGCTTAATGTTGATGGTGAAGCAAGCTG-3′ and ligated into pAcGFP-C1 (BD Clontech) using the XhoI and BamHI unique restriction enzyme sites. The entire fusion coding sequence was digested directly out of this vector using the existing XhoI and introduced AscI sites, and this ligated into pNEBR-X1Hygro (New England Biolabs) using the same enzyme sites to create pNEBR-X1HGK 3C5.

**Transfection and cloning**

P815 cells growing exponentially were diluted to 2 × 10⁷/ml in 5 ml and grown overnight. The next day cells were transfected with 200 ng/ml pNEBR-R1 in a 1 μg:3 μl liposomal context with FuGene 6 (Roche). After 6 h, the cells were washed and cloned by limiting dilution in selected antibiotic-containing medium. Effective, stably transfected clones were verified via transient transfection with a luciferase reporter construct (NEB). P815-R23 was a clone that exhibited good induction and titratability for luciferase and was diluted 1 day before secondary transfection as described above. P815-R23 cells were then transfected with pNEBR-X1HGK 3C5 at 200 μg/ml using 3 μl DNA:FuGene 6 and 6 h later the cells were washed and supplemented with 400 μg/ml hygromycin B. Stable transfectants were induced with 500 nM RSL1 overnight and sorted for high GFP-KOVAK expression using a FACSDiva (BD Biosciences). Cells exhibited good protein induction and dose-response kinetics to RSL1 and are subsequently referred to as P815-R23.

**Depletion of gp96 or β-gal**

Protein G beads (Sigma-Aldrich) were blocked with albumin, saturated with the relevant Ab for the protein to be depleted, and washed to remove excess Ab. A rabbit anti-gp96 antiserum (courtesy of Antigenics) was used for depletion of gp96. The antiserum against β-gal is described in a previous section. Ab-coated protein G beads were placed in protein sample and incubated for 8 h and the beads were removed by low-speed centrifugation. The process was repeated with fresh Ab-coated beads and depletion was monitored by immunoblotting of the protein sample with the relevant Ab. Typically, three sequential incubations with Ab-coated beads were sufficient for complete depletion.

**Results**

**Immunogenicity of apparently homogeneous gp96 preparations**

gp96 was purified from the β-gal-expressing P13.4 and parental P815 cells and analyzed for purity and identity (Fig. 1A). The major 96-kDa band on SDS-PAGE from both cell types reacts with the anti-gp96 mAb 9G10. gp96 constitutes nearly all detectable protein on SDS-PAGE in the preparations from both cell types. However, protein bands constituting <50 ng, the limit of detection by silver staining in our hands, may remain undetected. Hence, gp96 preparations were immunoblotted with a polyclonal antiserum to β-gal to detect contaminating β-gal. The choice of a polyclonal antiserum was made so that the largest possible repertoire of β-gal and its derivative fragments could be detected. No β-gal was detected in either of the gp96 preparations, even though control β-gal was easily detected (Fig. 1A). (One nanogram of β-gal can be easily detected under these conditions (data not shown).) To obtain another measure of contaminating β-gal, the gp96 preparations were tested for enzymatic activity of β-gal as described in Materials and Methods. Titrated quantities of commercially obtained β-gal were assayed in parallel in the same experiment. Under experimental conditions where as little as 5 femtograms (fg) of β-gal could be detected enzymatically, no enzymatic activity was detected in gp96 preparations from P13.4 or from the parental P815 cell line (Fig 1B). These observations indicate that the gp96 preparation applied to the SDS-PAGE (5 μg absolute quantity) contained <50 ng of intact β-gal or a fragment thereof (based on silver staining), or <1 ng of intact β-gal or a fragment thereof (based on immunoblotting), or <5 fg of enzymatically active β-gal (based on enzymatic assay). Since immunogenic contaminants need not be intact, nor enzymatically active, and as any number of fragments below the level of detection may still be present, this apparently homogenous gp96 preparation may still potentially contain immunogenic β-gal fragments.

