Vaccination with heat shock protein gp96-antigenic peptide complexes produces a powerful specific immune response against cancers and infectious diseases in some experimental animal models, and gp96-peptide complexes are now being tested in human clinical trials. gp96 appears to serve as a natural adjuvant for chaperoning antigenic peptides into the immune surveillance pathways. A fundamental issue that needs to be addressed is the mechanism of binding of antigenic peptide to gp96. Here, we show using scanning transmission electron microscopy that recombinant gp96 binds peptide in stable multimeric complexes, which may have biological significance. To open the possibility for genetically engineering gp96 for improved immunogenicity and to understand if molecular recognition plays a role in the binding of antigenic peptide, we mutagenized some specific aromatic amino acids in the presumed peptide-binding site. Replacement of Tyr-667 or Tyr-678 to Ala reduced affinity for peptide whereas conversion of Trp-654 to Tyr increased peptide binding. Similarly, changing Trp-621 to Phe or Leu or Ala or Ile negatively affected peptide binding whereas changing Trp-621 to Tyr or Val positively affected peptide binding. Probing the peptide microenvironment in gp96-peptide complexes, suggested that hydrophobic interactions (and perhaps hydrogen bonding/stacking interactions) may play a role in peptide loading by gp96.

The endoplasmic reticulum (ER) is the organelle responsible for constant peptide trafficking. The most abundant peptide-binding protein in the lumen of ER is the heat shock chaperone gp96 (GRP94, Ref. 1). gp96 has drawn the attention of many investigators for its induction by glucose starvation, androgen, and interferons and for its role in ischemia, tumor immunity, calcium, and peptide binding, as well as its role as a chaperone, a glycoprotein, and a phosphoprotein (reviewed in Ref. 2). gp96 and heat shock protein HSP90, its cytosolic paralog, share about 50% sequence homology (3). Both are dimeric proteins (4–6) and are also found as oligomers (7–10). Both HSP90 and gp96/GRP94 (1, 11) bind a variety of antigenic peptides in vitro and in vivo (for reviews, see Refs. 12–14). Intriguingly, gp96 acts as a chaperone of peptides and aids in provoking a strong immune response against peptide antigens (12). Its potential as a natural adjuvant for therapeutic cancer vaccines makes this protein highly significant for human health. The proposed role of gp96 is to ferry antigenic peptides into specialized antigen-presenting cells via receptor-mediated internalization of gp96-antigenic peptide complexes (15–17). The peptides are presumed to be transferred to and represented by MHC class I molecules (12, 18–22). Clearly, the primary event in the HSP-mediated immune response pathway appears to be the recognition and binding of antigenic peptides by gp96. In this context many questions remain unanswered; e.g. how are peptides selected, and what are the mechanisms of peptide binding by gp96? To address this, we developed a highly sensitive fluorescence-based assay for gp96/peptide binding (9) and mapped the minimal peptide-binding site to amino acid residues 624–630 in the C-terminal region of gp96 (23). A molecular model of the gp96 peptide-binding site was offered with the suggestion that the antigenic peptide may bind in a pocket. Here, to further explore the molecular interactions of peptide and gp96, we used STEM to show that gp96-peptide complexes exist in higher order multimeric complexes and used site-directed mutagenesis to understand the role of specific aromatic amino acid residues in the gp96 peptide-binding pocket.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Wild-type and mutant His-gp96 proteins were purified from plasmid-bearing *Escherichia coli* strain JM109 using published procedures (9, 10). Plasmid pHisGP96 encodes murine gp96 with an N-terminal His tag (10), and the mutants are described below. The purity and intactness of the proteins were assessed by electrophoresis in SDS-containing 10% polyacrylamide gels, silver staining, and immunoblotting (9).

**Preparation of Nanogold-labeled Peptide**—Peptide RGYVYQGLKS-GLRASLGRS is a known ligand of gp96 that contains a vesicular stomatitis virus-derived core sequence (23). It was synthesized (Alpha Diagnostic, San Antonio, TX) and purified by HPLC. The peptide was covalently labeled with nanogold using the amine-reactive reagent sulfo-N-hydroxysuccinimido nanogold according to the manufacturer's protocol (nanoprobes, Yaphank, NY). A 10-fold molar excess of peptide over nanogold was used to maximize the coupling efficiency. To purify nanogold-peptide from free peptide and nanogold, the reaction was subjected to ultrafiltration (10 kDa molecular mass cut-off) and washed repeatedly with 0.1 M triethylammonium acetate-water. The retentate was filtered (0.45 micron) and the nanogold-peptide conjugate was purified by C18 reverse phase HPLC. The following HPLC gradient was used: 0.1 M triethylammonium acetate-water, 0.1 M triethylammonium acetate-water/MeOH, and 0.1 M triethylammonium acetate-water/0.001 M TFA. The purity and intactness of the purified nanogold-peptide conjugate was assessed by mass spectrometry and confirmed by fast protein liquid chromatography (24).
Table I. Mutagenic oligonucleotides

