Poor Binding of a HER-2/neu Epitope (GP2) to HLA-A2.1 Is due to a Lack of Interactions with the Center of the Peptide

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Class I major histocompatibility complex (MHC) molecules bind short peptides derived from proteins synthesized within the cell. These complexes of peptide and class I MHC (pMHC) are transported from the endoplasmic reticulum to the cell surface. If a clonotypic T cell receptor expressed on a circulating T cell binds to the pMHC complex, the cell presenting the pMHC is killed. In this manner, some tumors cells expressing aberrant proteins are recognized and removed by the immune system. However, not all tumors are recognized efficiently. One reason hypothesized for poor T cell recognition of tumor-associated peptides is poor binding of those peptides to class I MHC molecules. Many peptides, derived from the proto-oncogene HER-2/neu have been shown to be recognized by cytotoxic T cells derived from HLA-A2+ patients with breast cancer and other adenocarcinomas. Seven of these peptides were found to bind with intermediate to poor affinity. In particular, GP2 (HER-2/neu residues 654–662) binds very poorly even though it is predicted to bind well based upon the presence of the correct HLA-A2.1 peptide-binding motif. Altering the anchor residues to those most favored by HLA-A2.1 did not significantly improve binding affinity. The crystallographic structure shows that unlike other class I-peptide structures, the center of the peptide does not assume one specific conformation and does not make stabilizing contacts with the peptide-binding cleft.

Class I major histocompatibility complex (MHC) proteins bind short peptides (9–11 amino acids) derived from cytosolically degraded proteins. These peptides are transported into the endoplasmic reticulum and bind to newly formed class I molecules. Peptide binding appears to be the final step in assembly of the complex (1). Following peptide binding, the complexes are transported to the plasma membrane. At the plasma membrane, clonotypic T cell receptors on the surface of circulating cytotoxic T lymphocytes (CTL) may recognize the peptide-MHC complex (pMHC). If the pMHC is recognized by the T cell receptor, the T cell is activated and the cell presenting the pMHC is killed. A normal cell will have a large assortment of pMHC on the cell surface that are not recognized by CTL. However, viral or mutated self-proteins are degraded by these same mechanisms, and many of the resulting pMHC are recognized by CTL. In this manner, virus-infected or mutated cells are targeted for lysis by cytotoxic T cells (reviewed in Ref. 2). Self-proteins that are expressed in abnormally high amounts or in abnormal cell types may also be targets for CTL (3).

Class I MHC molecules bind many peptides with diverse sequences and high affinity (4). To bind all these peptides, the class I protein primarily interacts with the invariant portions of the peptides, the N and C termini (5). Class I MHC also uses a subset of amino acid side chains within the peptide termed “anchors” to generate significant binding (6). These peptide anchor residues bind within “specificity pockets” that are primarily formed by the polymorphic residues within the peptide-binding cleft of the MHC molecule (7). Peptides that bind with high affinity to a given allotype are typically found to have one of a few preferred amino acids at each anchor position. The corresponding hypothesis is that peptides that do not have those preferred amino acids at the anchor positions will not bind well. The combination of amino acids that may bind at the anchor positions is known as the peptide-binding motif (8). These motifs have proven to be extremely valuable in predicting peptides that will bind to class I MHC. Other residues within the peptide besides the anchors may be used to generate increased binding affinity (9–11).

Interestingly, many peptides that appear to have the correct peptide-binding motif still bind poorly. Substituting the anchor residues of poor binding peptides with those that are most preferred by the allotype can generate high affinity binding. (10, 12). Some of these altered peptide ligands (APL) are even effective therapeutics (13). We show here that there are also peptides for which altering the anchor residues does not significantly increase binding affinity. It is not clear from the previous available data in the literature why these peptides bind poorly.

HER-2/neu (c-erb-2) encodes a receptor tyrosine kinase with homology to the epidermal growth factor receptor. Overexpression of HER-2/neu in many adenocarcinomas, including breast and ovarian tumors, correlates with a poor prognosis for remission and recovery (14). Tumor infiltrating lymphocytes have been found in cancer patients that overexpress HER-2/neu, and these tumor infiltrating lymphocytes are able to recognize and lyse the solid tumor (3, 15, 16), but these CTL do not eliminate the tumor. It has also been shown that several peptide epitopes derived from the gene product of HER-2/neu are presented by class I MHC molecules to circulating CTL. As with many other
tumor-associated antigens, most of these peptides bind poorly to HLA-A2.1 (A2). There are many potential reasons for the lack of immune removal of tumors including the down-regulation of class I MHC or down-regulation of the protein from which the peptide is derived. It has also been proposed that one reason for poor recognition by CTL is weak binding of the immunogenic peptides to class I MHC (3).