The gp96 preparation shown in Fig. 1A was tested for immunogenicity. C57BL/6 mice were immunized with PBS, or 1 or 10 μg of gp96, purified from P13.4 or P815 cells or with 1 or 10 μg of β-gal as a positive control. Mice immunized with 10 μg of P13.4-derived, but not P815-derived gp96 generated T cells specific for the K1-restricted β-gal epitope DPIYTNV as measured in a CTL assay (Fig. 1C). Mice immunized with 10 μg of β-gal also showed a CTL response. No CTL were detected in mice immunized with 1 μg of gp96 or β-gal. Intermediate doses were not tested. The requirement of ~10 μg of gp96 for specific immunogenicity of gp96 is consistent with previous studies (3, 26). With respect to β-gal, although the experiments in Fig. 1C do not establish the minimal quantity of free β-gal required for immunogenicity, they do establish that 1 μg is not sufficient, a point that is significant in future considerations (see Discussion).
Immunogenicity of gp96 preparations derived from 96-kDa proteins

To assess the contribution of enzymatically inactive intact β-gal and of β-gal fragments smaller than the intact β-gal, gp96 preparations from Fig. 1 were subjected to preparative SDS-PAGE. Previous studies have demonstrated that gp96, hsp90, and hsp70 preparations subjected to SDS-PAGE retain their immunogenicity (3, 19, 27). The SDS-PAGE fractions were tested for the presence of gp96 and β-gal. In parallel, purified β-gal preparations were also subjected to preparative SDS-PAGE and the fractions were tested for the presence of gp96 and β-gal. gp96 molecules eluted in fractions 45–51 and β-gal molecules eluted as a sharp peak (band) in fraction 114. The two preparations were analyzed by immunoblotting (Fig. 2A): 96-kDa bands in fractions 45–51 (of the gp96 preparation) were detected by the anti-gp96 Ab 9G10, but the polyclonal Ab to β-gal did not detect any bands in fraction 114 of the gp96 preparation. Conversely, fraction 114 of the β-gal preparation probed positively with the anti-β-gal antiserum, but fractions 45–51 of this preparation were not detected by the anti-gp96 Ab 9G10 (Fig. 2A).
Fractions 45–51 and, separately, fraction 114 of the gp96 and β-gal preparations were used to immunize C57BL/6 mice. Fractions 45–51 but not fraction 114 of the gp96 preparation elicited a clear CTL response to β-gal epitope DAPIYTNV (Fig. 2B), and the magnitude of the response was comparable to that seen with the corresponding amount of gp96 preparation before it underwent fractionation on preparative SDS-PAGE (Fig. 1C). As a positive control, fraction 114 from the β-gal preparation also elicited a comparable CTL response. These results show that the K/ DAPIYTNV-specific immunogenicity of gp96 preparations purified from P13.4 cells derives from gp96- and theoretically 96-kDa β-gal-β-gal fragments, but not from contaminating intact β-gal nor from β-gal fragments very different in size from ~96 kDa.

In a separate approach to address the contribution of putative β-gal contaminants to the immunogenicity of gp96 preparations, the gp96 preparations were repurified over anion exchange columns. Proteins were eluted over increasing salt gradients (Fig. 3A). Elution profiles of purified gp96 and β-gal did not overlap. gp96 eluted in fraction 30 while β-gal eluted in fraction 24 as analyzed by SDS-PAGE and immunoblotting with anti-gp96 and anti-β-gal Abs (Fig. 3, A and B). Importantly, no β-gal was detected in any of the fractions from the gp96 preparation. C57BL/6 mice were then immunized with fractions 24 or 30 from the gp96 or β-gal preparations. CTL responses against K/ DAPIYTNV were analyzed as in Figs. 1 and 2. Although mice immunized with fraction 30 of the gp96 preparation gave a robust CTL response, no response was detected in mice immunized with fraction 24 (where contaminating β-gal would have eluted) (Fig. 3C). Conversely, fraction 24 but not fraction 30 of the β-gal preparation provided a robust CTL response as expected. These results demonstrate that the immunogenic entity in gp96 preparations derived from gp96 or contaminating β-gal fragments with the same ionic charge as gp96. Along with results shown in Fig. 2, the immunogenic entity in P13.4-gp96 must be either gp96- or a β-gal-derived contaminant with the same size and charge as gp96.