| Codon change | Oligonucleotide sequence (5' to 3') |
|--------------|-----------------------------------|
| W485Y        | GTGCCGAACTCTTTAAGGAGGGTGCTGTTAAC |
| Y575A        | GGCTGATTCGAGCCTCCACAGGG          |
| W621A        | CTTATCTTCATGGAGTGGACGAGG         |
| W621F        | CTTATCTTCATGGAGTGGACGAGG         |
| W621I        | CTTATCTTCATGGAGTGGACGAGG         |
| W621L        | CTTATCTTCATGGAGTGGACGAGG         |
| W621Y        | CTTATCTTCATGGAGTGGACGAGG         |
| Y652A        | CAGACCACCCAGCCTGACTGCGCAC        |
| Y667A        | GGGCTCGTGGGCTGTGGTGGCTC          |
| Y677A        | GAGCTGGCATTGCGAGCTCTGC           |
| Y678A        | CTTTCTTCTCTGCGACGCGATTTGATAGAG  |
| Y677A + Y678A| CTTTCTTCTCTGCGACGCGATTTGATAGAG  |
| W654F        | ATGTGCCAGAGAAATCTCATGCTGG        |
| W654L        | ATGTGCCAGAGAAATCTCATGCTGG        |
| W654Y        | ATTTGCGAGAGAAATCTCATGCTGG        |

* Letters indicate original and mutated amino acid, respectively, and number indicates codon. Codons are numbered according to unprocessed gp96.

Oligonucleotides were purchased from Operon Technologies (Alameda, CA).

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**Binding of Peptide to gp96 as seen by STEM**—Recently, we used STEM to show that gp96 forms higher order multimers larger than dimers (10). It was shown that native mouse protein also forms higher order structures (10). We wished to know if peptide could be bound to gp96 in these large multimeric structures. Therefore we prepared antigenic peptide conjugated to 1.4 nm-nanogold and used STEM to analyze the peptide-gp96 complexes. STEM is particularly useful for studying higher order structures because one observes bare, unstained molecules and can obtain estimates of masses and dimensions.

For this purpose STEM is superior to solution-based techniques and other types of electron microscopy. Moreover, using STEM one can directly visualize and count nanogold-peptide particles (28, 29). Nanogold-peptide-gp96 complexes were prepared at concentrations approaching intracellular gp96 concentrations (5–10 μM, Ref. 1) and the complexes were diluted just prior to visualization by STEM (Fig. 1). Nanogold-peptide was seen as bright white spots against the background of gray gp96 multimeric structures resting on a black surface (Fig. 1, A–E).

It was not possible to determine whether a given nanogold-peptide resided on a distal or proximal surface of a complex because with STEM one sees a two-dimensional (transmission) projection of the particle. Isolated nanogold-peptide was also seen in some fields (Fig. 1, F–J) but at a much lower density than observed with or in close proximity to gp96 (Fig. 1, A–E).

In general, these examples of STEM images showing massive gp96 multimers are similar to ones seen earlier that were prepared without peptide (10) except that we did not observe many smaller complexes of gp96 such as monomer/dimer, tetramer etc. This suggested that gp96-peptide complexes were assembled in large multimers and that dilution and washing did not dissociate bound peptide, consistent with our previous findings (10). We manually counted the number of nanogold-peptide associated with gp96 complexes and found that the number of bound nanogold-peptide roughly corresponded to the number of gp96 monomer units in a complex (Fig. 1, below). With some types of samples, e.g. large viruses, nanogold-signal from a distal surface might be obscured because of sample thickness. However, this is unlikely to be the case with gp96 complexes. It is possible that some gp96 molecules in the multimeric complex failed to bind nanogold-peptide or lost their nanogold-peptide during sample processing. The dimensions of the multimeric complexes suggested no specific geometric configuration, only unordered shapes (Fig. 1, A–E and lower table), consistent with previous findings (10). Statistical analysis was not possible with this limited amount of data. Many of the molecules (not shown) appeared to be "denatured" because of unknown reasons.