Here we show that HER-2/neu-derived peptides, identified in the literature as recognized by CTL, bind with a range of affinities, but all are lower affinity than two index peptides of high affinity. One peptide was chosen for further study. This peptide, GP2 (IISAVVGLIL), binds very poorly to A2 but has anchor residues that are present in high affinity peptides (Ile at position 2 and Leu at position 9). Its inherently poor affinity is not significantly increased by substitution of its anchor residues. To understand why this peptide binds poorly, the crystallographic structure of the A2-GP2 complex was determined. Unlike all previously determined peptide-class I MHC (pMHC) structures, there is a large region of unresolved electron density in the center of the peptide. We interpret this to mean that the peptide assumes more than one conformation within the peptide-binding cleft. We hypothesize that the observed poor binding is due to the lack of important secondary interactions within the center of the peptide.

**EXPERIMENTAL PROCEDURES**

**Preparation of HLA-A2.1-Peptide Complexes—**Residues 1–275 of HLA-A2.1 (A2) and residues 1–99 of human β₂-microglobulin were expressed in *Escherichia coli*, produced as inclusion bodies, purified, and folded as described previously (17). Briefly, peptide, solubilized β₂-microglobulin and solubilized A2 heavy chain were rapidly diluted into folding buffer (10 mM Tris, pH 8.0, 0.4 M l-Arg, 10 mM reduced glutathione, 1 mM oxidized glutathione, and protease inhibitors) at molar ratios of 10:5:1, respectively. The final protein concentration was determined by amino acid analysis (Protein Chemistry Laboratory, Department of Chemistry, University of North Carolina, Chapel Hill). The list of peptides and references for immunogenicity are given in Table I.

**Determination of Thermal Stability—**Purified A2-peptide complexes were exchanged into a 10 mM KH₂PO₄ buffer, pH 7.5, and adjusted to a final protein concentration of 4–12 μM. The change in CD signal at 218 nm was measured as a function of temperature from 4 to 95 °C on a AVIV 62-DS spectropolarimeter (Aviv Associates Inc, Lakewood, NJ). The final melting curve was the average of at least three experiments for each A2-peptide complex. Tm values were calculated as the temperature at which 50% of the complexes are denatured using a two-state denaturation model (12).

**Cell Surface Stabilization Assay—**Cell surface stabilization of A2 was performed as described previously (11). Briefly, 2.5 × 10⁵ T2 cells (ATCC CRL-1992) were incubated overnight in AIM V serum-free medium at 37 °C, 5% CO₂ in the presence of 50 μM Brefeldin A (BFA, Sigma) to block the entry of new A2 molecules to the surface. Cells were incubated at 37 °C, 5% CO₂ in RPMI 1640, 15% fetal calf serum and 0.5 μg ml⁻¹ brefeldin A (BFA, Sigma). This concentration of BFA is toxic to the cells. Therefore, after 1 h the cells were then transferred to RPMI 1640, 15% fetal calf serum, and 0.5 μg ml⁻¹ BFA. At the indicated time points, 2.5 × 10⁶ cells were removed, incubated with BB7.2, and analyzed by flow cytometry as described above for cell surface stabilization assay. Each time point is evaluated as mean fluorescence with peptide minus mean fluorescence without peptide and normalized to the maximal level of fluorescence (at time zero) for each peptide.

**CRYSTALLIZATION, DATA COLLECTION AND PROCESSING—**Crystals were grown by hanging drop vapor diffusion as described previously (19).

**RESULTS**

**HER-2/neu-derived Peptides Bind Poorly to A2—**We began these studies to assess the correlation of immunological activity with peptide binding affinity to HLA-A2.1 (A2). Thermal stability of class I MHC-peptide complexes, as measured by
Table II
Summary of crystallographic data

The crystallographic structure of A2-GP2 was determined by molecular replacement using the A2-hepatitis B 10-mer (Protein Data Bank code 1HHH) as the search model. The structure was refined by a combination of X-PLOR and Refmac. Individual Be were refined in the penultimate cycle followed by the addition of waters.

| Data statistics         | Value |
|-------------------------|-------|
| Space group             | P1    |
| Cell Dimensions         |       |
| a                       | 56.34 Å |
| b                       | 63.61 Å |
| c                       | 75.14 Å |
| α                       | 81.95 Å |
| β                       | 76.25 Å |
| γ                       | 77.83 Å |
| Molecules/Asymmetric Unit | 2    |
| Resolution              | 30–2.4 Å |
| R<sub>merge</sub> (%)   | 9.3 (29.3)<sup>b</sup> |
| <I<sub>av</sub>/<I<sub>i</sub> > (%) | 7.80 (3.46) |
| Unique reflections      | 34,962 |
| Total reflections       | 66,839 |
| Completeness (%)        | 98.2 (97.6) |