**Immunodepletion of the active entity in gp96 preparations**

To probe further the relative contributions of the gp96 band and undetectable but putative contaminating β-gal fragments of ~96-

![Image 1](http://www.jimmunol.org/)

**FIGURE 2.** The immunogenic entity in gp96 preparations was of the 96-kDa size. A, gp96 or β-gal was loaded individually onto preparative SDS-polyacrylamide gels and fractions were collected according to size. gp96 and β-gal eluted in fractions 45–51 and 114, respectively, as detected by immunoblotting. B, Fractions 45–51 and 114 from either preparation were used to immunize mice. One week after immunization, spleen cells were removed and cultured with peptide for 1 wk. Cytotoxicity was tested 5 days thereafter as in Fig. 1. Each line represents the specific (peptide-pulsed minus un-pulsed) lysis of spleen cells from one mouse.

![Image 2](http://www.jimmunol.org/)

**FIGURE 3.** Repurified gp96 preparations retained immunogenicity. A, Purified P13.4-gp96 or purified β-gal was repurified over an ion exchange column with an increasing salt gradient from 200 to 800 mM. gp96 eluted in fraction 30 distinct from β-gal in fraction 24. B, Elution peaks for gp96 or β-gal were analyzed by SDS-PAGE and immunoblotting. gp96 and β-gal did not cross-contaminate each other. C, C57BL/6 mice were immunized with fractions 30 or 24 from the final repurification step (as in A) of P13.4-gp96 or β-gal or with PBS. CTL activity was tested on spleen cells as in Fig. 1.
Depletion of hypothetical contaminants did not deplete immunogenicity from gp96 preparations. A, Purified P13.4-gp96 preparations or β-gal were depleted with Abs to each protein. Depletions were monitored by SDS-PAGE and immunoblotting and were specific and complete. B, gp96 or β-gal, undepleted or depleted with the indicated Abs, were used to immunize mice. One week after immunization, spleen cells were removed and cultured with peptide for 1 wk. Cytotoxicity was tested 5 days thereafter as in Fig. 1. Each line represents the specific (peptide-pulsed minus unpulsed) lysis of spleen cells from one mouse.

gp96 does not act as an adjuvant to proteins mixed with it

gp96 provides adjuvanticity to the peptides that it chaperones (27). An argument has been made, without any accompanying evidence, that gp96 is such an excellent adjuvant for any proteins present in the same solution as gp96 and that this adjuvanticity of gp96 is responsible for eliciting immune response to “contaminating proteins” (21–24). The corresponding evidence with respect to peptides is clear; immunization with mixtures of gp96 and peptides does not elicit immunity specific for the peptide. The peptide must be noncovalently associated with it (27). Nonetheless, the premise that gp96 provides adjuvanticity to subimmunogenic quantities of intact proteins was tested experimentally. Mice were immunized with titrated quantities of β-gal, including subimmunogenic doses, mixed with gp96 purified from P815 cells. Splenocytes were tested for CTL activity as in Figs. 1–3. As shown in Fig. 1, 10 μg of β-gal is immunogenic while 1 μg is not. Mice were therefore immunized with 1 μg of β-gal mixed with 10 μg of gp96 as an experimental adjuvant. The quantity of gp96 was chosen as 10 μg because that quantity of gp96 purified from P13.4 cells was immunogenic while 1 μg is not. Mice were therefore immunized with 1 μg of β-gal with 10 μg of gp96 as an experimental adjuvant. The quantity of gp96 was sufficient to elicit Kb/DAPIYTNV-specific immunogenicity. Mice were also immunized with 10 μg of β-gal as a positive control. The results show that although all positive and negative controls behave as expected, mixing 10 μg of gp96 with 1 μg of subimmunogenic doses of β-gal does not confer immunogenicity (Fig. 5).