The finding that gp96-peptide complexes are assembled in massive multimers is potentially important because such a binding mode may facilitate the transport and receptor-mediated uptake of gp96-peptide complexes into antigen-presenting cells (15). These large complexes may sequester peptide more effectively than smaller ones (dimers, tetramers, etc.), resulting in more efficient delivery of antigenic peptides to their ligands. The finding that gp96-peptide complexes are assembled in massive multimers is potentially important because such a binding mode may facilitate the transport and receptor-mediated uptake of gp96-peptide complexes into antigen-presenting cells (15). These large complexes may sequester peptide more effectively than smaller ones (dimers, tetramers, etc.), resulting in more efficient delivery of antigenic peptides to their ligands.
subcellular destination in the antigen presenting cell for further processing. Also, higher order complexes may be more easily bound by the cognate gp96 receptors, because the high local concentration of gp96-peptide complexes could greatly enhance the frequency of collisional interaction with gp96 receptors. Thus we suggest a biological significance for the higher order assembly of gp96-peptide complexes.

**Rationale for Site-specific Mutagenesis**—We recently identified the peptide-binding site of gp96 based on photochemical cross-linking studies (23). Because no high resolution structure exists for the gp96- or HSP90-peptide complex, we constructed a computer model depicting the peptide-binding pocket of gp96 (for details and rationalization see Ref. 23). This model has served as a guide for further exploration of the molecular nature of gp96-peptide interactions. Crystallographic and NMR studies of other peptide-binding proteins have revealed that aromatic amino acids such as Tyr and Trp play an important, although nonexclusive role in optimal peptide-protein interactions (reviewed in Ref. 30). For example, in the MHC I molecule-antigenic peptide for their interactions (32, 33).

To better understand the microenvironment of the peptide-binding pocket of gp96, we turned to site-specific mutagenesis. The peptide-binding domain of gp96 contains five Tyr and three Trp residues. Site-specific mutagenesis was used to alter these codons in expression vector pHisGP96 (Table I). Each of the Tyr codons was individually changed to an Ala codon; a mutant Y677A and Y678A was also constructed. Alanine was selected as the replacement amino acid for Tyr because it lacks the large bulky hydrophobic side chain proposed to interface with peptide ligand but retains some hydrophobicity. Alanine was replaced with Tyr because nearly all HSP90 proteins have Tyr at the equivalent position. Trp-621 was substituted with Tyr at the equivalent position. Trp-621 was substituted with Tyr because nearly all HSP90 proteins have a binding pocket of gp96, we turned to site-specific mutagenesis. Crystalllographic and NMR studies of other peptide-binding proteins have revealed that aromatic amino acids such as Tyr and Trp play an important, although nonexclusive role in optimal peptide-protein interactions (reviewed in Ref. 30). For example, in the MHC I molecule-antigenic peptide for their interactions (32, 33).