**Refinement**

| Resolution              | 30–2.4 Å |
| R<sub>merge</sub> (%)   | 28.4 (1,714) |
| R<sub>work</sub> (%)    | 24.2 (31,969) |
| Rs fit<sup><sub>c</sub></sup> | 83.8% |
| No. of non-hydrogen atoms | 6,992 |
| No. of waters            | 103 |
| Average B factor         | 16.8 Å<sup>2</sup> |
| R.M.S. deviations from ideality |       |
| Bonds                    | 0.00 Å |
| Angles                   | 1.46Å<sup>2</sup> |

<sup>a</sup> R<sub>merge</sub> = Σ|I<sub>av</sub> − |I<sub>i</sub>|/Σ|I<sub>av</sub>|, where I<sub>av</sub> is the observed intensity and |I<sub>i</sub>| is the average intensity of multiple observations of symmetry related reflections.

<sup>b</sup> Number in parenthesis refers to the highest resolution shell (2.44–2.40) for A2-GP2 unless otherwise stated.

<sup>c</sup> Rs fit = the average real space fit of all atoms on an electron density map from DM with 2-fold noncrystallographic averaging, histogram matching, and solvent flattening.

<sup>d</sup> Error is the mean estimate of the coordinate error based on maximum likelihood methods (Refmac).

**DISCUSSION**

One hypothesis used to explain why tumors are not recognized and eliminated by the immune system is that potentially

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immunologically reactive peptides do not bind well to class I MHC molecules. If the peptides dissociate from class I MHC molecules too quickly, the cells presenting the peptides do not have a sufficient concentration of the specific pMHC at the surface of the cell to be recognized by circulating T cells. We examined binding of a selection of known immunologically recognized HER-2/neu-derived peptides bind poorly to A2, and anchor substitutions do not increase the stability of GP2-derived APL. The symbols and colors shown in A are also those used in B and C. Likewise, the symbols and colors shown in D are also those used in E and F. A, thermal stability of A2-peptide complexes as measured by CD. 4–12 μM protein was denatured by heat in a circular dichroism spectropolarimeter. The change in CD signal at 218 nm is an indication of the loss of secondary structure within the protein. Each curve is the average of three independent experiments. The error in the Tm is the sum of the curve fit error and the Peltier temperature controller error and is ±1 °C. B, cell surface measurements confirm relative affinities measured by circular dichroism. T2 cells were incubated with the indicated concentrations of peptide and the amount of cell surface A2 measured by flow cytometry using an A2-specific monoclonal antibody BB7.2. C, cell surface half-lives of A2-peptide complexes were determined by treating the peptide-pulsed cells (as in B) with BFA to halt vesicular transport. Aliquots of cells were removed at the indicated times and the remaining A2 on the cells determined by incubating with BB7.2. D, CD experiments show that anchor substitutions of GP2 do not greatly increase the stability. The best peptide is the double substitution I2L/L9V, but even it is deficient compared with ML. E, T2 cell surface stabilization confirms the CD data. F, the cell surface half-lives are moderately increased compared with GP2.
peptide ligands from the tyrosine kinase family member HER-2/neu. Despite the presence of CTL that recognize these peptides bound to A2, the tumors are not eliminated. These HER-2/neu peptides displayed a spectrum of binding affinities, but all were lower than the level observed for high affinity binders, such as ML or RT. Of particular interest to the immunology of tumor recognition was the clustering of many of these peptides in the “low affinity” category. Remarkably, all of these peptides, (GP2, CS4, and F56) have good anchor residues for A2.

There are two primary reasons to examine this phenomena in detail. The first is to understand how class I MHC binds peptides. There is a great deal known about how class I MHC binds many peptides with great sequence diversity, but there is very little information about how the protein binds any particular peptide well or poorly. There are now many examples of structural crystallography of high affinity peptides bound to class I MHC. GP2 is a perfect example of a poor binding peptide and as such offers the first opportunity to understand poor binding. The second reason to examine GP2 is that poor affinity peptides are potentially better targets for immunotherapy. The rationale for this has to do with T cell education. T cells are selected for survival by two mechanisms (positive and negative selection) in the thymus (35). If a self-peptide binds to class I MHC with high affinity, there is a larger concentration of pMHC in the thymus and thus a greater chance that T cells selected for survival by these mechanisms will also bind to class MHC molecule.

The averaged omit electron density map of the GP2 peptide with a cover radius of 1.5 Å. The map was calculated using modified phases from DM.

**FIG. 2. The center of the GP2 peptide is disordered.** The averaged omit electron density map of the GP2 peptide with a cover radius of 1.5 Å. The map was calculated using modified phases from DM.

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