Detection of MHC class I (MHC I) precursor peptides chaperoned by gp96

Since immunogenicity of gp96 preparations was determined to be associated with the gp96 molecule itself and not with contaminating Ag (as shown above), the presence of gp96-associated peptides was tested. Peptides were stripped off P13.4-derived gp96 as described in Materials and Methods and analyzed by mass spectrometry. Although a single 19-mer peptide (EVASDTPHPARIGLNQCLA) was found and fragmented by tandem mass spectrometry,
we were not sufficiently confident in assigning the sequence due to low accuracy of the precursor ion measurement (A. Tomlinson, unpublished data). Additionally, although major fragmentation ions were consistent with the 19-mer sequence, overall signal intensity was low and current efforts are underway to compare the sequence information collected for this peptide to synthetic analogs to increase confidence and verify this result.

Detection of MHC I epitope precursors (as opposed to precise epitopes) has traditionally been difficult because of the variability inherent in the precursors, and with rare exceptions (9) has not been made. Shastri and colleagues (25) devised an experimental system, precisely to address this question, using the OVA model. They modified the SIINFEKL epitope of OVA to SIINFEHL and flanked each end of this sequence by a lysine to generate trypsin and carboxypeptidase B cleavage sites on the termini of SIINFEHL. The OVA gene with these mutations was called KOVAK. A peptide pool obtained from KOVAK-expressing cells and treated with a combination of trypsin and carboxypeptidase B liberates the SIINFEHL epitope from all of the precursor peptides that might contain it (Fig. 6A). The SIINFEHL epitope, which is presented by H-2Kb, can be measured in a T cell-based assay.

We have created PIK23 cells (H-2Kb) that contain an RSL1-inducible KOVAK construct fused to GFP. Induction of KOVAK by RSL1 was monitored by GFP expression as described in Materials and Methods (Fig. 6B). PIK23 cells induced with RSL1 or uninduced were used as sources of purified gp96 of the same purities as used in Figs. 1–5. gp96 was purified to apparent homogeneity by the same procedure as used for P13.4 cells. gp96 preparations were immunblotted with polyclonal anti-GFP Ab to detect GFP-KOVAK or its fragments. No Ag was detected (data not shown) in a manner analogous to the data shown in Fig. 2 for the β-gal system. Peptides were stripped off gp96 and treated with a combination of trypsin and carboxypeptidase B. The resulting peptides were pulsed onto Kb-expressing EL.4 cells and tested for lysis by a T cell line specific for SIINFE(H/K)L. Peptides thus obtained from RSL1-induced, PIK23-derived gp96 were able to charge target cells for lysis while peptides obtained from control uninduced, PIK23-derived gp96 did not (Fig. 6C). This result shows the association of SIINFEHL peptides with gp96 and that these peptides include the direct MHC I binding peptide as well as the precursor peptides.

**Discussion**

Our results demonstrate that 1) apparently homogenous gp96 preparations from P13.4 cells elicit Kb/DAPIYTNV-specific CTL response, and this activity resides in proteins of ~96 kDa size; 2) if these preparations contain contaminating β-gal, its content is ~5

**FIGURE 5.** gp96 does not augment immune responses of suboptimal quantities of whole β-gal. Mice were immunized with 10 μg of P815-derived gp96 alone or mixed with 1 μg of β-gal protein. Spleens from immunized mice were cultured and tested in cytotoxicity assays for anti-β-gal responses as in Fig. 1. Control mice were immunized with 10 μg of P13.4-derived gp96 or 10 μg of β-gal.