**TABLE II**

| Item              | $a_1$ | $a_2$ | $a_3$ | $a_4$ | $\tau_1$ | $\tau_2$ | $\tau_3$ | $\tau_4$ | $\tau^{<\infty}$ | $\chi^2$ |
|------------------|-------|-------|-------|-------|-----------|-----------|-----------|-----------|-------------------|---------|
| Peptide-Pyr      | 0.70  | 0.29  | —     | —     | 77.4      | 10.5      | —         | —         | 90 87             | 1.1     |
| WT               | 0.19  | 0.25  | 0.22  | 0.33  | 19.6      | 35.5      | 92.8      | 105.6     | 181 177          | 1.2     |
| Y675A            | 0.17  | 0.14  | 0.60  | 0.08  | 11.9      | 60.4      | 105.6     | 181       | 177              | 1.2     |
| Y652A            | 0.13  | 0.05  | 0.79  | 0.03  | 0.2       | 0.2       | 56.4      | 56.6      | 114 112          | 1.5     |
| Y687A            | 0.11  | 0.01  | 0.88  | —     | 0.2       | 57.0      | —         | 58        | 56.6              | 1.0     |
| Y677A            | 0.01  | 0.02  | —     | 0.97  | 1.5       | 1.7       | 62.3      | 62.4      | 129 127          | 1.5     |
| Y675A            | 0.20  | 0.80  | —     | —     | 71.2      | —         | 72        | 70        | 1.0              |         |
| Y677A + Y678A    | 0.33  | 0.67  | —     | —     | 13.2      | 82        | —         | 96.2      | 94.0             | 1.1     |
| W485Y            | 0.27  | 0.22  | 0.55  | —     | 7.2       | 7.6       | 92.6      | 59.3      | 161 166          | 1.6     |
| W654F            | 0.12  | 0.12  | 0.61  | 0.13  | 9.9       | 56.5      | 116.0     | 183       | 181              | 1.0     |
| W654L            | 0.07  | 0.07  | 0.00  | 0.85  | 4.1       | 4.2       | 80.9      | 80.4      | 172 169          | 1.4     |
| W654Y            | 0.16  | 0.46  | 0.29  | 0.09  | 55.5      | 0.0       | 145.4     | 114.2     | 314 312          | 1.2     |
| W621A            | 0.32  | 0.68  | —     | —     | 108       | —         | 109       | 109       | 107 2.0          |         |
| W621F            | 1.00  |       |       |       | 4.5       | 99.5      | —         | 105       | 103              | 1.3     |
| W621I            | 0.04  | 0.96  | —     | —     | 24.7      | 109       | —         | 135       | 112              | 1.0     |
| W621L            | 0.09  | 0.91  | —     | —     | 24.9      | 222       | —         | 247       | 246              | 1.2     |
| W621Y            | 0.64  | 0.36  | —     | —     | 16.4      | 291       | —         | 308       | 309              | 1.1     |

$^a$ $a_i$ is the fractional amplitude normalized to sum of 1.
$^b$ $\tau_i$ is the lifetime (ns) associated with each component.
$^c$ $\tau^{<\infty}$ is the intensity-average lifetime (ns): $\Sigma_{a_i \tau_i} / \Sigma_{a_i}$.
$^d$ $\chi^2$ is a measure of the goodness of fit.

$^e$ The standard error for the average lifetime is $\pm 20–30$ ns.

FIG. 1. STEM analysis of higher order complexes of the nanogold-peptide-gp96 complex. Panels A–E show images of gp96 multimeric complexes with bound nanogold-peptide, whereas panels F–J show isolated free nanogold-peptide. The table below gives a semiquantitative analysis of the complexes. Total masses were computed using a PC mass program as described before (10). Visible nanogold particles were counted manually from onscreen images. A collective mass for nanogold-peptide (Au-pep) was estimated using a value of 20 kDa each. This value was subtracted from the total mass of the nanogold-peptide-gp96 complex, and the remainder was divided by the molecular mass of gp96 monomer (94.010 kDa) to estimate the number of gp96. The dimensions of the complexes were estimated by counting the number of pixels in enlarged views of images and were corrected for the appropriate magnification factor used to obtain the different images.
Characterization of Mutant gp96 Proteins—gp96 mutant proteins, which had N-terminal His$_6$ tags, were purified using Ni$^{2+}$-affinity chromatography and analyzed by SDS-gel electrophoresis and Western blotting (Fig. 2). Silver staining of the gel showed that the mutant proteins migrated as single bands similar to the WT gp96 protein (Fig. 2A). Western blotting with a C terminus-recognizing anti-KDEL antibody indicated that the proteins were full-length and intact (Fig. 2B). The results indicated that the mutant protein preparation was >95% pure gp96.

To find out if single mutations in the gp96 protein affected secondary structure, we used CD spectroscopy. CD is a sensitive method for measuring changes in protein conformation. An example of the CD data is shown in Fig. 3, top panel. Data for the other mutants are not shown because they were similar to Fig. 2A (bottom panel). The CD values were normalized as described before (10). The CD values were normalized as described before (10). All other mutant proteins not shown here showed similar results with these techniques.

Peptide Binding by WT and Mutant Proteins—To assay for peptide binding in solution and to quantitate the differences in peptide loading efficiencies of gp96 mutant proteins, we used a pyrene-labeled peptide ligand. The detailed procedure for synthesis of this fluorescent peptide and method of assay for peptide binding by gp96 have been described elsewhere (9, 10). Briefly, the fluorescent group pyrene was attached to the unique lysine residue in the peptide (SLSDLRGYVYQGLKS-GNVS) via an amide linkage. Here the pyrene serves as an optical sensor of the changes in the environment of the peptide-binding site. When peptide-pyrene binds to gp96 there is an increase in the excited-state lifetime of pyrene because it is in a hydrophobic environment (10). By measuring the fluorescence intensity and the intensity-averaged lifetime of pyrene, we can gauge peptide binding affinities as well as the extent of changes in hydrophobicity as previously demonstrated (9, 10).

This assay has several advantages over STEM for the present purpose. 1) The fluorescence assay allows for “sensing” the microscopic changes in pyrene environment (which may translate into peptide environment) brought about by alterations in amino acid side chains. 2) The fluorescence assay is quantitative; therefore potentially a hierarchical gradation of effects of the mutations is possible; and 3) it is much more rapid than STEM and is solution-based.

Fig. 3 (bottom panel) shows examples of pyrene emission spectra of peptide-pyrene-gp96 complexes. Spectra for other mutant gp96-peptide complexes are not shown because the basic changes were similar to those in Fig. 3. In all cases, binding of peptide-pyrene resulted in an increase in the intensity of emission bands at 378 and 396 nm compared with the free peptide-pyrene (Fig. 3, green line). To help describe the molecular interactions that the pyrene fluorophore experiences and to understand the local environment of the fluorophore we measured, in parallel to the emission spectra, the lifetime parameters (Table II). The intensity-averaged lifetime (\(<\tau>\)) of pyrene increased when WT gp96 bound to peptide, indicating that the pyrene moiety had moved into a hydrophobic environment in the peptide-binding site in gp96-peptide complexes (Table II). The increase in \(<\tau>\) is because of sequestration of pyrene from dynamic solute quenching of the excited state. Binding of peptide-pyrene to mutant gp96 proteins either increased or decreased the steady-state intensity and lifetime of pyrene compared with the WT gp96-peptide complex. Changes in steady-state intensities were quantitatively expressed by constructing binding isotherms, which reflected gross affinity of peptide to gp96 (Fig. 4). Here the steady-state fluorescence intensity at 378 nm was plotted against peptide-pyrene concentration. Intensity-averaged lifetime was used as a measure of changes in the peptide microenvironment (hydrophobicity).
Mutation of Tyr-667 or Tyr-678 to Ala caused a marked decrease in steady-state fluorescence and intensity-average lifetime (Fig. 4, top panel) and a corresponding lack of significant effect on the microenvironment, as indicated by the intensity-average lifetime parameter (Table II, t). Similarly, mutant proteins Y652A and Y677A did not show appreciable changes in the intensity-average lifetime (Table II, t). It is interesting to note that whereas Y678A affected both peptide binding and the pyrene environment, Y677A affected neither. Furthermore, with the double mutant protein, Y677A Y678A, the intensity-average lifetime decreased to a similar extent as seen for the single mutant protein Y678A (Table II). This clearly shows that the two neighboring tyrosines (Tyr-677 and Tyr-678) are nonequivalent and that Tyr-678 is perhaps closer to pyrene and may interact with peptide. These findings highlight the crucial role that tyrosines may play in the peptide-binding site and demonstrate that the environment of the bound peptide can be affected in such a way as to decrease peptide binding. Tyrosines closest to the peptide (at least based on our model Ref. 23) did indeed affect the peptide environment. In the MHC class I molecule–peptide complex, tyrosyl residues serve as anchors to restrict size-selectivity of antigenic peptide (31). Whereas any parallel between MHC class I molecules and gp96 is debatable, it is intriguing to note that tyrosines serve an essential role in peptide loading by gp96. The Tyr may be involved in stacking/hydrophobic interactions with peptide as observed in other types of protein–peptide interactions (30).

Next, we examined the potential role of another set of aromatic amino acids: the tryptophanyl residues. Earlier, we speculated based on resonance energy transfer between tryptophan and pyrene in peptide-pyrene-gp96 complexes that one or more Trp residues were close to the bound peptide (9). To examine the role of Trp residues in peptide loading, we focused on the three tryptophanyl residues (Trp-485, Trp-621, and Trp-654) closest to the peptide in the presumed peptide-binding pocket (23). Trp-654 is a highly conserved residue in gp96 paralogs, and HSP90 family of proteins and Trp-621 is closest to the minimal contact region (residues 624–630) that was identified by cross-linking peptide to gp96 (23). Mutation of Trp654 to Phe or Leu did not significantly alter gross peptide binding nor did these mutations affect the microenvironment as indicated by the relatively little change in the intensity-average lifetime (Fig. 4, bottom panel; Table II, t). The same was true for W485Y, which presumably lies farther away from the peptide-binding site (23). W654Y mutant gp96 appeared to bind peptide better than WT (Fig. 4, bottom panel) and the peptide-binding environment appeared to be affected as well, i.e. increased hydrophobicity because the <t> increased (Table II). These data suggest for the first time that mutagenesis can be used to engineer gp96 for greater peptide binding affinity. Furthermore, addition of a hydroxyl group (in W654Y, note that W654F...
has no effect) may facilitate new interaction such as H-bonding, which may have increased peptide affinity. The finding that the W654Y mutant protein (and others, see below) binds peptide better than WT gp96 opens the door to envisioning an improved gp96-peptide vaccine if one assumes that peptide affinity is correlated to immunogenicity, an assumption that needs further investigation.