**FIGURE 6.** MHC precursor peptides could be eluted from purified gp96 preparations. A, Schematic design for the KOVAK-GFP construct for detection of MHC I peptide precursor (based on Ref. 25). B, PIK23 cells were induced with RSL1 (solid line) and were observed expressing the KOVAK-GFP protein. The dashed line shows lack of KOVAK expression in uninduced cells. C, One milligram of gp96, purified from uninduced or RSL1-induced PIK23 cells, was stripped off associated peptides as described in Materials and Methods. Eluted peptides (~10 kDa) were treated with trypsin and carboxypeptidase to release SIINFEHL and pulsed onto EL-4 cells and used as targets in a CTL assay. Peptides from induced but not uninduced PIK23-derived gp96 were able to charge EL-4 cells. Synthetic SIINFEKL peptide (1 μM) was pulsed onto EL-4 cells as a positive control. (The T cells recognize equally SIINFEKL- or SIINFEHL-pulsed cells.) There was no lysis of unpulsed EL-4 cells.
pg/mg of gp96, and <50 fg per immunizing dose of gp96 preparations; 3) the specific immunogenicity of the gp96 preparation cannot be attributed to contaminating intact β-gal nor to any fragments of β-gal that may react with a polyclonal antisera against β-gal; 4) depletion of gp96 preparations of gp96 species depletes the specific immunogenicity of the gp96 preparation; however, depletion of these same preparations of any theoretically contaminating (but undetectable) β-gal has no influence on the specific immunogenicity of the gp96 preparation; 5) gp96 does not confer detectable adjuvanticity to subimmunogenic doses of β-gal; and 6) MHC I-binding peptides (and their precursors) can be eluted off gp96 molecules. These observations demonstrate that the K9/DAP1TVN-specific immunogenicity of gp96 preparations from P13.4 cells does not derive from contaminating β-gal. Conversely, the hypothesis that gp96 (and other HSPs) copurify with trace but undetectable quantities of intact proteins and that these contaminants are responsible for the specific immunogenicity of HSP preparations (21–24) cannot be substantiated.

The possibility that gp96 molecules are not associated with endogenous peptides was first raised from experiments that showed that truncated gp96 molecules lacking the carboxy terminus (where the first putative peptide binding site was previously reported to be (20)) could still confer protective immunity (21). While this article was under publication, a second peptide binding site of gp96 was identified on the amino terminus of gp96 (19). The presence of this second, N-terminally located peptide binding site invalidated the conclusion that the immunogenicity of gp96 is peptide independent. Furthermore, the immunogenicity of gp96 shown in that study was extremely weak and comparable to the nonspecific innate immune activity of gp96 as published by us previously (28, 29). Thus, the single study that purported to show evidence of the peptide independence of immunogenicity of gp96 could not be upheld after the identification of this second peptide binding site.

An extensive biochemical analysis of this scale has not been performed with other Ags; nonetheless, results of solid studies in the OVA system are consistent with the results shown here in the β-gal system. Thus, Berwin et al. (30) showed that gp96 constituted the immunogenic component of virally lysed cells. These authors showed through careful quantitative analyses that OVA-depleted but gp96-enriched fractions of the lysates contained the major immunogenic activity; conversely, gp96-depleted lysates had little activity. Binder and Srivastava (16) showed that cell lysates replete with OVA Ag but depleted of HSPs were devoid of immunogenic activity; a previous study (31) that had come to an opposite conclusion was shown to have done so because of dose restriction of the immunogenicity of cell lysates (16). Studies of Shastri and colleagues (25) have provided a definitive and independent verification of the original idea (4, 5) that selected HSPs chaperone precursors of MHC I epitopes in vivo. Using the KO-VAK system described in Results (25), they observed that the chaperones TriC (of the hsp90 family) and hsp90α associate with precursors of the K9-binding SIINFEKL peptide, and that extinction of expression of these chaperones through genetic means, leads to generation of empty MHC I molecules (32, 33). Collectively, these results argue unequivocally in favor of the observations reported in the present study.

The HSPs are the only molecules other than the MHC, to be peptide-binding proteins of immunological significance. The increasingly clear demonstration that the HSP-peptide complexes play a critical and essential role in Ag presentation (9, 32–35) and in cross-priming (7, 12, 13, 15, 16) have now begun to reveal that the two peptide-binding activities are in fact functionally related.

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