Because Trp-621 was predicted to be closest to the bound peptide (23) we wanted to examine in greater detail the effects of mutation at this position (Fig. 5). Mutation of Trp-621 to either Ala, Phe, Ile, or Leu caused a reduction in peptide binding compared with WT gp96 (Fig. 5, top panel). The intensity-average lifetime decreased somewhat, suggesting that these mutations moderately affect binding pocket environment (Table II, <t>). Interestingly, change to either W621Y or W621V significantly increased peptide binding relative to WT protein (Fig. 5, bottom panel). Concomitantly, the peptide-binding environment of the pocket also changed to greater hydrophobicity because the intensity-average lifetime of pyrene increased significantly (Table II, <t>). The fact that substitution of Trp-621 with Ile or Leu negatively affected peptide binding whereas substitution of Val affected peptide binding positively suggested that both hydrophobicity (which is similar for Ile, Leu, and Val) and the length of the side chain influenced peptide binding. Because substitution with Ala had a negative effect, the extra “greasy” methyls were important. The substitution of Trp-621 with Tyr affected peptide binding positively (Fig. 5, bottom; Table II). Putting all these results together, we suggest roles for hydrophobic interactions and hydrogen bonding (because W621F negatively affected peptide binding) in peptide binding to gp96.

The studies described here attempt to decipher the molecular nature of the interaction of antigenic peptide with gp96 by using site-specific mutagenesis of gp96. It is important to note that because a high resolution crystal structure is unavailable, a model-based approach was used for design of experiments. The finding that gp96 binds peptide in very large molecular complexes, as shown here (Fig. 1), could explain some of the difficulties in obtaining crystallographic models. The peptide-gp96 complexes appear to be organized in no specific geometric lattices, perhaps adding to the difficulty.

Steady-state fluorescence intensity and intensity-averaged lifetime of the pyrene probe are used to measure peptide affinity and changes in local environment of the peptide. It is likely that the affinity of peptide is altered by changes in the internal milieu (hydrophobicity) of the binding pocket. Indeed, these two properties viz. peptide affinity and hydrophobicity of the binding pocket, may be related and are simply two sides of the same coin. To get a better perspective, data from titration experiments were used to make our conclusions regarding the difference in gross peptide affinity. Differences in intensity-averaged lifetime may reflect changes in both affinity and hydrophobicity because the fluorescence quantum yield is related to the excited-state lifetime (26).

Our work suggests that hydrophobic interaction and hydrogen bonding and/or stacking interactions could play an essential role in peptide stability in the binding pocket. Some substitutions in the presumed peptide binding pocket that introduce Tyr residues (W654Y and W621Y) appear to positively influence peptide binding whereas changes to other residues such as Trp-621 to Phe, Leu, Ala, or Ile had a negative effect. This may suggest an important role for Tyr in peptide binding by gp96 (as seen with other peptide-binding proteins Ref. 31)). The observation that some specific substitutions positively or negatively modulate peptide binding could potentially be a useful way to engineer new mutant gp96 proteins and to investigate their immunogenic potencies. Such a study might help provide insights into the long standing and unresolved issue of whether peptide binding can be correlated to immunogenicity of gp96-peptide complexes. Furthermore, this work points to the possibility that there may be peptide selectivity by gp96 based on molecular recognition because specific alterations in key amino acids in gp96 do appear to affect peptide affinity for better or for worse. The fact that there are gp96 mutant proteins that appear to bind peptide better than WT suggests that the WT gp96 peptide-binding pocket may have evolved in a way that balanced peptide binding with the potential need to exchange/transfer bound peptide to other molecules such as co-chaperones and MHC.

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